Study of Mechanisms of Activation of the Interleukin-5 and Leptin Receptor

Lennart Zabeau

Promotor: Prof. Dr. Jan Tavernier
Co-promotor: Prof. Dr. Rudi Beyaert

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Department of Biochemistry, Institute Rommelaere, Faculty of Medicine and Health Sciences
For LZ and KdH, forever...
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<td>aa</td>
<td>amino acids</td>
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<tr>
<td>AgRP</td>
<td>Agouti Related Protein</td>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>$\beta_c$</td>
<td>$\beta_{common}$</td>
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<tr>
<td>BAL</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<td>Body Mass Index</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>Cocaine Amphetamine Regulated Transcript</td>
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<tr>
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<td>CNTF</td>
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<td>Dihydrofolate Reductase</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet Induced Obese</td>
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<td>dextrane sodium sulphate</td>
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<td>ER</td>
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<tr>
<td>FNIII</td>
<td>Fibronectin type III</td>
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<td>Fibroblast growth factor inducible Kinase</td>
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<td>Fluorescence Resonance Energy Transfer</td>
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<td>G-CSF</td>
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<td>Glycoprotein 130</td>
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<td>HRP</td>
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<td>Hematopoietin Receptor Response Element</td>
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<td>HUVECs</td>
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<td>IBD</td>
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<td>ICV</td>
<td>Intracerebroventricular</td>
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<td>IEL</td>
<td>intraepithelial lymphocyte</td>
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<td>IFN</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IGF</td>
<td>Insuline-like Growth Factor</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-1Ra</td>
<td>IL-1 receptor antagonist</td>
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<td>IP</td>
<td>Intraperitoneal</td>
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<td>Insulin Receptor Substrate</td>
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<td>JAK</td>
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<td>KIR</td>
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<td>LIFR</td>
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<tr>
<td>LPMC</td>
<td>lamina propria mononuclear cell</td>
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<td>LR</td>
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<td>LRlo</td>
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<tr>
<td>LRsh</td>
<td>LR short form</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MAPPIT</td>
<td>Mammalian Protein Protein Interaction Trap</td>
</tr>
<tr>
<td>MBP</td>
<td>Major Basic Protein</td>
</tr>
<tr>
<td>MC4R</td>
<td>MelanoCortin 4 Receptor</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>MRF</td>
<td>Modulator Recognition Factor</td>
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<tr>
<td>MSH</td>
<td>Melanocyte Stimulating Hormone</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>NF-AT</td>
<td>Nuclear Factor of Activated T cells</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localisation Signal</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
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<td>NP</td>
<td>Nasal Polyposis</td>
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<td>NPY</td>
<td>Neuropeptide Y</td>
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<td>New Zealand Obese</td>
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<td>OSM</td>
<td>Oncostatin M</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3 Kinase</td>
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<td>PIAS</td>
<td>Protein Inhibitor of Activated STAT</td>
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<td>PTP1B</td>
<td>protein tyrosine phosphatase 1B</td>
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<tr>
<td>PVN</td>
<td>Paraventricular Hypothalamic Nucleus</td>
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<tr>
<td>RDA</td>
<td>Representational Difference Analysis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rPAP</td>
<td>rat Panceatitis Associated Protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>scIL-5</td>
<td>single chain IL-5</td>
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<tr>
<td>SEAP</td>
<td>Secreted Alkaline Phosphatase</td>
</tr>
<tr>
<td>SH</td>
<td>Src Homology</td>
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<tr>
<td>SHP</td>
<td>SH2-containing Phosphatase</td>
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<tr>
<td>SIE</td>
<td>STAT inducible element</td>
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<tr>
<td>SOCS</td>
<td>Suppressors Of Cytokine Signalling</td>
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<tr>
<td>SOL</td>
<td>Soluble</td>
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<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<td>TGF</td>
<td>Transforming Growth Factor</td>
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<td>T_H</td>
<td>T helper subtype</td>
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<tr>
<td>TH</td>
<td>Thyroid Hormone</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>World Health Organisation</td>
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Cytokines are a large group of secreted proteins that are involved in intracellular communication in a multi-cellular organism. They transmit their signal to the inside of the target cell by binding and activation of membrane bound cytokine receptors. Although there is a growing body of biochemical and structural data regarding the ligands and their receptors, the precise mechanisms underlying the activation of these receptors are less understood. In this work, we use the receptors for interleukin-5 and leptin as a model to study the activation of class I cytokine receptors in more detail.

The first part of the thesis deals with interleukin-5 and its receptor. It is generally accepted that this cytokine plays a central role in the generation and propagation of allergic disorders, including bronchial asthma (chapter 3). We could show that neutralizing antibodies directed against IL-5 could potentiate proliferation of IL-5 responsive cells (chapter 4). These data do not only have implications for the stoichiometry of the activated receptor, but also warrant a careful analysis of neutralizing antibodies prior to their clinical use in IL-5 related disorders.

Leptin has been identified as the key player in the regulation of body weight and energy consumption. The last few years, it has become clear that besides this central function this hormone can act peripherally on several tissues. It is tempting to assume that leptin works as a control mechanism that links the body energy status to crucial processes like immune responses, reproduction, heart functions, and others (chapters 7-9). In this light, several studies provide evidence for the involvement of leptin in the onset of autoimmune and cardiovascular diseases. We used a signaling complementation assay to demonstrate the minimal requirements for leptin signal transduction (chapter 10). We furthermore show that receptor-receptor interaction are additionally needed for the formation of an activated receptor complex (chapter 11). Results from both approaches were used to propose a model for leptin induced receptor activation. These data could furthermore form the basis for the design of leptin or leptin
receptor antagonists that block the peripheral actions of the ligand, without interfering with the body weight regulation.
PART I: General Introduction
Chapter 1

General Introduction

1.1 Cytokines and their Receptors

1.1.1 Cytokines

Cytokines are by definition small and secreted regulatory proteins that are involved in intercellular communication in multi-cellular organisms. The family encompasses a broad range of growth factors, interleukins (IL), colony stimulating factors (CSF), lymphokines, monokines and interferons (IFN). They modulate cell growth, proliferation, differentiation and apoptosis and are involved in multiple processes such as hematopoiesis, immunity, and antiviral defense.

The biological actions of cytokines are characteristically redundant and pleiotropic. This redundancy implies that there are very few biological processes that are controlled by only one cytokine and that many responses can be achieved by a variety of cytokines. This does not suggest that specific cytokines are unimportant, but more that important cellular functions are backed up in a ‘fail-safe’ mechanism, in which one cytokine can compensate for the loss of another. Additionally, this feature allows coordinated responses between cytokines in specific cellular processes. Pleiotropy means that one cytokine can provoke a broad range of responses (both positive and negative) depending on the cell type or the differentiation stage.

Given the pleiotropic biological functions, cytokines are often secreted in very low amounts and thereby differ from classical hormones. Limitation of the sphere of action is achieved by several mechanisms: (i) cytokines are produced in small quantities, (ii) producer and responder cells are often physically located immediately to each other, (iii) spreading of some cytokines is limited by binding to the extracellular matrix of the responder cells, and (iv) soluble cytokine receptors can bind and inhibit the biological actions of cytokines.
Originally, cytokines were classified based on their biological responses. Given the apparent functional pleiotropy, cytokines are currently subdivided based on the structure of the cytokine ligands and their receptors.

1.1.1.1 Class I cytokines: 4-α-helical bundles
Cytokines of this family adopt an antiparallel 4-α-helical bundle structure, first observed for growth hormone. In this structure, two long over-hand loops (those between helices A, B and C, D) allow 4 α-helices to arrange in an up-up-down-down orientation. This group is further subdivided in short and long chain helical bundles. Short chain cytokines are generally smaller, have shorter helices and two short twisted antiparallel β-sheets are found in their A-B and C-D loops. Long chain cytokines have a longer overall chain length, and loops A-B and C-D contain additional helical segments. These structural features define the overall shape of the cytokine: ellipsoid or elongated cylinders for respectively short and long chain proteins (see figure 1).

![Figure 1: Examples of short and long chain 4-a-helical bundle cytokines](image)

structures of GM-CSF, a typical short chain cytokine (A), and G-CSF, a typical long chain cytokine (B) are shown (adapted from ‘The Cytokine Web’).

1.1.1.2 Class II cytokines: long chain β-sheet structures
Class II cytokines have an important role in inflammatory responses and are often produced as membrane bound precursors. β-Strands are the main feature of their structure. The tumor necrosis factor (TNF) family of cytokines has a β-jelly roll conformation, while IL-1 related cytokines adopt the β-trefoil fold.
1.1.1.3 Class III cytokines: mosaic structures, type I
This heterogeneous group encompasses the epidermal growth factor (EGF), insulin, insulin-related cytokines, and transforming growth factor β (TGF-β). Proteins of the first group contain an EGF domain, consisting of at least two antiparallel β-strands connected to the intervening loops by three disulphide bonds. Insulin, and insulin-related molecules consist of three α-helices linked by three disulphide bridges. Biological active TGF-β is a disulphide-linked dimer, with monomers containing three disulphide bonds in a threaded ring configuration termed ‘cysteine knot’.

1.1.1.4 Class IV cytokines: mosaic structures, type II
Chemokines are catalogued as class IV cytokines. They have unique chemotactic properties for leukocytes and are able to inhibit bone marrow progenitor cell growth. Chemokines are rather small (7-17 kDa.) basic heparin-binding proteins. They can be divided into four groups: CXC, CX3C, CC and C chemokines, in respect to the positioning of the first two closely paired and highly conserved cysteines.

1.1.2 Cytokine receptors
Cytokines are unable to pass the cellular membrane and therefore use a highly diverse group of transmembrane proteins to transmit their signal inside the cell. Based on their (predicted) secondary and tertiary structure and their biochemical functions, cytokine receptors can be divided into 4 classes. There appears to be a clear correlation between structural classes of the cytokine ligands and their receptors.

1.1.2.1 Class I cytokine receptors: Hemopoietin/interferon receptors
Hemopoietin and interferon receptors are single membrane spanning glycoproteins. Characteristic for these receptors is the presence of an extracellular cytokine receptor homology (CRH) domain (illustrated in figure 2). This CRH domain consists of two subdomains, each approximately 100 amino acids long and with a barrel-like structure of 7 β-strands resembling the fibronectin type III fold. The major conserved features are a pair of cysteine residues in the first subdomain and proline residues preceding each subdomain. Hemopoietin receptors are further characterized by a canonical Trp-
Ser-Xxx-Trp-Ser motif and a stretch of aromatic residues.

**Figure 2: Structure (A) and topology (B) of the cytokine receptor homology (CRH) domain of gp130**

(A) Ribbon representation of the structure of gp130-CRH (residues 100-303), with helices and β-strands shown in red and green respectively (B) Topology diagram of the two domains of gp130-CRH. Helices are represented by cylinders and β-strands by arrows. The positions of the five cysteine residues are marked by black bars. (Adapted from (Bravo et al., 1998)).

Relative simple class I receptors are dimers composed of a single subunit, like the receptors for erythropoietin (Epo) or growth hormone (GH). The majority of receptors however are built up of different receptor subunits. As mentioned, the activities of cytokines are redundant. One explanation for this biological redundancy is the observation that several receptor subunits are utilized by different cytokines. Examples of such shared receptors are β_common (β_c), gp130 and γ_c. The β_c receptor is shared in the receptors for IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF). These cytokines bind to a ligand-specific α-chain with low to intermediate affinity. Recruitment by this complex of the β_c receptor results in the generation of a high affinity receptor complex capable of signaling. A similar mode of receptor activation occurs for the receptors for IL-6 related cytokines (IL-6, IL-11, leukemia inhibitory factor (LIF) and oncostatin M (OSM), and others). Here, gp130, devoid of any affinity for the ligand, is recruited by a complex of the cytokine bound to a (soluble or membrane-bound) ligand-specific α-chain. A third group, which contains IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15, utilizes the γ_c receptor.

Class I cytokine receptors have no intrinsic enzymatic activity, but use intracellular
associated enzymes to trigger signaling. Receptors of this family typically activate the JAK/STAT signaling pathway, which will be discussed in more detail in chapter 1.2.

1.1.2.2 Class II cytokine receptors: NGF/TNF receptors
The nerve growth factor (NGF)/tumor necrosis factor (TNF) receptors are characterized by a repeat of a domain that contains 6 cysteine residues in the extracellular part. Often, a death domain, involved in induction of apoptosis, is found in the cytoplasmic tail. The extracellular part of the IL-1 receptor has a typical immunoglobulin fold.

1.1.2.3 Class III cytokine receptors: Receptor kinases
In contrast to class I and class II, receptors of class III have an intrinsic kinase activity. Based on the substrate specificity (tyrosine versus serine/threonine), this class is further divided in two subfamilies. Some of the tyrosine kinase receptors (e.g. receptors for macrophage colony stimulating factor and EGF) can also activate the JAK/STAT pathway. Example of a serine/threonine kinases is the receptors for TGF-β.

1.1.2.4 Class IV cytokine receptors: Serpentine receptors
Chemokine receptors differ from other cytokine receptors in that they traverse the cellular membrane seven times. C-terminal serine and threonine residues act as phosphorylation sites for receptor regulation. Intracellular signaling is dependent on hetero-trimeric G-proteins. These receptors are activated by a ligand-induced conformational change which results in a reshuffling of the transmembrane regions and the association and activation of G-proteins. An example of a chemokine receptor is CXCR3, which is activated by IP-10, MIG and CXCL11. RANTES (regulated on activation, normal T-cell expressed and secreted) activates receptors CCR1, CCR3, and CCR5. Some chemokines, like RANTES, can also signal via the JAK/STAT pathway, implying cross-talk between G-protein mediated signaling and the JAK/STAT pathway.
1.2 The JAK/STAT Pathway

Class I cytokine receptors lack any intrinsic enzymatic activity, but use cytoplasmic associated Janus kinases (JAKs) for signaling. The initial step in cytokine signaling is the ligand induced receptor oligomerisation (step 1 in figure 3). Several models of receptor clustering will be discussed in chapter 1.3. Receptor clustering brings the cytoplasmic receptor domains, and hence the docked JAKs, in close proximity so that the kinases can activate each other by cross-phosphorylation (step 2). Step 3 in the signaling cascade is the phosphorylation of cytoplasmic tyrosines. These phosphorylated residues serve as docking sites for various signaling molecules. Signal transducers and activators of transcription (STATs) typically dock to these phosphotyrosines containing motifs (step 4). Once recruited, these STAT factors become a substrate for the JAKs. Phosphorylated STAT (hetero)dimer dissociate from the receptor, translocate to the nucleus and activate gene transcription (step 5).

![Figure 3: Schematic representation of the JAK/STAT pathway](image)

**Figure 3: Schematic representation of the JAK/STAT pathway**
For more details, see text.

1.2.1 The Janus Kinases (JAKs)

To date, four mammalian members of the JAK family have been identified, JAK1, JAK2, JAK3 and Tyk2. JAKs are relative large proteins, with a molecular weight ranging from 120 to 130 kDa. JAK1, JAK2 and Tyk2 are expressed ubiquitously, while the expression of JAK3 is restricted to cells of the myeloid and lymphoid lineages (for an overview, see (Leonard and O'Shea, 1998). Different JAKs are activated by different receptors, and one receptor complex can use more than one kinase. JAK kinases associate with
proline-rich, membrane proximal sequences in the receptors. Based on the use of JAKs
and STATs, cytokine receptors are divided into five subfamilies (see table 1).

**Tabel 1: Cytokine specific JAK and STAT activation**
See text for details (adapted from (Schindler and Strehlow, 2000))

<table>
<thead>
<tr>
<th>Ligands</th>
<th>JAK kinase</th>
<th>STATs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFN family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-α/β</td>
<td>Tyk2, JAK1</td>
<td>STAT1, STAT2 (STAT3, STAT4, STAT5)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>JAK1, JAK2</td>
<td>STAT1, (STAT5)</td>
</tr>
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<td>IL-10</td>
<td>Tyk2, JAK1</td>
<td>STAT3</td>
</tr>
<tr>
<td><strong>gp130 family</strong></td>
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<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>JAK1, JAK2</td>
<td>STAT3, STAT1</td>
</tr>
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<td>IL-11</td>
<td>JAK1</td>
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</tr>
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<td>OSM</td>
<td>JAK1, JAK2</td>
<td>STAT3, STAT1</td>
</tr>
<tr>
<td>LIF</td>
<td>JAK1, JAK2</td>
<td>STAT3, STAT1</td>
</tr>
<tr>
<td>CNTF</td>
<td>JAK1, JAK2</td>
<td>STAT3, STAT1</td>
</tr>
<tr>
<td>G-CSF</td>
<td>JAK1, JAK2</td>
<td>STAT3</td>
</tr>
<tr>
<td>Leptin</td>
<td>JAK2, (JAK1)</td>
<td>STAT3, (STAT1, STAT5)</td>
</tr>
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<td>IL-12</td>
<td>Tyk2, JAK2</td>
<td>STAT4</td>
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<td><strong>γc family</strong></td>
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<td>IL-2</td>
<td>JAK1, JAK3</td>
<td>STAT5, (STAT3)</td>
</tr>
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<td>IL-7</td>
<td>JAK1, JAK3</td>
<td>STAT5, (STAT3)</td>
</tr>
<tr>
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<td>JAK1, JAK3</td>
<td>STAT5, STAT3</td>
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<td>JAK1, JAK3</td>
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</tr>
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<tr>
<td><strong>βc family</strong></td>
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<td>IL-3</td>
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</tr>
<tr>
<td>IL-5</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td><strong>Single chain family</strong></td>
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<td></td>
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<tr>
<td>Epo</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>GH</td>
<td>JAK2</td>
<td>STAT5, (STAT3)</td>
</tr>
<tr>
<td>PRL</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>TPO</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
</tbody>
</table>

1.2.1.1 Structure of JAKs

A schematic representation of the JAK structure is shown in figure 4. Comparison of JAK
sequences reveals seven regions of high homology, JH1-JH7. JH1 and JH2 encode
respectively the kinase and the pseudokinase domain.

**Figure 4: Schematic representation of the JAK structure**
JAKs share seven regions of high homology, JH1-7. N-terminal domains are involved in receptor binding,
JH2 and JH1 encode respectively a pseudokinase and a kinase domain.
Domains JH3-JH7 are involved in receptor association. The **kinase domain** has structural features found in classical tyrosine kinases, like the presence of tyrosine residues which are a critical component of the activation loop (Y1038/Y1039 in JAK1, Y1007/Y1008 in JAK2, Y980/Y981 in JAK3 and Y1054/Y1055 in Tyk2). Phosphorylation of these residues induces a conformational reorganization that allows substrate binding. The presence of a **pseudokinase domain** is a unique feature of the JAKs. Although this domain has all structural features of a bona fide tyrosine kinase, it is devoid of any catalytic activity. Although the precise function remains elusive, it is suggested that this domain regulates the activity of the JH1 encoded kinase. In this light, Saharinen et al. could show that deletion of the pseudokinase domain results in hyperactivation of JAK2 (Saharinen et al., 2000). Although similar mutations in the other JAK members did not lead to hyperreactivity, JH2 domains appear to modulate kinase activity (Velazquez et al., 1995; Yeh et al., 2000). The **amino-terminal** region of JAKs (JH3-7) is approximately 550 amino acids long and is most likely involved in receptor binding. JH6-7 appears to be the minimal receptor interaction element in JAK2 and 3 (Chen et al., 1997; Kohlhuber et al., 1997). Structural modeling illustrated that the JH3-4 shares similarity with SH2 domains, while the structure of JH4-7 resembles that of a FERM (Four-point-one, Ezrin, Radixin, Moesin) domain. FERM domains are known to mediate protein-protein interactions (Girault et al., 1998).

1.2.1.2 Biological functions – lessons learned from knock-out experiments

Insights in the biological functions of the different JAKs come from knock-out experiments. An overview is given in table 2.

It is of note that the phenotype of JAK3\(^{-}\) mice is in strong contrast to that of JAK3\(^{-}\) humans. These patients have severe combined immunodeficiency (SCID), and suffer from a profound T cell defect, although B cells seem unaffected (Leonard and O'Shea, 1998). This difference seems to be attributed to IL-7. This cytokine seems to be essential for pre-B cell growth in mice, but not in humans.
<table>
<thead>
<tr>
<th>Kinase</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK1</td>
<td>early postnatal lethal, neurological lesions, defects in thymocyte and B cell development, impaired response to type I and II interferons, impaired response to IL-10</td>
</tr>
<tr>
<td>JAK2</td>
<td>embryonic lethal, defects in erythropoiesis</td>
</tr>
<tr>
<td>JAK3</td>
<td>normal growth in a pathogenic-free environment, severe defects in lymphopoiesis, T cells defective in negative selection</td>
</tr>
<tr>
<td>Tyk2</td>
<td>impaired antiviral response</td>
</tr>
</tbody>
</table>

### 1.2.2 Signal Transducers and Activators of Transcription (STATs)

The family of mammalian STAT factors consists of seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. The genes coding for these factors are segregated in three clusters, with each cluster representing tandem duplication. STAT1 and 4 map to chromosome 1, STAT2 and 6 to chromosome 10 and STAT3 and 5 are located on chromosome 11. More recently in evolutionary history, the STAT5 gene has undergone an additional duplication, resulting in the STAT5a and 5b genes (Copeland et al., 1995). Drosophila contains only one STAT (d-STAT), with STATs 3, 5a, and 5b being the closest functional relatives (Miyoshi et al., 2001). Further analysis of STAT3 and 5 gene structures suggest that these STATs may have been the first mammalian STATs (Miyoshi et al., 2001). Additional STAT isoforms can be generated by alternative mRNA splicing: STAT1β, STAT3β, STAT5a1 and STAT5a2 (Shuai et al., 1993; Kazansky et al., 1995; Schaefer et al., 1997), or by protein processing (Azam et al., 1997).

#### 1.2.2.1 Structure of STATs

The mammalian STAT proteins range in size from 750 to 850 amino acids. Six conserved domains have been identified through structural and functional analysis (figure 5).
The **SH2 domain and tyrosine activation motif**: the SH2 domain is involved in the recruitment to the activated receptor. It consists of an anti-parallel β-sheet flanked by two α-helices, which form a pocket. A conserved arginine residue involved in binding of the phosphate group of a phosphotyrosine lies in this pocket. The crystal structure of STAT1 illustrates that the SH2 domain is involved in dimerisation; one STAT SH2 binds the phosphorylated tail of a second STAT molecule (see figure 6). The **DNA-binding domain** is a β-barrel with an immunoglobulin fold, a structure resembling that of NF-κB and p53 DNA binding domains (Chen et al., 1997). A linker domain connects the SH2 domain and the DNA binding domains. The crystal structure illustrates that the linker domain helix α10 interacts with the SH2 domain, while helix α6 docks into the DNA binding domain. This suggests that the DNA binding capacity can be regulated by structural changes in the SH2 domain after phosphotyrosine binding (Yang et al., 1999). Four α-helices make up the **coiled-coil domain**. This domain can interact with other transcription factors (Horvath et al., 1996), but is also involved in receptor binding, tyrosine phosphorylation and nuclear export (Begitt et al., 2000; Zhang et al., 2000). A **transcriptional activation domain (TAD)** is located at the C-terminus of a STAT protein. Given its regulatory role in unique transcriptional responses, this domain is poorly conserved among STATs. STAT isoforms lacking this domain through alternative splicing or protein processing, appear to function as dominant-negative regulators of transcription. This domain can be serine phosphorylated, which enhances the transcription of some but not all genes (reviewed in (Decker and Kovarik, 2000)).
1.2.2.2 Mode of action

In unstimulated cells STATs predominantly localize to the cytoplasm, and are recruited to phosphotyrosines in activated cytokine receptors. Phosphorylation by the JAKs results in a conformational change and dissociation of the STATs from the receptor. STAT4, 5 and 6 form homodimers, while for STAT1 and 3 homo- and heterodimers are observed. STAT2 can only trigger signaling when complexed to STAT1. How the STAT complexes are transported to the nucleus is only partially understood. STAT1 translocation appears to be independent of the actin cytoskeleton or microtubules (Lillemeier et al., 2001). Using a STAT1-GFP (green fluorescent protein) chimera Lillemeier and co-workers could show that non-activated as well as activated cytoplasmic STAT1 exhibit a high energy-independent mobility resulting in a random walk from the plasma membrane to the nucleus (Lillemeier et al., 2001).

Active nuclear import of activated STATs is mediated by importin NPI-1. Importin proteins recognize, bind and translocate nuclear localization signal (NLS) containing
proteins. To date, no classical NLS sequences have been identified for the STAT proteins. Alternatively, receptor chains can be involved in targeting STATs to the nucleus. Putative NLS sequences have been identified in some cytokine receptors (Subramaniam et al., 2000). Energy required for the STAT translocation is provided by the hydrolysis of GTP by Ran/TC4. In the nucleus, STATs typically bind to palindromic sequences in the promoter of target genes: 5'-TTCN₃GAA-3' for STAT1, 3, 4, 5a, and 5b, while STAT6 binds to 5'-TTCN₄GAA-3' (Leonard and O'Shea, 1998).

1.2.2.3 Biological functions – lessons learned from knock-out experiments

**STAT1** deficient mice confirmed the primary role of STAT1 in IFN signaling. These mice are defective in IFN dependent immune responses to both microbial and viral agents. STAT1 is no longer able to induce target gene expression in response to IFNs. STAT1 seems also to be involved in non-immune responses, like chondrocyte proliferation (Sahni et al., 1999) and regulation of apoptosis through induction of caspases (Chin et al., 1997).

Studies in **STAT2** null mice illustrated that the type I IFN autocrine/paracrine loop regulates the basal expression level of STAT1 in primary embryonic fibroblasts (MEFs), but not in macrophages (Park et al., 2000). STAT1/STAT2 double knock-out mice are completely unresponsive to both classes of IFNs and are more susceptible to infections than either single knock-out mice.

Disruption of the **STAT3** gene leads to an early embryonic lethal phenotype. Embryos develop normally to the egg cylinder stage, but rapidly regress and die around day 7.5. Tissue specific knock-out of the gene illustrates that T cells no longer proliferate in response to IL-6, which has been shown to suppress apoptosis in these cells (Takeda et al., 1998). Macrophages depleted of STAT3 exhibit an impaired immune reaction in response to IL-10 (Takeda et al., 1999). Hepatocytes lacking this protein have defects in the induction of acute phase genes in response to IL-6 (Alonzi et al., 2001). Finally, a tissue specific elimination of STAT3 in keratinocytes results in both skin and hair defects (Sano et al., 1999). More detailed analysis showed that these observations are due to
an impaired response to EGF, TGF-α and IL-6 in wound healing assays.

### Table 3: Overview of STAT knock-out phenotypes

<table>
<thead>
<tr>
<th>STAT</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>STAT1</td>
<td>defective macrophage activity high sensitivity to viral infections</td>
</tr>
<tr>
<td>STAT2</td>
<td>high sensitivity to viral infections</td>
</tr>
<tr>
<td>STAT3</td>
<td>embryonic lethality</td>
</tr>
<tr>
<td>STAT4</td>
<td>impaired T&lt;sub&gt;1&lt;/sub&gt; responses</td>
</tr>
<tr>
<td>STAT5a</td>
<td>impaired mammalian gland development partial defect in T-cell growth</td>
</tr>
<tr>
<td>STAT5b</td>
<td>impaired expression of sexual dimorphism defects in T-cell growth defect in NK cell development</td>
</tr>
<tr>
<td>STAT6</td>
<td>impaired T&lt;sub&gt;2&lt;/sub&gt; responses</td>
</tr>
</tbody>
</table>

**STAT4** expression is restricted to natural killer (NK) cells, dendritic cells and T lymphocytes, and is mainly activated in response to IL-12. The phenotypes of mice deficient in IL-12 or STAT4 are therefore very similar: lymphocytes fail to differentiate into Th1 cells and to produce IFN-γ. In contrast to mice, STAT4 in humans is also activated by type I IFNs.

**STAT5a and 5b** are very homologues (96% identity) and are only divergent in their C-termini. In vitro studies showed that STAT5a and 5b display a functional redundancy, although their knock-out phenotypes are different. STAT5a deficient mice are predominantly defective in mammary gland development in response to prolactin (Liu et al., 1997). STAT5a/b exhibit a more severe phenotype. The phenotype of STAT5b single knock-out mice resembles that of growth hormone deficient mice (Udy et al., 1997). While female mice are largely unaffected, STAT5b<sup>-/-</sup> males are significant smaller, and exhibit a loss in the expression of male-specific liver genes. Again, defects are more pronounced in double knock-out mice. Rather unexpected, STAT5a/b<sup>-/-</sup> mice have normal peripheral blood counts (Teglund et al., 1998). Only under conditions of stress can defects in the development of myeloid and lymphoid lineages be observed.
**STAT6** is widely expressed and involved in signaling by IL-4 and IL-13, two cytokines with important functions in regulating acquired immunity. STAT6\(^{-/-}\) mice are affected in their ability to develop Th2 cells (Kaplan et al., 1996; Takeda et al., 1996), and to class switch antibody production to the IgE isotype. IL-4 stimulation of these mice fails to upregulate expression of MHC class II, the IL-4 receptor and the low IgE receptor in resting B-cells (Takeda et al., 1996).

### 1.2.3 Negative regulation of JAK/STAT signaling

JAK/STAT signaling is involved in many important processes including cellular growth, proliferation and differentiation. It is therefore important that cytokine signals are rapid and finely tuned, in order to avoid toxic side effects. Thus far, five general mechanisms of inhibition have been documented: (i) receptor internalization, (ii) phosphatase activity, (iii) expression of SOCS proteins, (iv) protein inhibitors of activated STATs (PIAS), and (v) dephosphorylation of STATs in the nucleus. **Receptor internalization and degradation** is the most effective form of turning off the cytokine signal. Ligand-induced endosomal degradation via clathrin-coated pits has been demonstrated for gp130, which contains an intracellular internalization motif (Dittrich et al., 1996; Govers et al., 1997). Alternatively, receptor recruited STAT molecules form a target for ubiquitination and subsequent proteasome-mediated degradation (Kim and Maniatis, 1996; Wang et al., 2000).

Since JAK phosphorylation is a prerequisite for its activity, cytokine signaling can be stopped by JAK **phosphatases**. Two such enzymes have been identified, the SH2 domain containing phosphatase 1 (SHP1), which is expressed exclusively in haemopoietic cells, and SHP2, which has a much broader expression pattern. Mutations that diminish recruitment of SHP1 and 2 to the activated receptor prolong JAK/STAT activation in the case of Epo (Klingmuller et al., 1995), βc (Yi et al., 1993; Ohtani et al., 2000) or gp130 signaling (Ohtani et al., 2000). Mice with a naturally occurring mutation in the SHP1 gene, die short after birth due to an accumulation and over-activation of macrophages in the lung (Kamata et al., 2003). SHP2 knock-out mice are embryonic lethal due to defects in EGF signaling (Qu et al., 1999). Other phosphatases are the
membrane-bound CD45, PTP1B and PTPξC.

A well studied family of cytokine signaling inhibitors are the **suppressors of cytokine signaling (SOCS)**. Eight members have been identified thus far: SOCS1-7 and cytokine-inducible SH2 containing protein (CIS). Structurally, these proteins are characterized by a SH2 domain and a highly conserved so-called SOCS box. This latter domain is most likely involved in the proteasome degradation (Nicola and Greenhalgh, 2000). Biochemical studies have shown that the SOCS box associates with the elongin B/C complex, which binds a E3-like ubiquitin ligase to regulate degradation (Kamura et al., 1998). The SH2 domain is involved in recruitment to the activated receptor or JAK kinase, and provides thereby an alternative mechanism of inhibition, i.e. competition with STAT molecules for receptor phosphotyrosines (Yoshimura et al., 1995). SOCS1 and 3 have an additional kinase inhibitory region (KIR), which acts as a pseudo-substrate for the JAK kinase thereby blocking signaling (Yasukawa et al., 1999).

Cytokine signaling can also be regulated by STAT interacting proteins, including the protein inhibitor of activated STATs (PIAS). This family consists of five members: PIAS1, PIAS3, PIASy, PIASxα and PIASxβ. PIAS proteins are constitutively expressed. PIAS1 and 3 bind directly to activated STAT1 and 3 respectively, thereby blocking DNA binding (Chung et al., 1997; Liao et al., 2000). In the case of STAT1, inhibitory properties can be blocked by methylation of the STAT molecule (Mowen et al., 2001).

Finally, STAT activity can be stopped by dephosphorylation in the nucleus. TC45 splice variant of the T-cell protein tyrosine phosphatase TCPTP, is located in the nucleus (Lorenzen et al., 1995).
1.3. Activation of Class I Cytokine Receptors

It was accepted cytokine receptors become activated upon ligand induced oligomerisation. This receptor clustering brings the cytoplasmic associated JAKs in close proximity, which allows them to activate each other by cross-phosphorylation and to trigger intracellular signaling pathways. However, the situation seems more complex. First, several cytokine receptors are pre-assembled at the plasma membrane. This has been demonstrated for the receptors for Epo (Livnah et al., 1999; Remy et al., 1999), GH (Frank, 2002), IFN-γ (Krause et al., 2002), the IL-6 receptor α-chain (Schuster et al., 2003), and the βc signaling component in the receptors for IL-3, -5 and GM-CSF (Muto et al., 1996). Second, crystal structures of some ligand bound receptors illustrate that receptor-receptor interactions help to stabilize activated receptor complexes. These observations of pre-assembled complexes suggest that ligand binding induces a spatial reorganization of the preformed receptor complexes, more than a simple oligomerization, thereby triggering intracellular signaling.

In the remainder of this chapter, insights in the mechanisms of class I cytokine receptor activation will be discussed based on findings for three receptor-models: the homodimeric Epo and GH receptors, the G-CSF receptor and the heterodimeric gp130 receptors.

1.3.1 ‘Simple’ homodimeric receptors: receptors for Epo and GH, ...

The receptors for Epo and GH represent the most simple cytokine-receptor complexes: the extracellular part of these receptors consists of only a CRH domain, and the receptors are activated upon ligand induced dimerisation.

1.3.1.1 The GH receptor (GHR)

The GHR was the first cytokine receptor to be crystallized in complex with its ligand (de Vos et al., 1992). In this 2:1 complex (GHR:GH), one GH molecule binds to two GHR chains, via binding site I and II (see figure 7). Site I is located at the C-terminus of the D helix, while site II comprises residues from helices A and C. Mutagenesis and binding studies showed that the affinity of site I is much higher compared to that of site II.
(Cunningham et al., 1991; Fuh et al., 1992). This observation could explain the bell-shaped GH biological activity in cell culture systems. It was suggested that the initial interaction between GH and its receptor occurs via site I. The resulting 1:1 complex exhibits an increased affinity for residues of site II. Remarkably, both sites interact with very similar epitopes in the GHR, located in the elbow formed between both subdomains in the GHR CRH domain. The 2:1 complex is further stabilized by receptor-receptor interactions near the plasma membrane.

![Figure 7: Crystal structure of the 2:1 GHR:GH complex](image)

GH is shown in green, GHR1 and GHR2 in red and blue. (Adapted from (de Vos et al., 1992)

1.3.1.2 The Epo receptor (EpoR)

Due to difficulties in crystallizing the ligand and the receptor, the Epo-EpoR complex was resolved more than six years later than the GH-GHR complex (Syed et al., 1998). Like with GH, a 2:1 ligand:receptor complex was observed (see figure 8). Epo binds also to its receptor with two binding sites I and II. Furthermore, the interaction sites on the EpoR are similar to those in the GHR. Besides these overlaps, there are also two major differences in the complexes. First, the difference in affinity between site I and II in Epo is much more pronounced (a 1000-fold) when compared to GH (Philo et al., 1996). Second, while there is a large receptor-receptor interface between GHR chains, only one intermolecular binding in the Epo-EpoR was observed.
Livnah and co-workers compared the structure of unliganded and liganded EpoR (Livnah et al., 1999). They could show that even in the absence of Epo, the EpoR exists as a dimer (figure 9). The dimer interface consists of the self association of the ligand binding sites. In this configuration, the C-terminal parts of both CRH domains, and hence the cytoplasmic domains are quite far apart, approximately 73 Å (arrows in figure 9). In the liganded (the agonistic peptide EMP1 was used here) structure, these C-terminal regions are brought in closer proximity (39 Å). This leads to the conclusion that Epo binds to a pre-formed EpoR dimer, thereby inducing a conformational change which brings the cytoplasmic receptor tails in closer proximity.

**Figure 8: Crystal structure of the 2:1 EpoR:Epo complex**
Helices, β-strands and loops (L) are shown in red, green and blue respectively. (Adapted from Syed et al., 1998)

**Figure 9: Crystal structure of unliganded (A) and liganded EpoR (B)**
The EpoR chains are shown in gold and cyan, the EMP1 peptide in purple. D1 and D2 represent the EpoR CRH sub-domains. Arrows indicate the C-terminus. (Adapted from Livnah et al., 1999)
More evidence for this model was provided using a dihydrofolate reductase (DHFR) complementation assay (Remy et al., 1999). In this assay, two interacting proteins are coupled to two inactive enzyme fragments (F[1,2] and F[3]) using linkers of different length. Interaction between the proteins can be measured by binding of the fluorescent labeled methotrexate (fMTX) inhibitor of the DHFR enzyme. In case of the EpoR, complementation was observed in the absence of Epo when linkers of 30 Aa were used, indicating that the EpoR exists as a dimer on the cellular surface (panel C in figure 10). When 5 Aa linkers were inserted, only Epo dependent complementation occurred (panel B).

Alternatively, EpoR dimerization could be mediated by interactions between the receptor transmembrane domains. Constantinescu and colleagues used a fluorescent copatching technique to show that the EpoR transmembrane domain, and not the extracellular and cytoplasmic parts, allows ligand-independent copatching of the prolactin receptor with the EpoR (Constantinescu et al., 2001).

1.3.2 ... the more complex homomeric G-CSF receptor ...

When compared to the EpoR and GHR, the G-CSF receptor has a more complex topology. The extracellular domain is composed of an immunoglobulin-like (Ig) domain, a CRH module and three membrane-proximal fibronectin type III (FNIII) structures (figure 11, panel A). A crystal structure of G-CSF bound to its receptor showed a 2:2 (G-CSF:G-CSFR) complex (Aritomi et al., 1999). The interaction via site II was very similar to these found in the EpoR and GHR complexes: ligand residues of this site are located at helices A and C, and site II on the receptor localizes to the elbow formed
between the sub-domains of the G-CSFR CRH. This site II interaction allows the formation of a 1:1 complex. The 2:2 complex is then formed due to interaction of site I (at the N-terminus of G-CSF) and a region in the C-terminal half of the G-CSFR (figure 11, panel C). These findings however are not consistent with mutagenesis studies on the ligand (Reidhaar-Olson et al., 1996) or the receptor (Layton et al., 2001). These studies, combined with the behavior of neutralizing antibodies, showed that there is likely no site I, but an additional site III (located in helix E) which interacts with the Ig domain of the G-CSFR (panel B). Layton et al suggested that the observed 2:2 crystal structure is an artifact or represents only an intermediate complex.

Figure 11: Structure of G-CSFR (A), the tetrameric receptor complexes (B, C) and the G-CSF binding sites (D)

(A): schematic representation of the G-CSFR is shown. Ig: immunoglobulin-like, CRH: cytokine receptor homology, FNIII: fibronectin type III, TM: transmembrane, cyto: cytoplasmic. (B) G-CSF-G-CSFR tetrameric complex with binding sites II and III. (C) schematic representation of the crystal structure of the receptor complex. (D) ribbon diagram of G-CSF showing the main helices labeled A-D and the additional E helix in the A-B loop. (Adapted from Layton et al., 2001)

1.3.3 ... and the heteromeric gp130 receptor family

The IL-6 family of cytokines comprises a set of related long chain cytokines: IL-6, its viral counterpart vIL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neutrophic factor (CNTF), and cardiotrophin. These cytokines share the glycoprotein 130 (gp130) in their receptors. A schematic representation of this receptor is shown in figure 11. Next to the shared component, the receptors also consist of a ligand specific chain. The LIF and OSM receptors have intrinsic signaling capacities,
while the α-chains specific for IL-6 and IL-11 have a shortened cytoplasmic tail. The receptor for CNTF lacks a transmembrane and intracellular domain, but is membrane bound by a GPI anchor. Mutagenesis combined with binding studies identified three binding sites in IL-6 (Simpson et al., 1997), IL-11 (Barton et al., 1999; Tacken et al., 1999), LIF (Hudson et al., 1996) and CNTF (Di Marco et al., 1996). Sites I (at the C-terminus of the D-helix) and II (residues from helices A and C) exhibit strong similarities with those found in Epo and GH. Site III on the other hand is believed to interact with the Ig domain of gp130 and is located at the N-terminus of the cytokine.

Detailed structural data comes from the crystal structure of vIL-6 complexed to gp130. In contrast to mammalian IL-6, this Kaposi's sarcoma-associated herpesvirus IL-6 binds directly to gp130, even in the absence of the ligand-specific IL-6Rα chain. A tetrameric complex is observed in which two ligand molecules interact via sites II and III with respectively the CRH and Ig domain of two gp130 molecules (Chow et al., 2001). In this orientation, site I remains accessible.

**Figure 12:** The IL-6/IL-6Rα/gp130 hexameric complex

(A) the hexameric complex with positioning of interaction sites I, IIa, IIb, IIIa and IIIb. IL-6 molecules are shown in red, IL-6Rα in green and purple, gp130 in light and dark blue. (B) Close-up from the five different interaction sites. (Adapted Boulanger et al., 2003)

More recently, the crystal structure of the hexameric IL-6 receptor complex (two IL-6, two IL-6Rα and two gp130 molecules) was solved (Boulanger et al., 2003). The overall shape resembles a table, with the tabletop composed of IL-6, the IL-6Rα CRH and the
gp130 CRH and Ig domains, resting on the gp130 FNIII legs (figure 12, panel A). Up to five protein-protein interaction sites can be observed per structure half: site I: IL-6 site I (helix D) and the elbow of the IL-6Rα CRH; site IIa: IL-6 site II (helices A and C) and the gp130 CRH; site IIb: between the Ig domains of IL-6Rα and gp130; site IIIa: a rather unexpected interaction between gp130 Ig domain and the N-terminal half of the IL-6Rα CRH domain; and site IIIb: IL-6 site III (N-terminus of the D-helix) and the Ig domain of gp130. A more detailed representation of these interfaces is given in figure 12, panel B.
1.4. References


Chapter 1


PART II:
Receptors for IL-3, IL-5, and GM-CSF
Chapter 2

IL-3, IL-5 and GM-CSF

Class I cytokines are further subdivided based on the shared receptor chains they use for signaling. The β common (βc) family comprises three structurally and functionally related short chain cytokines; IL-3, IL-5 and GM-CSF. These cytokines play an important role in hematopoiesis by promoting survival, growth and differentiation of myeloid precursor cells (see figure 1). They also act on terminal differentiated cell types in inflammation and protective immunity. Finally, recent studies demonstrate that they can modulate T\textsubscript{H}1 and T\textsubscript{H}2 responses.

![Figure 1: Major effect of IL-3, IL-5 and GM-CSF on the generation of leukocytes](image)

Positive and negative regulation are shown in respectively green and red. Biological importance is illustrated by the cytokine letter size. Adopted from (Martinez-Moczygemba and Huston, 2003).

2.1. Interleukin-5

2.1.1. IL-5: from DNA to protein

The cDNA that encodes murine IL-5 was cloned in 1985 (Takatsu et al., 1985), rapidly followed by isolation of the human IL-5 cDNA from a T-cell leukemia line (Azuma et al., 1986). The hIL-5 gene, composed of four exons and three introns (Campbell et al., 1987), has been mapped to chromosome 5 in humans (Sutherland et al., 1988), as part of a cluster of genes encoding the related cytokines IL-3, IL-4, and GM-CSF. The human IL-5 promoter spans a 1.2 kb region that controls the specific expression of IL-5.
IL-5 gene transcription can be further enhanced by binding of nuclear factors of activated T-cells (NFAT) (Prieschl et al., 1995). Besides regulation of gene activation, IL-5 synthesis is also regulated at the level of mRNA stability (Umland et al., 1998).

The hIL-5 precursor is 134 amino acids residues long, including a signal peptide of 19 amino acids (Azuma et al., 1986), and has a calculated molecular mass of 13 kDa for the mature protein. It has been shown that IL-5 exists as a cross-linked homodimer, with both monomers arranged in an anti-parallel manner (i.e. head-to-tail) (McKenzie et al., 1991). This dimer formation is essential for biological activity (Tavernier et al., 1989; McKenzie et al., 1991). The variable high-molecular-weight range of IL-5 is explained by the heterogeneous addition of carbohydrate (Takahashi et al., 1990; McKenzie et al., 1991). Deglycosylation illustrates that the presence of sugar groups is not essential for receptor binding and biological activity (Takahashi et al., 1990; Kunimoto et al., 1991). X-ray diffraction at 2.4 Å resolution revealed a two-domain structure in which each domain adopts the typical cytokine fold, consisting of a four-helical bundle in an up-up-down-down array (Milburn et al., 1993). Unique to IL-5 however is D-helix swapping, whereby each bundle is built up of three helices of one monomer, and a fourth helix of the second monomer.

Figure 2: Structure of IL-5
The dimeric structure of IL-5 is shown, with monomers in pink and blue. Cylinders represent the α-helices. Cysteine bridges are shown in orange.
2.1.2. The IL-5 Receptor: IL-5Rα and βc – it takes two to tango.

IL-5 mediates its biological effects upon binding and activation of a membrane bound receptor (IL-5R). This receptor is composed of two subunits: a ligand-specific α-chain (IL-5Rα) and a shared βc-chain (Tavernier et al., 1991, Miyajima et al., 1993). The use of this latter receptor is shared with the receptors for IL-3 and GM-CSF, which both also have their own specific α-chains. This observation provides the molecular basis for the overlapping biological activities of these three cytokines.

2.1.2.1. The ligand-specific IL-5Rα-chain

The IL-5Rα-chain is expressed mainly on eosinophils and basophils, activated murine B-cells (Mita et al., 1988; Rolink et al., 1989), and on certain muscle cells (Rizzo et al., 2002). Human eosinophils express through alternative splicing three different transcripts from the same IL-5Rα gene (Tavernier et al., 1992; Tuypens et al., 1992), two soluble (SOL) and one membrane-bound (TM) receptor variants. Remarkably, one of the soluble variants is the predominant (>90%) transcript detected in mature, circulating eosinophils. The SOL isoform has antagonistic properties in vitro: it interferes with IL-5 binding on its surface-anchored receptor, inhibits proliferation of IL-5 dependent cell-lines, and blocks IL-5-induced eosinophil differentiation from human cord blood cultures (Tavernier et al., 1991). It has become clear that IL-5 can affect the expression of its own receptor, at the mRNA, splicing and the protein level. Northern blot analysis of normal blood eosinophils illustrates that IL-3, IL-5 and GM-CSF down-regulate IL-5Rα expression at the mRNA level (Wang et al., 1998). IL-5 itself, but not IL-3 or GM-CSF, could stimulate a reversible switch from SOL towards TM hIL-5Rα isoform expression in cell culture systems (Tavernier et al., 2000). On the other hand, IL-5Rα and βc were markedly reduced on airway eosinophils, SOL IL-5Rα concentrations were significantly elevated in bronchoalveolar lavages, and airway eosinophils exhibited a lack of responsiveness to IL-5, in strong contrast to circulating eosinophils (Liu et al., 2002). It is postulated that the reduced expression is due to an IL-5 driven proteolytic cleavage of the membrane bound isoform (Liu et al., 2002).
The nucleotide sequence of the human IL-5Rα (hIL-5Rα) cDNA predicts a polypeptide of 420 amino acids (Tavernier et al., 1991; Murata et al., 1992; Tavernier et al., 1992). The mature protein has a molecular mass of 60 kDa, characterised by N- and O-linked glycosylations. Three domains can be identified: an extracellular domain of 322 Aa, a 20 residue-long transmembrane domain, and a cytoplasmic tail of only 58 Aa. The extracellular domain consists of one CRH and an additional N-terminal FNIII domain. The receptor binds IL-5 with intermediate (Kd ~ 500 pM - 1 nM) and low (Kd ~ 5-10 nM) affinity in man and mouse, respectively. Upon association with the βc-chain, high affinity binding of approximately 150 pM is observed for both species. One IL-5 binding pocket is present in the hinge region between the two sub-domains of the CRH (Czabotar et al., 2000). The N-terminal FNIII-like domain provides a second ligand binding site. This domain also determines the species-specificity of IL-5 binding, and its deletion completely abolishes IL-5 binding (Cornelis et al., 1995). This domain contains a free cysteine residue, which may be involved in disulfide-linked receptor heterodimerisation with the βc upon ligand binding (Stomski et al., 1998).

2.1.2.2. The βc-chain
The βc-chain is a 897 Aa protein, including a 16 residue-long signal peptide, an extracellular part of 424 Aa, a 27 residue-long transmembrane domain, and a cytoplasmic tail of 430 Aa. The mature protein has a molecular mass of 120-130 kDa, and is also likely to be glycosylated. The human receptor has no detectable affinity for one of the three cytokines, but plays an essential role in the formation of the high affinity complex and in signal transduction. The extracellular part of this latter receptor is built of two complete CRH modules. Using chemical cross-linking and immunoprecipitation, it has been shown that the βc-chain forms ligand-independent homodimers on the cell surface (Muto et al., 1996). These results were confirmed by unravelling the structure of the extracellular part of the receptor at 3.0 Å resolution (Carr et al., 2001), showing that βc forms an interlocked dimer in which residues of domain 1 hydrogen bonds into the β sheet of domain 3 of a second receptor. A ligand binding pocket in this receptor is formed between domain 1 of the first βc-chain, and domain 4 of the second βc-chain in the intertwined dimer (Murphy et al., 2003). As with
the IL-5R α-chain, IL-5 is able to regulate expression of the βc-receptor. Activation of
the IL-5 receptor results in proteasomal degradation of the βc cytoplasmic domain in
the activated receptor complex (Martinez-Moczygemba and Huston, 2001), which
results in termination of the signal. The remnant of the βc is endocytosed and further
degraded in the lysosomes.

2.1.3. Biology of IL-5
IL-5 is primarily produced by the type 2 subset of helper T-cells (T\textsubscript{H2}) (Altman et al.,
1990). T-cells do not store IL-5, but upon appropriate stimulation, synthesize and
immediately release the cytokine. IL-5 can also be found in mast cells and eosinophils,
which can rapidly release pre-formed IL-5 from storage granules upon cellular
activation (Bradding et al., 1994; Dubucquoi et al., 1994). It is of note that sensitized
mast cell-deficient mice are fully capable of producing high amounts of IL-5 (Takeda et
al., 1997), whereas T cell-deficient mice lack the ability to produce a substantial IL-5
driven cellular response (Hamelmann et al., 1997). As a third source of IL-5, malignant
and virally transformed cells have been identified (Paul et al., 1990; Gruss et al., 1994).

Due to the restricted expression pattern of its receptor, human IL-5 selectively
functions on eosinophils and basophils, two predominant effector cell types in allergic
inflammation (Clutterbuck et al., 1987; Hirai et al., 1990). IL-5 has numerous effects on
precursors of eosinophils and on the mature eosinophils. (i) It stimulates growth and
differentiation of myelocytes of the eosinophil lineage in the bone marrow (Sanderson
et al., 1986; Clutterbuck and Sanderson, 1988). (ii) It regulates the pro-inflammatory
function of eosinophils by promoting the release of the eosinophil granular proteins:
major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase
(EPO) (Kita et al., 1992). (iii) IL-5 is weakly chemotactic on mature eosinophils, mostly
by priming the cells for enhanced responsiveness to other chemotactic mediators, like
eotaxin, IL-8, RANTES or platelet-activating factor (Sehmi et al., 1992; Warringa et al.,
1992; Warringa et al., 1993; Schweizer et al., 1994). IL-5 up-regulates the adhesion of
eosinophils to endothelium, thereby further increasing eosinophilic accumulation (Walsh
et al., 1990). (iv) Eosinophils present at sites of inflammation, persist because of IL-5-
induced inhibition of programmed cell death or apoptosis (Sakai and Kraft, 1997; Simon et al., 1997; Wedi et al., 1997). Apart from its actions on eosinophils, IL-5 also increases histamine and leukotriene release in basophils (Bischoff et al., 1990; Hirai et al., 1990).

Generation of mice lacking the IL-5 gene has demonstrated the obligatory role of IL-5 in eosinophil development (Foster et al., 1996; Kopf et al., 1996). IL-5−/− mice have normal baseline eosinophil and immunoglobulin levels and generally exhibit normal T- and B-cell development. However, IL-5−/− mice do not develop eosinophilia in response to helminth and nematode infections. Although eosinophils are thought to play a crucial role in parasite immunity, the parasite burden in these mice was not affected. It is of note that in the case of IL-5 deficiency, IL-3 or GM-CSF, which can also act on eosinophils in vitro, cannot take over the function of IL-5 in vivo. This might suggest the existence of a strictly IL-5-dependent eosinophilic progenitor, or of IL-5Rα-specific signaling pathways. The central role of IL-5 in eosinophil biology is further supported using mice constitutively expressing IL-5 (IL-5 transgenic mice). These mice have high numbers of eosinophils in the peripheral blood and eosinophil infiltration in many organs, including lung, liver and lymphoid tissue (Dent et al., 1990; Tominaga et al., 1991). Despite this ‘eosinophil overload’, IL-5 transgenic mice are healthy, demonstrating that the expression of IL-5 or the presence of eosinophils per se may not be pathogenic.

IL-5 has a variety of functions on the murine immune system ranging from stimulation of immunoglobulin (Ig) release to enhancing B-cell growth and differentiation (Gleich and Adolphson, 1986; Bertolini et al., 1993). Although IL-5 is able to induce in vitro IgA and IgM production in human B-cells, a physiologically functional role in controlling antibody production in the human immune system is yet to be determined (Jaffe et al., 1995). The role of IL-5 in eosinophil, basophil (and murine B-cell) function is well documented and is generally accepted. Interestingly, Rizzo et al. recently demonstrated an exaggerated contraction of IL-5-treated, isolated normal human bronchus smooth muscle cells in response to acetylcholine (Rizzo et al., 2002). This cholinergic
contractility was blocked by neutralizing anti-IL-5 and anti-IL-5 receptor antibodies, and occurred independently of an eosinophil influx. The authors further showed a clear expression of the IL-5 receptor in bronchus muscle, and to a lesser extent in trachealis, saphenous vein, and atrial muscle.

2.1.4. IL-5, eosinophils and asthma

IL-5 and eosinophils are believed to play a critical role in allergic disorders including bronchial asthma. Bronchial asthma is a chronic inflammatory disease defined by a reversible airway obstruction and non-specific airway hyper-responsiveness (AHR). An extensive infiltration of the bronchial mucosa with lymphocytes and eosinophils provokes the pathology. This inflammation increases mucus production, airway smooth muscle hyperplasia and airway remodeling.

The asthmatic response can be divided into two steps: an early response leading to airway obstruction, and a late response which results in the observed AHR. T cells from atopic patients exhibit an increased production of Th2-type cytokines, like IL-4, IL-5, IL-13 and IL-6, after allergen sensitization. A second allergen challenge induces the production of allergen specific IgE, the predominant antibody isotype in the asthmatic cascade. The isotype switch is mainly under control of the cytokines IL-4 and IL-13. Binding of IgE to its specific receptor on mast cells induces in the release of pro-inflammatory mediators (like leukotrienes, prostaglandins, histamine and a panel of cytokines), eventually leading to the early asthmatic response. IL-5 activation of eosinophils results in the degranulation and release of the cationic proteins ECP, EPO and MBP. All these proteins are toxic to the lung epithelial cells and lead to the late asthmatic response.

The role of IL-5 and eosinophils in the asthmatic response has been well established. In serum and bronchoalveolar lavage (BAL) fluid of patients suffering from bronchial asthma, upregulation of IL-5 expression could be shown at the mRNA (Hamid et al., 1991) and protein level (Robinson et al., 1992). These IL-5 serum levels correlate well with the number of eosinophils in the peripheral blood and the disease severity
(Robinson et al., 1993). In animal model systems, intratracheal administration of IL-5 increases airway reactivity (Iwama et al., 1993), and pre-treatment with anti-IL-5 (Coffman et al., 1989) or anti-IL-5R (Hitoshi et al., 1991) mAbs significantly reduced parasite- or allergen-induced allergic inflammation in guinea pigs. Furthermore, allergen-provoked bronchial hyper-reactivity is strongly reduced in IL-5 knock-out mice (Foster et al., 1996). In Ascaris-responsive primates, the humanised anti-hIL-5 mAb Sch 55700, reduces reactive pulmonary eosinophilia to 75% up to six months after the statum dose (Egan et al., 1999), and in Cynomolgus-monkeys the humanised anti-hIL-5 mAb SB-240563 strongly suppresses the eosinophil count (Zia-Amirhosseini et al., 1999). In men, administration of recombinant IL-5 to atopic patients during bronchoscopy resulted in a significant eosinophilic infiltration and cellular activation. Furthermore, inhalation of IL-5 by asthmatic patients increased the observed AHR, eosinophil counts and concentrations of the basic proteins (Shi et al., 1998).

### 2.2. Interleukin-3 and GM-CSF

#### 2.2.1. Interleukin-3

IL-3 is a second cytokine involved in hematopoiesis. It controls the growth and terminal differentiation of basophils, mast cells, and myeloid-derived dendritic cells (see figure 1). IL-3 knock out mice exhibit basal hematopoiesis, also growth, development and longevity of the animals are not affected (Lantz et al., 1998). IL-3 appears however necessary for increased numbers of tissue mast cells, enhanced basophil production, and protective immunity in response to parasites. In vitro, IL-3, but not IL-5 or GM-CSF, is able to prevent apoptosis of basophils through the activation of phosphatidylinositol 3-kinase (Zheng et al., 2002). IL-3 and its receptor play an important role in the generation and prolongation of acute myeloid leukemia (Testa et al., 2002). Increased expression of IL-3Rα is associated with enhanced blast proliferation, increased cellularity and a poor prognosis. Although the IL-3 receptor is not expressed on B-cells under normal conditions, its expression is observed in approximately 40% of patients with B cell-acute lymphocytic leukemia.
IL-3 has also biological affects apart from the hematopoietic compartment, with dendritic and microglial cells as the main targets. A novel role for the cytokine has been reported in modulating the actions of type I and II dendritic cells. In vitro, IL-3 derived dendritic cells tend to promote a $T_H2$ response, while GM-CSF grown cells preferentially induce $T_H1$ responses (Ebner et al., 2002). This difference was due to the decreased production of IL-12 in the presence of IL-3. A potential role for IL-3 in the development of diseases resulting from inflammation of the central nervous system has also been suggested (Lee et al., 1993; Araujo and Lapchak, 1994; Appel et al., 1995). This is in accordance with the observation in rats that microglial cells express the $\beta_c$ receptor, and that these cells can produce and respond to IL-3 in a putative autocrine feedback loop (Appel et al., 1995). IL-3 can stimulate the proliferation of microglial cells in vitro as well as the formation of multinucleated giant cells (Lee et al., 1993). Finally, this is further supported by the observation that IL-3 can be detected in postmortem brain tissue from patients with Alzheimer's disease (Araujo and Lapchak, 1994).

2.2.2. GM-CSF

Of the three cytokines discussed in this chapter, GM-CSF has the most diverse effects. It is produced and secreted by a broad panel of different cell types, including activated T lymphocytes, eosinophils, mast cells, macrophages, endothelial cells, and bone marrow stromal cells. It controls the terminal differentiation of nearly all myeloid cell lines, as illustrated in figure 1, and the clinical use of this cytokine to promote myeloid cell recovery has been proposed (Armitage, 1998). In contrast, GM-CSF can block the generation of mast cells (Welker et al., 2001), or the cellular differentiation of CD34$^+$ progenitors into lymphoid progenitors or type II dendritic cells (Iwasaki-Arai et al., 2003). Like with IL-3, deletion of the GM-CSF gene in mice did not result in a decrease in progenitor or terminally differentiated myeloid blood cells. However, infection of these mice did not result in an accelerated production of granulocytes, macrophages and eosinophils (Stanley et al., 1994; Nishinakamura et al., 1995; Robb et al., 1995).

A rather unexpected observation with GM-CSF$^{-/-}$ mice are symptoms of the pulmonary alveolar proteolysis (PAP) disease (Robb et al., 1995). PAP is characterized by abnormal
surfactant metabolism resulting in an accumulation of phospholipids and surfactant proteins within alveolar spaces. It is suggested that PAP is caused by a lack of GM-CSF since auto-antibodies directed against the cytokine have been detected in patients with PAP (Bonfield et al., 2002b) and the antibody levels correlate with the disease severity (Bonfield et al., 2002a).

Expression of the receptor for GM-CSF can result in cellular transformation of both non-hematopoietic and hematopoietic cell types. Its expression could be shown in malignancies including chronic and acute myeloid leukemia, juvenile myelomonocytic leukemia, small-cell lung carcinoma, melanoma, certain breast cancer cell lines, and prostate cancer. These observations make GM-CSF an important molecular target for clinical treatment of GM-CSF responsive cancers. Treatment with a GM-CSF antagonistic mutant unable to interact with $\beta_c$ in its receptor, resulted in a transient improvement of patients with myelomonocytic leukemia (Bernard et al., 2002).
2.3. References


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Chapter 3

Interleukin-5, Eosinophilic Disease and Therapeutic Intervention

Zabeau L., Gevaert P., Bachert C., and Tavernier J.

Interleukin-5, Eosinophilic Diseases and Therapeutic Intervention

Lennart Zabeau\textsuperscript{1,1}, Philippe Gevaert\textsuperscript{2,2}, Claus Bachert\textsuperscript{2} and Jan Tavernier*\textsuperscript{1}

\textsuperscript{1}VIB09 Dept. Medical Protein Research, Ghent University, Belgium
\textsuperscript{2}Department of Otorhinolaryngology, Ghent University Hospital, Belgium

\textbf{Abstract}: Interleukin 5 (IL-5) is the key cytokine in an eosinophil’s life span: it supports eosinophilopoiesis and eosinophil differentiation, contributes to eosinophil migration, tissue localisation and function, and prevents eosinophil apoptosis. Given the likely role of eosinophils in chronic inflammatory diseases, a lot of research over the past decade was aimed at antagonising IL-5 function. It appears from recent studies that, although this can easily be achieved \textit{in vitro}, blocking IL-5 function \textit{in vivo} is much more difficult than originally anticipated. Here, we review the current status of IL-5 and IL-5 receptor research, with emphasis on strategies to interfere with IL-5 function.

\textbf{BIOLOGY OF INTERLEUKIN 5 (IL-5)}

IL-5 is primarily produced by the type 2 subset of helper T-cells (Th2) \cite{1}. These cells are involved in the control of growth and effector functions of cell types that contribute to allergic inflammatory responses \cite{1-5}. T-cells do not store IL-5, but upon appropriate stimulation, synthesize and immediately release this cytokine. Immunohistochemical staining revealed that besides in T-cells, IL-5 can also be found in mast cells and eosinophils. However, regulation of its production is different in these latter cell types, which rapidly release preformed IL-5 from storage granules upon cellular activation \cite{6,7}. Thus, in allergic diseases eosinophils could maintain the inflammation by autocrine stimulation. It is of note that sensitized mast cell-deficient mice are fully capable of producing high amounts of IL-5 \cite{8}, whereas T-cell-deficient mice lack the ability to produce a substantial IL-5 driven cellular response \cite{9}. As a third source of IL-5, malignant and virally transformed cells have been identified. Reed-Sternberg cells in patients with Hodgkin’s disease express mRNA and proteins of various cytokines and growth factors, including IL-5 \cite{10}. Similarly, cell supernatants from Epstein-Barr virus transformed B-cells contain large amounts of IL-5 \cite{11}.

The cDNA that encodes murine IL-5 (mIL-5) was cloned in 1986 \cite{12,13}, rapidly followed by isolation of the human IL-5 (hIL-5) cDNA from a T-cell leukemia line \cite{14,15}. The hIL-5 gene, composed of four exons and three introns \cite{16}, has been mapped to chromosome 5 in humans \cite{17}. It is part of a cluster of genes encoding the related cytokines IL-3, IL-4, and granulocyte macrophage-colony stimulating factor (GM-CSF): CEN - IL-4 - IL-13 - IL-5 - GM-CSF - IL-3 - TEL \cite{18}. A common regulator of transcription of IL-4, IL-5 and IL-13 was recently identified by Loots \textit{et al.} based on cross-species sequence comparison \cite{19}. This conserved non-coding sequence, 400 bp in length, is located in the intergenic region between IL-4 and IL-13. Transgenic mice lacking this sequence do not have altered expression of IL-4 and IL-13 per cell, but the number of Th2 cells expressing these cytokines is significantly reduced. This observation might suggest that this sequence does not act as a classical enhancer or suppressor, but might be involved in modulation of the overall chromatin structure.

The human IL-5 promoter region is a 1.2 kb region that controls the specific expression of IL-5, and contains several GATA and CLEO (conserved lymphokine element O) sites. Thus far, three GATA3 binding sites have been identified in the IL-5 promoter. The -70 and -152 sites positively regulate transcription, while binding to the -400 site results in strong inhibition of IL-5 expression \cite{20}. The CLEO element, positioned between nucleotides -42 and -56, acts as an on/off switch for IL-5 expression. It binds the transcription factor activator protein-1 (AP-1), as well as Oct-1 (Octamer-1) and Oct-2 \cite{21,22}. The AP-1 complex is composed of Fra-2 and JunD, and \textit{de novo} synthesis of both these factors is necessary for AP-1 control of expression \cite{23}. This might explain the sensitivity of IL-5 transcription to protein inhibitors, which is in clear contrast to other related genes. IL-5 gene transcription can be further enhanced by binding of nuclear factors of activated T-cells (NFAT) at position -110 \cite{24,25}. Next to regulation of gene activation, IL-5 synthesis is also regulated at the level of mRNA stability \cite{26}.

Due to the restricted expression pattern of its receptor, human IL-5 selectively functions on eosinophils and basophils, two predominant effector cell types in allergic inflammation \cite{27-29}. IL-5 has numerous effects on precursor of eosinophils and on the mature eosinophils: (i) it stimulates growth and differentiation of myelocytes of the eosinophil lineage in the bone marrow \cite{30,31}. (ii) it regulates the pro-inflammatory function of eosinophils by promoting the release of the eosinophil granular proteins: major basic protein, eosinophil cationic protein and eosinophil peroxidase. Eosinophils incubated for four days with IL-5 released up to 60% of their granule proteins \cite{32}. (iii) IL-5 is weakly chemotactic on mature eosinophils, mostly by priming the cells for enhanced responsiveness to other chemotactic mediators, like IL-8, RANTES/CCL5 or platelet-activating factor \cite{33-36}. IL-5 up-regulates the adhesion of eosinophils to endothelium, thereby further increasing eosinophilic accumulation \cite{37}. (iv) Eosinophils...
present at sites of inflammation persist because of IL-5-induced inhibition of programmed cell death or apoptosis [38-40]. Apart from its actions on eosinophils, IL-5 also increases histamine and leukotriene production in basophils [28,41].

Generation of mice lacking the IL-5 gene has demonstrated the obligatory role of IL-5 in eosinophil development [42,43]. IL-5−/− mice have normal baseline eosinophil and immunoglobulin levels and generally exhibit normal T- and B-cell development. However, IL-5−/− mice do not develop eosinophilia in response to helminth and nematode infections. Although eosinophils are thought to play a crucial role in parasite immunity, the parasite burden in these mice was not affected. It is of note that in the case of IL-5 deficiency, IL-3 or GM-CSF, which can also act on eosinophils in vitro, cannot take over the function of IL-5 in vivo. This might suggest the existence of a strictly IL-5-dependent eosinophilic progenitor. Alternatively, IL-5-specific signaling pathways may also explain this observation (see further). The central role of IL-5 in eosinophil biology is further supported using mice constitutively expressing IL-5 (IL-5 transgenic mice). These mice have high numbers of eosinophils in the peripheral blood and eosinophil infiltration in many organs, including lung, liver and lymphoid tissue [44,45]. Despite this ‘eosinophil overload’, IL-5 transgenic mice are healthy, demonstrating that the expression of IL-5 or the presence of eosinophils per se may not be pathogenic.

IL-5 has a variety of functions on the murine immune system ranging from stimulation of immunoglobulin release to enhancing B-cell growth and differentiation [46,47]. Although IL-5 is able to induce in vitro IgA and IgM production in human B-cells, a physiologically functional role in controlling antibody production in the human immune system is yet to be determined [48].
Chapter 3

Interleukin-5, Eosinophilic Diseases and Therapeutic Intervention

The role of IL-5 in eosinophil, basophil (and murine B-cell) function is well documented and is generally accepted. Interestingly, Rizzo et al. recently demonstrated an exaggerated contraction of IL-5-treated, isolated normal human bronchus smooth muscle cells in response to acetylcholine [49]. This cholinergic contractility was blocked by neutralizing anti-IL-5 and anti-IL-5 receptor antibodies, and occurred independently of an eosinophil influx. The authors further showed a clear expression of the IL-5 receptor (IL-5R) in bronchus muscle, and to a lesser extent in trachealis, saphenous vein, and atrial muscle.

THE IL-5 PROTEIN

The hIL-5 precursor is 134 residues long and includes a signal peptide of 19 amino acids [13,50]. Mature hIL-5 has a calculated molecular mass of 13,149 kDa, which is in accordance with the observation that the E. coli-derived protein migrates as a single 13-kDa band on SDS-PAGE gels in the presence of reducing agents. However, under non-reducing conditions, IL-5 is detected as a molecule of 27 kDa. It has been shown that IL-5 exists as a cross-linked homodimer [51]. Mutagenesis of cysteine residues on position 44 and 86 identified both residues as essential in the generation of a 27-kDa bridge, and showed that both monomers are arranged in an anti-parallel (i.e. head-to-tail) manner [52]. Reduction and alkylation of these two residues in recombinant IL-5 lead to a biologically inactive monomer [51,53], illustrating that dimer formation via disulfide bonds is essential for biological activity [51,52,54,55].

Recombinant IL-5, expressed in a variety of eukaryotic cells, has native molecular masses between 35 and 60 kDa, or between 16 and 32 kDa after treatment with reducing agents. This variable high-molecular-weight range is explained by the heterogeneous addition of carbohydrate (O-linked glycosylation on threonine 3 and N-linked glycosylation on asparagine 28) [51,56]. Deglycosylation illustrates that the presence of sugar groups is not essential for receptor binding and biological activity [56,57]. Evidence was presented that O-linked glycosylation might act as a suppressor of biological activity, while N-linked glycosylation might contribute to thermo-stabilization of the protein [58].

X-ray diffraction at 2.4 Å resolution revealed a two-domain structure in which each domain adopts the typical cytokine fold, consisting of a four-helical bundle in an up-up-down-down array [59]. Unique to IL-5 however is D-helix swapping, whereby each bundle is built up of three helices of one monomer, and a fourth helix of the second monomer (Fig. 1). In addition, IL-5 contains two short β-strands (between helices A and B, and helices C and D), forming a small anti-parallel β-sheet.

Despite the necessity for dimer formation, three monomeric variants of IL-5 have been described thus far: mono 5 [60], GM1 and DABC [61] (Fig. 1). Superimposition of the high-resolution structures for IL-5 and the closely related Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF) illustrated that the loop between helices C and D is significantly shorter in case of IL-5. Introduction of 8 amino acid residues in this CD-loop resulted in the stable formation of biologically active monomeric mono 5 and GM1 IL-5-variants. Both molecules display slightly lower affinity for the receptor, and exhibit minor decrease in biological activity. The DABC-IL-5 variant has been constructed by a circular permutation. Circularly permuted variants are generated by connecting their normal termini (here helices A and D) covalently and introducing breaks at other sites (in the case of DABC in the CD-loop) to produce new termini [62,63]. This monomeric IL-5 variant has biochemical and biological properties comparable to that of GM1.

A cDNA encoding single-chain IL-5 (sIL-5) was constructed by linking two IL-5 coding regions with a glycine-glycine linker [64]. sIL-5 is expressed as a stable protein with a molecular mass corresponding to that of dimeric IL-5. Receptor affinity and biological activity of sIL-5 are comparable to that of wild type IL-5. This variant allows asymmetric mutations of the symmetrical IL-5 molecule (see below).

PROPERTIES OF THE INTERLEUKIN 5 RECEPTOR (IL-5R) SYSTEM

IL-5 mediates its biological effects upon binding and activation of a membrane bound receptor (IL-5R). This receptor is composed of two subunits: a ligand-specific α-chain (IL-5Rα) and a shared β-chain [65]. The use of this latter receptor is shared with the receptors for IL-3 and GM-CSF, which both also have their own specific α-chains. For this reason, the β-subunit is often referred to as β common or βc. This observation provides the molecular basis for the overlapping biological activities of these three cytokines.

The Ligand-Specific IL-5Rα-Chain

The IL-5Rα-chain is expressed mainly on eosinophils and basophils, on activated murine B-cells [66,57], and on certain muscle cells [49]. Human eosinophils express through alternative splicing three different transcripts from the same IL-5Rα gene [68,69], two soluble and one membrane-bound receptor. Remarkably, one of the soluble variants is the predominant (>90%) transcript detected in mature, circulating eosinophils. This (SOL) isoform has antagonistic properties in vitro. It interferes with IL-5 binding on its (TM) surface-anchored receptor, and inhibits proliferation of IL-5 dependent cell-lines, and blocks IL-5-induced eosinophil differentiation from human cord blood cultures [70].

The nucleotide sequence of the human IL-5Rα (hIL-5Rα) cDNA predicts a polypeptide of 420 residues [68,79,71]. It contains a 29 residue-long signal peptide, an extracellular domain of 322 Aa, a 20 residue-long transmembrane domain, and a cytoplasmic tail of 38 Aa. The receptor belongs to the class I cytokine receptor subfamily [72]. Members of this family are characterised by the presence of a so-called cytokine receptor homology (CR1) domain. This domain is formed by two barrel-like structures, each 100 Aa in length, and resembling the fibronectin type III (FN III) fold. Two conserved disulfide bridges are found in the N-terminal part, while a WXSWS motif is typical of the C-terminal module. Typical of the receptor α-subunits for IL-3, IL-5 and GM-CSF is the presence of one additional N-terminal FN III-like domain.
One IL-5 binding pocket is present in the hinge region between the two domains of the CRH [73]. In addition, the N-terminal FN III-like domain provides a second ligand-binding site. This domain determines the species-specificity of IL-5 binding, and its deletion completely abolishes IL-5 binding [74]. This domain contains a free cysteine residue, which may be involved in disulphide-linked receptor heterodimerisation with the βe upon ligand binding [75]. The IL-5Rα-chain is identified as a 60 kDa mature protein, characterised by N- (and perhaps also O-) linked glycosylation of one or more of the potential N-glycosylation sites. This receptor binds IL-5 with intermediate (Kd ~ 500 pM - 1 nM) and low (Kd ~ 3-10 nM) affinity in man and mouse, respectively. Upon association with the βe-chain, a high affinity binding of approximately 150 pM is observed for both species.

The βe-Chain

The βe-chain is a 897 Aa protein, including a 16 residue-long signal peptide; an extracellular part of 424 Aa, a 27 residue-long transmembrane domain, and a cytoplasmic tail of 430 Aa. The mature protein has a molecular mass of 120-130 kDa, and is also likely glycosylated. This receptor has no detectable affinity for one of the three cytokines, but plays an essential role in the formation of the high affinity complex and in signal transduction. Like the IL-5Rα-chain, the βe-chain belongs to the class I cytokine receptor family. The extracellular part of this latter receptor is built up of two complete CRH modules. Using chemical cross-linking and immunoprecipitation, it could be shown that the β-chain forms ligand-independent homodimers on the cell surface [76]. These results were confirmed when the structure of the extracellular part of the receptor was unravelled at 3.0 Å resolution [77], showing that the β-chain formed an interlaced dimer in which the G-strand of domain 1 hydrogen bonds into the β-sheet of domain 3 of the dimer related molecule. A ligand binding pocket in this receptor is formed between domain 1 of the first β-chain, and domain 4 of the second β-chain in the intertwined dimer [78].

IL-5 and Regulation of Expression of Its Receptor Subunits

Recent publications have demonstrated a complex pattern of regulation of IL-5Rα expression. Clearly, IL-5 can affect the expression of its own receptor, and hence of eosinophil responsiveness, at the transcriptional, splicing and the protein level. Northern blot analysis of normal blood eosinophils illustrates that IL-3, IL-5 and GM-CSF downregulate IL-5Rα expression at the mRNA level [79]. These authors showed that this regulation occurs very rapidly (reaching maximum inhibition within 2 hours), in a dose-dependent manner which does not require protein synthesis. We generated a stable promyelocytic FDC-P1 cell-line containing a hIL-5Rα-chain minigene, in which cDNA and genomic DNA segments were combined [80]. Using this cell-system, we showed that IL-5 itself, but not IL-3 or GM-CSF, could stimulate a reversible switch from SOL toward TM hIL-5Rα isoform expression. These results were confirmed using primary human cord blood-derived CD34+ cells. On peripheral blood eosinophils, Hellman et al. recently demonstrated that surface-anchored TM IL-5Rα was strongly downmodulated by recombinant IL-5, immediately with both IL-5 and GM-CSF, and weakly with only GM-CSF, indicating that GM-CSF binding partially affects surface IL-5 receptor expression [81]. Furthermore, the proportion of CD69-positive eosinophils was significantly up-regulated at the same level by IL-5, GM-CSF as well as by the combination of both cytokines, indicating that activation of eosinophils, judged by CD69 upregulation, is not mandatory linked to TM IL-5Rα downregulation. Liu and co-workers could recently show a different control mechanism for expression of the membrane bound IL-5Rα on airway or circulating eosinophils [82]. After allergen challenge, IL-5Rα and βe were markedly reduced on airway eosinophils, and thus in contrast to circulating cells, soluble IL-5Rα concentrations were significantly elevated in bronchoalveolar lavages, and airway eosinophils exhibited a lack of responsiveness to IL-5. These authors postulate that the reduced expression is due to an IL-5 driven proteolytic cleavage of the membrane-bound isoform [83]. Finally, IL-5 induced activation of its receptor chain also results in a posttranslational degradation of the βe cytoplasmic domain in the activated receptor complex [84]. As a consequence, signalling is terminated by deletion of the cytoplasmic phosphorylated residues. The remnant of the βe is endocytosed and further degraded in the lysosomes.

IL-5R SIGNAL TRANSDUCTION

Like all members of the cytokine receptor family, neither subunit of the IL-5R possesses inherent tyrosine kinase activity, and signalling therefore requires receptor associated kinases. Physical association of kinases JAK2 (Janus kinase 2) [85,86] Lyn [87] and Fes [88] with the β-chain could be shown, and a membrane proximal region between amino acids 451 and 517 was identified as essential for these interactions [88]. The IL-5Rα-chain on the other hand binds JAK2 [89], and Lyn [90]. Stimulation of IL-5R carrying cells with IL-5 leads to the phosphorylation of tyrosine residues in the cytoplasmic tail of the βe receptor subunit, hereby providing docking sites for adaptor and signalling proteins like Shc, Grb2, SHP-2, Crkl and the STAT (signal transducer and activator of transcription) proteins [91-93]. Two different signal-cascades are well documented: the JAK/STAT and Ras/MAPK pathway. Members of the STAT family utilized in βe JAK/STAT signalling are mainly the STAT5 homologues STAT5A and STAT5B [94-96], but also STAT1 [85,97], STAT3 [85,97,98], and STAT6 [99]. Once recruited to the activated receptor, STAT molecules themselves become a substrate for the JAK kinase. After phosphorylation, STAT's dimerize into an activated STAT complex, and after translocation to the nucleus, participate in transcriptional regulation. Recruitment of the adaptors Shc and Grb2 to the βe in the activated receptor leads to propagation of signalling via the Ras/MAPK pathway, through the activation of Ras [100]. Ras translocates Raf-1 kinase to the juxtamembrane compartment, where the latter is activated by tyrosine kinases, protein 14-3-3, and possibly by PKC. Raf-1 regulates ERK (extracellular signal-regulated kinase) a member of the MAPK family, through activation of MAP or ERK kinases. ERK is a positive regulator of c-fos induction. The precise role of the α subunit in IL-5 signalling is less clear. Apart from ligand binding, this subunit is essential for
signal transduction, since deletion of its cytoplasmic part, which contains a critical JAK binding site, completely abolishes signalling [101]. There is a growing body of evidence that, although IL-3, IL-5 and GM-CSF use the same β subunit, they also provoke cytokine-specific signals. One example is the regulation of isoform expression of the IL-5Rα chain (see above). Recently, syntenin was described as an adapter molecule that associates with the IL-5Rα subunit, but not with the IL-5Rβ or GM-CSFRα subunits [102].

**CLINICAL POTENTIAL FOR INTERLEUKIN-5 BLOCKAGE**

IL-5 is normally not found at high levels in healthy individuals. A variety of diseases of the respiratory tract, skin, gut and of the hematopoietic system are associated with eosinophilia and with elevated levels of IL-5 mRNA and protein in bone marrow, in circulation and in tissue. Examples include allergic and non-allergic respiratory diseases, aspirin sensitivity, atopic dermatitis, hyperergic eosinophilic syndrome, Churg-Strauss syndrome, food and drug allergies, and helminth infections ([103]; see also table 1). Many studies have provided evidence for an eosinophil involvement in the development or maintenance of asthma. Compared to healthy subjects, asthmatic patients show increased numbers of eosinophils in peripheral blood, in BAL fluid and in bronchial biopsies. The number of eosinophils correlates with severity of asthma symptoms and with lung function [104-106]. Upon recruitment and activation, pro-inflammatory cationic proteins and lipid mediators are released from the eosinophil granules, which may contribute to damage and dysfunction of tissues and may lead to structural abnormalities with the duration of the disease [104,107]. Many studies of the inflammatory infiltrate of patients with asthma show that, amongst all present cytokines, IL-5 was highly represented and that the majority of the cells were eosinophils [108]. Indeed, segmental bronchial provocation of asthmatic patients results in eosinophil infiltration and accumulation in lungs and also in the nose [109,110]. Similarly, nasal provocation resulted in eosinophil infiltration in the nasal and bronchial mucosa [111].

There is a large body of evidence suggesting that IL-5 is obligatory for the development of the prominent eosinophil lung inflammation, known to correlate with increased airway responsiveness in human asthma. Sensitized guinea pigs respond to allergic challenge with bronchial hyper-reactivity and infiltration of eosinophils into lung tissue and bronchoalveolar lavage (BAL) fluid [112-114]. Monoclonal antibodies to interleukin-5 inhibit these pulmonary responses [114]. Notably, larger doses of an anti-interleukin-5 antibody are needed to block the hyper-reactivity than are needed to block eosinophilia. It has recently been shown that administration of recombinant IL-5 to isolated airway smooth muscle from both rabbits and humans results in increased reactivity to acetylcholine [115]. Furthermore, recent publications have demonstrated eosinophil-independent airway hyper-responsiveness and IL-5Rα gene expression in human bronchus smooth muscle [49,115].

Despite recent controversies, there is still strong evidence for an important role of IL-5 in airway eosinophilia and in chronic asthma. Although the activity of IL-5 may not be restricted to eosinophils, it remains a prominent target to interfere with several allergic diseases, such as allergic

**Table 1. Eosinophil-Associated Diseases**

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<th>Parasitic infections</th>
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<tr>
<td>Allergic diseases</td>
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<td>Asthma, allergic rhinitis,</td>
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<td>urticaria, allergic bronchopulmonary aspergillosis (ABPA), drug hypersensitivity reactions</td>
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<th>Respiratory tract</th>
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<tr>
<td>Nasal polyposis, eosinophilic pneumonia, Loeffer's syndrome, hypersensitivity pneumonitis</td>
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<th>Toxic reactions</th>
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<td>Eosinophil myalgic syndrome</td>
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<td>(L-tryptophan), toxic oil syndrome</td>
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<th>Neoplastic and myeloproliferative disorders</th>
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<tr>
<td>Idiopathic hyperergic eosinophilia syndrome (IHEIS), eosinophilic leukemia, lymphoma, angioimmunoplastic lymphadenopathy, immunotherapy with interleukin-2</td>
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<th>Connective tissue diseases</th>
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<td>Hypersensitivity vasculitis,</td>
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<td>Churg-Strauss syndrome,</td>
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<tr>
<td>hypereosinophilic fasciitis</td>
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<th>Skin disorders</th>
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<td>Atopic dermatitis, seborrheic,</td>
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<td>eosinophilic cellulitis</td>
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<th>Immunodeficiency syndromes</th>
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<td>Wiskott-Aldrich syndrome,</td>
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<td>hyper IgE syndrome</td>
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<th>Gastrointestinal diseases</th>
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<td>Eosinophil gastroenteritis,</td>
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<td>inflammatory bowel diseases</td>
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rhinitis, nasal polyps, asthma, idiopathic eosinophilic syndromes, and atopic dermatitis.

**INTERFERING WITH IL-5 OR IL-5R SYNTHESIS**

The biological actions of IL-5 can be inhibited either by blocking the interaction of IL-5/IL-5R using antibodies or IL-5 mutants, or by preventing expression of IL-5 or components of its receptor. One way to achieve this is the use of antisense oligonucleotides. These are short, synthetic DNA sequences, which specifically hybridise to the mRNA of the target protein. This hybridisation results in the prevention of mRNA transport, splicing, transcription, and also leads to degradation of the mRNA by endogenous ribonuclease H. Usually, phosphorothioate oligonucleotides are used to prevent their degradation by nucleases. Additionally, 2'-O-methoxyethyl modification further enhances RNA binding affinity and decreases the intrinsic toxic effects of the oligonucleotides. Karras and co-workers were the first to introduce antisense technology in the biology of IL-5 [116]. These authors showed that administration of an antisense oligonucleotide directed against IL-5 resulted in decreased IL-5 protein expression, and furthermore led to reduced lung eosinophilia and Agammediated late-phase airway hyper-reactivity in a mouse model of asthma. The fact that the abrogation of the late-phase airway response was not complete suggested that, besides IL-5, additional pathways may contribute to airway hyper-reactivity. The IL-5Rα chain is another interesting target for antisense therapy. Antisense oligonucleotides were designed to reduce expression of the TM and SOL IL-5Rα isoforms in vitro, and to suppress eosinophilia in vivo upon IL-5 treatment or in a ragweed-induced allergic pleuritis model [117]. Furthermore, proof-of-concept data for an antisense strategy diverting the expression pattern from the TM to the SOL hIL-5Rα isoform has also been obtained [118]. A final example of the use of antisense oligonucleotides is provided by the group of Renzi [119]. These authors designed an oligonucleotide for the β2 subunit, thereby targeting the actions of IL-3, IL-5 and GM-CSF simultaneously. Intra-tracheal injection resulted in significant and sequence-specific inhibition of β2 mRNA and protein expression in rat lungs. Administration in vivo leads to reduced eosinophilia upon ovalbumin challenge, and reduced airway hyper-reactivity to leukotriene D4. It can be expected that similar results will be obtained using RNAi (small interfering RNA). In this technique, double-stranded RNAs homologous to the gene of interest initiate sequence-specific post-transcriptional gene silencing.

**INTERFERING WITH LIGAND BINDING**

A number of protein-based IL-5 antagonists have been reported, including single point mutants of IL-5 that occupy the receptor but fail to initiate receptor activation [120,121], neutralizing antibodies directed against IL-5 [122], and the SOL IL-5Rα isoform that sequesters the ligand in solution [123].

**IL-5 Mutants**

Over the past decade, the interaction between IL-5 and its receptor has well been established at the molecular level. We reported a detailed structure-function analysis of hIL-5 showing that most residues affecting binding to the α-chain are clustered in a loop connecting β-strand 1 and helix B (His 38, Lys 39, and His 41), in β-strand 2 of the CD-loop (Glu 89, Arg 91, and Glu 90) and close to the C terminus of helix D (Thr 109, Glu 110, Trp 111, and Ile 112) [120]. These findings were confirmed using similar approaches by two other groups [124,125]. Using single-chain IL-5 (see above), Li and co-workers extended these observations [126]. Asymmetric mutation of residues Arg91, Glu110, and Trp111, in only one of the two monomers in IL-5, was used to illustrate that the IL-5/IL-5Rα interaction is preferentially stabilized by one of the two monomers. Wu et al. recently reported a phage display study using the biologically active single-chain (wt/A5)scIL-5 mutant [127]. In this IL-5 variant, only the N-terminal EERRR92 sequence in the CD-loop is intact (wt), while the same sequence in the C-terminus is mutated to five alanine residues (A5). Random mutagenesis of this EERRR sequence illustrated that electrostatic balance of one net positive charge, more than the sequence itself is important for binding to the α-chain.

A glutamate residue on position 13 in hIL-5 was identified as essential for interaction with the β2-chain [120,125]. A homologous glutamate residue is also found in GM-CSF (Glu21) [128] and IL-3 (Glu22) [129]. Mutation of this IL-5 residue to a glutamine residue (E13Q mutant) resulted in a complete loss of biological activity on the erythroblastic leukemia cell line, and proved to have antagonistic properties. Interactions of this mutant with both receptor subunits were nevertheless indistinguishable from those of wild type hIL5 by cross-linking and Scatchard plot analysis of transfected COS-1 cells [120]. We used two early myeloid FDC-P1 derived cell-lines to clarify this discrepancy [130]. FDC-P1-Clone7 cells express the wild type IL-5Rα, while IL-5 responsiveness of FPC-P1-CA1 cells was obtained by stable transfection of a human-mouse hybrid α-chain. Scatchard plot analysis and neutralizing anti-hIL-5 antibodies were used to show that the expression level of the IL-5Rα-chain determines the agonistic/antagonistic balance of this E13Q mutant. Mutation of this same glutamate residue to lysine (E13K) by McKinnon and co-workers showed wild type α-chain binding, and only a rather moderate reduction (2.6 fold) in high-affinity binding. This mutant not only is a selective antagonist of IL-5-dependent TF-1 proliferation and eosinophil activation, but also a full agonist capable of eliciting a maximal biological response in an eosinophil survival assay, albeit with a 50,000-fold reduction in potency. These authors argue that the E13K mutant can be used to dissect different signaling pathways from the same activated IL-5R [121].

Finally, as an alternative for IL-5 mutants, peptides can be used to block IL-5 induced biological activities. England et al. identified a peptide with no primary sequence homology to IL-5, but which binds to the IL-5Rα with an affinity equal to that of IL-5 [131]. This peptide can act as a potent and specific antagonist of IL-5 activity in a human eosinophil adhesion assay. When dimerized via a disulfide linkage, it can cluster two α-chains in solution, and is able to activate a chimeric receptor consisting of the IL-5Rα extracellular domain fused to the intracellular domain of the epidermal growth factor receptor.
Chapter 3

Interleukin-5, Eosinophilic Diseases and Therapeutic Intervention

Monoclonal Antibodies

Humanized monoclonal antibodies (mAbs) have recently been used in several human clinical trials. mAbs offer clear advantages over traditional small molecular weight drugs. First, specific mAbs can be rapidly developed. Second, mAbs are highly selective for their targets and do not compete with the same drug metabolism, resulting in predictable biological effects and low risk of unfavorable drug interactions, respectively. Moreover, the pharmacokinetics of mAbs are predictable and the circulating half-life is often between days and weeks. Third, mAbs can interfere with the binding of polypeptide hormones and cytokines with their receptors, which appears to be extremely difficult for small molecular weight compounds. Adversely, production costs are high, tissue penetration of mAbs may be restricted due to their size and oral delivery. Currently, therapeutic mAbs are given through parenteral administration, although technologies allowing biological agents be delivered through the inhaled route are under development.

The profound inhibitory effect of anti-IL-5 mAbs on the development of lung eosinophilia and on airway hyperresponsiveness was demonstrated in several animal studies. Intraperitoneal as well as intranasal application of the anti-IL-5 mAb, TRFK-5, led to a long lasting inhibition of pulmonary eosinophilia and bronchial obstruction in mice [114]. In a monkey model of Ascaris-allergic asthma, Egan and coworkers could abolish inflammatory cell migration and airway hyperactivity for up to three months by treating the monkeys with a single dose of a neutralizing humanized monoclonal antibody against hIL-5 [132]. The promising results of treatment with IL-5 mAbs observed in animal models made this approach a favorite candidate for targeting eosinophilic inflammation in humans. Similar anti-IL-5 treatment in humans, however, failed to demonstrate clinical efficacy in two independent studies. A single-dose phase I clinical trial was conducted with SCH55700 in patients with severe persistent asthma and demonstrated a significant decrease in peripheral blood eosinophils lasting up to 90 days and a trend towards improvement in lung function at the higher doses (30 days after dosing) [133]. SB240563 (Mepolizumab) was tested in mild asthmatics and caused a significant reduction in peripheral blood eosinophils and in post-challenge sputum eosinophils but did not alter FEV1 values upon allergen challenge [134]. Completely blocking IL-5 activity in vivo therefore appears to be much more difficult than in vitro. Perhaps, the local tissue microenvironment, and/or the existence of IL-5-dependent autocrine activation mechanisms [135,136] may limit in vivo antagonism [137]. Furthermore, only very limited amounts of IL-5 may be required to maintain eosinophil action and survival in the local tissue microenvironment. Hence, the in vivo levels of humanized anti-IL-5 mAbs may be insufficient to block local effects. Therefore, complete detailed reports on the phase II trials in asthma, but also in other eosinophil-associated diseases such as nasal polyps and atopic dermatitis are required to fully evaluate and understand the underlying mechanisms of anti-IL-5 treatment.

Remarkably, some anti-IL-5 mAbs can potentiate IL-5 activity. We recently analysed in more detail the behaviour of two anti-IL-5 monoclonal antibodies (mAbs): 5A5 and H30 [138]. 5A5 exhibits strong neutralising activity, while H30 only inhibits IL-5 induced proliferation at very high concentrations. Both mAbs were tested on FDC-P1-CAL cells, which express a human-mouse hybrid IL-5Rα-chain. We observed that, at low concentrations of hIL-5, both mAbs, within a certain concentration window, were able to significantly increase proliferation above the level induced by hIL-5 alone. Using Fab-fragments and the IL-5 variants, GM-1 and sell-5, we could show that the enhanced IL-5 activity was due to the Ab driven cross-linking of activated IL-5R complexes. Altough our results were restricted to cells expressing a hybrid IL-5Rα-chain, those observations warrant careful selection of anti-IL-5 mAbs for clinical applications. It is possible that anti-hIL-5-mAb administration causes neutralisation of hIL-5 activity in circulation, but diffusion may lead to local mAb concentrations in tissues causing enhanced hIL-5 activity.

Soluble IL-5Rα

We recently demonstrated the presence of SOL-IL-5Rα protein in clinical samples including serum, nasal secretion, tissue homogenates, urine, and peripheral blood fluid [139]. So far, expression of SOL-IL-5Rα protein was only reported in sera and ascitic fluids of mice bearing chronic B-cell leukemia [140]. In human serum, the SOL-IL-5Rα protein was detectable in all samples with a median concentration of 210 pg/ml in healthy controls, whereas IL-5 was not detectable in serum of healthy controls. Notably, we found that SOL-IL-5Rα mRNA and protein were upregulated in nasal polyps and were correlated with eosinophil counts, ECP and IL-5 concentrations. In addition, SOL-IL-5Rα levels in nasal tissue or blood of NP patients were related to disease severity, more specifically with the involvement of local (NP) or systemic airway disease (NP with concomitant asthma). We observed abundant SOL-IL-5Rα expression in NP with concomitant asthma, often dramatically exceeding the IL-5 concentrations: in nasal tissue SOL-IL-5Rα levels up to 1200 times higher than IL-5 concentrations could be found. If therefore appears that in the most severe disease manifestations, a regulatory system may operate to downregulate the ongoing eosinophilic inflammation by enhanced synthesis of SOL-IL-5Rα. Therefore, the precise in vivo role of the SOL-IL-5Rα protein requires more detailed study. Secreted receptor variants are a hallmark of the receptor family to which the IL-5Rα belongs. This mere evolutionary conservation underscores their functional importance, but the precise physiological role may differ from case to case. Models in which the antagonistic effects prevail include surface receptor downmodulation and ligand capture. On the other hand, secreted receptors may bind their ligands in circulation, protecting them from proteolytic breakdown and prolonging their serum half-life or facilitating ligand-mediated signaling. On the other hand, SOL-IL-5Rα protein has antagonistic properties in vitro as demonstrated in eosinophil differentiation assays as well as in proliferation assays using IL-5 responsive cell lines [70,123]. It captures IL-5 in solution, but this IL-5/IL-5Rα complex is unable to associate and activate membrane bound βe chains [68]. Recently, the antagonistic effect of recombinant SOL-IL-5Rα was demonstrated using nasal tissue explants. Culturing tissue with recombinant SOL IL-5Rα almost completely attenuated the ragweed-induced
decrease in eosinophil precursors and increase in MBP-immunoreactive cell numbers [141]. This observation leads one to consider that endogenous production of soluble receptor may regulate IL-5 function in vivo. Indeed, the observed correlation between SOL IL-5Rα levels and disease severity may suggest an antagonistic role for the SOL IL-5Rα in vivo.

INTERFERING WITH SIGNAL TRANSDUCTION

An intrinsic disadvantage of interfering with IL-5 function at the extracellular level is that all cellular responses are indiscriminately eliminated, resulting in possible unwanted side effects. As an alternative, intervention at the level of intracellular signalling following receptor activation should be considered. Blockage of only a subset of (cell-specific) signals, or signal cascades, may be achieved by interfering with specific protein-protein interactions in a given pathway, or by blockage of specific modifying enzymes, such as kinases and phosphatases. An example of cascade specific inhibition of IL-5 signalling was recently provided by Alam and co-workers [142]. They characterised the binding site of Lyn kinase on the β5 receptor, and used this sequence information to design a cell-permeable β5-derived peptide. This peptide specifically eliminated binding and activation of the Lyn kinase. This inhibitor furthermore blocked IL-5-dependent eosinophil differentiation and survival, but not eosinophilic degranulation in vitro. When applied in vivo, the Lyn-binding peptide significantly inhibited airway eosinophil influx in a mouse model of asthma.

CONCLUDING REMARKS

Interleukin 5 remains an interesting molecular target for pharmacological intervention in allergic diseases. Its role as a central, non-redundant player in eosinophil function in vivo has been well documented over the past years. Furthermore, within the hematopoietic system, it delivers specific signals to the eosinophilic and basophilic cells. Recent observations point to an additional function on airway smooth muscle cells, suggesting that IL-5 may directly contribute to bronchial hyper-responsiveness. Since IL-5-deficient mice are perfectly viable, it can be anticipated that blocking IL-5 function in vivo will not lead to severe unwanted side effects. Yet, initial studies using humanised anti-IL-5 monoclonal antibodies have been disappointing. In this context, the recent observation that the local environment in tissues may antagonise IL-5 function is intriguing. Indeed, reduced levels of the hIL-5Rα TM isoform and enhanced levels of its SOL hIL-5Rα variant were recently observed. It therefore appears that more refined future strategies should be aimed at directly interfering with IL-5 responsiveness of its target cells.

ABBREVIATIONS

mIL-5 = Murine Interleukin-5
hIL-5 = Human Interleukin-5
GM-CSF = Granulocyte-macrophage colony-stimulating factor

REFERENCES

Chapter 4

Neutralizing Monoclonal Antibodies can Potentiate IL-5 Signaling


4.1. Introduction

Interleukin-5 (IL-5) and eosinophils play a critical role in allergic diseases including bronchial asthma. Asthma is characterized by an increased number of eosinophils in circulation, bronchoalveolar lavage (BAL) and bronchial biopsies. The number of cells correlates well with the severity of the disease (Bousquet et al., 1990; Gleich, 1990; Djukanovic et al., 1992). IL-5 is highly expressed in the lungs of asthmatic patients (Sanderson, 1992), and may account from the release of pro-inflammatory cationic proteins and lipid mediators for the eosinophil granules. This contributes to the damage and dysfunction of the lung tissue, to the observed airway obstruction and hyper-responsiveness (Flavahan et al., 1988; Bousquet et al., 1990). Administration of neutralizing antibodies directed against IL-5 in mice and primates can block the airway cellular infiltration and airway hyperreactivity (Mauser et al., 1993; Egan et al., 1999). In contrast, two clinical trials with humanized antibodies did not result in improvement of the bronchial obstruction in asthmatic patients (Leckie et al., 2000; Greenfeder et al., 2001).

In this study, we show that non-neutralizing as well as neutralizing antibodies to IL-5 can potentiate IL-5 signaling, i.e. increase proliferation of responsive cell lines above the level induced by IL-5 alone, and this at every sub-optimal hIL-5 concentration
analyzed. Since $F_{Ab}$ fragments do not potentiate IL-5 signaling, we propose that the observed potentiation is dependent on mAb-mediated cross-linking of activated IL-5 receptor complexes. This may help to explain why humanized antibodies fail to block IL-5 functions in lung tissue of asthmatic patients. It is possible that the administration of antibodies results in neutralization in circulation, but due to a restricted tissue penetration creates circumstances in which potentiation of IL-5 effects is possible.

4.2. References


4.3. Article
Neutralizing monoclonal antibodies can potentiate IL-5 signaling

Lennart Zabeau1, José Van der Heyden1, Daniël Broekaert1, Annick Verhoe1, Joël Vandekerckhove1, Sheng-Jiun Wu2, Irwin Chaiken3, Peter Heinrich3, Iris Behmann4 and Jan Tavernier1

1 Flanders Interuniversity Institute for Biotechnology, Department of Medical Protein Research (VIB09), Ghent University, Ghent, Belgium
2 Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, USA
3 The Institute of Biochemistry, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany

IL-5 is a major determinant in the survival, differentiation and effector-functions of eosinophils. It mediates its effect upon binding and activation of a membrane bound receptor (R), composed of a ligand-specific α-chain and a β-chain, shared with the receptors for IL-3 and granulocyte-macrophage colony-stimulating factor. We have generated and mapped the epitopes of three monoclonal antibodies (mAb) directed against this cytokine: the strong neutralizing mAb 5A5 and 1E1, and the very weak neutralizing mAb H30. We found that H30 as well as 5A5 can increase proliferation above the level induced by human (h)IL-5 alone, in a JAK-2-dependent manner, and at every sub-optimal hIL-5 concentration analyzed. This effect is dependent on mAb-mediated cross-linking of IL-5R complexes, and is only observed on cell lines expressing a hybrid human/mouse IL-5Rα-chain. We discuss these findings in view of the stoichiometric and topological requirements for an activated IL-5R. Since humanized anti-IL-5 mAb are currently in clinical testing, our findings imply that such mAb should be carefully evaluated for their potentiating effects.

Key words: IL-5 / IL-5 receptor / Antibody / Cellular proliferation

1 Introduction

Human IL-5 is a disulfide-linked homodimeric glycoprotein, with monomers of 115 amino acids [1]. Each subunit adopts a four α-helix bundle-structure, the so-called “cytokine fold”, which has been described for many other cytokines, including IL-3 and GM-CSF [2]. The four helices are arranged in a typical up-up-down-down array and the two monomers are organized in an anti-parallel manner. Unique to IL-5, however, is the phenomenon of D-helix swapping, whereby one bundle is built up of three helices of the first monomer and a fourth helix which is contributed by the second monomer. IL-5 contains both O- and N-linked glycosyl groups, but deglycosylation does not affect the biological activity [3].

hIL-5 mediates its effects upon binding and activation of a membrane-bound receptor (IL-5R), which is composed of two different subunits, the IL-5Rα-chain (IL-5Rα) and β-chain [4, 5]. The IL-5Rα-chain is ligand-specific and binds IL-5 with intermediate affinity in man (Kd = 4x10^10 M) or low affinity in mouse (Kd = 6x10^-9 M). Binding affinity is increased two- to threefold and tenfold upon association with the β common (βc)-chain in man and mouse, respectively. Besides the transcript for the membrane-bound form, two other mRNA encoding soluble human IL-5Rα-subunit splicing variants have been described [6]. One of these splicing variants, s-IL-5Rα, has antagonistic properties in vitro. It can block IL-5 binding and can interfere in a hIL-5-driven eosinophil differentiation or proliferation assay [4]. Fortunately, one hIL-5 homodimer binds only one hIL-5Rα in solution [7].

An induced fit model may explain this 1:1 stoichiometry, as suggested by Verschelde et al. [8]. The βc-subunit is shared with the receptors for IL-3 and GM-CSF, and hence called β common. This feature explains the overlap in biological functions for these cytokines. Despite the lack of any detectable binding affinity for IL-5, this chain is critical for conversion to a high-affinity complex. In mice, a second highly related chain exists which is
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specific for IL-3, the β_{1.5}-chain. In contrast to the β₁-chain, the β_{1.5}-chain can bind IL-3 with low affinity (reviewed in [9–11]).

We previously performed a detailed structure-function analysis of hIL-5 showing that most of the residues that interact with the α-chain were clustered in the loop connecting β-strand 1 and helix B, in the CD loop between helices C and D and close to the C terminus of helix D [12]. Two other groups confirmed these observations [13, 14]. In contrast, Wu et al. [15] showed that electrostatic balance of one net positive charge in the CD loop, more than the sequence itself, is important for binding of the α-chain. Using single-chain hIL-5 Li and co-workers [16] extended these findings and showed that the IL-5/IL-5Rα interaction is stabilized preferentially by one of the two four-helix bundles of IL-5. For the interaction with the β-chain, only the glutamate on position 13 (E13) scored in an alanine-scan [12, 13].

T helper cells, mast cells and eosinophils are the main cellular sources of IL-5. Due to the expression pattern of the IL-5/IL-5Rα-chains, the effects of IL-5 are essentially restricted to the eosinophil/basophil lineages in humans. In vitro, IL-5 induces eosinophil outgrowth from bone marrow or cord blood, and plays a central role in eosinophil survival, motility and effector-functions [9] and references therein). IL-5 also plays a crucial role in the differentiation and activation of eosinophils in vivo: increased IL-5 synthesis correlates with increased numbers of eosinophils in humans [17], and mice [18], and conversely, administration of anti-IL-5 mAb abolishes eosinophilia [19, 20]. It is of note that in the case of IL-5 deficiency, as in IL-5−/− knockout mice [21], IL-3 or GM-CSF cannot take over the function of IL-5.

IL-5 and eosinophils are believed to play a critical role in allergic disorders including bronchial asthma. Supporting a crucial role for IL-5, a number of studies in asthmatics have provided evidence for activation of CD4⁺ T lymphocytes and increased expression of both IL-5 mRNA and protein [22, 23]. Biopsy studies in patients with atopic or intrinsic asthma provide evidence for regulated expression of hIL-5/IL-5Rα secreted and membrane-bound isoforms, which may influence local eosinophil responsiveness, and which may relate to airflow limitation [24]. Furthermore, we recently showed that IL-5 itself upregulates the expression of the membrane-bound hIL-5Rα-chain [25]. In animal model systems, intratracheal administration of IL-5 increases airway reactivity [26] and pretreatment with anti-IL-5 [20] or anti-IL-5R [27] mAb significantly reduced parasite- or allergen-induced allergic inflammation in guinea pigs. Furthermore, allergen-provoked bronchial hyper-reactivity is strongly reduced in IL-5 knockout mice [28]. In Ascaris-responsive primates, the humanized anti-hIL-5 mAb Sch 55700, reduces reactive pulmonary eosinophilia to 75% up to 6 months after the statum dose [29], and in Cynomolgus-monkeys the humanized anti-hIL-5 mAb SB-240563 strongly suppresses the eosinophil count [30].

Based on these observations with neutralizing anti-hIL-5 Ab, strategies using humanized mAb are currently being evaluated in the clinic for treatment of diseases involving IL-5 and eosinophilia. Here we show that Ab, even with neutralizing properties, can potentiate the hIL-5 induced proliferation of cells expressing a hybrid IL-5Rα-chain. We show that the underlying mechanism is an Ab-mediated cross-linking of activated receptor complexes. These results help to define possible limitations of monoclonal Ab in therapy, but may also lead to increased understanding of the process of receptor activation.

2 Results

2.1 Epitope mapping of mAb directed against hIL-5

We have studied the behavior of three anti-hIL-5 mAb: 5A5, 1E1 and H30. The former two are IgG1 and IgA, respectively, and of mouse origin, the latter is a rat IgG2b. Based on hIL-5-dependent growth of the TF-1-hIL5Rα cell line, mAb 5A5 and 1E1 were found to exhibit a strong neutralizing effect, while H30 neutralized hIL-5 activity only weakly. hIL-5 epitopes recognized by these mAb were determined using a panel of hIL-5 mutants [12]. In a first approach, hIL-5-specific ELISA were set up, and results are shown in Table 1. In case of the 5A5/5A5 ELISA, detection of hIL-5 mutatins was done with 5A5 as capture Ab and HRP-conjugated 5A5 as secondary Ab, taking advantage of the dimeric structure of hIL-5. For all other ELISA, detection was done with a sandwich ELISA using the capture mAb H30 or 1F1, and a polyclonal rabbit anti-hIL-5 serum (column H30/poly) or 1F1 (column H30/1F1), and 1E1 (column 1F1/1E1) as secondary Ab, followed by an HRP-conjugated anti-rabbit or anti-mouse Ab for revelation.

The 5A5/5A5 ELISA shows that the 5A5 epitope consists of residues R32, E89, R90 and R91. This explains its neutralizing capacity since the epitope overlaps with the IL-5Rα-chain binding pocket (Fig. 1, panels A and B). Despite the fact that analysis of the hIL-5 binding sites of 5A5 and H30 points to only 1 amino acid difference (E89 interacts only with 5A5 while R92 interacts only with H30), H30, in contrast to 5A5, has only a very weak neutralizing capacity. This major difference in neutralizing
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Table 1: Epitope mapping of mAb 5A5, H30, 1F1, and 1E1:
A summary of the results from four different ELISA experiments is shown. Immunoassays were coated with the mAbs 5A5, H30 and 1F1, followed by binding of the hIL-5 muteins.

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<th>5A5/5A5*</th>
<th>H30/POLY*</th>
<th>H30/1F1*</th>
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1F1-epitope

5A5-epitope  H30-epitope  H30-epitope  1E1-epitope

* In case of 5A5, detection was done using labelled 5A5, taking advantage of the dimeric structure of hIL-5
* b: the capture and secondary Abs are shown on top, separated by a slash character
* c: +++ indicates binding comparable to wild-type hIL-5; + indicates binding less than 10% vs. wild-type hIL-5; O indicates complete loss of binding

Keeping in mind the epitope for H30, the H30/1F1 ELISA (Table 1; column H30/1F1) led to the conclusion that K70 is a major determinant of the 1F1-epitope. This result is confirmed in the 1F1/1E1 ELISA (Table 1; column 1F1/1E1), and further showed that residues K12 and E13 make up the 1E1-epitope (Fig. 1, panel E). Since it was previously demonstrated that E13 is the key residue in the interaction with the βc-chain [12, 13], the neutralizing activity of 1E1 can be explained by the physical overlap with the βc-chain interacting residue E13. We confirmed the delineated epitopes for the mAb 5A5 and H30 by immunoprecipitation studies (data not shown).
2.2 Neutralizing anti-hIL-5 mAb can potentiate IL-5 signaling

We next analyzed the behavior of these mAb in the hIL-5-driven FDC-P1-CA1 proliferation assay. This FDC-P1-derived cell-line was made responsive to hIL-5 by the stable expression of a human/mouse hybrid IL-5Rα-chain, consisting of the human extracellular and the mouse transmembrane and cytoplasmic domains [4]. Similar neutralizing characteristics, as on TF-1-hIL5Rα cells, were observed. However, we also found that, at low concentrations of hIL-5 (here 50 pg/ml), the mAb 5A5 or H30, within a certain concentration window, were also able to significantly increase proliferation above the level induced by hIL-5 alone (Fig. 2). This potentiating effect was most pronounced when hIL-5 was first pre-incubated with a dilution of mAb, and cells cultivated in the absence of growth factor (6 h; 37°C). In the absence of hIL-5, nor 5A5 or H30 were capable to induce a proliferative response in the FDC-P1-CA1 cells (data not shown).

As expected, 5A5 was capable to neutralize the hIL-5 induced growth of the FDC-P1-CA1 cells, while at low mAb concentration (< 0.1 ng/ml) no effect was observed (Fig. 2, panel B). However, at concentrations around 10 ng/ml we observed a significant potentiation, which was a 3.4 times higher compared to hIL-5 alone. A similar neutralization/potentiation profile was seen for the H30 mAb (Fig. 2, panel A), with a potentiating effect of 5.8 (H30 + hIL-5 vs. hIL-5 alone); however at a much higher concentration range (maximal effect ± 1 μg/ml). This potentiating effect was not found for 1E1 or TRFK-5 (Fig. 2, panel B), when compared to 5A5, which was tested as a positive control in the same experiment. Also, no cross-talk by Fc-receptor activation was observed and the mAb-dependent potentiation was completely suppressed by addition of the Jak-2 inhibitor AG 490 (data not shown).

Optimal potentiating activity by 5A5 was observed at a concentration of 10 ng/ml. We next determined the optimal hIL-5 concentration for potentiation keeping the 5A5 level at 10 ng/ml. As can be seen in Fig. 3, 5A5 is capable to potentiate the hIL-5 mediated proliferation at every sub-optimal hIL-5 concentration. This effect increases with decreasing hIL-5 concentrations (up to a factor 13.7, at a hIL-5 concentration of 0.5 pg/ml).
Neutralizing Monoclonal Antibodies can Potentiate IL-5 Signaling

Fig. 2. Potentiating activities of the mAb H30, 5A5, 1E1 and TRFK-5. The mAb H30 (panel A) and 5A5, TRFK-5 and 1E1 (panel B) were tested for their ability to potentiate the effect of hIL-5 on FDC-P1-CA1 cells. 1E1 and TRFK-5 were tested in the same assay as 5A5, which acted as a positive control. hIL-5 (50 pg/ml) was pre-incubated with or without indicated dilutions of mAb and incubated for 6 h. Cells were washed to remove hIL-5 and resuspended in medium depleted of growth factors for the same period of time. Then, cells were added to the hIL-5/mAb mixture. After 4 days, growth was measured by monitoring [3H]thymidine incorporation (0.5 μCi per well; 4–6 h; 37°C). Lines represent proliferation of FDC-P1-CA1 cells in the presence of hIL-5 and a dilution of the indicated mAb. Proliferation in the presence of hIL-5 alone is also indicated. Error bars represent SD of triplicate samples. Similar results were obtained in several independent assays.

In addition, we evaluated whether the potentiating effect is dependent on IL-5/IL-5R clustering as suggested by the bell-shaped dose/response curve (Fig. 2). To test this hypothesis, we compared the behavior of 5A5 Fαβ-fragments with intact 5A5. As can be seen in Fig. 4 (panel A), a complete loss of potentiating ability is observed for the 5A5 Fαβ-fragments, while the neutralizing capacity is even increased. Further support for the critical role of receptor complex cross-linking is illustrated in panel B. Addition of polyclonal rabbit anti-mouse IgG Ab to 5A5 Fαβ-fragments restores the 5A5-dependent potentiating effect. This effect is partial (ratio = 2.6), likely because of random binding of the Fαβ-fragments by the polyclonal anti-mouse Ab.

To further confirm this cross-linking hypothesis, we compared the potentiating properties of 5A5 with sclIL-5 versus the derived wt/A5 variant, and also tested monomeric GM-1 IL-5. In the wt/A5 asymmetrical sclIL-5 mutant, the original five charged residues EEERRR in the CD loop of only the C-terminal half was mutated to five alanine residues [15]. This results in an IL-5 mutein with only one functional IL-5Rα-binding domain and also only one 5A5 binding epitope, both in the N-terminal half. Biological activities of these three IL-5 variants were tested on FDC-P1-CA1 cells. Activities, compared to wild-type IL-5, were similar as on the human TF-1 cells [15, 16]. As illustrated in Fig. 5, panel A, 5A5 is capable of potentiating sclIL-5 activity. In contrast, with wt/A5, only neutralization by 5A5 could be observed (Fig. 5, panel A). An analogous situation occurs with the monomeric GM-1. This IL-5 variant also has only one functional α-binding site and only one 5A5 binding epitope. As illustrated in Fig. 5 (panel B), 5A5 is unable to potentiate the activity of this monomeric IL-5. These data further underscore the Ab-mediated cross-linking of IL-5 receptors.

2.3 Topological constraints of the potentiating effect

We investigated whether these mAb are capable to potentiate the IL-5 effect in other cell-systems. Surprisingly, no potentiation was observed on the human TF-1 cells expressing the complete human IL-5Rα-chain
Fig. 4. Comparison of potentiating activities of intact 5A5 and 5A5 Fab-fragments. (A) 5A5 Fab-fragments were assayed for their ability to potentiate the hIL-5 mediated growth of FDC-P1-CA1 cells. The Fab-fragment concentrations are indicated on the lower X-axis. 5A5 was also tested in the same experiment as a positive control (5A5 concentrations indicated on the upper X-axis), and the hIL-5 concentration used was 50 pg/ml. Experimental procedures were as described in Fig. 2. (B) Recovery of the potentiation effect by IgG-mediated cross-linking of 5A5 Fab-fragments. Indicated concentrations of rabbit anti-mouse IgG were incubated with 50 pg/ml hIL-5 and in the presence or absence of 1 ng/ml 5A5 Fab-fragments. After 6 h, FDC-P1-CA1 cells, which were depleted of hIL-5, were added. Four days later, growth was measured by \[^{3}H\]thymidine incorporation. Error bars represent SD of triplicate samples. Similar results were obtained in several independent assays.

(5A5 and hIL-5), nor on murine B13 cells with the wild-type murine IL-5Rα-chain (using H30 and mlL-5) (data not shown). We also included FDC-P1-Clone7 cells in our investigation, which differ in two aspects from FDC-P1-CA1: (i) instead of a human/mouse hybrid, Clone7 expresses a wild-type IL-5Rα-chain, and (ii) the expression level of the IL-5Rα is about six fold higher in Clone7 compared to CA1 cells [31]. Again, no potentiation was observed (data not shown).

Fig. 5. Comparison of 5A5 potentiation with sclL-5 and wtA5. 5A5 was tested for potentiation with IL-5 variants. Therefore indicated concentrations of sclL-5, wtA5 (panel A), and GM-1 (panel B) were incubated with a serial dilution of 5A5. After 6 h, FDC-P1-CA1 cells were added and growth was measured 4 days later with \[^{3}H\]thymidine incorporation. Error bars represent SD of triplicate samples.

To investigate whether the mAb potentiation is intrinsic to the human/mouse hybrid IL-5Rα-chain, we transfected its cDNA in the murine IL-3-dependent Ba/F-3 cell-line. Different clones were selected for their ability to grow in response to hIL-5. In three independently isolated clones tested, 5A5 potentiating activity was observed. Data for one of these clones are given in Fig. 6, and confirms that expression of this hybrid receptors makes cells responsive to potentiation by 5A5 and H30.

3 Discussion

In this study we have analyzed the behavior of three anti-hIL-5 mAb; 5A5 (mouse IgG1), H30 (rat IgG2b) and 1E1 (mouse IgA). The binding epitopes for these mAb were determined using a series of hIL-5 mutants and hIL-5-specific ELISA (Table 1). The 5A5 epitope consists of the residues R32, E89, R90 and R91. Therefore, the neutralizing properties of 5A5 can be explained by the physical overlap of its epitope with the hIL-5Rα-chain binding site.
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on hIL-5 (Fig. 1, panels A and B). Compared to 5A5, the H30 epitope differs only in one amino acid (R92 instead of E89), and lies more to the apical end of hIL-5 (Fig. 1, panel C). The weak neutralizing effect of H30 may support the critical role of residue E89 in the hIL-5/hIL-5Rα interaction. The difference in neutralizing properties is unlikely due to the binding affinities of both mAb for hIL-5 (Kd = 8.7 x 10^-11 and Kd = 4 x 10^-10, for respectively 5A5 and H30). The 1E1 epitope was mapped to the residues K12 and E13 (Fig. 1; panel E), which explains the neutralizing capacity by the physical overlap with the β-chain interaction site [12, 13].

When we analyzed the behavior of these mAb in the hIL-5-driven proliferation of FDC-P1-CA1 cells, we noticed that mAb 5A5 and H30 were able to potentiate the effect of hIL-5, with maximal effects at 10 ng/ml and 1 μg/ml antibody concentrations, respectively. We found that 5A5 enhances hIL-5 activity at every sub-optimal hIL-5 concentration (Fig. 3). The bell-shaped dose/response curve (see for example Fig. 2) in the presence of mAb indicated that potentiation is most likely due to Ab-mediated cross-linking of two hIL-5 molecules bound to their receptor. For verification, 5A5 Fab-fragments were prepared, and were found to be unable to potentiate the hIL-5 induced growth of FDC-P1-CA1 cells. Nevertheless, they retained their ability to neutralize the hIL-5 effect, and were even more effective than intact 5A5. A balance between neutralization and potentiation in case of the intact Ab may explain this, while Fab-fragments are only capable to neutralize. With a polyclonal rabbit anti-mouse IgG Ab we were able to partially restore the potentiation of 5A5 Fab-fragments, which strongly supports this Ab-mediated cross-linking hypothesis. Further confirmation comes from the comparison of IL-5 variants; scIL-5, wt/5A5 and monomeric GM-1 IL-5. 5A5 is able to potentiate the activity of scIL-5, but not of wt/5A5, nor GM-1 (Fig. 5, panels A and B, respectively). In the wt/5A5 scIL-5 variant only the N-terminal 8EERRR86 sequence is intact, resulting in only one functional IL-5Rα-chain binding domain, and one overlapping 5A5 binding epitope. The monomeric GM-1 variant has also only one functional α-interaction site. This explains why 5A5 only neutralizes, and cannot potentiate wt/5A5, or GM-1 activity. The observed potentiating activity was Jak-2 dependent, since this effect could be completely suppressed by the addition of tyrphostin AG 490.

The exact stoichiometry of active hIL-5R complexes remains elusive. Based on the observations that (i) several constitutive mutants of the β-chain lead to ligand independence and tumorigenicity, (ii) clustering of the cytoplasmic domains of the β-chains leads to a proliferative signal, (iii) inactive β-homodimers could be shown to exist on the cell-surface (reviewed by Bagley et al. [32]),

Fig. 6. SA5 potentiation on Ba/F-3 cells transfected with the hybrid IL-5Rα. Representative data for one out of three tested Ba/F-3 clones expressing the hybrid human/mouse IL-5Rα-chain are shown. Line and point at the right indicate growth with 100 pg/ml hIL-5 in the presence or absence of a serial dilution of 5A5, respectively. Growth was measured using [3H]thymidine incorporation after 3 days.

Fig. 7. Model for potentiation by 5A5 and H30. Panel A, a schematic representation of the dimeric hIL-5 molecule with the α- and β-chain pharmacophores. Panels B and C show a possible model for the mAb 5A5 and H30 potentiating effect, respectively. For reasons of clarity, only the IL-5R α-chains are shown, highlighting the different modular domains. For the β-chain, only the interaction sites are represented on the hIL-5 dimer.
the idea of two β-chains being involved in an activated IL-5 receptor complex was put forward. Besides these findings, it could be shown that homodimerization of the α-chains may be sufficient for prevention of apoptosis or a weak growth stimulation signal [33]. Recently, Stomski and co-workers [34] showed that ligand binding induced covalent disulfide bridge formation between the α- and β-chains in the receptor for IL-3, IL-5 and GM-CSF, leading to the suggestion of an α2/β2 activated receptor. In contrast, Orban et al. [35] concluded that, using chimeric receptors consisting of CD8 or CD16 and the cytoplasmic domains of the IL-3Rα-chain and βm, simple heterodimers of α and β cytoplasmic domains, rather than higher-order oligomeric complexes, are sufficient for IL-3 signaling, and hence likely also for IL-5.

Since potentiation could be explained by Ab-mediated cross-linking, we believe that this effect is due to the formation of higher-order complexes of the IL-5Rα- and β-chains, resulting in enhanced local concentrations of signaling components. Given the neutralizing properties of 5A5, only one hIL-5Rα-chain binding site can be bound per hIL-5 molecule in the activated, cross-linked receptor complex. Thus, our findings imply that one hIL-5Rα-chain per IL-5 molecule is sufficient for formation of an activated receptor complex (Fig. 7; panel B). This 1:1 stoichiometry of the hIL-5/hIL-5Rα interaction is in line with binding data in solution [7], and was also suggested by Verschelde et al. and Li et al. [8, 16], based on an induced fit model or physical occlusion, respectively. In the case of H30, given its weak neutralizing capacity, two molecules of H30 are capable of binding to a hIL-5 dimer without interfering with hIL-5Rα-chain binding, and consequently, H30 can induce the formation of higher order complexes (Fig. 7; panel C). This difference may reflect the higher potentiating factor of H30, compared to 5A5 (5.8 vs. 3.4 potentiating effect at 50 pg/ml hIL-5, for H30 and 5A5, respectively). Such effects may be further influenced or enhanced by mAb-mediated stabilization of the interaction between IL-5 and its receptor. This latter possibility is less likely since 5A5 Fab'-fragments, functional as illustrated by the ability to neutralize, show no potentiation at all.

Testing mAb 5A5 and H30 in complete human (5A5 + hIL-5 + human TF-1-hIL-5Rα cells) or complete murine (H30 + mIL-5 + murine B-13 cells) cell-systems, or in the closely related FDC-P1-Clone7 cell-line, revealed that the potentiating effect is restricted to the FDC-P1-CA1 cell-line. These cells express a human/mouse hybrid IL-5Rα-chain, where the 314 N-terminal amino acids of the soluble splice-variant sIL-5Rα, are coupled to the 29 membrane-proximal amino acids of the extracellular, the complete transmembrane and cytoplasmic domain of the mIL-5Rα-chain. Expression of this hybrid IL-5Rα in the murine IL-3-dependent Ba/F3 cell line, made these cells sensitive to the 5A5 potentiation. The different results with hybrid versus wild-type IL-5Rα-chains illustrate that the potentiating effect is most probably sensitive to topological constraints. This implies that the mAb 5A5 or H30 do not allow simultaneous binding of two IL-5 molecules to the wild-type receptor in such a way that two, or more, activated receptor complexes are brought together. One could argue that this hybrid receptor has a higher intrinsic flexibility compared to its wild-type counterpart, and hence is more susceptible to the Ab-mediated potentiation. We believe that this is not the case, since: (i) the human and mouse parts of the hybrid receptor are fused at a homologous region, without insertion of an additional hinge region. (ii) Molecular modeling indicates that this fusion point is located within a loop connecting two β-strands in the membrane-proximal Cytokine Receptor Module (CRM2, data not shown), whilst the rigidity of this CRM module is determined by the β-pleated sheet. Finally, (iii) a gain in flexibility would conceivably lead to an increased constitutive signaling in cells expressing the hybrid receptor. Comparison of the proliferation of FDC-P1-CA1 and the closely related FDC-P1-Clone7 cells illustrates that this is not the case (data not shown). Although our observations are restricted to the use of a chimeric α-receptor chain, it remains likely that other mAb may exhibit potentiating activities on wild-type receptors. Topological restrictions are further illustrated by the inability of mAb TRFK-5, which has an overlapping epitope [36] with 5A5 and H30, to potentiate the hIL-5 response. Topology may also underlie the inability of mAb 1E1 to potentiate the hIL-5 induced proliferation. Alternatively, given its neutralizing capacity by inhibiting interaction with βm, this may also indicate that two β-chains are necessary for an activated receptor complex.

The observed potentiation by ligand neutralizing mAb, is a new phenomenon, which to our knowledge has not been described before for any other growth factor. However, this effect may not be restricted to hIL-5, mAb directed against other dimeric cytokines such as IL-10, platelet-derived growth factor, stem cell factor, IFN-γ, colony stimulating factor-1 or the epidermal growth factor, or cytokines which are believed to dimerize upon receptor binding, such as members of the IL-6 family, could also potentiate the growth stimulus of their ligand.

A consequence of our findings is that, whilst anti-hIL-5-mAb administration may cause neutralization of hIL-5 activity in circulation, diffusion may lead to local mAb concentrations in tissues causing enhanced hIL-5 activity. This could lead to the local expansion and/or differentiation of e.g. hIL-5Rα+, CD34+ progenitor cells in bone marrow or inflamed tissues. These observations there-
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fore warrant careful selection of anti-IL-5 mAb for clinical applications.

4 Materials and methods

4.1 Cytokines

hIL-5 and mIL-5 were produced in Cos-1 and ST-9 cells, respectively, and purified using H30 affinity-chromatography. Purified glycosylated recombinant mouse IL-3 was a kind gift from Dr. Y. Furui and Dr. R. Devos (Roche). ScCl-5, the derived wt/A5 mutant, and monomeric GM-1 IL-5 were constructed, expressed, and purified as described before [15, 16].

4.2 Cell culture

TF-1-hIL5Rα cells are derived from the human erythroleukemic TF-1 cell-line, by stable expression of the hIL-5Rα-chain. These cells are cultured with hIL-5 (20 ng/ml) in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS (Gibco BRL).

FDC-P1-CA1 cells are derived from the murine, early myeloid FDC-P1 cell-line. hIL-5 responsiveness was obtained by stable transfection of a chimeric IL-5Rα-chain, consisting of the human extracellular part fused to the transmembrane and intracellular parts from the mouse IL-5Rα-chain (mIL-5Rα) [4]. Cells were cultured in RPMI 1640 medium containing 10% FBS and 1 mg/ml hIL-5.

Ba/F-3, an IL-3-dependent pre-B cell line, was cultured in Dulbecco’s MEM medium with glutamax (Gibco BRL) supplemented with 10% FBS and 10% WEHI-3 cell conditioned medium as a source of IL-3.

4.3 Monoclonal antibodies

5A5, 1E1 and 1F1 are mouse mAb (BALB/c × SP2/0) of isotypes IgG1, IgA and IgG2b, respectively, raised against hIL-5, which was produced in Saccharomyces cerevisae (5A5) or ST9 insect cells (1E1 and 1F1) [3].

H30 is a IgG2b rat mAb (Wistar × SP2/0) raised against hIL-5, which was purified from ST9 insect cell medium as described [3].

TRFK-5 is an IgG1 rat mAb (Lewis × P3X63Ag8.653), raised against mIL-5 [37].

4.4 5A5 Fas-fragment production and purification

5A5 (2.5 mg/ml) was incubated with 8 mg ∼ 6 units of enzyme) papaine-agarose beads (Sigma), 50 mM cysteine and 1 mM EDTA in 0.1 M acetate buffer (pH = 5.5) for 4.5 h at 37°C. The reaction was stopped by centrifugation and removal of the beads, followed by addition of iodoacetamide (ICH2CONH2) to a final concentration of 75 mM. The supernatant was then switched to 20 mM phosphate buffer (pH = 7.0) by means of a NAP™-10 column (Pharmacia Biotech). The Fas−-fragments were finally purified over a protein A Sepharose 4 Fast Flow column (Pharmacia Biotech) to remove undigested Ab and Fc-fragments. Purity was checked by SDS-PAGE using 10% gels under nonreducing conditions.

4.5 Epitope-mapping and immunoprecipitation

hIL-5 mutants were constructed and expressed as described before [12]. Immunoplates (Nunc) were coated with capture antibody. Plates were washed and incubated with IL-5 or IL-5 mutants (in 10% FBS RPMI 1040 medium plus 0.1% Tween-20), for 1–2 h at room temperature. After removal of excess IL-5, or IL-5 mutants, revelation was done using HRP conjugated 5A5 (5A5/5A5), polyclonal rabbit anti-hIL-5 plus anti-rabbit IgG-HRP (H30/poliv), 1F1 plus anti-mouse IgG-HRP (H30/1F1), or 1E1 plus anti-mouse IgA-HRP (1F1/1E1). The substrate used for the HRP enzyme was TMB (KPL). Reaction products were measured by colorimetry.

4.6 Cell proliferation and potentiation assay

To determine the neutralizing characteristics of mAb, TF-1-hIL5Rα cells were seeded at a density of 5 x 10⁵ cells/well in the presence of serial dilutions of antibody, starting at 50 µg/ml, and a constant concentration of hIL-5 at 20 ng/ml. Cells were cultured for 3 days at 37°C, and ³Hthymidine (0.5 µCi/well; for 4–5 h) incorporation was measured using a TopCount scintillation counter (Canberra Packard). To measure the potentiating activities, serial dilutions of mAb were pre-incubated in the presence of hIL-5 at concentrations as indicated, for 4–6 h at 37°C to allow binding. During this period cells were cultured in medium without hIL-5. Next, 10⁵ cells/well were added to hIL-5 with or without the Ab dilution series. After the indicated period of time, cell growth was measured as described above.

4.7 Transfection of hybrid human/mouse IL-5Rα-chain in Ba/F3 cells

The hybrid IL-5Rα-chain was constructed in the pCDM8 expression vector, as described [4]. In brief, the extracellular part of the mIL-5Rα in the pCDM8 vector was exchanged for the cDNA encoding the soluble hIL-5Rα cDNA [8], by means of a SacI digest. The SacI recognition sequence is common to human and mouse IL-5Rα. 5 µg of this DNA was electroporated in 5 x 10⁶ Ba/F3 cells. Cells were selected for their ability to grow in medium supplemented with 20 ng/ml hIL-5.
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Correspondence: Jan Tavernier, Flanders Interuniversity Institute for Biotechnology, Ghent University, Faculty of Medicine and Health Sciences, VIB00, Dept. Medical Protein Research, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium Fax: +32-9-264.52.93 e-mail: Jan.Tavernier@rug.ac.be
Chapter 5

Heteromeric MAPPIT: a novel strategy to study modification-dependent protein-protein interactions in mammalian cells


5.1. Introduction

Our lab recently developed a novel protein-protein interaction trap in mammalian cells, MAPPIT (Eyckerman et al., 2001). In this system, a bait protein is fused to a (chimeric) receptor in which all tyrosine residues in the cytoplasmic domain are mutated to phenylalanine, thereby eliminating all STAT and negative regulator recruitment (figure 1). Ligand binding still leads to normal activation of the intracellular associated JAK kinases. A prey protein is fused to the cytoplasmic tail of the gp130 protein of the IL-6 receptor. The bait-prey interaction results in the recruitment of the gp130 tail to the activated JAK kinases, tyrosine phosphorylation and STAT activation. Activated STAT dimers translocate to the nucleus and activate the STAT3 dependent rPAP (rat pancreatitis associated protein 1) promter, ultimately leading to activation of transcription of the luciferase gene or expression of an antibiotic resistance marker. For an overview of this system and comparison with other protein-protein interaction detection systems, we refer to (Eyckerman and Tavernier, 2002).
Figure 1: Outline of the MAPPIT protein-protein interaction trap
The extracellular part of the bait containing receptor can be of the LR itself, or of other class I cytokine receptors (like the EpoR). Tyrosine mutation in this receptor blocks STAT recruitment, while mutation of the other tyrosines eliminates negative regulation of the signal. Bait-prey interaction leads to recruitment of the gp130 cytoplasmic tail, containing four STAT docking sites. Activated STAT dimers can induce transcription from the rPAP1 promoter. (adapted from Eyckerman et al., 2001).

The bait protein itself can be a substrate for JAK activity, allowing detection of phosphorylation dependent interactions. Therefore, this system allows the study of (JAK mediated) phosphorylation dependent interaction between two proteins, or screening for interaction partners of a specific bait protein in a complex cDNA library. We next wanted to expand the technology to other modification dependent interactions, like these based on non-JAK tyrosine and serine phosphorylation, acetylation, acylation, methylation, and others. This can be achieved by the use of a heteromeric chimeric receptor in which the bait protein is fused to one receptor and the modifying enzyme to the second receptor. In this study, we compared the signaling properties of chimeric receptors containing the extracellular parts of the human receptors for IL-3, IL-5 and GM-CSF, and the cytoplasmic tail of the mouse leptin receptor.
5.2. Material and methods

5.2.1. DNA constructs
Four chimeric receptors were constructed in the pSV-SPORT expression vector containing the extracellular part of the hIL-3Rα, hIL-5Rα, hGM-CSFRα and hβc and the cytoplasmic tail of the mouse leptin receptor (mLR). The extracellular parts of the four receptors were PCR amplified using the Pfu polymerase. A KpnI and PacI digestion removes the extracellular part of the EpoR in the pSV-SPORT EpoR-mLR construct (Eykereman et al., 2001). Amplified cDNA fragments were cloned in this opened vector. The resulting constructs were checked by DNA sequence analysis.

5.2.2. Transfection procedure and luciferase assay
For transfection experiments, 2.10^5 human embryonic kidney (HEK) 293T cells per 10 cm^2 well are freshly seeded and cultured overnight. Transfections were done overnight with a standard calcium phosphate precipitation procedure. One day after transfection, cells were washed with PBS-A, and cultured overnight until further use. Two days after transfection, cells expressing different combinations of LR chimeric proteins were resuspended with cell dissociation agent (Invitrogen) and seeded in a 96 black well plate (Costar). Cells were stimulated overnight with a serial dilution of the appropriate cytokine as indicated, or left unstimulated. Lysates were prepared (lysis buffer: 25 mM Tris, pH 7.8; 2 mM EDTA; 2 mM DTT; 10% glycerol; 1% Triton X-100), and 35 µl luciferase substrate buffer (20 mM Tricine; 1.07 mM (MgCO_3)_4Mg(OH)_2.5H_2O; 2.67 mM MgSO_4.7H_2O; 0.1 mM EDTA; 33.3 mM DDT; 270 µM Coenzyme A; 470 µM Luciferin; 530 µM ATP; final pH 7.8) was added per 50 µl lysate. Light emission was measured for 5 seconds in a TopCount Chemiluminescence Counter (Packard).

5.3. Results
HEK293T cells were transfected with (i) the hEpoR-mLR or with the combinations (ii) hIL-3Rα-mLR + hβc-mLR, (iii) hIL-5Rα-mLR + hβc-mLR, and (iv) hGM-CSFRα-mLR + hβc-mLR. Cells were stimulated overnight with a serial dilution of the appropriate cytokine, or left unstimulated. Results of triplicate luciferase measurements are shown in figure 2. These data illustrate that signaling via the GM-CSFR chimera is most
efficient when compared to the other three receptors. With this chimera a STAT3 dependent signal can be observed at GM-CSF concentrations as low as 50 pg/ml. The maximal luciferase signals are also significant higher.

The GM-CSF receptor chimera will therefore be used for the design of the heteromeric MAPPIT strategy, as discussed in the remainder of this chapter.

![Figure 2: Comparison of signaling properties of chimeric receptors](image)

**Figure 2: Comparison of signaling properties of chimeric receptors**
See text for more details.

### 5.4. References

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Heteromeric MAPPIT: a novel strategy to study modification-dependent protein–protein interactions in mammalian cells

Irma Lemmens, Sven Eyckerman, Lennart Zabeau, Dominiek Catteeuw, Els Vertenten, Kristin Verschueren¹, Danny Huylebroeck¹, Joël Vandekerckhove and Jan Tavernier*

Department of Medical Protein Research VIB09, Faculty of Medicine and Health Sciences, Ghent University, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium and ¹Department of Developmental Biology VIB07 and Laboratory of Molecular Biology (Celgen), University of Leuven, Herestraat 49, B-3000 Leuven, Belgium

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ABSTRACT
We recently reported a two-hybrid trap for detecting protein–protein interactions in intact mammalian cells (MAPPIT). The bait protein was fused to a STAT recruitment-deficient, homodimeric cytokine receptor and the prey protein to functional STAT recruitment sites. In such a configuration, STAT-dependent responses can be used to monitor a given bait–prey interaction. Using this system, we were able to demonstrate both modification-independent and tyrosine phosphorylation-dependent interactions. Protein modification in this approach is, however, strictly dependent on the receptor-associated JAK tyrosine kinases. We have now extended this concept by using extracellular domains of the heteromeric granulocyte/macrophage colony-stimulating factor receptor (GM-CSFR). Herein, the bait was fused to the βc chain and its modifying enzyme to the GM-CSFRα chain (or vice versa). We demonstrate several serine phosphorylation-dependent interactions in the TGFβ Smad pathway using the catalytic domains of the ALK4 or ALK6 serine/threonine kinase receptors. In all cases tested, STAT-dependent signaling was completely abolished when mutant baits were used wherein critical serine residues were replaced by alamines. This approach operates both in transient and stable expression systems and may not be limited to serine phosphorylation but has the potential for studying various different types of protein modification-dependent interactions in intact cells.

INTRODUCTION
Post-translational modifications of proteins play a critical role in cellular functions as diverse as gene transcription, protein synthesis and degradation, cell cycle control, signal transduction and apoptosis. Often these modifications affect protein–protein interactions and the mechanisms underlying these regulated processes are the focus of intensive research.

Genetic methods to study protein–protein interactions were first explored in yeast cells using the ‘yeast two-hybrid’ method and have become an extremely useful tool for analyzing protein–protein interactions in vivo (1). In the original method, reconstitution in the nucleus of the DNA-binding domain (fused to bait) and activation domain (fused to prey) of the yeast transcription factor GAL4 lead to activation of a GAL4-sensitive promoter directing a reporter/selector gene. To circumvent the requirement for nuclear translocation, alternative yeast-based methods were developed, including the SOS or Ras recruitment system, wherein the bait–prey interaction is artificially tethered to the yeast cell membrane (2,3), and the ubiquitin-based split sensor system (USPS), which detects interactions occurring in the cytosol (4). An intrinsic disadvantage of using yeast cells is that interactions are detected in a lower eukaryote, implying a sub-optimal context for studying protein interactions of higher organisms. In particular, post-translational modifications are often hard to reproduce in yeast, although a ‘yeast tri-hybrid’ system, whereby a modifying enzyme is co-expressed in the yeast cell, was developed. However, expression of the mammalian modifying enzyme in yeast cells is often cytotoxic, and it should be carefully verified whether the post-translational modification is carried out correctly inside such yeast cells (5).

Some of these limitations can be overcome by the use of mammalian cell systems. As such, the incorporation of β-galactosidase (6) or dihydrofolate reductase mutants in hybrid proteins (7) and fluorescence/bioluminescence resonance energy transfer (FRET/BRET) (e.g. using chimeras containing green fluorescent protein variants; reviewed in 8) may be methods of choice to trace interactions that occur in the cytosol. Modification-dependent interactions have been monitored via these mammalian complementation assays, but they rely on the presence and activity of endogenous modifying enzymes (9–11).

*To whom correspondence should be addressed. Tel: +32 9 331 33 01; Fax: +32 9 331 35 99; Email: jan.tavernier@rug.ac.be

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We recently reported a novel method to study modification-dependent protein interactions in intact human cells using type I cytokine receptors. Ligands for these transmembrane glycoproteins include many interleukins, colony stimulating factors and hormones. Type I cytokine receptors lack intrinsic enzymatic activity but are constitutively associated with cytosolic tyrosine kinases of the JAK family. Ligand-induced clustering and/or reorganization of receptor subunits leads to trans-phosphorylation and activation of the JAKs, which subsequently phosphorylate tyrosine residues in the intracytoplasmic tail of the receptors allowing recruitment and activation by phosphorylation of signaling molecules. Among these, signal transducers and activators of transcription (STATS) play an essential role in the direct transmission of signals to the nucleus (12). Based on these insights, we developed a mammalian protein–protein interaction trap (MAPPIT) wherein the bait is fused to a STAT recruitment-deficient receptor, while the prey is coupled to a receptor fragment containing functional STAT recruitment sites. Bait–prey interaction leads to ligand-dependent STAT activation, which can be detected by the use of a STAT-responsive reporter/selector gene. Using this approach we were able to demonstrate modification-dependent recruitment of STATs in complex with tyrosine phosphorylation-dependent interactions. In the latter case, tyrosine phosphorylation of the bait is, however, strictly dependent on the receptor-associated JAK tyrosine kinase activity (13). To broaden the application field of the MAPPIT method to other types of protein modifications, we explored the use of a more complex heteromeric receptor system wherein the bait can be fused to one of the receptor chains and its modifying enzyme to the other. In such a configuration, bait and enzyme are brought into close proximity, favoring modification of the bait. We apply this concept here to serine phosphorylation-dependent Smad interactions in transforming growth factor β (TGFβ) family signaling.

**MATERIALS AND METHODS**

**Constructs**

The basic MAPPIT bait, prey, reporter and pSVsport-EpoR-LR constructs were generated as previously described (13). The leukaemia receptor (LR) fragment obtained from pSVsSpot-EpoR-LR by PacI and XbaI digestion was used to replace the IFNAR-1 fragment in pSVsSpot-hIFNα/IFNAR-1 construct to give pSVsSpot-Bc-LR. The hIL-3Rα-LR chimeric receptor was obtained by transfer of the bait fragment derived from the erythropoietin receptor (EpoR)-LR construct (13) by Pacl and Notl digestion into the pSVsSpot-IL-3Rα/IFNAR-2/2 construct (14). The pSVsSpot-IL-3Rα-LR and pSVsSpot-GM-CSFRe-LR constructs were generated by replacing the extracellular domains by PCR with Pfu polymerase and exchanging the EpoR domain in the EpoR-LR construct by KpnI and PacI digestion. Replacing the LR domain by the mutant LRF3 fragment by PacI and Notl digestion generated the chimeric GM-CSFRα-LRF3 and Bc-LRF3 receptors. Full-length human Smad3 and Smad5 cDNA fragments (cloned into pcde1 plasmids, gifts from Dr Miyazono) were obtained by EcoRI and XhoI digestion, treated with Klenow polymerase and cloned into the SalI and Klenow polymerised blunted Bc-LRF3 or GM-CSFRα-LRF3 vector. cDNA fragments encoding the cytoplasmic tail of ALK4 or its constitutive active variant (T200D, generated by site-directed mutagenesis) were amplified by PCR with Pfu using the PTZ18R-ALK4 plasmid (15) as a template. These fragments were cloned into pGAD424 or pGBT9, respectively, and subsequently transferred into the GM-CSFRα-LRF3 or βc-LRF3 vectors by ligating blunted (Klenow) EcoRI–BamHI fragments into a blunted SalI restriction site. The constitutively active variant of ALK6 contains a Q203D substitution and the relevant fragment was amplified by PCR using pCS2-CA-ALK6 (a gift from Dr Tylzanowski) and was cloned by SalI and Notl digestion into the chimeric receptor chains. The pMG2 vector originates from the pMGL vector (13) and contains amino acids 905–918 from the gp130 chain in duplicate. The Smad-binding domain (SBD) of Smad-interacting protein-1 (SIP1) was derived from a pCS3-SIP1SBD construct encoding a 183 amino acid long segment encompassing the SBD (16) corresponding to amino acids 315–498 in full-length mouse SIP1. The cDNA fragment encoding this polypeptide was isolated by SmaI and XhoI digestion of the plasmid and cloned into the pMG2-SVT and pME7-Flag vectors with a blunt EcoRI and a XhoI site. Human Smad4 (missing the first 3 amino acids, a gift from Dr Miyazono) was obtained by EcoRI and XhoI digestion and cloned into EcoRI and XhoI digested pMG2-SVT and pME7-Flag vectors. The Smad3/5 S–A mutants and the insertion of extra leucine residues (1, 2 or 3 at position 851 within the predicted transmembrane region of the leukaemia receptor) in the transmembrane domains from the chimeric receptor chains were generated using the Quick Change site-directed mutagenesis procedure (Stratagene). The pRK5-JAK2 construct was a kind gift from Dr Constantinescu. The dual expression vector is based on the pcDNA5/FRT vector (Invitrogen). A CMV-newMCB-SBH polyadenylation signal was amplified and inserted into the BgIII and MfeI digested pcDNA5/FRT vector, resulting in a pcDNA5/FRT vector with two CMV-MCS-BGH cassettes. The βc-LRF3+1L-Smad3 chain was inserted in the dual expression vector by KpnI and Notl digestion, the GM-CSFRα-LRF3+1L-CA-ALK4 chain using SbfI/NsiI and SmaI/SmaI digestions.

**Cell lines, transfections, reporter assays and FACs analysis**

The dual expression vector pcDNA5/FRT containing the GM-CSFRα-LRF3+1L-CA-ALK4 and βc-LRF3+1L-Smad3 chains was stably integrated in the HEK293-16 cell line, as previously described (13), using the Flp-In recombine reaction (Invitrogen) and after selection on hygromycin (100 µg/ml) for 10 days. Culture conditions, transfection procedures and luciferase and β-galactosidase assays for HEK293T cells are described elsewhere (17). For a typical luciferase experiment, 4 × 10^5 cells were transfected with the desired constructs in the presence of luciferase and β-gal reporter genes. After 48 h, cells were left untreated (UN) or were treated with 10 ng/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) or with the corresponding ligands at doses as indicated. After another 24 h, luciferase activity from triplicate samples was determined and normalized against β-galactosidase activity. The results shown are representative of at least three independent transfections.
experiments. Recombinant hGM-CSF and hIL-3 were purchased from PeproTech Inc. (Rocky Hill, NJ). hIL-5 was produced in COS-1 cells and purified using HiTrap affinity chromatography as described before (18). hEpo was obtained from R&D Systems. GM-CSF was typically used at 10 ng/ml; the concentrations of interleukin 3 (IL-3), interleukin 5 (IL-5) and Epo used are as indicated. GM-CSF/R-α-LRF3+IL- CA-ALK4 expression was monitored with 2 μg/ml of an anti-human GM-CSF receptor monoclonal antibody (sc-456; Santa Cruz) and 4 μg/ml Alexa488-conjugated goat anti-mouse IgG (Molecular Probes). The βc-LRF3+IL-Smad3 chain was stained with 2 μg/ml of biotin-conjugated mouse anti-human monoclonal antibody against the common β-chain (CD831; PharMingen International) and 4 μg/ml Alexa488-conjugated goat anti-mouse IgG (Molecular Probes). FACScalibur analysis was performed on a FACScalibur (Becton Dickinson).

**Immunoprecipitation and western blot analysis**

To demonstrate Smad3 serine phosphorylation, we transiently transfected ~3 × 10^5 HEK293T cells with plasmid vectors encoding the GM-CSF receptor and βc-LRF3- Smad3 receptor chains with one extra leucine in their transmembrane part or the GM-CSF receptor and βc-LRF3- Smad3 S→A (critical serines of Smad3 mutated to alanines) receptor chains or empty vector. Sixty-five hours after transfection, cells were harvested in serum-free medium for 5 h and were left untreated or were stimulated with 10 ng/ml GM-CSF for 10 min. Cleared lysates (in modified RIPA buffer, 50 mM Tris–HCl pH 8.0, 200 mM NaCl, 1% NP-40, 0.5% DOC, 0.05% SDS, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 20 mM β-glycerophosphate and Complete protease inhibitor cocktail [Roche]) of the untreated and stimulated cells were incubated with 2 μg mouse anti-human β common antibody (CD831; PharMingen International) and Protein G-Sepharose (Amersham Pharmacia Biotech). After immunoprecipitation, polyacrylamide gel electrophoresis and western blotting, serine phosphorylation of Smad3 was detected with a monoclonal anti-phosphoserine Smad3 antibody (a gift of Dr ten Dijke). The presence of the chimeric chain was visualized using an anti-LR antibody directed against residues 942–953 of the cytoplasmic domain of the leptin receptor.

For STAT3 and JAK2 phosphorylation, 4 × 10^5 HEK293T cells were transfected with the desired constructs. After 65 h, the cells were starved in serum-free medium for 5 h, left untreated or were stimulated with 10 ng/ml GM-CSF for 5 or 10 min, respectively. Cells were lysed in 200 μl modified RIPA buffer, of which 50 μl was loaded on a 7.5% polyacrylamide gel for gel electrophoresis and subsequent western blotting. STAT3 phosphorylation was detected with the Phospho-STAT3 Tyr705 antibody (Cell Signaling) and expression levels were verified using an anti-STAT3 antibody (Transduction Laboratories). To detect JAK2 phosphorylation, extra JAK2 was transfected (0.001 μg pRK5-JAK2, except for the mock transfection) and was revealed using an anti-phospho-JAK2 antibody (Upstate Biotechnology) and expression levels were checked using an anti-JAK2 antibody (Upstate Biotechnology).

**RESULTS**

**Design of the heteromeric MAPPT approach**

The MAPPT system is outlined in Figure 1A. A chimeric cytokine receptor, composed of the extracellular part of the homodimeric EpoR fused to the transmembrane and cytosolic domains of the LR, can activate STAT3, but is made inactive by a Y1138F mutation, which is known to eliminate a critical STAT3 recruitment site. Ligand administration leads to JAK activation, but in the absence of a functional recruitment site.
Chapter 5

Detection of serine phosphorylation-dependent interactions

To test the heteromeric MAPK1P concept, serine phosphorylation-dependent Smad interactions were investigated. Smad proteins are components of the TGFβ signalling machinery, which regulate a wide range of biological and cellular processes. In one proposed and generally accepted model, signal transduction via TGFβ receptors is initiated when the ligand induces assembly of a heteromeric complex of type II and type I transmembrane serine/threonine kinase receptors. The type II kinase then phosphorylates the type I receptor in a conserved glycine-serine-rich (GS) domain. This activates the type I kinase, which subsequently recognizes and phosphorylates members of the intracellular Smad signal transduction pathway. The type I receptors specifically recognize the Smad subgroup known as receptor-activated Smad5 (R-Smads). These include Smad1, Smad2, Smad3, Smad5 and Smad8. The R-Smads have two conserved and well-characterized functional domains, i.e. the MH1 and MH2 domains, which are separated by a proline-rich domain. Receptor-mediated phosphorylation of the C-terminally located SSXS motif contributes to the relief of a mutually inhibitory interaction between these two domains and leads to Smad activation and subsequent accumulation in the nucleus. Prior to nuclear translocation, phosphorylated R-Smads bind to a common Smad, Smad4. Such R-Smad–Smad4 complexes participate in DNA binding and/or recruitment of transcriptional cofactors, thereby modulating gene transcription (21).

To create a signaling-deficient heteromeric receptor complex, the cytoplasmic tail from the wild-type LR in the GM-CSFRα-LR3 and βc-LR3 chimeras was replaced by its signaling-deficient LRF3 variant. These chimeras were used to probe interactions within the context of Smad signaling. Therefore, the cytoplasmic tail of a constitutively active form of the mouse activin-like kinase 6 type I receptor, carrying a Q203D mutation in its GS domain (CA-ALK6), and the bait, full-length human Smad5, were fused in-frame to the GM-CSFRα-LR3 and βc-LR3 receptor chains. Optimal bait–prey interaction-dependent signaling required two optimization steps. First, comparison of the two reciprocal combinations showed that more efficient signaling was obtained when the modifying enzyme CA-ALK6 was fused to the βc-LR3 chimeric receptor and the bait, Smad5, to the GM-CSFRα-LR3 receptor chain. Second, based on recent reports that optimal signaling by class I cytokine receptors may depend on the relative orientation between the extracellular and intracellular domains within a receptor complex (22,23), we inserted one, two or three extra leucine residues within the transmembrane parts of each of the receptor chimeras. The combination where both chimeric receptor chains contained two extra leucines appeared most favorable for the CA-ALK6/Smad5 combination, although other combinations also showed enhanced efficacy. Results for the optimal combination together with negative controls are shown in Figure 2A. Importantly, the negative control, whereby the serine residues in the SSXS phosphorylation motif of Smad5 were replaced by alanines, was completely signaling-deficient. Additional controls included absence of the ALK6 catalytic domain, absence of Smad5 bait, absence of Smad4 (i.e. the presence of an irrelevant gp130–SV40 large T prey) and absence of the gp130 chain (wild-type Smad4). In all these cases no signal was obtained, indicating that STAT3 activation was strictly dependent on the interaction between the serine-phosphorylated bait and the gp130–Smad4 prey. Signaling was also strictly ligand-dependent.

Similarly, the cytoplasmic tail of a constitutively active form of the mouse activin-like kinase 4 type I receptor (15) (obtained by a T206D mutation in the GS domain, and further referred to as CA-ALK4) and full-length human Smad3 were fused in-frame to the GM-CSFRα-LR3 and the βc-LR3 chimeric receptors. Serving as prey were either full-length Smad4 or a polypeptide encompassing the SBD of the transcriptional repressor SIP1 (16), which were fused C-terminally to the STAT3 recruitment sites of the gp130 chain. Here, for both preys, the combination whereby CA-ALK4 was fused to the GM-CSFRα-LR3 receptor chain and Smad3 to the βc-LR3 chimeric receptor and whereby both receptor chimeras contained a single additional leucine residue within the transmembrane region gave best results (shown in Fig. 2B for the Smad3–Smad4 interaction, with the Smad3 S→A mutant as negative control).
Figure 2. Detection of phosphoserine-dependent Smad interactions using the heteromeric MAPPIT procedure. (A) HEK293T cells were transfected with plasmids encoding: 1, βc-LRF3+2L-CA-ALK6, GM-CSFRα-LRF3+2L-Smad5 and gpl30-Smad4; 2, βc-LRF3+2L-CA-ALK6, GM-CSFRα-LRF3+2L-Smad5 and gpl30-Smad4; 3, βc-LRF3+2L (no CA-ALK6), GM-CSFRα-LRF3+2L-Smad5 and gpl30-Smad4; 4, βc-LRF3+2L-CA-ALK6, GM-CSFRα-LRF3+2L (no Smad5) and gpl30-Smad4; 5, βc-LRF3+2L-CA-ALK6, GM-CSFRα-LRF3+2L-Smad5 and gpl30-SVT (no Smad4); 6, βc-LRF3+2L-CA-ALK6, GM-CSFRα-LRF3+2L-Smad5 and Smad4 (no gpl30). Averaged values for relative luciferase activity (± fold increase, luciferase values obtained from stimulated cells with respect to values derived from untreated cells) are shown. (B) HEK293T cells were transfected with plasmids encoding: 1, GM-CSFRα-LRF3+1L-CA-ALK4, βc-LRF3+1L-Smad3 and gpl30-Smad4; 2, GM-CSFRα-LRF3+1L-CA-ALK4, βc-LRF3+1L-Smad3 S→A and gpl30-Smad4; (C) HEK293T cells were transfected with plasmids encoding: 1, GM-CSFRα-LRF3+1L-CA-ALK4, βc-LRF3+1L-Smad3, and gpl30-Smad4; 2, GM-CSFRα-LRF3+1L-CA-ALK4 and βc-LRF3+1L-Smad3 S→A (critical serines of Smad3 mutated to alanines) and gpl30-Smad4; 3, mock control with empty vector. Cells were either left untreated (+) or were stimulated with 10 ng/ml GM-CSF (+). α phospho-Smad3, serine phosphorylated Smad3; α lepin receptor, the chimeric chain detected using an anti-lepin receptor antibody; α phospho-JAK2, phosphorylated JAK2; α JAK2, total JAK2; α phospho-STAT3, phosphorylated STAT3; α STAT3, total STAT3.

Analysis of bait and prey modifications

Serine phosphorylation of Smad3 by the CA-ALK4 kinase was checked by western blot analysis using an antibody specific for serine-phosphorylated Smad3 (Fig. 2C, α phospho-Smad3). Smad3 phosphorylation was ligand-independent, suggesting the existence of preformed receptor complexes. Similar ligand-independent serine phosphorylation of Smad3 was observed in an isogenic cell population stably expressing the chimeric receptor chains (as discussed below; data not shown). Importantly, although bait modification was independent of ligand, JAK activation as well as subsequent tyrosine phosphorylation of STAT3 was strictly dependent on GM-CSF administration (see Fig. 2C, α phospho-JAK2 and α phospho-STAT3).

Analysis of the Smad3-Smad4 interaction in stably transfected cells

Since the above mentioned experiments were performed by transient overexpression, we subsequently analyzed whether heteromeric MAPPIT could also be set up in a stable cell system with more physiological expression levels of the receptor chimeras. For this purpose a dual expression vector was generated based on pcDNAs/FRT (Invitrogen). This plasmid vector facilitates a Flp-In recombinase reaction, allowing fast and easy selection of a so-called isogenic cell population by stable integration into a transcriptionally active locus in the cell genome. This basic vector contains a CMV promoter followed by a multiple cloning site (MCS) and a bovine growth hormone polyadenylation signal (BGH). A second, similar expression cassette, but with a different MCS, was inserted, allowing expression of both heteromeric receptor chimeras from a single vector. As shown by FACS analysis in Figure 3B and C, stable expression of both receptor chimeras was obtained after recombinase-assisted integration. Upon transient expression of the gpl30-Smad4 prey, a clear ligand-dependent signal was obtained. Significantly, this signal was inhibited by coexpressing a receptor chimeral containing the S→A mutated Smad3 or a receptor chimeral lacking the modifying enzyme and by the coexpression of wild-type Smad4 not linked to gpl30 (Fig. 3A).
DISCUSSION

The complexity of the human proteome is estimated to exceed its encoding genome by at least one order of magnitude. This enhanced molecular variation is mostly attributed to post-translational modification of proteins, combined with alternative splicing. The best-studied type of protein modification is phosphorylation, whereby covalent attachment of phosphate groups can act as an activity modulator or even as an on/off switch controlling the activity of receptors, signaling molecules, enzymes or transcription factors. As such, protein phosphorylation is involved in a wide range of cellular processes, including growth, proliferation, differentiation, motility, membrane transport, and in their control by extracellular signals. Together, this ultimately results in a wide range of controlled physiological processes at the body level. Understanding the importance of protein phosphorylation is the complexity of the human kinase, which with 518 known kinases represents ~1.7% of the complete protein encoding gene set of the genome, and the estimation that about one-third of all proteins can be a target for phosphorylation (24). Even further understanding the importance and the complexity of controlled protein modifications is the fact that besides phosphorylation, many other types of functionally important post-translational modifications have been described, including proteolytic cleavage, ubiquitination, sumoylation, acetylation, and methylation.

Here, we describe a novel method to facilitate the study of modification-dependent protein–protein interactions in the physiologically optimal context of intact mammalian cells. We previously reported a two-hybrid approach based on type I cytokine receptor signaling. Using this MAPPT approach we were able to demonstrate tyrosine phosphorylation-dependent interactions between the pY402 motif of the EpoR and the cytokine-inducible SH2-containing protein (CIS) and suppressor of cytokine signaling-2 protein (SOCS-2) (13). Additional interaction-dependent signals were also demonstrated using other preys, including PLCγ, PI3K, Grb2, SHP-2 and Vav (to be reported elsewhere). Given the homodimeric structure of the EpoR, MAPPT applications are restricted to homodimeric baits. We therefore explored the use of the extracellular domains of the heteromeric GM-CSF receptor (GM-CSFRT þ βc chains), whereby the bait is fused to one of the receptor chains and its modifying enzyme is fused to the other. In such a configuration, receptor complex formation brings bait and modifying enzyme into close proximity, favoring modification of the bait. In principle, any type of protein modification can be studied, as long as the modifying enzyme can be functionally coupled to the chimeric receptor chain. As model interactions, we selected several previously documented protein interactions in the TGFβ family signaling pathways, i.e. between Smad5 and Smad4, Smad3 and Smad4 and Smad3 and SIPP, which are all strictly dependent on correct serine phosphorylation of the R-Smad. Interestingly, as shown in Figure 2C, Smad3 serine phosphorylation is observed in the absence of ligand, suggesting that the GM-CSFRT+βc chimeric receptors likely exist as preformed complexes allowing spontaneous bait modification. Preformed complex formation in this cytokine receptor family is well documented for the EpoR (25–28). It is unclear at present which parts of the GM-CSFRT+α/LRF3 and βc/LRF3 receptor chimeras induce complex formation, however, this bait modification assay can be used as read-out for structure/function analyses. It should be stressed that the heteromeric MAPPT read-out is completely independent of such preformed receptor complexes.

Given the availability of numerous constitutively active kinases, heteromeric MAPPT is very well suited to study most, if not all, phosphorylation-dependent protein–protein interactions. It should be stressed that the substrate–enzyme proximity induced upon formation of the chimeric receptor complex is also likely to suffice for other types of protein modifications, e.g. in cases where this is controlled by regulated expression or localization of the modifying enzyme.

As has been shown for gp130 and for the EpoR, proper orientation of signaling subunits within a cytokine receptor complex is necessary for efficient activation and for triggering downstream signaling cascades. Constantinescu et al. concluded that orientation-dependent signaling is transmitted through the transmembrane domain toward the cytosolic juxtamembrane domain, suggesting that the transmembrane segment is important for preserving the orientation between the extracellular domain and the cytoplasmic domain (22). Greiser et al. reported two distinct requirements for efficient signaling through the gp130 chain: first, JAKs have to be located close to the membrane for full activation and, secondly, the cytoplasmic regions of the receptors have to be precisely oriented to allow STAT activation (23). Here we observed that inserting extra leucines into the transmembrane part of the chimeric GM-CSFRT+LRF3 and βc-LRF3 chains also clearly affected signaling efficiency. In the case of heteromeric MAPPT, however, optimal receptor configurations appear to differ from case to case: insertion of two leucine residues in either receptor chain led to optimal signaling for the CA-ALK6/Smad5 set-up, whereas insertion of one single leucine residue was required for optimal signaling in the case of the CA-ALK4/Smad3 combination. It should be stressed that signaling in heteromeric MAPPT depends on, besides optimal JAK activation, modification of the bait. A likely explanation for this bait-dependent signaling variability is therefore that the efficiency of bait modification may also depend on the relative orientations of the target serine residues in the Smad baits and the catalytic domains of the ALK modifying enzymes.

An important advantage of MAPPT over other mammalian two-hybrid methods is the possibility to perform interactors hunts in complex cDNA libraries (13). We therefore explored the use of this heteromeric approach in stable cell systems. A dual expression vector was constructed, allowing rapid selection of a cell population stably expressing both receptor chimeras. Since both receptor chimeras are preferably expressed at comparable levels, we used a vector containing two expression cassettes based on the same CMV promoter. This is not the case for bis-cistronic vectors that make use of an internal ribosomal entry site (IRES) sequence since a lower expression level of the gene cloned behind the IRES sequence is often observed (29). In this isogenic cell pool, which stably expresses the GM-CSFRT+LRF3+IL-CA-ALK4 and βc-LRF3+IL-Smad3 chains, serine phosphorylation-dependent interaction between Smad3 and Smad4 was clearly detected. Furthermore, overexpression of βc-LRF3+IL-Smad3 S→A, of GM-CSFRT+LRF3+IL lacking the modifying enzyme or of
wild-type Smad4 inhibited this signal, showing that the read-out is strictly dependent on proper Smad3 serine phosphorylation and on JAK-dependent tyrosine phosphorylation of the gp130 prey. Clearly, screening of complex cDNA libraries may yield preys interacting with either the (modified) bait or with the modifying enzyme. Albeit the latter may prove interesting by themselves, preys specifically interacting with the bait can easily be discerned by the use of receptor chimeras lacking the bait or containing a mutant bait lacking the modification site, as was demonstrated before (13).

In conclusion, we have developed a novel method to study modification-dependent protein–protein interactions in intact mammalian cells. We demonstrated efficient detection of phosphoserine-dependent protein–protein interactions, however, the use of such engineered heteromeric receptor systems has the potential to be extended to many other types of modification-dependent protein–protein interactions.

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REFERENCES

PART III: Leptin and its Receptor
Chapter 6

Leptin and its Receptor

The human body is evolutionary conditioned to be able to pass periods of low feeding or fasting. This is mainly achieved by energy storage in adipose tissue, but also by lowering thermogenesis and reduction of physical activity. It is of vital importance that these energy stores, reflecting the difference between energy uptake and energy expenditure, are maintained throughout the individual’s life. This is achieved by a rigorous balance between eating, physical activity and body metabolism. This control mechanism is regulated within the brain and ultimately results in the regulation of one’s body weight. Over the past decade, it became clear that the adipocyte derived hormone leptin and its receptor play a central role in the control of body weight and energy expenditure. Mutations in the genes encoding the ligand or receptor result in marked obesity phenotypes in both mice and men. There is a growing body of evidence that energy stores, or the nutritional status, influence important physiological processes like immune reactions, the onset of puberty and reproduction. The role of leptin in these processes will be discussed at the end of this chapter.

1.1 Leptin...

Mice with the spontaneous and recessive ob/ob (ob: obese) mutation display an early onset obese phenotype which is associated with a number of endocrinological disorders. These mice weigh three times more than their wild type friends and exhibit a five-fold increase in their body fat content (see figure 1). Friedman and colleagues used a positional cloning strategy to identify the product of this ob gene and named it leptin, referring to the Greek leptos, meaning thin (Zhang et al., 1994). Administration of recombinant leptin to ob/ob mice resulted in a decreased food intake, increased energy expenditure and weight loss, thereby supporting a central role for leptin in the regulation of body weight.

The leptin gene is located on chromosome 7 and 6 in respectively men and mice, and
Introduction: Leptin and its receptor

Leptin encodes a 167 Aa long polypeptide. Leptin is expressed as a 16 kDa non-glycosylated protein with an intra-molecular disulphide bond necessary for biological activity (Rock et al., 1996). The structure shows it is a type I long-chain cytokine (figure 1, and see general introduction for more details). The hormone is mainly secreted by white adipose tissue, and its circulating level correlates well with the body fat mass (Frederich et al., 1995; Maffei et al., 1995; Considine et al., 1996). Low leptin expression could also be shown in placenta, stomach, mammary epithelium and skeletal muscle (Senaris et al., 1997; Bado et al., 1998; Wang et al., 1998), although the physiological importance remains unclear. Serum leptin levels show a clear gender difference, since they are higher in females than males matched by age and body weight (Kennedy et al., 1997).

Figure 1: Phenotype of the ob/ob mouse (A) and structure of leptin (B)
(A) A wild type mouse (left) and an obese ob/ob mouse (right) are shown. (B) Structure of leptin is shown. Helices are in pink, loops and β-strands in green.

1.2 ...and its receptor

The leptin receptor (LR) was first isolated as the product of the db (db: diabetes) gene from a choroid plexus cDNA library using an expression cloning strategy (Tartaglia et al., 1995). Thus far, six isoforms of the LR generated by alternative mRNA splicing have been recognized and termed LRa through LRf. These isoforms have the same extracellular domain, but differ in the length of their cytoplasmic tail, or represent a secreted receptor (LRe) (see figure 2). The LR long form (LRlo, or LRb) has an intracellular chain length of 302 Aa, and is the only isoform capable of efficient
signaling. It is this LRlo isoform that is primarily expressed in specific nuclei of the hypothalamus (Mercer et al., 1996; Schwartz et al., 1996b; Fei et al., 1997), a region of the brain that is known to be involved in regulating body weight. Expression of LRlo has also been observed at lower levels in many other cell-types (Ghilardi et al., 1996; Mercer et al., 1996; Dyer et al., 1997). A second isoform, LRa, is a short variant lacking most of the cytosolic domain (LRsh), and is much more widely expressed, often at higher levels compared to LRlo, e.g. in the choroid plexus, kidney, lung, and liver (Tartaglia, 1997). The precise function of this and the other three short forms remains elusive. It was suggested that, at least LRa, could play a role in the transport of leptin through the blood-brain-barrier. This will be discussed in more detail later in this chapter. It is believed that the soluble LR form (LRe) delays leptin clearance, thereby increasing its half life and thus the available leptin pool in circulation (Liu et al., 1997). In murine pregnancy, massively elevated levels of LRe are observed that may deliver leptin to target tissues like the feto-placentar unit (Gavrilova et al., 1997; Lammert et al., 2002). Finally, overexpression of this isoform in the ob/ob mice could be shown to enhance the weight-reducing effects of leptin in vivo (Huang et al., 2001).

Like with the ligand leptin, mutations in the gene encoding the receptor result in an extreme phenotype. The three best characterized animal models are the db/db mice, the fa/fa (Zucker) and f/f (Koletsky) rats. In obese db/db mice, the mutation creates a new splice donor site in the exon encoding the cytoplasmic tail LRlo form. This results in the generation of a premature stop codon, and replacement of the LRlo by the LRsh form. The fa/fa mutation includes a 880 A → C nucleotide missense mutation, resulting in glutamine to proline substitution (at position 269) in the extracellular domain of the receptor. This leads to a marked decrease in cell surface expression, leptin binding properties and signaling capacities. The f/f mutation in Koletsky rats leads to the generation of a premature STOP codon mutation resulting in expression of only a secreted LR variant (Wu-Peng et al., 1997). Finally, Cohen and co-workers showed that conditional knock-out of the LR in only the brain, but for example not in liver, resulted in the obese phenotype (Cohen et al., 2001). This result further supports the idea that the organism’s body weight is centrally regulated by leptin.
**Figure 2: Schematic representation of the six LR isoforms**

The extracellular domain consists of a membrane distal cytokine receptor homology (CRH1) domain, an immunoglobulin (Ig), CRH2 and two fibronectin type III (FNIII) domains. Differences in the cytoplasmic tail are represented by the N-terminal amino acids. Numbers are the length of the different isoforms. Box1 represents the proline rich region necessary for JAK binding.

The LR was identified as a member of the class I cytokine receptor family and binds leptin with nanomolar affinity. The receptor shares highest sequence similarity with the granulocyte colony-stimulating factor (G-CSF) receptor and the glycoprotein 130 (gp130) family receptors, including gp130, the leukemia inhibitory factor (LIF) and oncostatin M (OSM) receptors (Nakashima et al., 1997; Zabeau et al., 2003). Like all class I cytokine receptors, the LR is characterized by the extracellular CRH module. A rather unique feature of the LR is the presence of two such domains (figure 2). Other receptors containing two CRH modules are the βc chain (shared in the receptors for IL-3, -5, and GM-CSF), the LIF receptor, IFN receptor IFNaR1, and the thrombopoietin receptor. In the LR the membrane distal CRH1 and a membrane proximal CRH2 domain are separated by an Ig-like domain and followed by two FNIII domains (reviewed in Zabeau et al., 2003).

So far, relatively little is known on the precise mechanisms of activation of the LR. Fong and co-workers showed that the membrane proximal CRH2 domain is sufficient and
absolutely necessary for leptin binding (Fong et al., 1998). Despite the lack of any
affinity for the ligand, the FNIII domains are also needed for activation of the receptor.
More recently, we could show that a LR deletion-variant, which lacks both CRH1 and Ig-
like domains, is unable to activate the JAK kinases and therefore cannot generate a
STAT3-dependent signal in response to leptin. We also could demonstrate that the
membrane distal CRH1 is not strictly required for signalling, but allows optimal
signalling (Zabeau et al., 2004).

Several lines of evidence suggest that the LR exists as a preformed (i.e. in the absence
of the leptin ligand) complex. Chemical cross-linking and Western blot analysis indicate
that the receptor forms dimers in solution and on the cell-surface (Devos et al., 1997;
White and Tartaglia, 1999). Couturier et al. further extended these findings using a
quantitative Bioluminescence Resonance Energy Transfer (BRET) approach. They could
show that 60% of the receptors are expressed as dimers, and this dimerisation is not
increased after addition of leptin (Couturier and Jockers, 2003). It is therefore
reasonable to assume that the LR becomes activated upon conformational changes,
more than by a simple leptin-induced receptor oligomerisation. We recently showed
that both the membrane proximal CRH2 module as well as the FNIII domains could be
involved in this ligand-independent dimerisation. These results will be discussed in
chapter 11.

1.3 Body weight regulation
The body weight of an individual is to a significant extent under endocrine control. The
system that balances feeding behavior and energy stores appears to be composed of a
short-term and long-term system. Changes in plasma glucose concentrations, body
temperature, plasma amino acids, cholecystokinin and several other hormones
determine the food intake and hence the short-term control. The long-term system
balances food intake and energy consumption, ultimately regulating the body energy
stores. Leptin appears to be a key player in this long-term control mechanism. As
mentioned, the hormone is mainly produced by adipocytes, and functions as a negative
feedback adipostat, signaling the brain of the fat reserves in the body. Administration of
leptin to rodents decreases food intake and increases energy expenditure (Halaas et al., 1995; Campfield et al., 1997), demonstrating that leptin acts as an afferent satiety signal or as an anti-obesity hormone. Leptin plays also a role in the adaptive response to starvation. Food restriction is rapidly followed by a decrease in circulating leptin levels, resulting in a set of neuroendocrine responses that favor survival in periods of limited energy supplies. Both actions of leptin are represented in figure 3.

**Figure 3: Leptin functions as an adipostat, signaling the body energy stores to the brain.**

As mentioned, peripherally produced leptin exerts its weight reducing effects upon binding and activation of the LR in certain nuclei of the hypothalamus. In order to do this, leptin must past the blood-brain-barrier (BBB). This BBB actually consists of several layers: the vascular barrier, the blood-cerebrospinal fluid barrier, and the circumventricular organs. Endothelial cells in these barriers are modified to prevent the unrestricted movement of circulating proteins between the blood and brain interstitial fluid. Given its high expression pattern in the choroids plexus, it has been proposed that the LRsh form plays a role in the transport of leptin trough the BBB. This idea was supported by the observation that expression of this LR isoform in Madin-Darby canine kidney cells results in the transport of labeled leptin in a transwell transport system.
(Hileman et al., 2000). This is further in line with BBB transport experiments performed in db/db mice, which do not express the LRlo form. Transport across the BBB of intravenously administrated radioiodinated leptin was not affected as compared to the lean wild type mice (Maness et al., 2000). More recently, Banks and colleagues used the Koletsky rat to study this issue in more detail (Banks et al., 2002). These rats are extremely obese and lack expression of all membrane-bound LR splice variants. The brain perfusion technique, which eliminates the influence of the high endogenous leptin levels, was used to illustrate that in the Koletsky rats leptin was transported at the same rate as in wild type rats. This observation suggests that there is a yet to be identified leptin transporter and might illustrate that the short form has only a modulating role in the transport.

Leptin exerts its central effects upon stimulation of two distinct populations of neurons in the hypothalamic nuclei (see figure 4). The first population includes the orexigenic neurons that response to absent (or low) leptin levels. These cells express neuropeptide Y (NPY) and Agouti-related transcript (AgRP). The second subset express the satiety factors $\alpha$-melanocyt-stimulating hormone ($\alpha$-MSH) and cocaine-amphetamine-regulated transcript (CART). Leptin inhibits the expression of the orexigenic neuropeptides NPY and AgRP, while inducing CART and $\alpha$-MSH. The secondary target neurons carry the MelanoCortin4 receptor (MC4R). This receptor is directly involved in the inhibition of food intake. $\alpha$-MSH and AgRP act respectively as an agonist and an antagonist of the receptor (Ebihara et al., 1999; Fehm et al., 2001).
Introduction: Leptin and its receptor

Figure 4: Schematic overview of the melanocortin circuit
After transfer through the BBB, leptin stimulates primary target neurons in the hypothalamus. This results in an decrease in expression of the orexigenic neuropeptides neuropeptide Y (NPY) and Agouti-related transcript (AgRP), and an increase in expression of POMC, and cocaine-amphetamine-regulated transcript (CART). These peptides act as agonists and antagonist of the MelanoCortin4 receptor (MC4R).

1.4 Obesity
The body mass index (BMI) is used to correlate one's body weight with one's length. It is defined as the weight in kilograms divided by the square of the height in meters. The classification of overweight and obesity by the world health organization (WHO) is given in table 1.

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI</th>
<th>Risk of comorbidities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt; 18.5</td>
<td>Low</td>
</tr>
<tr>
<td>Normal range</td>
<td>18.5 - 25</td>
<td>Average</td>
</tr>
<tr>
<td>Overweight</td>
<td>≥ 25.0</td>
<td></td>
</tr>
<tr>
<td>Preobese</td>
<td>25.0 - 29.9</td>
<td>Increased</td>
</tr>
<tr>
<td>Obese class I</td>
<td>30.0 - 34.9</td>
<td>Moderate</td>
</tr>
<tr>
<td>Obese class II</td>
<td>35.0 - 39.9</td>
<td>Severe</td>
</tr>
<tr>
<td>Obese class III</td>
<td>≥ 40.0</td>
<td>Very severe</td>
</tr>
</tbody>
</table>

Table 1: Classification of adults according to their BMI

Obesity is defined as a condition of abnormal or excessive fat accumulation in adipose tissue. It is estimated that nearly a quarter of the North Americans today are obese (i.e. have a BMI over 30) (Flegal et al., 1998). In Europe, the situation is less severe but nevertheless increasing. One explanation for the increasing prevalence of obesity is
undeniable our ‘western way of life’. The fast food industry is kindly providing us with fast and ‘nutritious’ meals. Television, video games and the internet allows us spent our free time with a minimum of efforts. And most of the today's jobs in the western world require hardly any physical activity.

The most obvious consequence of obesity is the difficulty to ‘fit in’ our modern society, ultimately resulting in an unpleasant lack of self acceptance. The media are entertaining us with ‘make-over’ programs, in which liposuction is a common feature. Highly underweight models are used to show us what one's ideal life should look like. And traveling by plane, or a simple visit to the theater, reminds a ‘fat’ person how small and uncomfortable a chair can be. Besides these esthetic considerations, obesity is also, and even more importantly, often associated with a number of disorders or co-morbidities, resulting in an enhanced risk for mortality. Disorders range from premature death to several non-fatal health problems, ultimately leading to a decrease in the quality of life. Overweight can be a predispose for a number of cardiovascular risks, like high blood pressure, elevated cholesterol levels and impaired glucose intolerance. There appears to be a strong correlation between obesity and the onset and development of type II (i.e. non-insulin-dependent) diabetes. Obese women exhibit a greater risk of endometrial, ovarian and breast cancer, while obese men have an increased prevalence of prostate cancer. Other obesity affected disorders are: gallbladder disease, endocrine disturbances, pulmonary disease and others.

Mutations in the genes for leptin or its receptor result in extreme obesity in men. However, these mutations are rarely found. Nonetheless, two cousins of Pakistani origin exhibit an extreme obese phenotype due to the lack of any circulating leptin (Montague et al., 1997). Sequence analysis illustrated that this is a result of a homozygous frame shift mutation in the leptin coding gene. A similar missense mutation was found in a Turkish family (Strobel et al., 1998). Seven out of eleven children with this homozygous mutation died in childhood following an infectious disease (Ozata et al., 1999), indicating a crucial role for leptin in the immune response (see below). In these rare cases of leptin deficiency, repetitive administration of recombinant leptin was, as
expected, clinically very effective in reducing body weight (Farooqi et al., 1999). Three obese members of a French family carry a mutation in the LR (Clement et al., 1998). Finally, also mutations in other genes, such as the effectors of leptin signaling in the hypothalamus, are associated with human obesity. It is estimated that approximately 1 to 2% of morbidly obese patients have a mutation in the MC4R receptor (see above) (Yeo et al., 1998).

In the majority of the obesity cases, leptin levels are significantly elevated, suggesting that there is a resistance to leptin. This resistance can result from defects in one of the three levels of the leptin action: **(i) an impaired transport of leptin trough the BBB.** It could be shown that obese humans who have a 300% increase in leptin serum levels, have only a 30% elevation in the cerebrospinal fluid (Caro et al., 1996; Schwartz et al., 1996a). Furthermore, some rodents resistant to peripherally administrated leptin, loose weight when the hormone is directly injected into the brain (Halaas et al., 1997; El-Haschimi et al., 2000). **(ii) defects in LR activation and signal transduction.** Defects in expression of the receptor result in a marked obese phenotype (e.g. db/db, fa/fa and f/f rodents). Defects in signal transduction involve an abnormal expression of the negative regulators of leptin signaling. For example, SOCS-3 is known to be expressed in hypothalamic nuclei in response to leptin. An elevated expression of this inhibitor accounts for the obese phenotype of some rat and mice strains (Bjorbaek et al., 1998; Wang et al., 2000). **(iii) defective secondary leptin signaling.** This comprises mutations in the genes encoding components of the neural circuits activated by leptin (see above). Examples are the obese agouti mice (Duhl et al., 1994) and the MC4R knock out mice (Farooqi et al., 2000).

**1.5 Other leptin functions**

There is a growing body of evidence that leptin also plays a role in modulating other responses and processes than regulation of body weight. Some of them will be discussed briefly in the remainder of this chapter. For a more detailed overview of the functions of leptin, we refer to chapters 8 and 9.
1.5.1 Leptin and immune responses

Initial experiments with mice illustrated that low leptin concentrations (like in ob/ob mice, or evoked by starvation) appear to correlate with impaired immune responses. Since administration of leptin in ob/ob mice normalised these immune dysfunctions, a direct role for leptin was implied (Howard et al., 1999; Faggioni et al., 2000). Likewise, rare mutations in leptin or in the leptin receptor also lead to immune deficiencies and to a high risk of mortality due to infectious diseases during early childhood (Clement et al., 1998; Ozata et al., 1999). Major observations from in vivo and in vitro experiments are summarized in Tables 2 and 3. The cellular targets of leptin in the immune system are: lymphocytes, natural killer (NK) cells, macrophages, and monocytes. This may help explain the altered cell-mediated immunity, the atrophy of the thymus, phagocyte function, and differences in cytokine and antibody production seen in human and rodents upon malnutrition.

<table>
<thead>
<tr>
<th>Cell-type</th>
<th>Effects of leptin in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>phagocytosis (Loffreda et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>production of GM-CSF and G-CSF (Gainsford et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>secretion of TNF-α, IL-6 and IL-12 (Loffreda et al., 1998)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>enhanced expression CD38, IL-2 receptor, and transferrin receptor (Santos-Alvares et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>secretion IFN-α, IL-6, IFN-γ (Zarkesh-Esfahani et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>elevated levels of IL-1 receptor antagonist (Gabay et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>secretion of IFN-γ-inducible protein IP-10 (Meier et al., 2003)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>proliferation of circulation blood T-lymphocytes (idem)</td>
</tr>
<tr>
<td></td>
<td>enhanced secretion of Th1 cytokines IL-2, IFN-γ, TNF-α and IL-18 (idem)</td>
</tr>
<tr>
<td></td>
<td>decreased production of Th2 cytokines IL-4, IL-5 and IL-10 (idem)</td>
</tr>
<tr>
<td></td>
<td>enhancement of anti-CD3 driven proliferation of naïve T-cells (Lord et al., 2002)</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2: Effects of leptin on immune cells**

These reports indicate that leptin has pro-inflammatory effects by supporting T-cell populations, shifting the T helper balance towards a Th1 phenotype, by inducing the release of pro-inflammatory cytokines, and by stimulating macrophage and natural killer cell function. This is in line with the observation by Heymsfield et al. that virtually all patients in a clinical trial showed signs of inflammatory reaction at the site of recombinant leptin injection (Heymsfield et al., 1999). Since cell-mediated immunity is
an energy-demanding process, leptin may provide an important link between the body's energy status and the immune system. This control mechanism thus may allow saving energy for other vital body functions in periods of malnutrition. Given its crucial role in regulating CD4 T-cell mediated immune responses, leptin plays a role in the onset and maintenance of T-cell controlled autoimmune diseases, like Crohn’s disease, rheumatoid arthritis, multiple sclerosis and type I diabetes (see also chapter 9).

<table>
<thead>
<tr>
<th>Cell-type</th>
<th>Observations in ob/ob and db/db mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>reduced phagocytic activity, administration of leptin restores this phagocytosis defect (Loffreda et al., 1998)</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>thymic atrophy (Faggioni et al., 2000, Madel et al., 1978, Chandra et al., 1980) reduced numbers of circulating lymphocytes (idem) protection from T-cell mediated hepatotoxicity (Faggioni et al., 2000)</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>decreased number of cells in liver, spleen, lung and blood (Tian et al., 2002) normal levels after leptin treatment (idem) suppression of the CD4+ NK population (Li et al., 2002)</td>
</tr>
</tbody>
</table>

1.5.2 Leptin and development

Given its production in placenta and expression of its receptor in a broad range of fetal tissues, a role for leptin in development was put forward (Hoggard et al., 1997; Masuzaki et al., 1997). The hormone may also be involved in the development of the brain: brains of ob/ob and db/db mice are smaller, exhibit structural neuronal defects, and impaired myelination (Bereiter and Jeanrenaud, 1979; Sena et al., 1985; Ahima et al., 1999; Steppan and Swick, 1999). Continuous administration of leptin greatly restores these defects (Ahima et al., 1999; Steppan and Swick, 1999).

1.5.3 Leptin and reproduction

Leptin appears to be essential for the onset of puberty since its administration accelerates puberty in wild type mice (Chehab et al., 1997), and restores fertility in ob/ob mice (Chehab et al., 1996).
1.6 References


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Chapter 7

The Ins and Outs of Leptin Receptor Activation

Zabeau L., Lavens D., Peelman F., Eyckerman S., Vandekerckhove J., and Tavernier J.

Minireview

The ins and outs of leptin receptor activation

Lennart Zabeau\(^1\), Delphine Lavens\(^1\), Frank Peelman, Sven Eyckerman, Joël Vandekerckhove, Jan Tavernier\(^*\)

The Flanders Interuniversity Institute for Biotechnology, Department of Medical Protein Research (VIB), Ghent University, Faculty of Medicine and Health Sciences, Baerlochnerlaan 3, B-9000 Ghent, Belgium

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Abstract The adipocyte-derived hormone leptin signals the status of body energy stores by activating its receptor in hypothalamic nuclei. In contrast to the initial expectations, leptin treatment of human obesity was largely unsuccessful. One explanation for this is the marked leptin resistance, which likely operates at the receptor level. The leptin receptor is a member of the class I cytokine receptor family, which uses the Janus kinase/signaling transducer and activator of transcription pathway as a major signaling route. In this review, we focus on the molecular mechanisms underlying leptin receptor activation. Different modes of leptin-induced clustering of the ectodomains and the subsequent signaling events will be discussed. © 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Leptin; Leptin receptor; Receptor clustering; Signal transduction

1. Introduction

Leptin and its receptor are essential components in the complex genetic wiring diagram underlying energy homeostasis and body weight. Spontaneous mutations in leptin or in its receptor result in marked obesity in both mice [1,2] and man [3,4]. Leptin is mainly produced in white adipose tissue [2] although expression has also been demonstrated in the fundus of the stomach [3] and in skeletal muscle [6]. The long, signaling-competent isoform of the leptin receptor (LR) shows high expression peaks in the feeding centers of the hypothalamus [7], consistent with leptin being the afferent signal informing the central nervous system of the body fat status. This concept is further supported by the observation that leptin-deficient (ob\(^{-/-}\)) mice and men can be successfully treated with leptin [8,9]. Leptin was therefore initially considered a miracle drug for treatment of obesity. However, obese people often have elevated leptin levels [10] and leptin administration showed only very limited effects [11]. Recent data have indicated that this is likely due to desensitization for the leptin signal, a phenomenon now often referred to as leptin resistance. This may be situated at least at two distinct levels: saturable transport of leptin across the blood–brain barrier, and abnormalities at the level of LR activation and/or signal transduction [12]. Besides its role via the central nervous system, leptin also has direct effects on a series of peripheral tissues, implying a much more complex leptin axis than was originally anticipated [13].

To date, six splice variants of the LR have been identified. The long isoform or Ob-Rb (further referred to as LR1l) consists of 1162 amino acids and is the only LR isoform with clearly demonstrated signaling capability. It is highly expressed in hypothalamic centers although expression at functional levels has also been demonstrated in a number of other tissues including liver, lung, testis, etc. Neuronal-specific ablation of Ob-Rb results in obesity, clearly indicating that the weight-reducing properties of leptin are exerted centrally [14]. Four short isoforms (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf; Ob-Ra will be further referred to as LRsh) with shortened intracellular tails have been identified. High expression levels of Ob-Ra and Ob-Rc can be found in choroid plexus and brain microvessels [7], suggesting their role in blood-brain barrier transport. This idea is further supported by observations in mouse models for obesity [15] and by the use of an in vitro leptin transport assay [16]. A secreted isoform can be generated either by alternative splicing (Ob-Re) or by ectodomain shedding, and may be involved in modulating leptin activity [17].

2. Structure and evolutionary relationships of the LR extracellular domain

The LR was first cloned from a mouse choroid plexus cDNA library using an expression cloning strategy by Tartaglia and co-workers [7]. Based on sequence homology, this receptor belongs to the class I cytokine receptor family, which typically contains a so-called CRH (cytokine receptor homol-
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ogy) domain in its extracellular domain. This structure consists of two barrel-like domains, each approximately 100 amino acids in length, which resemble the fibronectin type III (FN III) fold. Two conserved disulfide bridges are found in the N-terminal domain, while a WXXWS motif is characteristic for the C-terminal part. The LR shares highest sequence similarity with the granulocyte colony-stimulating factor (G-CSF) receptor and the glycoprotein 130 (gp130) family receptors, including gp130, the leukemia inhibitory factor (LIF) and oncostatin M (OSM) receptors. Moreover, structural superposition shows that also leptin is structurally most similar to G-CSF and cytokines of the gp130 family, such as interleukin-6 (IL-6).

Fig. 1A shows a consensus phylogenetic tree, based upon multiple sequence alignment of the membrane-proximal CRHs. The tree shown in Fig. 1 was calculated using the neighbor-joining distance method [18] with the Seqboot, Protdist and Neighbor programs of the PHYLYP package [19]. In this tree, the LR is most closely related to the OSM and LIF receptors, although the bootstrap support value for this evolutionary relationship is very low (40%). A parsimony tree, calculated using PAUP [20], shows an identical image, and supports the close evolutionary relationship between the LR and the LIF and OSM receptors (bootstrap support value 39%). A parsimony tree, calculated using the Seqboot and Protdist programs of the PHYLIP package [19] however, suggests that the LR would be more closely related to the G-CSF receptor and gp130 (bootstrap support value = 25%). This relationship is also clearly reflected in the overall architecture of the ectodomains of these receptors (Fig. 1B): besides a canonical CRH domain, the receptors for leptin, G-CSF, LIF, OSM and gp130 all contain an immunoglobulin-like (Ig-like) domain (Fig. 1B). The LR and LIF receptors have an additional N-terminal CRH module, the OSM receptor has a truncated N-terminal CRH module. While gp130 and the G-CSF, LIF and OSM receptors each contain three membrane-proximal FN III domains, the LR stands out, having only two.

Fig. 1. A: Protdist phylogenetic tree of leptin and other long-chain cytokine receptors, based on an alignment of the membrane-proximal CRH. Numbers under the branches indicate the percent bootstrap support values out of 1000 bootstraps. PRLR: prolactin receptor; GHR: growth hormone receptor; EpoR: erythropoietin receptor; OSMR: oncostatin M receptor; LIFR: leukemia inhibitory factor receptor; G-CSFR: granulocyte colony-stimulating factor receptor; CNTFRα: ciliary neurotrophic factor receptor; IL-6Rα: interleukin 6a receptor; IL-11Rα: interleukin 11 receptor. B: Schematic presentation of the overall structure of long-chain cytokine receptors. The different domains are represented by colored squares or ovals, as explained below the figure.
3. Models for leptin/LR complex formation

Fong and co-workers generated a panel of LR deletion and substitution mutants, and showed that the membrane-proximal CRH domain is necessary and sufficient for leptin binding. These authors further provide evidence that the two FN III domains have no affinity for the ligand, but nevertheless are essential for receptor activation [21]. Recently, we could define a critical role for the Ig-like domain in receptor activation. Receptors lacking this domain are properly expressed on the cell surface, and bind leptin comparable to the wild type receptor, but are unable to activate the associated Janus kinases (JAKs) and fail to generate a signal transducer and activator of transcription (STAT)-3-dependent signal (see below; Zibbau et al., submitted).

Like all other class I cytokines, leptin adopts a four helical bundle structure. These cytokines usually interact with their receptor with two binding sites located on the helical faces of helices D (site 1) and A and C (site II). A unique feature of G-CSF and of the gp130 family of cytokines is the presence of an additional binding site III, at the N-terminus of helix D, in one of the four helix bundle [22]. The function of this binding site III and of the Ig-like domain in the gp130 receptor systems was recently clarified by the crystal structure of the membrane-proximal CRH and Ig domain of gp130 in a 2:2 complex with Kaposi sarcoma herpesvirus IL-6 (vIL-6) [23]. This complex contains two copies of the gp130 fragment and of vIL-6. Each vIL-6 molecule interacts with two gp130 molecules by two interactions: binding site II in vIL-6 interacts with the CRH of one gp130 molecule, while binding site III in vIL-6 interacts with the Ig-like domain of a second gp130 molecule. This 2:2:2 tetrameric type of complex formation is very likely also a good model for the G-CSF/G-CSFR complex (Fig. 2A) [24]. Human IL-6 binds to its receptor in a 2:2:2 hexameric complex consisting of IL-6, IL-6Rα and gp130. In a generally accepted model, two human IL-6 molecules first bind to the CRH domains of dimeric IL-6Rα with their binding site I, and then bind to two gp130 receptor molecules with binding sites II and III [23,25] (Fig. 2B). It is tempting to speculate that similar clustering mechanisms also take place in the leptin/LR complex (Fig. 2C,D).

In gp130, the G-CSF receptor and the LIF receptor, the Ig-like domain interacts with binding site III of their cognate cytokine ligands. It is therefore very likely that a similar binding site III in leptin will interact with the Ig-like domain of the LR, which would help explain the deleterious effect of removal of this domain in the LR (Zibbau et al., submitted). Comparison with the IL-6 and G-CSF receptor systems suggests that leptin will bind its receptor either by its site I in the helical face of helix D, or by its site II in the helical faces of helices A and C. One of these sites probably interacts with the membrane-proximal CRH, as this constitutes the major leptin binding site [21]. Interactions between a binding site II in leptin and the membrane-proximal CRH were also suggested by molecular modeling of the leptin/LR complex [26,27].

Like the OSM and LIF receptors, LR contains an additional N-terminal CRH. In the LIF receptor, deletion of this CRH leads to a constitutively active receptor [28]. This is reminiscent of the constitutive activation seen in the fatty Zucker rat LR, which carries a mutation in this domain [29]. In the LIF receptor, this N-terminal CRH is able to interact with the cholinergic neurotransmitter receptor [30], suggesting a possible role for receptor/receptor interaction for the N-terminal CRH in the LR.

The membrane-proximal FN III domains of gp130 and of the LIF receptor interact with each other [31]. Although Devos et al. [32] showed that the membrane-proximal FN III domains are not necessary for dimerization of a soluble recombinant extracellular LR domain, similar interactions in the membrane-anchored LR cannot be excluded.

An important, unresolved question in LR biology is how LR10 can signal in the presence of excess LR3h. In many cell types, mRNA for the latter may account for up to 95% of all LR transcripts [33]. One possible explanation for this relative signaling insensitivity of LR10 to the effect of co-expression of dominant negative LR isoforms may be the formation of higher order clusters [29]. Based on the behavior of signal-

Fig. 2. Schematic representation of the complexes between cytokines and the extracellular domains of their receptors. A: G-CSF/G-CSF receptor 2:2 complex. B: IL-6/gp130/IL-6Rα receptor 2:2:2 complex. C: Model for a 2:2 leptin/LR complex. D: Model for a 2:4 leptin/LR complex.
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Fig. 3. LR signaling. The LR carries three conserved tyrosines in its cytoplasmic domain. JAK2, which associates membrane-proximally with the receptor, becomes activated upon ligand binding and phosphorylates these tyrosine residues. The membrane-distal Y1138 functions as a docking site for STAT3 which itself is a substrate of JAK2. Upon subsequent dimerization, it translocates to the nucleus and induces expression of SOCS3 and other genes. The membrane-proximal Y985 and Y1077 are involved in regulation and attenuation of the lepin signal. SOCS3 is taking part in a feedback loop inhibiting leptin signaling by binding to both tyrosines although binding affinity for Y1077 is much weaker. SHP2 is recruited to the Y985 position and activates the MAPK pathway through the adapter protein Grb-2, ultimately inducing c-fos expression. PTP1B is localized on the surface of the endoplasmic reticulum, and is also involved in negative regulation of LR signaling through dephosphorylation of JAK2 after internalization of the LRo complex.

4. Activation of the JAK/STAT signaling pathway

A general overview of cellular events following LR activation is shown in Fig. 3. Leptin signaling occurs typically through the JAK/STAT pathway (see above). After ligand-induced clustering, the LRo predominantly activates JAK2 [35], although JAK1 activation has also been demonstrated in some settings [36]. JAKs associate constitutively with a conserved box 1 motif, which is characterized by two essentially invariant prolines, in the class 1 cytokine receptors. Some receptors also contain an additional sequence, box 2, which is required for (maximal) JAK activation. In the murine LRo, a box 1 motif (intracellular amino acids 6-17) critical for JAK2 activation and a putative box 2 motif (intracellular amino acids 49-60) have been identified. The latter may be required for maximal activation [35,37].

The LRb has three conserved tyrosines in its cytoplasmic domain, which correspond in the murine receptor to positions Y985, Y1077 and Y1138. Y1138 is situated in a typical STAT3 recruitment or YxxQ motif, similar to motifs found in the gp130 family of receptors. After phosphorylation of this site, STAT3 is recruited via its SH2 (Src homology) domain. Activation, homo-dimerization and nuclear translocation of STAT3 will then lead to specific gene induction. The critical role of this site is underscored by the dramatic obese phenotype observed in knock-in mice containing a Y1138S mutation in LRb [38]. It is unclear whether STAT3 is the only STAT factor that is activated upon stimulation. Vaisse and colleagues could demonstrate the activation of STAT3, but not of other STAT factors in the hypothalamus of leptin-treated ob mice [39]. In cell lines however, STAT1 and STAT5 activation was also shown [40]. The fact that LRb Y1138S knock-in mice clearly have defects in body weight regulation but not in fertility, as opposed to mice lacking functional LRs, suggests alternative pathways, possibly via other STAT factors [38].

5. Role of phosphatases and of SOCS proteins

Mutation of the Y985 site in the receptor leads to enhanced signaling after leptin stimulation [36,41,42]. The Y985 site was identified as a recruitment site for the receptor-associated SH2-containing phosphatase-2 (SHP-2) [36,41]. It remains unclear whether negative effects are exerted via the phosphatase activity of SHP-2, or via the suppressor of cytokine signaling (SOCS)-3 protein, as it was later shown that this strong inhibitor of cytokine signaling also binds to the Y985 site [43,44]. SHP-2-dependent dephosphorylation of JAK2, how-
ever, also suggests an inhibiting function for SHP-2 in regulation of LIR1o signaling [45]. SOCS proteins act in a typical negative feedback loop: they are rapidly induced after cytokine stimulation, and directly inhibit the receptor via various mechanisms, including receptor targeting to the proteasome. Both SOCS1 and SOCS3 can inhibit leptin signaling, and the observation that SOCS3 expression levels are elevated in the lethal yellow (A/v/a) obese mouse strain makes it a potential mediator of leptin resistance in vivo [46]. SOCS3 gene transcription is very rapidly induced in vitro and in vivo after leptin treatment [46,47], and even serves as a marker to map leptin-responsive neurons in the hypothalamus [48].

The function of the highly conserved Y1077 site is still unclear, mainly because phosphorylation of this site remains to be demonstrated. Remarkably, the Y985 and Y1077 sites are highly similar, suggesting binding of common signaling molecules. The Y1077 site shows weak interaction with SOCS3 and can therefore have an additive effect in signal termination [44].

SHP-2 is proposed as a positive regulator of leptin signaling through mitogen-activated protein kinase (MAPK) activation. Docking to Y985 is followed by recruitment of the adapter protein growth receptor bound 2 (Grb-2) and activation of the Ras/Raf pathway. A secondary pathway for leptin-induced MAPK signaling, directly via JAK2, probably requires the phoshatase activity of SHP-2. The MAPK pathway is responsible for leptin-induced c-fos activation [49]. It is of note that in LIR1o signal transduction SOCS3 may also function as an adapter protein to other pathways [50].

Mice lacking PTPIB (phosphotyrosine phosphatase 1B) are hypersensitive to insulin and leptin and exhibit resistance to high fat diet obesity [51]. PTPIB recognizes a specific consensus substrate motif (E/D)pVYpY-R/KpX, which was identified in JAK2 and the kinase activation loop of the insulin receptor [52,53]. Over-expression of PTPIB resulted in hyperphosphorylation of endogenous JAK2 and blocked the leptin-induced transduction of endogenous SOCS3 and c-fos in a hypothalamic cell line. PTPIB appears to be a negative mediator of both the JAK/STAT and MAPK pathways in LR signaling and may be implicated in leptin resistance [54]. PTPIB is localized exclusively on the endoplasmic reticulum [55]. How PTPIB interacts with its substrates is not yet clear, although prior internalization of the receptor complex is suggested, especially since JAK2 has been detected at the endoplasmic reticulum [56].

6. Role of PI3 kinase

A strong correlation is assumed between the leptin and insulin signaling pathways since leptin and insulin resistance occur coincidentally in the majority of obese humans. Cross-talk between these pathways can be readily observed in various cell lines and in vivo. Phosphorylation of the insulin receptor substrates 1 and 2 (IRS1 and 2) as well as their interaction with Grb-2 and phosphatidylinositol 3-kinase (PI3K) show clear modulation by leptin in various hepatocytic cell lines [13,57]. Kim and colleagues performed a detailed in vivo study in rats showing STAT3 activation in insulin-responsive tissues after intraperitoneal injection of leptin. They also observed modulation of the PI3K and Grb-2 interaction with both IRS1 and IRS2 [58]. The apparent discrepancies described by various authors suggest that leptin and insulin signaling pathways interact in different ways depending on tissue type and cell line.

PI3K is also activated via IRS2 in the hypothalamus of rats, and appears to be crucial for the weight-reducing properties of leptin. Impaired PI3K signaling in peripheral tissues of obese individuals may also contribute to obesity-induced insulin resistance. A similar mechanism may explain the desensitization of leptin signaling in the hypothalamus, ultimately resulting in leptin resistance and obesity [59].

7. Concluding remarks

It is generally accepted that leptin plays a central role in regulating body weight. However, treatment of obesity using recombinant leptin seems to be only effective in individuals with a rare homozygous mutation in the gene for leptin or its receptor, or who exhibit subnormal secretion of the hormone, ruling out its current use as a generic drug. In most cases, obese humans have elevated leptin levels, indicative of leptin resistance. Unraveling the molecular mechanisms underlying this leptin resistance is therefore of great clinical interest. Given the complexity of the physiological processes controlling body weight, many defects may underlie leptin resistance. One possibility is a defect in the passage of leptin through the blood-brain barrier. It was suggested that the LR short isoform plays a role in this transport, but the precise mechanism is still a matter of debate and some evidence points to an hitherto uncharacterized leptin transporter in the brain capillary endothelium. Also, leptin resistance might result from a defect at the level of LR activation in the hypothalamic nuclei causing inappropriate sensing of the leptin levels. A better understanding of LR activation may help in understanding this, and may also provide a molecular explanation for the relative insensitivity of LR signaling in the presence of excess dominant negative LRSh receptors. Defects in LR signal transduction could be important as well, and in this light, negative regulators of signaling like SOCS3 and PTPIB are of special interest and may represent targets for the treatment of human obesity.

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References

Chapter 8

**Leptin: linking adipocyte metabolism with cardiovascular and autoimmune diseases.**

Peelman F., Waelput W., Iserentant H., Lavens D., Eyckerman S., Zabeau L., and Tavernier, J.

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8.1. Abstract

Leptin was originally discovered as an adipocyte-derived hormone involved in the central control of body weight and energy homeostasis. It is now clear that leptin is a pleiotropic cytokine, with activities on many peripheral cell types. These findings may help explain the surprising role of leptin in pathophysiological processes. Recent evidence suggests that leptin contributes to atherosclerosis and to the increased risk of cardiovascular disease in obese people. Leptin also appears to be involved in T-cell-dependent immunity and possibly in the development and maintenance of certain autoimmune diseases. Here, we review the role of leptin in cardiovascular and autoimmune diseases, and also briefly address the potential therapeutic use of leptin antagonists.

8.2. Introduction

Leptin (from the Greek leptos - meaning thin) was identified by positional cloning in 1994 [1] as a key molecule in the regulation of body weight and energy balance. Subsequent research revealed that leptin’s effects are not restricted to appetite and food intake demonstrating that leptin is a pleiotropic molecule with a broad variety of different biological actions, including reproductive function, regulation of the Hypothalamic – Pituitary – Adrenal (HPA) axis, glucose and insulin metabolism, lipolysis, sympathetic nerve activity, immune response, haematopoiesis and angiogenesis.
Leptin is a 167 amino acid secreted protein encoded by the ob gene. It is predominantly expressed by adipocytes and its plasma levels correlate well with the body fat mass [2,3]. The protein is comprised of four $\alpha$-helices and two short $\beta$-strands, containing an intra-chain disulfide bond necessary for its biological activity [4]. Adipocyte leptin expression is transcriptionally regulated, with the status of the energy stores in white adipose tissue and the adipocyte size as major determinants. In addition, leptin expression and serum levels increase after food intake. In contrast, leptin expression is rapidly suppressed with food restriction, exceeding the rate at which fat mass and adipocyte size is reduced [5-8].

Homozygous leptin null mutations in the ob/ob mice are associated with early-onset morbid obesity. Ob/ob mice weigh three times more than wild type mice and exhibit a fivefold increase in body fat content. In addition, affected mice exhibit hyperphagia, diabetes, hypothermia, hypercortisonemia, decreased linear growth and infertility [9]. Administration of recombinant leptin reduces body weight and normalizes all aspects of the obesity and diabetes syndrome and restores reproductive function in ob/ob mice [10-12]. Leptin ensures body energy homeostasis, and controls body weight and lipid storage by three different mechanisms: control of food intake, energy partitioning and energy expenditure [13]. Increased lipid content in adipocytes increases their leptin secretion, and elevated leptin levels appear to induce a switch from carbohydrate to lipid oxidation. Indeed, leptin has direct autocrine or paracrine effects on adipocytes and other cell types, inducing increased lipolysis and reduced fatty acid and triacylglycerol synthesis, as summarized in Table 1. A more detailed discussion is provided in references [14,15].

Human ob gene mutations are rare and were described in children from highly consanguineous parents [16,17]. The human phenotype is very similar to that of the mouse and is characterised by morbid obesity, hypogonadism, sympathetic system dysfunction, postural hypotension, mild hypothyroidism, hyperinsulinemia and alterations in growth hormone and parathyroid hormone-calcium function [17-19]. Human leptin deficiency is also associated with a low T-cell count and a severe
Leptin: linking adipocyte metabolism with cardiovascular and autoimmune diseases.

immune-deficiency as was described in a pedigree of a Turkish family where 7 out of 11 homozygous affected individuals died in childhood after an infectious episode [18].

<table>
<thead>
<tr>
<th>Effect</th>
<th>Cell type or tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipogenesis</td>
<td></td>
</tr>
<tr>
<td>inhibition of acetyl coenzyme A carboxylase</td>
<td>muscle, preadipocytes, aortic endothelial cells, adipocytes, liver, pancreatic islets</td>
</tr>
<tr>
<td>acetyl coenzyme A carboxylase mRNA ↓</td>
<td></td>
</tr>
<tr>
<td>fatty acid synthase mRNA ↓</td>
<td></td>
</tr>
<tr>
<td>glycerol-3-phosphate acyltransferase (GPAT) mRNA ↓</td>
<td></td>
</tr>
<tr>
<td>Lipolysis, fatty acid oxidation</td>
<td></td>
</tr>
<tr>
<td>hormone sensitive lipase mRNA ↑</td>
<td>adipocytes</td>
</tr>
<tr>
<td>acyl coenzyme A oxidase mRNA ↑</td>
<td>adipocytes, pancreatic islets</td>
</tr>
<tr>
<td>carnitine palmitoyl transferase-1 mRNA ↑</td>
<td>adipocytes, pancreatic islets, aortic endothelial cells, muscle</td>
</tr>
<tr>
<td>inhibition of acetyl coenzyme A carboxylase</td>
<td></td>
</tr>
<tr>
<td>→ malonyl-CoA decreases</td>
<td></td>
</tr>
<tr>
<td>→ disinhibition of carnitine palmitoyltransferase</td>
<td></td>
</tr>
<tr>
<td>→ increased fatty-acid oxidation</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Effects of leptin on different key enzymes of lipogenesis, lipolysis and fatty acid oxidation

The leptin receptor (LR) is encoded by the db gene and was first isolated from mouse choroid plexus by expression cloning [20]. It was identified as a member of the class I cytokine receptor family and binds leptin with nanomolar affinity. The LR shares highest sequence similarity with the granulocyte colony-stimulating factor (G-CSF) receptor and the glycoprotein 130 (gp130) family receptors, including gp130, the leukemia inhibitory factor (LIF) and oncostatin M (OSM) receptors [21,22]. The LR exists in several isoforms due to alternative splicing [20]. Five isoforms (originally termed Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Rf) have a same large extracellular domain, a transmembrane domain and a variable intracellular domain. Only the long form of the leptin receptor (Ob-Rb, further referred to as LRlo) is thought to mediate leptin signalling. This isoform is highly expressed in specific nuclei in the hypothalamus, known to be important in regulating body weight [20], but expression was also demonstrated throughout the whole body, including endothelial cells, CD4+ and CD8+ T-helper lymphocytes, monocytes, natural killer cells, pancreatic β-cells and enterocytes (reviewed in [23]). A short form of the leptin receptor (Ob-Ra, here further referred to as LRsh) is highly expressed in the choroid plexus and is thought to participate in transport of leptin from
the blood to the cerebrospinal fluid [24,25]. Transfection experiments suggested that Ob-Ra may also be involved in signal transduction [26], but no definitive demonstration of the signalling role of this receptor has been reported. Ob-Re is a soluble isoform that may modulate leptin activity (see below).

Leptin was originally considered as a good candidate drug for the treatment of obesity. However, obese people often have elevated leptin levels [2] and leptin administration showed only very limited effects [27]. This is quite reminiscent of insulin resistance in type 2 diabetes and may be explained by desensitisation of the leptin signal, hence the term “leptin resistance”. This phenomenon may be situated at least at two distinct levels: the system transporting leptin across the blood-brain barrier and LR activation and signalling.

Leptin’s regulatory function in the neuro-endocrine network controlling body weight and energy balance has been extensively reviewed elsewhere [28,29]. Here, we will discuss the role of leptin on peripheral cell types, with focus on the role of leptin in cardiovascular and autoimmune diseases.

**8.3. Leptin and the pathogenesis of cardiovascular diseases**

Obesity, and particularly, visceral obesity, is a key risk factor for cardiovascular disease. At present, it remains unclear whether obesity is an independent risk factor for cardiovascular disease or whether the connection is indirect and dependent on increased prevalence of hyperinsulinemia/insulin resistance, type 2 diabetes, hypertension, and dyslipidemia. The clustering of obesity with the abovementioned cardiovascular risk factors is often referred to as Metabolic Syndrome X, and in developed nations, this syndrome has a high prevalence and contributes strongly to morbidity and premature mortality [30]. The reasons for the clustering of these risk factors and the etiology of the syndrome remain unclear. High circulating leptin levels were shown to be an independent predictor of cardiovascular morbidity and mortality [31-33], and hyperleptinemia has been suggested to play a central role in Metabolic Syndrome X [34]. Below, we review how leptin can contribute to the increased
cardiovascular risk, either directly or indirectly, by influencing insulin resistance, type 2 diabetes, atherogenesis, ventricular hypertrophy and blood pressure.

8.3.1. Leptin and insulin resistance

In population-based studies, serum leptin concentrations are not only positively correlated with Body Mass Index (BMI), but also with fasting insulin concentrations [35]. In obesity, hyperinsulinemia and hyperleptinemia often coexist. In rats, overfeeding leads to insulin resistance and leptin resistance after just 3 days, and both resistances seem to be tightly coupled, and probably causally related [36]. It is clear that leptin influences the actions of insulin at various levels, and vice versa. Insulin influences leptin mRNA expression and increases leptin secretion by adipocytes [37,38]. While insulin has a general lipogenic effect, stimulating fatty acid and triacylglycerol synthesis and suppressing fatty acid oxidation, leptin has the exact opposite role and can inhibit insulin’s effects on lipid oxidation and synthesis [15,39,40]. Leptin can enhance insulin sensitivity and insulin’s inhibition of hepatic glucose production in rats [41,42]. In adipocytes, leptin inhibits insulin’s actions on glucose metabolism, by decreasing glucose transport and glycogen synthase activity [40]. Leptin has been shown to have anti-diabetic effects in several diabetic rodent models [43-45]. Insulin signalling decreases with excesses of intracellular lipid [46]. The net lipolytic effect of leptin in peripheral tissues might thus play a role in prevention of insulin resistance. Unger proposes that leptin’s net lipolytic effects may protect pancreatic β-cells against over-accumulation of intracellular lipids and subsequent lipo-apoptosis, thus reducing development of type 2 diabetes [47].

Several experiments in hepatocytes, muscle cells and pancreatic β-cells along with in vivo studies show intense overlap and marked redundancy in the signalling components recruited to both the leptin and insulin receptor systems. For more details, we refer to the section on leptin receptor signalling below.
8.3.2. Leptin and atherogenesis

In man, plasma leptin concentrations are independently associated with the intima-media thickness of the common carotid artery, an early atherosclerosis marker [48]. Elevations in leptin concentrations in adolescents are associated with decreased arterial distensibility [49]. Ob/ob mice are markedly resistant against diet-induced atherosclerosis [50]. Wild type mice on an atherogenic diet show increased leptin levels and develop enhanced neointimal wall thickening after carotid artery injury. The lesions show a high LR expression. Ob/ob mice do not show this diet-induced wall thickening despite the clear presence of atherosclerosis risk factors like diabetes, obesity and hyperlipidemia. However, wall thickening can be induced in ob/ob mice after leptin administration [51]. Leptin thus probably plays a direct role in the development of atherosclerotic plaques.

Leptin receptors are found on the endothelium [52], macrophages and foam cells [53] and on vascular smooth muscle cells [54]. Leptin seems to affect the function of each of these cell types, and probably plays a pro-atherogenic role in almost every step of atheroma formation, as summarized in Table 2.

<table>
<thead>
<tr>
<th>Cell type / event</th>
<th>Effect of leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cells</td>
<td>Induces endothelial cell migration by acting as chemoattractant</td>
</tr>
<tr>
<td></td>
<td>Increases secretion of VEGF in HUVECs and endothelial cell proliferation</td>
</tr>
<tr>
<td></td>
<td>Increases expression of matrix metalloproteinases</td>
</tr>
<tr>
<td></td>
<td>Increased accumulation of reactive oxygen species</td>
</tr>
<tr>
<td></td>
<td>Increased expression of monocyte chemotactant protein-1, attracting monocytes</td>
</tr>
<tr>
<td></td>
<td>Increased endothelin-1 expression</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>Stimulation of proliferation and migration of vascular smooth muscle cells</td>
</tr>
<tr>
<td></td>
<td>Increases expression of matrix metalloproteinases</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Increased lipoprotein lipase (LPL) expression</td>
</tr>
<tr>
<td></td>
<td>Increased hormone sensitive lipase expression in J774</td>
</tr>
<tr>
<td></td>
<td>→ Increased cholesterol ester hydrolysis</td>
</tr>
<tr>
<td></td>
<td>→ Decreased foam cell formation</td>
</tr>
<tr>
<td></td>
<td>Increased expression of acyl-CoA: cholesterol O-acyltransferase (ACAT) in J774</td>
</tr>
<tr>
<td></td>
<td>→ Increased cholesterol ester synthesis</td>
</tr>
<tr>
<td></td>
<td>→ Increased foam cell formation</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Increases IP-10 production → attracts activated T-cells</td>
</tr>
<tr>
<td>Neovascularisation</td>
<td>Promotes angiogenesis</td>
</tr>
<tr>
<td></td>
<td>Enhances neovascularisation and formation of capillary tubes</td>
</tr>
<tr>
<td>Calcification</td>
<td>Enhances calcification of calcifying vascular cells (in vitro)</td>
</tr>
</tbody>
</table>

Table 2: Effects of leptin on different cell types and events in atheroma formation
Leptin also contributes to arterial thrombosis after vascular injury, and its prothrombotic effects are probably mediated through the platelet LR [55].

### 8.3.3. Leptin and heart function

Leptin deficiency in the ob/ob mouse leads to ventricular hypertrophy, independent of body mass, and administration of leptin rapidly reduces the ventricular hypertrophy [56]. Clinically, increased myocardial wall thickness is seen in association with increased leptin levels [57], and possibly leptin resistance leads to ventricular hypertrophy. Leptin activates fatty acid oxidation and decreases triglyceride content in isolated working rat hearts [58]. This decreases the cardiac efficiency. Hyperleptinemia might thus lead to cardiac dysfunction, although it might also protect the heart against lipotoxicity [58].

### 8.3.4. Leptin and blood pressure

Leptin also has direct effects on blood pressure [59]. A positive correlation is found between mean blood pressure and leptin serum levels in lean subjects with essential hypertension [60]. The effects of leptin on blood pressure can vary between chronic and acute administration. Chronic intravenous injection of leptin in Sprague-Dawley rats increases their arterial pressure [61], while acute intravenous injection of leptin in sympathectomized rats decreases their arterial pressure [62]. Intracerebroventricular leptin administration in rats or in rabbits increases blood pressure through an increased lumbar and renal sympathetic nerve activity [63,64]. An observation that may help explain this apparent effect of leptin on blood pressure is that in vitro treatment of human umbilical vein endothelial cells (HUVECs) with leptin induces endothelin-1, a known vasoconstrictor [65].

### 8.4. A role for leptin in immune response and in autoimmune diseases

It is well established that genetic defects in the leptin / leptin receptor system in mice are associated with thymus atrophy [66,67], impairment of the Delayed-Type Hypersensitivity (DTH) reaction [68], a decreased number of circulating lymphocytes
[69], intraepithelial lymphocytes in the intestinal mucosa [70] and natural killer cells (NK) cells in the liver spleen, lung and peripheral blood [71]. Accordingly, leptin-deficient children have a low T-cell count and increased incidence of infectious diseases and associated mortality [18,66,69,72]. Recently, new insights into the immunologic actions of leptin have been provided by studies exploring the in vitro effects of leptin on different cellular targets including macrophages, monocytes, lymphocytes and NK cells (see Table 3). In vivo studies in experimental mouse model systems for human diseases further designate a determining role for leptin in the development and/or maintenance of autoimmune diseases like inflammatory bowel disease (IBD), rheumatoid arthritis (RA), multiple sclerosis (MS) and type 1 diabetes. For recent reviews, we also refer to Matarese et al. and Peelman et al. [73,74].

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Effects observed upon leptin stimulation</th>
</tr>
</thead>
</table>
| Peritoneal macrophages in mice                  | Increased phagocytosis  
Increased GM-CSF, G-CSF, TNF, IL-6 and IL-12 production                                               |
| Cultured human peripheral blood mononuclear cell| CD38, CD25, CD71 expression  
Secretion of IFN-alpha, IFN-gamma, IL-6  
Secretion of IP-10  
Secretion of IL1-R-antagonist                                                                   |
| Cultured mouse peripheral blood mononuclear cell | Secretion of IL1-R-antagonist                                                                       |
| Isolated intraepithelial lymphocytes of the intestinal mucosa | Increased IFN-y production                                                                         |
| Intestinal lamina propria mononuclear cells      | Decreased apoptosis                                                                                 |
| Cultured T-lymphocytes                          | Proliferation  
Shift toward T_{H}1 population with increased production of pro-inflammatory cytokines like IL-2, IFN-y, TNF-α and IL-18 and decreased T_{H}2 cytokines IL-4, IL-5 and IL-18 |

**Table 3: Effects of leptin on isolated immune cells in vitro**

In the dextrane sodium sulphate (DSS) model for IBD it was demonstrated that leptin deficient mice were protected against DSS-induced colitis in comparison with wild type mice. Intraperitoneal treatment with recombinant leptin restored the sensitivity to develop the intestinal inflammation. Similar results were obtained in the trinitrobenzene sulphonic acid (TNBS) colitis model where the intestinal inflammation is induced by rectal administration of the hapten TNBS [70].
Antigen-induced arthritis is a generally accepted model for RA and is established by administration of methylated bovine serum albumin (BSA) in the knees of mice. In this model, ob/ob and db/db mice develop less severe arthritis in comparison with wild type mice and display decreased interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in the knee synovial fluid and decreased serum levels of anti-methylated BSA antibodies. In ob/ob mice, a decreased antigen-specific T-cell proliferative response, with a lower interferon-γ (IFN-γ) and a higher IL-10 secretion, typical for a shift towards an anti-inflammatory T\textsubscript{H}2-type response is observed [75]. The increased leptin production in RA patients further argues for a role of leptin in the pathogenesis of RA [76]. In a group of ten RA patients it was shown that a 7-day fasting period results in a significant clinical improvement, accompanied by reduced serum leptin concentrations, reduced CD4+ lymphocyte activation and increased mitogen-induced IL-4 production [77].

The clinical onset of experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, in disease-susceptible C57BL/6J (H-2\textsuperscript{b}) and SJL/J (H-2\textsuperscript{s}) mice is preceded by an increase in serum leptin concentrations [78]. This increase is correlated with disease susceptibility. Acute starvation, which reduces serum leptin levels, delays disease onset and attenuates the EAE symptoms. Leptin-deficient C57BL/6J ob/ob mice are resistant against EAE. This resistance is abolished by administration of leptin, which is accompanied by a switch from a T\textsubscript{H}2 to T\textsubscript{H}1 pattern of cytokine release [79]. These data strongly indicate that leptin is a required factor for development of EAE.

Type 1 diabetes is an autoimmune disease, in which the pancreatic β-cells are destroyed by inflammatory processes. In the Non-Obese Diabetic (NOD) mouse, an animal model for type 1 diabetes, an increased serum level of leptin precedes diabetes in susceptible females, while injection of leptin accelerates the autoimmune destruction of the pancreatic β-cells and increases the IFN-γ production in peripheral T-cells, arguing that leptin promotes the development of type 1 diabetes through T\textsubscript{H}1 responses [80].
In addition to the above-mentioned diseases, leptin might also be involved in the pathogenesis of other human autoimmune diseases such as endometriosis or systemic lupus erythematosus. Endometriosis is an autoimmune disease with ectopic implantation of endometrial tissue outside the uterine cavity and musculature, a gynaecological disorder that can cause infertility in women. Matarese et al. found that leptin levels in serum and peritoneal fluid of 13 pelvic endometriosis patients are significantly elevated compared to 15 age- and BMI-matched controls. The leptin levels seem to be more elevated during early stages of endometriosis development [81]. The increased leptin levels could thus play a role in the inflammatory processes and angiogenesis associated with endometriosis. De Placido et al. found that the presence of peritoneal endometriosis, but not ovarian endometriosis increases leptin concentrations in the peritoneal fluid [82].

In 41 women with systemic lupus erythematosus, serum leptin levels are significantly higher than in an age-, sex- and BMI- matched control group [83].

8.5. The mechanism of action of leptin
8.5.1. The leptin / leptin receptor interaction
The single membrane spanning LR is a member of the class I cytokine receptor family. All members of this family are characterized by the presence of a so-called cytokine receptor homology domain (CRH), a combination of two barrel-like domains with a structure resembling that of fibronectin type III (FNIII) and immunoglobulin (Ig) folds (Figure 1). The LR contains two CRH domains. Other receptors containing two CRH modules are the βc chain (shared in the receptors for IL-3, -5, and granulocyte-macrophage colony stimulating factor), the LIF receptor and the thrombopoietin receptor. In the LR the membrane distal CRH1 and a membrane proximal CRH2 domain are separated by an Ig-like domain and followed by two FNIII domains (reviewed in [22]).

So far, relatively little is known on the precise mechanisms of activation of the LR. Fong and co-workers constructed a panel of LR deletion and substitution variants, and tested
these receptors for the ability to bind leptin and to signal in response to leptin. They showed that the membrane proximal CRH2 domain is sufficient and absolutely necessary for leptin binding [84]. Despite the lack of any affinity for the ligand, the FNIII domains are also needed for activation of the receptor. More recently, we could show that a LR deletion-variant, which lacks both CRH1 and Ig-like domains, is unable to activate the JAK kinases and therefore cannot generate a STAT3-dependent signal in response to leptin. We also could demonstrate that the membrane distal CRH1 is not strictly required for signalling, but allows optimal signalling [85].

Several lines of evidence suggest that the LR exists as a preformed (i.e. in the absence of the leptin ligand) complex. Chemical cross-linking and Western blot analysis indicate...
that the receptor forms dimers in solution and on the cell-surface [86,87]. Couturier et al. further extended these findings using a quantitative Bioluminescence Resonance Energy Transfer (BRET) approach. They could show that 60% of the receptors are expressed as dimers, and this dimerisation is not increased after addition of leptin [88]. It is therefore reasonable to assume that the LR becomes activated upon conformational changes, more than by a simple leptin-induced receptor oligomerisation. We recently showed that both the membrane proximal CRH2 module as well as the FNIII domains could be involved in this ligand-independent dimerisation [89].

A model whereby the LR becomes activated as a simple homodimer, as is the case for the erythropoietin (Epo) and growth hormone receptor, has been questioned. A higher order complex was first proposed by White and co-workers [90]. These authors showed that a signalling-deficient LR variant lacking almost the complete cytoplasmic tail is unable to efficiently repress signalling by LRlo. This is in strong contrast with observations using the related G-CSF receptor, and deletion variants thereof. We provided clear evidence for receptor oligomerisation using a JAK-STAT complementation strategy [85]. Two signalling-deficient receptors, one unable to activate the JAK kinases, the other lacking a functional STAT3 recruitment site, are only able to signal when they are co-expressed. Given the inherent requirements for JAK-STAT signalling (at least two receptors able to activate JAK, and one STAT recruitment site), the observed complementation can only be explained by higher order LR clustering. This complementation strategy also allowed us to demonstrate a non-redundant role for the different receptors in the activated complex. It is not unlikely that binding of leptin to a preformed LR homodimer results in the recruitment of a second homodimer thereby forming an activated receptor complex. The membrane distal CRH1 module may perhaps further increase the degree of clustering, since its presence enhances signalling. This formation of complex LR clusters might explain why LRlo signalling is only to a moderate extent sensitive to the presence of excess signalling deficient LR short forms.
8.5.2. Leptin receptor signalling

8.5.2.1. The JAK-STAT pathway

The JAK-STAT pathway plays a prominent role in leptin signalling. Leptin binding to the preformed receptor complex induces JAK2 activation in several cell systems [88,91,92]. Three tyrosines are conserved in the cytoplasmic domain of the LRlo, situated at positions Y985, Y1077 and Y1138 in the murine LR. The Signal Transducers and Activators of Transcription (STATs) are recruited to phosphorylated tyrosines through their SH2 (Src Homology 2) domain. In vivo, only STAT3 action was demonstrated in the hypothalamus of mice after leptin stimulation [93]. Observations in COS cells showed, besides STAT3, also leptin-induced activation of STAT1 and STAT5B when the latter were co-expressed with LRlo [94]. The membrane-distal tyrosine Y1138 was identified as a typical STAT3 docking site. After phosphorylation, activated STAT3 dimers translocate to the nucleus, leading to induction of leptin target genes [95]. The critical role of this tyrosine for STAT3 activation was confirmed by knock-in mice expressing a Y1138S mutated LRlo. The obese phenotype observed in these mice underscores an essential role for Y1138 and thus also for STAT3 in leptin-regulated energy homeostasis. However, infertility and other defects observed in the LR-deficient db/db mice are not found in the Y1138S knock-in mice, implying STAT3-independent LR signalling cascades [96].

8.5.2.2. Ras-Raf, PI-3K and other pathways

The SH2-containing tyrosine phosphatase SHP-2 is recruited to position Y985 in the activated LRlo. Although SHP-2 may exert an inhibitory function [97], negative regulation associated with position Y985 is mainly due to the activity of suppressor of cytokine signalling 3 (SOCS-3) [98]. SHP-2 may in fact positively regulate leptin signalling through activation of the MAPK pathway. To this effect, the adaptor Growth receptor bound protein 2 (Grb2) is recruited to phosphorylated and activated SHP-2, providing a physical link to the Ras-Raf pathway. In addition, an alternative pathway involving JAK2 and SHP-2 phosphatase activity but independent of LR tyrosines may exist, since phosphatase-inactive SHP-2 completely abolishes MAPK activation while
MAPK activity remains upon mutation of the intracellular tyrosines [99].

Coincidence of insulin resistance and obesity in humans suggests important interactions between leptin and insulin signalling. Discrepancies observed between separate studies likely reflect the use of different cell lines and experimental context, suggesting complex tissue-dependent interactions between the two pathways. Leptin appears to have a strong influence on insulin-dependent activation of IRS-1 and -2 (insulin receptor substrate 1 and 2) and their downstream signalling molecules Grb2 and phosphatidylinositol 3-kinase (PI-3K) [100-102]. Neuronal PI-3K is activated through IRS-2 in the hypothalamus of rats upon leptin stimulation and appears to be essential for its weight-reducing properties. The mechanism by which hypothalamic PI-3K dysfunction leads to leptin resistance may be reminiscent of the role of peripheral PI-3K in insulin resistance [103,104].

Invasiveness of colonic and kidney epithelial cells may also be modulated by leptin. Thus, leptin resistance in obese individuals may lead to a higher risk of fatal colon and other cancers. Rho A is essential in this signalling cascade and other factors like JAK2 and PI-3K may also be involved [105]. Migration of endothelial cells and angiogenesis is promoted by leptin via activation of the PI-3K pathway and its downstream Akt target [106]. Leptin induces ROS (reactive oxygen species) as a signalling messenger in vascular endothelial cells, activating the JNK pathway and the transcription factors NF-κB and AP-1. Elevated plasma leptin levels associated with obesity may thus generate chronic oxidative stress that may also contribute to the development of vascular pathology [107].

8.5.2.3. Down modulation of leptin signalling
Leptin resistance is likely situated at various levels in the leptin pathway including attenuated signalling via the leptin receptor in the hypothalamus. Inhibitors of signal transduction can therefore, in theory, contribute to leptin resistance.
Protein Tyrosine Phosphatase-1B (PTP1B) knockout mice show leptin and insulin hypersensitivity and consequently do not develop an obese phenotype when subjected to a high fat diet [108]. Moreover, while leptin-deficient mice suffer from severe obesity, mice lacking leptin as well as PTP1B show attenuated weight gain and increased sensitivity to administered leptin [109]. Leptin-dependent JAK-STAT signalling is down regulated by PTP1B, as shown by dephosphorylation of JAK2 and decreased leptin-induced expression of SOCS-3. Reduced c-fos expression suggests that PTP1B also inhibits the MAPK pathway [110]. PTP1B thus appears involved in leptin resistance and has emerged as a promising therapeutic target for obesity [111]. The fact that PTP1B is also involved in insulin signalling [112] again underscores the cross wiring and redundancy in these two pathways.

How PTP1B precisely exerts its dephosphorylating function is yet to be clarified. Internalisation of the receptor complex is probably required since PTP1B is situated on the cytoplasmic side of the endoplasmatic reticulum [113]. Detection of JAK2 and several cytokine receptors near the ER support this model [114,115].

SOCS proteins, a family of potent inhibitors of cytokine signalling, carry a highly conserved C-terminal SOCS-box and in some cases a KIR (kinase inhibitory region) domain. The former may help SOCS proteins in targeting signalling proteins to the proteasome for degradation, while the latter, present in SOCS-1 and SOCS-3, is able to directly inhibit JAK kinase activity - possibly by acting as a pseudosubstrate. SOCS-1 directly binds to JAKs, while SOCS-3 has a much lower affinity for the kinase but binds the receptor in close proximity to the JAKs [116,117]. In some settings, SOCS proteins may also enhance specific signalling pathways. SOCS-3, for example, positively modulates the Ras pathway after IL-2 stimulation [118]. For a recent review on SOCS proteins, we refer to Krebs et al. [117]

SOCS-3 is rapidly up regulated both in vitro and in vivo in response to leptin stimulation [95,119], reflecting its role in a typical negative feedback circuit of leptin receptor signalling. SOCS-3 was shown to be implicated in desensitisation of IL-6 signalling.
If the same holds true for the leptin pathway in the hypothalamus, SOCS-3 may be a causal factor in the development of leptin resistance and obesity. SOCS-3 is recruited to the membrane proximal Y985 in the LR and accordingly, mutation of this tyrosine results in augmented signalling after leptin stimulation. Both Y985 and Y1077 are embedded in highly conserved motifs of great resemblance. Although phosphorylation of Y1077 is yet to be shown, this site can probably recruit similar signalling molecules. Indeed, weak association of SOCS-3 with Y1077 was demonstrated [121].

It is of note that dietary fat can affect leptin receptor signalling in the hypothalamus. [122]. Fatty acids can also downregulate leptin dependent JAK-STAT signalling in pancreatic β-cells [123].

### 8.6. Strategies to antagonise leptin activity

Given the likely role of leptin in important pathogenic processes, it will be interesting to evaluate whether antagonising its activity could result in therapeutic benefit, e.g. in autoimmune or cardiovascular diseases. In this section we will briefly discuss some strategies.

A first possibility is the use of the secreted leptin receptor (sLR). Such receptor variant occurs naturally as a consequence of alternative splicing and/or proteolytic cleavage [124-127]. For several cytokines, secreted receptor variants have been shown to act as antagonists in vitro and in vivo. An example is etanercept: a fusion protein comprising the extracellular part of the TNF receptor and the constant region of human IgG1, which is currently used for treating rheumatoid arthritis and psoriatic arthritis [128]. At first sight, this also appears a viable strategy to counter the undesired effects of leptin. The precise in vivo role of the sLR, however, remains unclear. Increasing evidence suggests a role as a buffering system that controls the amount of bio-available leptin [129,130]. In morbidly obese subjects, leptin levels are high while sLR levels are decreased. After surgically induced weight loss, the concentration of leptin decreases
and sLR levels increase till they reach normal values. Since leptin bound to the sLR has a longer half-life, this could be explained by a feedback mechanism wherein the sLR aims to reduce the increase in leptin levels [130]. Consequently, sLR administration might result in either leptin inhibition or, adversely, in extended leptin half-life in circulation, depending on its effective concentration. To tip this subtle balance towards leptin inhibition, it is likely that the affinity of the receptor for its ligand will have to be artificially increased to assure irreversible leptin binding. A comparable strategy was recently developed for cytokines using multi-component receptor systems, whereby sufficient ligand binding affinity was obtained with “cytokine traps” [131].

Successful therapeutic intervention in cytokine systems can be achieved by the use of humanised monoclonal antibodies (mABs), since they combine high affinity binding and high specificity. Given the fact that leptin research was so far primarily focussed on weight regulation, little attention has been given to the development of anti-leptin or anti-leptin receptor mABs. It can be anticipated that the recent observations supporting a causal role for leptin in autoimmune and cardiovascular diseases may spur interest in the development of such mABs.

Modulation of leptin’s activities might also be achieved through the use of leptin muteins with antagonistic properties. For practical use, such a molecule should be able to bind the leptin receptor complex with high affinity, but no subsequent activation of the complex may occur. One candidate human leptin mutant, R128Q, has been described [132]. This R128Q mutant causes weight gain when injected in normal mice [132] and induces an increased sensitivity to the lethal effect of TNF (which is also seen in ob/ob and db/db mice)[133], demonstrating its antagonistic effects in vivo. Using molecular modelling in combination with site directed mutagenesis, we recently mapped the interaction sites between leptin and its receptor [134]. It appears that there are two binding sites: a primary, high affinity binding site interacting with the CRH2 domain of the LR, and a secondary site which is not involved in high affinity binding. The latter is however strictly required for activation of the receptor, by interacting with the Ig-like domain of another LR subunit. Introducing disruptive mutations in this secondary
binding site generates leptin muteins that cannot activate the receptor, while their high affinity binding to the LR remains unaffected. These muteins show no receptor activation, even at very high doses - in contrast to the R128Q mutant. In fact, R128 is not part of either receptor binding site. The effect of the R128Q mutation is therefore probably due to indirect disturbing of the secondary site. Injection of an antagonistic leptin mutein results in weight gain in mice [134]. Another type of leptin-derived antagonist was reported by Gonzalez and Leavis [135]. The synthetic peptide LPA-2, corresponding to residues 70-95 of leptin, also binds with high affinity to the LR and blocks LR activation in vitro. Since administered recombinant leptin has a half-life of less than two hours [136], it is reasonable to assume that leptin muteins suffer from rapid clearance in vivo. To overcome this problem, strategies to extend their half-life, e.g. by PEGylation, hyperglycosylation or by the use of fusion proteins, should be employed.

The use of protein-based anti-cytokine therapeutics has inherent disadvantages, and small molecules are preferred by the pharmaceutical industry. Small organic molecules can be orally administered, do not evoke an immune response and offer the possibility of easy and low-cost manufacturing. All the abovementioned protein-based antagonists target the extracellular leptin / leptin receptor interaction. Although strategies to select and optimise compounds capable of disrupting such interactions characterised by high affinity binding due to large interacting protein surfaces are constantly being refined [137,138], targeting intracellular proteins may offer an interesting alternative.

It should be stressed that any (small) antagonistic molecule should not cross the blood-brain barrier (BBB): the main effects of leptin regarding food intake and energy expenditure occur at the hypothalamic level, while most of the effects on the immune and vascular systems are mediated directly by receptors on peripheral target cells. Although it cannot be put so extreme that body weight control occurs only centrally and immune effects only peripherally, it clearly will be beneficial to specifically target leptin’s peripheral effects.
8.7. Concluding remarks

The discovery of leptin a decade ago incited research on the adipose tissue, which is now considered to function as an endocrine organ rather than a simple fat storage system. Besides leptin, adipocytes were found to produce many other hormones or cytokines including adiponectin, IL-6 and TNF-α, with roles in metabolism, reproduction, cardiovascular function and immunity. Like these other cytokines, leptin also appears to be a very pleiotropic cytokine with many effects on peripheral tissues and affecting many different processes including reproduction, immune response, blood pressure homeostasis and insulin action. Leptin secretion levels correlate with adipocyte mass and lipid load and leptin therefore provides an interesting link between obesity and pathophysiological processes such as insulin resistance and atherosclerosis, and disorders such as autoimmune and cardiovascular diseases and the metabolic syndrome. Increased serum leptin levels in obesity and metabolic syndrome may well fit into the view that these disorders are in fact low-grade systemic inflammatory diseases, characterized by increased concentrations of pro-inflammatory cytokines like IL-6, TNF-α and leptin. Its pro-inflammatory role suggests that leptin may link energy homeostasis to the immune system. Indeed, adequate energy stores may be required to generate an appropriate immune response against pathogens; adversely, high nutrition stores (reflected in elevated leptin levels) may support long-term, detrimental immune reactions such as those observed in autoimmune diseases.

There is increasing evidence that leptin contributes to the development of cardiovascular and autoimmune diseases. The observation that leptin-deficient mice are protected against several autoimmune diseases and against atherosclerosis supports the notion that a strategy aimed at blocking leptin’s peripheral actions on immune cells or on the vascular wall could have therapeutic benefit. Importantly, a major challenge will be to uncouple the peripheral and central, weight controlling actions of leptin. The requirement for leptin transport across the blood-brain barrier may offer a unique opportunity for the development of such a selective leptin antagonist.
8.8. Acknowledgements

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8.9. References

Leptin: linking adipocyte metabolism with cardiovascular and autoimmune diseases.

Leptin: linking adipocyte metabolism with cardiovascular and autoimmune diseases.


Chapter 9

Leptin, immune responses and autoimmune disease. Perspectives on the use of leptin antagonists

Peelman F., Iserentant H., Eyckerman S., Zabeau L., and Tavernier, J.

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9.1. Abstract

The pivotal role of leptin in regulating body weight and energy homeostasis is very well established. More recently, leptin also emerged as an important regulator of T-cell-dependent immunity. Reduced leptin levels, as observed during periods of starvation, correlate with an impaired cellular immune response, whereby especially the T\textsubscript{H}1 pro-inflammatory immune response appears to be affected. Physiologically, this could reflect the high energy demand of such processes, which are suppressed in animals or people with nutrient shortage. Several autoimmune diseases are T\textsubscript{H}1 T-cell dependent. In line with a pro-inflammatory role for leptin, animal models of leptin deficiency are markedly resistant to a variety of T-cell dependent autoimmune diseases. Here, we review the role of leptin in immune responses, with emphasis on autoimmune diseases. The design and potential use of leptin antagonists is also discussed.

9.2. Introduction

Mice carrying the ob/ob mutation suffer from a complex syndrome primarily characterised by severe obesity and endocrinological disorders. In 1994, Friedman and co-workers identified leptin as the product encoded by the ob gene [1]. Leptin is 167 amino acids long and elucidation of its structure revealed it as a $\alpha$-helical-bundle

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cytokine [2]. Other members of this large cytokine family include growth hormone, interleukins such as interleukin-6 (IL-6) and most colony stimulating factors. Leptin is predominantly produced by adipocytes, and its circulating level positively correlates with white adipose tissue mass. As part of a feedback mechanism, leptin regulates energy expenditure and food intake by activating its receptor in certain nuclei of the hypothalamus. Leptin administration to ob/ob mice increases basal metabolism and reduces food intake, leading to a marked rapid weight loss [3-5].

Leptin mediates its effects upon binding and activation of its oligomeric leptin receptor (LR) complex [6]. This receptor is composed of a single subunit encoded by the db gene and belongs to the class I cytokine receptor family [7,8]. Thus far, at least five isoforms of the LR generated by alternative mRNA splicing have been recognised: one long (LRb or LRlo), three short (LRa, -c, and -d), and one secreted (LRe) LR variant. The LRlo has a predicted 306 amino acids long intracellular domain in man and is the only isoform capable of efficient signalling [9,10]. Besides its crucial role via hypothalamic neurons, substantial expression of the LRlo is also observed on other cell-types [11]. A second isoform, LRa, is a variant lacking most of the cytosolic domain. This LR short form (LRsh) is much more widely expressed, often at higher levels compared to LRlo, e.g. in the choroid plexus, kidney, lung, and liver [12].

Since leptin injection leads to rapid weight loss in ob/ob mice, it was initially speculated that leptin could be used to treat obesity in humans. Contrary to ob/ob mice however, most cases of human obesity are not due to the absence or lowered levels of leptin. In fact, the reverse is true: in most overweight individuals, serum leptin levels are elevated, indicating inappropriate leptin sensing. This so-called leptin resistance may be caused by impaired blood-brain barrier (BBB) transport of leptin and/or defects at the LR activation level or further down in the signal transduction or effector pathways [13-16].

Besides its evident role in regulating body weight, it has become clear in the past few years that leptin also has important activities on various peripheral cell types, thereby
regulating reproduction, haematopoiesis and the immune system. This is reminiscent of the pleiotropic nature characterising most α-helical bundle cytokines. In this review, we focus on recent insights in the immune functions of leptin.

9.3. Leptin and immune responses

The role of leptin on the immune system was initially recognised from in vivo observations in the leptin- or LR-deficient ob/ob and db/db mouse strains and from a series of in vitro experiments, demonstrating an altered immune phenotype. Since administration of leptin in ob/ob mice normalised these immune dysfunctions, a direct role for leptin was implied [17,18]. Along the same line, low leptin concentrations evoked by starvation appear to correlate with impaired immune responses in mice. Similar findings have been reported in man, and the rare mutations in leptin or in the leptin receptor also lead to immune deficiencies and to a very high mortality due to infectious diseases during early childhood [19,20]. As discussed below, these studies have documented a complex immuno-regulatory role of leptin by concerted activities on different cellular targets: macrophages, monocytes, lymphocytes and natural killer (NK) cells. This may help explain the altered cell-mediated immunity, phagocyte function, cytokine and antibody production and the atrophy of the thymus seen in human and rodents upon malnutrition.

Cultured peritoneal macrophages from ob/ob mice display a lower phagocytic activity, compared to macrophages of normal mice. Administration of leptin restored this phagocytosis defect [21]. In vitro, leptin induces phagocytosis of parasites by intraperitoneal LRlo-expressing macrophages [22]. Furthermore, the production of granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) [22] and the pro-inflammatory cytokines Tumor Necrosis Factor α (TNF-α), IL-6 and IL-12 [21] by murine macrophages is enhanced after treatment with leptin.

Human monocytes express both the LRsh and LRlo isoforms [23,24]. In vitro, human peripheral blood mononuclear cells (PBMC) express the early activation marker (CD38),
interleukin-2 receptor (CD25) and the transferrin receptor (CD71) [25], and secrete interferon α (IFN-α) IL-6 and IFN-γ when cultured with leptin [24]. Leptin-stimulated PBMCs secrete elevated levels of the IL-1Receptor antagonist (IL-1Ra), an acute-phase protein with anti-inflammatory properties by virtue of blocking IL-1α and IL-1β activity [23,26]. This observation is consistent with the fact that the weight-reducing properties of leptin depend in part on IL-1 activity, and a role for IL-1Ra in leptin resistance has been suggested [27]. Luheshi et al. showed that injection of IL-1Ra in cerebral ventricles could inhibit leptin-induced thermogenesis and feeding reduction [28]. Leptin also stimulates the secretion of the IFN-γ-inducible protein IP-10 in a human monocytic cell line, as well as in PBMCs [29]. This IP-10 protein acts as a chemo-attractant for lymphocytes and monocytes [30], recruiting activated T-cells to the site of inflammation [30,31].

Ob/ob and db/db mice display clear defects in the lymphoid compartment: they suffer from thymic atrophy and have reduced numbers of circulating lymphocytes [32-34]. Impaired T-cell immunity in these mice pointed to a direct effect of leptin on T-lymphocytes. This was substantiated by the demonstration of functional expression of LRlo on CD4+ as well as on CD8+ T-cells [35,36]. In vitro, leptin enhances proliferation of circulating blood T-lymphocytes in a dose-dependent manner [35,36]. Importantly, leptin appears to affect the T helper (T<sub>H</sub>) subsets, tipping the balance towards the T<sub>H1</sub> subtype: it stimulates production of the T helper T<sub>H1</sub> pro-inflammatory cytokines IL-2, IFN-γ, TNF-α and IL-18, with concomitant decreased production of the T<sub>H2</sub> cytokines IL-4, IL-5 and IL-10 [35,36]. These effects are not seen with T-lymphocytes from db/db mice, supporting the notion that this effect directly involves the LR, expressed on the T-lymphocytes. More recently, it was shown that leptin inhibits anti-CD3 driven proliferation of memory T-cells, while that of naïve T-cells was significantly enhanced [37]. Faggioni et al. showed that ob/ob mice are protected from T-cell mediated hepatotoxicity. This effect apparently depends on the leptin-mediated production of the pro-inflammatory IL-18 and TNF-α cytokines [32]. We further elaborate on the effect of leptin on the T<sub>H</sub>-balance in the section below.

Leptin also seems to be a regulator of NK cell development and activation. Db/db mice
show decreased numbers of NK cells in the liver, spleen, lung and peripheral blood, and leptin administration increases the basal or induced lysis of splenocytes in normal mice, but not in db/db mice [38]. Similarly, the hepatic CD4+ NK T-cell population is suppressed in ob/ob mice. This effect is possibly a consequence of altered production of IL-12 and IL-15 by Kupffer cells [39].

Taken together, an extensive dataset indicates that leptin has pro-inflammatory effects by supporting T-cell populations, shifting the T helper balance towards a TH1 phenotype, by inducing release of pro-inflammatory cytokines, and by stimulating macrophage and natural killer cell function. This is in line with the observation by Heymsfield et al. that virtually all patients in a clinical trial showed signs of inflammatory reaction at the site of recombinant leptin injection [40]. On the other hand, the induction of IL-1Ra by leptin suggests an anti-inflammatory role. Elevated IL-1Ra levels may help explain the protective effect by leptin against normally lethal doses of LPS or TNF-α [26,41]. Also, inflammatory agents and pro-inflammatory cytokines such as IL-1β and TNF-α stimulate leptin secretion [42-46]. For a more detailed review of the crosstalk between leptin and pro-inflammatory cytokines, we refer to Waelput et al. [47].

Leptin appears to be part of the complex network that coordinates immune responses to various stimuli. Its unique contribution may lie in integrating the body's energy status and thus adjusting the immune response to an appropriate level. Indeed, cell-mediated immunity is an energy-demanding process, and impairment of this immunity during starvation may save energy necessary for vital body functions. Such crosstalk between energy homeostasis and the immune system appears to be bi-directional. This is illustrated in IL-6−/− mice, which develop an obese phenotype with increased leptin levels and decreased responsiveness to leptin [48].

9.4. Leptin and autoimmune disease

Leptin apparently plays a role in CD4+ T-cell mediated immune responses, promoting a pro-inflammatory TH1 T-cell phenotype. Evidence is accumulating that leptin also plays
a determining role in the development and/or maintenance of CD4+ T-cell-mediated autoimmune diseases including Crohn’s disease, rheumatoid arthritis, multiple sclerosis and type I diabetes.

In experimental mouse model systems for human inflammatory bowel disease (Crohn’s disease), where acute and chronic colitis is induced by dextran sulphate sodium or trinitrobenzene sulphonic acid, leptin-deficient ob/ob mice show a 72% reduction of colitis severity and a marked decrease of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-18, IL-6) in colon cell culture supernatants, compared to wild-type mice. Administration of leptin in ob/ob mice abolishes this resistance against experimentally induced colitis [49]. Leptin directly stimulates intraepithelial lymphocytes (IEL) and lamina propria mononuclear cells (LPMC). In ob/ob mice, the number of IELs is reduced and IELs show a decreased IFN-γ secretion, while the LPMCs of ob/ob mice show increased apoptosis [49]. In another murine model of inflammatory bowel disease, administration of Clostridium difficile toxin A induces severe colitis. Ob/ob as well as db/db mice are partially protected against the toxin A induced intestinal secretion and inflammation. Again, leptin administration in ob/ob, but not in db/db mice reverses this protection [50]. Finally, Barbier et al. showed that plasma leptin concentrations rise during early stages of experimental intestinal inflammation in rats [51].

Administration of methylated bovine serum albumin (BSA) in the knees of mice leads to the development of antigen-induced arthritis. As compared to wild type mice, ob/ob and db/db mice develop less severe arthritis, with decreased IL-1β and TNF-α in the knee synovial fluid and decreased serum levels of anti-methylated BSA antibodies. In addition, a decreased antigen-specific T-cell proliferative response, with a lower IFN-γ and a higher IL-10 secretion, typical for a shift towards an anti-inflammatory T_{H2}-type response is also observed [52]. These data may also suggest a role for leptin in the development of rheumatoid arthritis in man. Bokarewa et al. found that leptin production is significantly increased in rheumatoid arthritis patients, compared to controls. These authors found lower leptin levels in synovial fluid, compared to plasma
and suggested consumption of leptin in the synovium [53]. Clinical trials, where ten rheumatoid arthritis patients underwent a 7-day fast showed that this fasting leads to significant clinical improvement, accompanied by reduced serum leptin concentrations, reduced CD4+ lymphocyte activation and increased mitogen-induced IL-4 production [54].

The clinical onset of experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, in disease-susceptible C57BL/6J (H-2b) and SJL/J (H-2s) mice is preceded by an increase in serum leptin concentrations [55]. This increase is correlated with disease susceptibility. Acute starvation, which reduces serum leptin levels, delays disease onset and attenuates the EAE symptoms. Leptin-deficient C57BL/6J ob/ob mice are resistant against EAE. This resistance is abolished by administration of leptin, which is accompanied by a switch from a T\textsubscript{H2} to T\textsubscript{H1} pattern of cytokine release [56]. Interestingly, the same group also provides evidence for an autocrine effect of leptin on the proliferation of activated T-cells. Together, these data strongly indicate that leptin may be required for development of EAE, and thus, possibly also for multiple sclerosis in man.

Type 1 diabetes is an autoimmune disease, in which the pancreatic β-cells are destroyed by inflammatory processes. In the Non-Obese Diabetic (NOD) mouse, an animal model for type 1 diabetes, an increased serum level of leptin precedes the diabetes in susceptible females, while injection of leptin accelerates the autoimmune destruction of the pancreatic β-cells and increases the IFN-γ production in peripheral T-cells, indicating that leptin promotes the development of type 1 diabetes through T\textsubscript{H1} responses [57].

Endometriosis is an autoimmune disease with ectopic implantation of endometrial tissue outside the uterine cavity and musculature, a gynaecological disorder that accounts for infertility in 10 to 15% of women of reproductive age. Matarese et al. found that leptin levels in serum and peritoneal fluid of 13 pelvic endometriosis patients are significantly elevated compared to 15 age- and body mass index-matched controls. The leptin levels
seem to be more elevated during early stages of endometriosis development [58]. The increased leptin levels could play a role in the inflammatory processes and angiogenesis associated with endometriosis. De Placido et al. found that the presence of peritoneal endometriosis, but not ovarian endometriosis increases leptin concentrations in the peritoneal fluid [59].

Garcia-Gonzalez et al. reported that serum leptin levels in 41 women with systemic lupus erythematosus are significantly higher than in an age-, sex- and body mass index-matched control group [60].

Autoimmune diseases affect women more than men: in multiple sclerosis and rheumatoid arthritis, 60 to 75% of the patients are female, while in other autoimmune diseases (thyroiditis, scleroderma, lupus erythematosus, Sjögren’s disease), 85% or more of the patients are female [61]. This gender effect may reflect the higher average leptin concentrations in women [62]. Matarese et al. propose that leptin helps to determine the balance between predisposition to infections and predisposition to autoimmune diseases: higher circulating leptin levels predispose to autoimmune diseases, and lower circulating leptin levels to infection. This could help explain why autoimmune diseases show an increasing incidence in industrialised countries, and a decreasing incidence in less-developed countries [63].

9.5. Strategies towards antagonising leptin

As detailed above, a growing body of evidence points towards a regulatory role for leptin in the development and/or maintenance of autoimmune diseases. To further corroborate this association, and perhaps even as a future therapeutic tool, there is a clear need for leptin antagonists. Several strategies towards the development of such antagonists can be envisaged and will be discussed below.

A major challenge, and opportunity, in the design of a successful leptin antagonist will be to uncouple central and peripheral effects. This would allow interfering with the adverse effects of leptin on the immune system in case of autoimmune diseases, without concomitant activation of the metabolic feedback loop inevitably leading to
considerable, unwanted weight gain. The central, hypothalamic function of leptin depends on its ability to cross the blood-brain barrier (BBB). This occurs via a specific and saturable transport system [13,64], which is mostly studied in the context of leptin resistance. In this condition, inappropriate sensing of circulating leptin levels occurs, possibly by impaired leptin transport over the BBB. The LRsh isoform is likely involved in leptin transport [65-67]. Interestingly, recent evidence indicates that the LRsh could function as a modulator and not as the main transport protein [68]. This may open strategies towards the design of leptin (receptor) antagonists incapable of passing the BBB. It is beyond the scope of this review to detail the possible ways to modulate BBB passage of therapeutic polypeptides or organic molecules. For recent reviews on this subject, we refer to Misra et al. [69], and specifically for leptin, to Banks et al. [70].

9.5.1. Leptin receptor variants

The level of circulating cytokines and hormones is tightly regulated. One mechanism relies on the expression of secreted receptor variants. For several pro-inflammatory cytokines, such receptors seem to act as natural antagonists. Examples for which this is amply proved include members of the IL-1 and TNF families. The prototype members IL-1α and β and TNF-β exert well-described immune functions, and for each there exists at least one soluble receptor isoform, the soluble type II IL-1 receptor (IL-1sRII) and soluble TNF receptor (sTNF-R) 1 & 2 respectively. These receptors appear to function as natural scavengers of their cognate ligand, which they prevent from binding to their membrane bound receptors and thus from generating a biologic response (reviewed in [71,72]). The ability of soluble receptors to act as cytokine inhibitors, in combination with their specificity, makes them very interesting for use as immunotherapeutic agents. Probably the best studied example is etanercept (Enbrel®), a fusion construct between the extracellular domain of the p75 TNF-α receptor and the Fc domain of human IgG, which has proven to be successful in treating rheumatoid arthritis [73,74].

As previously mentioned, several differentially spliced isoforms of the LR have been described, including a secreted receptor variant. In addition, shedding of the
extracellular domain of the human receptor by proteolytic cleavage has also been described [75]. In contrast to the IL-1 and TNF receptor systems, the precise functions of these secreted leptin receptors (sLR) is not yet as well documented. sLR circulates in mouse [76] and human [77] plasma and is able to bind leptin [76] – indeed; a significant amount of circulating leptin is found bound by it [78]. It comes as no surprise then that a negative regulatory role for the sLR has been proposed [79, 80]. In a majority of studies, however, the sLR has been attributed another function. In ob/ob mice, the effects of administrated leptin on food intake and body weight are enhanced when its soluble receptor is over-expressed. The reason for this is probably that bound leptin is continuously released, resulting in increased leptin levels [81]. In this scenario, sLR rather functions as a carrier protein, binding leptin and thus extending its half-life in circulation. It is of note that these two hypotheses are not mutually exclusive. Illustrating this dichotomy, two comparable datasets were recently interpreted in opposing ways [80, 82]. All in all, it seems most likely that the sLR acts as a buffer by regulating the concentration of leptin, arguing against the mere use of the sLR as a functional leptin antagonist. Rational design of receptor mutants with increased binding affinity, eventually even coupled to extended half-life, may lead to the development of engineered variants with a profoundly altered biological behaviour. Much along the same line, “cytokine traps” have recently been developed for leptin-related interleukins [83].

9.5.2. Leptin muteins

Another strategy to neutralise cytokine activity is the use of mutant cytokine variants with antagonistic properties. Some examples of antagonistic α-helical bundle cytokine muteins are growth hormone [84], IL-5 [85], IL-11 [86] and IL-15 [87]. A good antagonistic molecule should bind to the LR with high affinity, without activating it. Verploegen et al. found that the R128Q human leptin mutant binds to the LR, but fails to transduce a signal. When injected into normal mice, this leptin antagonist induces weight gain, indicating the R128Q mutant has leptin antagonistic properties in vivo [88]. Takahashi et al. treated mice with the R128Q mutant, and found that this antagonist induces an increased sensitivity towards the toxic effects of TNF, similar to
what is observed in ob/ob and db/db mice [41]. α-helical bundle cytokines typically have two or three different binding sites for binding two or three receptor chains. Binding of multiple receptor chains by a single cytokine molecule brings the receptor chains in the correct orientation for receptor activation. We very recently mapped interaction sites of leptin with its receptor complex, using molecular modelling combined with site directed mutagenesis of residues at the solvent accessible surface of leptin, and found that leptin has two interaction sites with its receptor [89]. These were found in similar locations as binding site 2 and 3 in cytokines of the gp130 family of cytokines [90]. A primary high affinity binding site is involved in the initial high affinity binding to cytokine receptor homology domain 2 (CRH2) of the LR. A secondary binding site is not involved in high affinity binding of leptin to the receptor, but is strictly required for activation of the receptor, most likely by interacting with another leptin receptor chain during formation of an active leptin/receptor complex. The R128Q mutation is not part of either site, but probably indirectly disturbs the secondary binding site. Introduction of disruptive mutations in the secondary binding site of leptin have no effect on the high affinity binding to the LR, but such mutants show no receptor activation, even at very high doses (in contrast to the R128Q mutein). Administration of such leptin mutein induces weight gain in mice [89]. Gonzalez and Leavis showed that the synthetic peptide LPA-2, corresponding to residues 70-95 of leptin binds with high affinity to the LR, and that this peptide completely blocks LR activation in rabbit endometrial cells in vitro. No in vivo effect was reported [91]. Leptin muteins were shown to be active antagonists in vivo, but probably suffer from rapid clearance by the kidneys, as administered recombinant leptin has a half-life of less than two hours in circulation [92]. The LPA-2 peptidic antagonist is likely to suffer even more from rapid clearance. To turn antagonistic leptin muteins into useful tools for anti-leptin therapy, ways of extending their half-life, such as PEGylation [93], hyperglycosylation [94] or the use of fusion proteins will need to be tested.

9.5.3. Antibodies
One of the most extensively studied approaches when it comes to antagonising cytokines is the design of antibodies, since they link high binding affinity and specificity.
A typical anti-cytokine antibody-based treatment already in clinical use is infliximab (Remicade®) targeting TNF-α for treatment of rheumatoid arthritis, and many others are being developed [95,96]. A number of studies described the use of anti-leptin or anti-LR antibodies for detection and quantification purposes [78,80-82], but virtually none aimed at antagonising leptin. To our knowledge, only four studies are available today in which leptin's function was counteracted in vivo by antibodies, of which only one suggests clinical use [97]. These authors used a neutralising anti-LR antibody to suppress rat leukemic cell growth by inhibiting angiogenesis. The other groups antagonise leptin to study its effects on various processes: energy and glucose homeostasis [98], prolactin and luteinizing hormone secretion [99] and regulation of wound healing [100].

While antibodies are certainly promising to oppose detrimental effects of pro-inflammatory cytokines, some general considerations must be made. One aspect is safety in long-term clinical use. Long-term effects of such antibody treatment are still the object of further study, in particular regarding the risk of opportunistic infections [101]. Also, therapeutic use of even humanised monoclonal antibodies might elicit immune responses and be neutralised after repeated therapy [102]. In case of neutralising monoclonal antibodies targeting IL-5, we observed quite opposite IL-5 potentiating effects under certain circumstances [103] warranting careful examination of the biological behaviour of such antibodies. Nevertheless, given the success seen for quite a number of therapeutic applications in other cytokine systems, selection of useful monoclonal antibodies blocking leptin activity is quite likely.

**9.5.4. Small Molecule Antagonists**

Protein-based therapeutics have clearly found clinical application [104]. Examples for treating autoimmune diseases include the abovementioned etanercept (Enbrel®) and infliximab (Remicade®) targeting TNF-α for treatment of rheumatoid arthritis and IFN-γ (Betaseron) for multiple sclerosis. Yet, the pharmaceutically preferred track undoubtedly is the development of small molecular weight antagonists given their oral availability, easy and low-cost manufacturing and lack of immunogenicity.
The number of small organic molecules capable of disrupting larger protein-protein
interactions is increasing. Most of such antagonists have been identified using a
screening strategy, followed by optimisation. Inhibitors of the extracellular receptor-
ligand interactions that were recently identified using this approach include antagonists
to gp130 [105] and to the IL-6 receptor α chain [106].

The increasing availability of protein structures together with the recognition of
structure activity relationships (SAR) led to more structure-based approaches. These
allow a more oriented search: throughout the process, a reference is available that can
be used for rational design of the antagonists. One of these new strategies is tethering
[107], a site-directed screening method that searches for complementary small ligand
fragments at a specific sub-site of a protein. Fragments for different sub-sites that have
been identified in this way can then be combined with each other to obtain a lead
molecule with enhanced affinity. This method has been used to study the binding
modus of an IL-2 binding compound [108]. Another example of particular interest is the
interaction between the adhesion molecule lymphocyte function-associated antigen-1
(LFA-1) and its ligand (or counterreceptor) intercellular adhesion molecule-1 (ICAM-1)
(reviewed in [109]).

Depending on the availability of detailed structural data, preferably of a ligand/receptor
complex, a strategy using the in silico screening of virtual compound databases can be
used. This technique allows narrowing down huge libraries of chemicals to a few likely
lead compounds on which in vitro screening can be started (reviewed in [110]).

To our knowledge, no small molecule antagonist that prevents binding of leptin to its
receptor has been reported. This is not surprising since the main objective of leptin
research until recently was to enhance its effects in order to achieve weight reduction.
Given the increasing success in identifying such compounds for other ligand/receptor
interactions and the development of more refined selection strategies [109], and given
the successful identification of a non-peptidic antagonist directed against gp130, a receptor chain closely related to LR [105], selection of compounds interfering with the leptin/LR interaction may be possible.

Besides interfering with the extracellular leptin/LR interaction, enzymes or protein-protein interactions in intracellular signalling could also be targeted. Being a member of the type I cytokine receptors, the leptin receptor signals via the JAK/STAT pathway. Upon clustering of the homomeric receptor complex by leptin, the associated Janus kinase 2 (JAK2) tyrosine kinase molecules become activated by cross-phosphorylation [111]. Subsequently, three highly conserved tyrosines within the LR tail are likely phosphorylated, providing recruitment sites for a variety of signalling molecules. The Signal Transducer and Activator of Transcription 3 (STAT3) docks to the membrane distal tyrosine Y1138 (mouse LR numbering) and is critical for the weight-reducing effects of leptin. However, this Y1138 site and concomitant STAT3 activation may not be required for reproduction in mice, suggesting the existence of other critical pathways initiating from the LR [112]. The membrane proximal tyrosine Y985 binds Suppressors Of Cytokine Signalling 3 (SOCS-3) [113], a potent inhibitor of leptin signal transduction implied in leptin resistance [114]. The tyrosine phosphatase SHP-2 (Src homology 2-containing phosphotyrosine phosphatase 2) is also recruited via the Y985 site and was originally described as an inhibitor [115] although recent evidence suggests a positive role in leptin signalling by acting as an adaptor molecule for the Ras/Raf pathway [116]. Although phosphorylation of the Y1077 site has not been formally demonstrated yet, this tyrosine apparently acts as an accessory site for the recruitment of SOCS-3, and possibly also for other signalling molecules. The high conservation of this site throughout evolution supports its critical function [117].

Mice lacking the protein tyrosine phosphatase-1B (PTP-1B) gene are more resistant to obesity [118]. Further research showed that these mice are hyper-responsive to leptin, including increased leptin-dependent STAT3 activation in the hypothalamus [119,120]. Inhibitors of this phosphatase may increase central responsiveness to leptin leading to weight loss. Search for PTP1B inhibitors is current pursued by several companies [121].
Various reports describe the activation of phosphatidylinositol 3-kinase (PI3K) after leptin treatment both in vitro [122] and in vivo [123]. A central role was also established by intracerebroventricular (ICV) injection of known PI3K inhibitors, resulting in prevention of leptin-induced weight loss [124]. Other pathways, such as the c-Jun Kinase/Stress-Activated Protein Kinase pathway [125] and the 5'-AMP Protein Kinase pathway [126] are also activated by leptin.

As discussed above, leptin has clear immuno-modulatory properties, through direct effect on immune cells. Although activation of STAT3 and the resultant induction of Bcl-xL and fatty acid amide hydrolase by leptin has been shown in T-lymphocytes [127,128], other signalling pathways have not been studied yet in these cells. In PBMCs, activation of the JAK/STAT, PI3K and the Ras/Raf pathways by leptin has been demonstrated (reviewed in [129]). Leptin-dependent tyrosine phosphorylation of the RNA-binding protein Sam68 was also shown in PBMCs. This results in increased association of Sam68 with the p85 regulatory subunit of PI3K and with STAT3, as well as the dissociation from RNA. The implications of leptin-induced Sam68 phosphorylation on transcription and/or splicing remain to be defined [130]. STAT3 activation by leptin has also been shown in the murine macrophage cell line J774.2 [131].

Given the redundancy in signal transducers recruited/activated via many receptor systems, selection of compounds selectively interfering with leptin signalling is challenging. One strategy may be to target the specific determinants of the cytosolic domain of the LR interacting with downstream molecules, as has been shown for the βc-Lyn interaction in the case of IL-5 signalling [132]. The recruitment of STAT3, Ras/Raf and PI3K may prove to be interesting targets. Alternatively, negative feedback mechanisms could be targeted such as SOCS-3 or PTP-1B. This can perhaps be obtained by modulating their expression levels or binding affinities or by stimulating downstream pathways such as the proteasome activity associated with SOCS-3 [133]. As mentioned before, compounds also should only interfere with peripheral leptin activities, and therefore not cross the blood-brain barrier.
9.6. Concluding remarks

The hypothalamic leptin/leptin receptor system is a determining component in the control of energy homeostasis and body weight. Leptin has more recently also emerged as a pleiotropic cytokine with important functions on several peripheral tissues. Its activities on immune cells point to an integrating role between the body energy supplies and the immune system. Indeed, adequate nutrition is a prerequisite for generating appropriate immune responses against pathogens. Adversely, sufficient energy stores may be one of the factors required for long-term, detrimental immune reactions, as observed in autoimmune diseases. The link between leptin and immune processes may therefore offer a new therapeutic strategy against autoimmune diseases. It must be stressed however that, as is the case for many other $\alpha$-helical bundle cytokines, leptin exerts both pro- and anti-inflammatory activities. Detailed studies of the role of leptin in in vivo models of autoimmune disease will be required to confirm its clinical potential. For that purpose, there is a clear need for leptin antagonists.

So far, given the focus on the link between leptin and obesity, little attention was given to the development of leptin antagonists. In analogy to other cytokines, a range of straightforward strategies exists including the use of secreted receptor variants, neutralising antibodies and antagonistic muteins. Besides protein-based antagonists, a better understanding of LR activation and of the mechanisms triggering the intracellular signal transduction pathways may provide researchers with new targets to search for small molecular weight drugs. A main concern will be not to interfere with the regulation of body weight; in other words to block the peripheral, and not the central effects of leptin. Leptin’s central activities imply transport across the blood-brain barrier, suggesting a molecular basis for selective antagonists. Insights into the precise leptin transport mechanism are however still far from complete. Arguably, leptin antagonists, e.g. leptin muteins, may provide novel tools to unravel the underlying mechanisms. Local delivery of leptin antagonists offers an alternative possibility. Several autoimmune diseases are limited to a specific tissue, for example Crohn’s disease to the intestine. In this particular case, local delivery of a leptin antagonist can be envisaged using a Lactococcus or Lactobacillus delivery system, as has been demonstrated using...
recombinant IL-10 secreting Lactococcus lactis [134,135]. In conclusion, recent work on the function of leptin on the immune system suggests its detrimental role in autoimmune processes. Leptin antagonists may therefore have therapeutic value, but in addition, their development will lead to novel research tools allowing a better understanding of leptin biology.

9.7. Acknowledgements

We wish to thank J. Vandekerckhove for continued support. Given the very extensive research field covered in this review, we inevitably could not refer to all published data. We apologise to those whose work was not mentioned.

9.8. References


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10.1. Introduction

In strong contrast to other well studied class I cytokine receptors, little is known about the mechanisms underlying the activation of the leptin receptor (LR). Crystal structures of (ligand bound) receptors and several mutagenesis studies on the receptor or on the ligand, are available for the receptors for Epo, GH, G-CSF and IL-6. In this study we examine the requirements for LR activation in more detail.

The LR has a CRH1-Ig-CRH2-FNIII2 structure, as discussed in chapter 6. Using a panel of deletion and substitution mutants, Fong and co-workers showed that the membrane proximal CRH2 domain is necessary and sufficient for leptin binding, whereas the two FNIII domains are not involved in ligand binding, but are needed for receptor activation (Fong et al., 1998). Here we demonstrate that the membrane distal CRH1 is not directly involved in ligand binding or receptor activation, but its presence is needed for optimal leptin signaling. The Ig-like domain appears to be essential for activation of the cytoplasmic kinases and hence also for signaling.

The model in which the LR becomes activated by a simple homodimerization has been questioned (White et al., 1997; Couturier and Jockers, 2003). In this study, we provide
for the first time biochemical evidence for this higher order clustering of the LR. We therefore used a JAK/STAT complementation strategy. In this assay, two signaling deficient LR receptors mutants (one defective in JAK activation, the other unable to recruit STAT molecules) are only able to generate a STAT3 dependent signal when they are co-expressed. Co-expression of LR mutants in which the extracellular part is replaced by that of the EpoR, is not sufficient to restore JAK/STAT signaling. This experimental set-up was also used to demonstrate a non-redundant role for the different receptors in the activated LR complex. These results were used to propose a model for LR activation.

10.2. References


10.3. Article
Functional Analysis of Leptin Receptor Activation Using a Janus Kinase/Signal Transducer and Activator of Transcription Complementation Assay

LENNART ZABEAU, DELPHINE DEFAEU, JOSÉ VAN DER HEYDEN, HANNES ISERTANT, JOËL VANDEKERCKHOVE, AND JAN TAVERNIER

Flanders Interuniversity Institute for Biotechnology, Department of Medical Protein Research (VIB09), Ghent University, Ghent, Belgium

The leptin receptor (LR), a member of the class I cytokine receptor family, is composed of a single subunit. Its extracellular domain consists of two so-called cytokine receptor homology domains, separated by an Ig-like domain, and two additional fibronectin type III modules. Requirements for LR activation were examined using a complementation strategy. Two LR mutants, LR-FFY-Dbox 1 and LR-F3, deficient in Janus kinase or signal transducer and activator of transcription (STAT) activation, respectively, were only able to generate a STAT3-dependent signal when coexpressed. Based on the requirements for Janus kinase/STAT signaling, and on the lack of complementation with similar receptor constructs, but containing the extracellular domain of the homodimeric erythropoietin receptor, this observation can be explained only by higher order LR clustering. Using a panel of deletion mutants we were able to define a role for the cytokine receptor homology 1 and Ig-like domains in leptin signaling. Moreover, we demonstrate a nonredundant function for the individual receptor chains within the homomeric LR complex. Based on these data, we propose a possible model for LR clustering. (Molecular Endocrinology 18: 150–161, 2004)

LEPTIN, THE PRODUCT of the ob gene (1), is a key player in energy homeostasis and body weight control. It is a 16-kDa circulating protein with a structure resembling 4 α-helical bundle cytokines (2). It is secreted mainly by adipose cells, and the circulating level of this hormone strongly correlates with white adipose tissue mass. Leptin regulates energy expenditure and food intake by activating its receptor in certain nuclei of the hypothalamus (3–5). Loss-of-function mutations within the genes for leptin (1, 6) or for its receptor (7–9) cause complex syndromes characterized by morbid obesity, hyperglycemia, hyperinsulinemia, and reduced fertility. Numerous data suggest that leptin also has direct effects on tissues outside the brain, which may help explain its role in basal metabolism, reproduction, and hematopoiesis (10–13).

The leptin receptor (LR) is composed of a single subunit, encoded by the ob gene (7, 8, 14, 15), and is a member of the class I cytokine receptor family. It contains two so-called cytokine receptor homology (CRH) modules, which are formed by two barrel-like domains, each 100 amino acids (aa) in length, and which resemble the fibronectin type III (FN III) and Ig folds. Two conserved disulfide bridges are found in the N-terminal domain, whereas a WSXWS motif is characteristic for the C-terminal domain. Both LR CRH modules are separated by an Ig-like domain and are followed by two membrane-proximal FN III domains (Fig. 1). Using a panel of deletion and substitution mutants, Song et al. (16) showed that the membrane-proximal CRH domain is necessary and sufficient for leptin binding, whereas the two FN III domains are not involved in ligand binding but are needed for receptor activation. Thus far, six isoforms of the LR generated by alternative mRNA splicing have been recognized and termed LRα and LRβ through LRγ. The LR long form (LRα or LRβ) has an intracellular chain length of 302 aa and is the only isoform capable of efficient signaling. It is this LRβ isoform that is primarily expressed in specific nuclei of the hypothalamus (17–19), but expression at lower levels in other cell types has also been observed (20–22). A second isoform, LRα, is a variant lacking most of the cytosolic domain. This LR short form (LRβ) is much more widely expressed, often at higher levels compared with LRβ, e.g., in the choroid plexus, kidney, lung, and liver (23).

Like all members of the class I cytokine receptor family, the LR has no intrinsic kinase activity, and uses cytoplasmic-associated Janus kinases (JAKs) for intracellular signaling. Two short conserved sequences in the membrane-proximal region (box 1 and a putative box 2) are thought to mediate binding and activation of
the JAK kinases [i.e. JAK2 in the case of the LR1o (24)]. In a generally accepted model, leptin binding leads to formation of a receptor complex, allowing activation of JAK2 by cross-phosphorylation. Activated JAK2 then rapidly phosphorylates several tyrosine residues in the cytosolic domain of the receptor (i.e. in the case of the mouse LR1o, tyrosines at positions 985, 1077, and 1138). Phosphorylated tyrosine 1138 provides a binding site for STAT3 (signal transducer and activator of transcription 3) (25). STAT3s themselves are also substrates for JAKs and homo- or heterodimerize upon phosphorylation, translocate to the nucleus, and modulate transcription of target genes. The STAT molecule primarily involved in leptin signaling is STAT3. Leptin-mediated STAT3 activation has been confirmed by Vaisse et al. (26) in the hypothalamus of wild-type and ob/ob mice after injection of the leptin. In cell lines, however, leptin-mediated STAT1 and STAT5B activation was also shown (27). The other mouse LR1o cytoplasmic tyrosine residues (on position 985 and 1077) are involved in negative feedback of LR signaling, by recruitment of the SH2-containing tyrosine phosphatase-2 (25, 28), or suppressor of cytokine signaling-3 (29-31), or in coupling to other signaling molecules such as MAPK (32) and phosphoinositide 3 kinase (33).

The model in which the LR becomes activated upon mere ligand-induced dimerization, i.e. one leptin molecule clusters two receptor chains, has been questioned (34, 35). To address this issue, two signaling-deficient LR mutants were constructed. Based on complementation of these two mutants, we here provide direct evidence for leptin-induced higher order clustering of its receptor. Using a panel of deletion mutants, we further define the role of individual LR subunits and of LR extracellular subdomains in LR complex formation and functioning.

**RESULTS**

**Construction and Expression of LR Mutants**

To study the requirements for LR activation in more detail, we constructed two types of signaling-deficient LR mutants. In the LR-F3 mutant, all three cytoplasmic tyrosine residues were mutated to phenylalanine, thereby blocking recruitment of STAT3 molecules to the activated receptor. Signaling by the second mutant, LR-FFY-Δbox 1, was abolished by two proline to serine mutations at positions 876 and 878 in the box 1 motif. These mutations prevent binding and activation of the associated JAK kinases (36). To reduce negative feedback by regulators like SH2-containing tyrosine phosphatase-2 or suppressor of cytokine signaling-3, and activation of other signaling pathways such as activation of MAPK and phosphoinositide 3 kinase, we also introduced tyrosine to phenylalanine mutations at positions 985 and 1077. The only intact tyrosine, Y1138, in this mutant allows activation of and signaling via STAT3. This LR-FFY mutant shows an approximately 2- to 3-fold increased induction of STAT3-dependent reporter activity when compared with the wt LR (37). A schematic representation of both mutant receptors is given in Fig. 1A. Also shown is the expression of both mutants upon transfection in human
embryonic kidney [HEK]293T cells, using Western blot analysis (Fig. 1B) and a binding assay with a leptin-secreted alkaline phosphatase (SEAP) chimera (Fig. 1C). Both data sets illustrate that mutations in the cytoplasmic portion of the receptor have no significant effect on the overall expression and cell surface anchoring of the mutant receptors.

To address the role of the membrane distal CRH (also referred to as CRH1) and Ig-like domains in LR signaling, a panel of deletion mutants was constructed. In the first set, we deleted this CRH1 module in the wild-type LR receptor (LR ΔCRH1) and in both signaling-deficient mutants (LR-F3 ΔCRH1 and LR-FFY-Δbox 1 ΔCRH1). Western blot analysis and leptin-SEAP binding data on transfected HEK293T cells illustrate that this deletion results in a minor, but significant, decrease in LR cell surface expression (see Fig. 1, B and C). In a similar way, receptors lacking both the CRH1 and Ig-like domains (LR ΔCRH1,ΔIg; LR-F3 ΔCRH1,ΔIg; and LR-FFY-Δbox 1 ΔCRH1,ΔIg) were constructed. In contrast, additional deletion of the Ig-like domain may lead to a slightly enhanced LR expression (Fig. 1). Binding of leptin-SEAP was performed at room temperature, and NaN3 was added to prevent internalization of the ligand-bound receptors. Similar results were obtained when the binding experiment was done on ice (data not shown). It is of note that the expression patterns are in the same range for each set of extracellular deletion mutants, irrespective of the mutations in the cytosolic domains.

Complementation with Signaling-Deficient LR Mutants

We next analyzed leptin-induced signaling via the mutant LR constructs upon (co-)transfection in HEK293T cells. As a read-out, the STAT3-responsive rPAP1 (rat pancreatitis-associated protein 1)-luciferase reporter construct was used as described previously (31). Using a subtractive cloning strategy, rPAP1 was identified as a late target gene upon leptin stimulation (38). As expected, both LR-F3 and LR-FFY-Δbox 1 mutant receptors were inactive, because neither STAT3-dependent activation of a rPAP1-luciferase reporter construct nor STAT3 phosphorylation could be observed (Fig. 2A). Interestingly, when both receptor mutants were coexpressed, we observed functional complementation leading to a clear increase (≥70-fold) of the leptin-induced rPAP1-luciferase reporter activation (Fig. 2A) and a concomitant marked STAT3 phosphorylation (Fig. 2B). The complementation-dependent signal was less sensitive when compared with the LR-FFY, a control receptor also not subjected to negative feedback (31); approximately 10-fold higher leptin concentrations were required, and a 70% decrease in plateau value was also observed (Fig. 2C). This is not unexpected because inevitably, cotransfection leads to the formation of several inactive LR complexes (e.g. composed of only one mutant receptor). Importantly, the decrease in sensitivity is limited, and all experiments described below were performed using physiological leptin concentrations (10 ng/ml, or as indicated).
Because two LR-F3 chains are needed for reciprocal activation of JAK kinases, and because at least one LR-FFY-Δbox 1 chain with a phosphorylated tyrosine residue is necessary for the recruitment of STATs, our complementation data may be explained by formation of a higher order LR complex.

No Complementation Observed with Erythropoietin Receptor (EpoR)-LR Chimeras

To test this hypothesis, we constructed EpoR-LR chimeras containing similar LR-F3 and LR-FFY-Δbox 1 mutations, but with the extracellular part of the LR replaced by that of the EpoR. It is generally accepted that the EpoR functions as a homodimer. As observed for the full-length LR mutants, cytoplasmic mutations in the EpoR chimeras did not significantly alter expression, as could be shown by immunoprecipitation with an antibody directed against the extracellular domain of the EpoR, and Western blot analysis with an anti-LR antibody (data not shown). As shown in Fig. 3, neither EpoR chimera separately, nor in combination, were able to generate a STAT3-dependent signal even at an erythropoietin (Epo) concentration of 50 ng/ml. As a positive control, EpoR-LR, with an intact box 1 motif and the critical tyrosine 1138, showed a clear Epo-mediated activation of the rPAP1-luciferase reporter, illustrating that simple dimerization of the LR cytoplasmic domains was sufficient for JAK2 and STAT3 activation. It is of note that stimulation of cells expressing the Epo-LR chimera with 50 ng/ml Epo, the concentration used to test complementation signaling, results in an optimal STAT3-dependent signal. Together these data show that receptor dimerization does not allow complementation and lends further support to the LR being a higher order complex. Furthermore, the behavior of the EpoR chimeras most likely illustrates that the formation of higher order LR complexes is mediated by the extracellular domain of the receptor.

Role of the CRH1 and Ig-Like Modules in LR Activation and Clustering

We next investigated the role of specific subdomains of the LR extracellular part in LR binding, clustering, and activation. Deletion of the CRH1 module in the wild-type receptor (LR ΔCRH1) results in a limited, but clear, decrease in receptor cell surface expression and on leptin binding (both shown in Fig. 1). As with the full-length mutants, the ΔCRH1 deletion in LR-F3 and LR-FFY-Δbox 1 mutants did not affect the ability to signal in the complementation assay (Fig. 4A), excluding a strict requirement for this module in higher order clustering of the LR. However, a lower leptin-induced response was clearly observed when the CRH1 domain was deleted (Fig. 4B). Similar data were obtained with receptor constructs lacking cytosolic mutations (Fig. 4E).

We next analyzed whether CRH1 deletion reduced signaling only as a consequence of lowered LR cell surface expression. In a titration experiment, different amounts of DNA encoding full-length LR-F3 and LR-FFY-Δbox 1 (1, 0.75, 0.5, and 0.25 μg each) were cotransfected in HEK293T cells. Leptin-dependent signaling in the transfected cells was measured using the rPAP1-luciferase reporter and compared with cells cotransfected with 1 μg LR-F3 ΔCRH1 and 1 μg LR-FFY-Δbox 1 ΔCRH1. As expected, decreasing the amount of transfected DNA caused reduced leptin binding (Fig. 4C) and signaling (Fig. 4D). Clearly, the combination LR-F3 and LR-FFY-Δbox 1 showed at least an equal (when 0.5 μg DNA was used) or a significantly higher response (0.75 μg) when compared with the LR-F3 ΔCRH1 and LR-FFY-Δbox 1 ΔCRH1 combination (1 μg transfected), even at lower LR cell surface expression levels (Fig. 4C). These results indicate that the membrane-distal CRH1 also contributes to optimal LR activation.

Interestingly, additional deletion of the Ig-like domain in the wild-type LR completely abolished STAT3-dependent activation of the rPAP1-luciferase reporter (Fig. 4E). Western blot analysis clearly illustrates that this is due to the inability of the LR ΔCRH1,Ig variant to activate JAK2, and this in strong contrast to LR and LR ΔCRH1 (Fig. 4F). Thus far, the function of this Ig-like domain remained unexplored and was not yet related to LR clustering and/or signaling.
Fig. 4. Involvement of CRH1 and Ig-Like Domains in LR Signaling

A, Signaling via LR-F3 ΔCRH1 and LR-FFY-Δbox 1 ΔCRH1. Both mutant receptors were transfected alone (2 ng) or in combination (1 ng each), and transfected HEK293T cells were treated with leptin, or were left unstimulated (NS). Mean luciferase values and error bars of triplicate measurements are plotted. B, Deletion of CRH1 leads to a lower leptin signal. Combinations of LR-F3 + LR-FFY-Δbox 1 (solid bars) and LR-F3 ΔCRH1 + LR-FFY-Δbox 1 ΔCRH1 were transiently transfected in HEK293T cells. Stimulations were overnight with a serial dilution of leptin (as indicated). Mean luciferase values and error bars of triplicate measurements are plotted. C, Effect of the amount transfected DNA on LR expression. Different quantities of DNA encoding LR-F3 and LR-FFY-Δbox 1 or LR-F3 ΔCRH1 and LR-FFY-Δbox 1 ΔCRH1 were transfected in HEK293T as indicated. Leptin binding was measured as described in Fig. 1. Bars represent the mean values and SD of triplicate chemiluminescence measurements. D, Complementation analysis of full-length vs. ΔCRH1 signaling-deficient mutants. HEK293T cells were transfected with 1 ng (solid circles), 0.75 ng (open circles), 0.5 ng (solid squares), 0.25 ng (open squares) DNA encoding full-length LR-F3 and LR-FFY-Δbox 1, or 1 ng (open triangles) LR-F3 ΔCRH1 and LR-FFY-Δbox 1 ΔCRH1. Cells were stimulated overnight with a serial dilution of leptin, as indicated. E, Role for the LR Ig-like domain in leptin signaling. Signal via the wild-type LR and two deletion variants thereof, LR ΔCRH1 and LR ΔCRH1 ΔIg, were analyzed in HEK293T cells as described. Bars represent mean luciferase counts of triplicate measurements. F, JAK2 and STAT3 phosphorylation. Phosphorylated and total amounts of JAK2 and STAT3 proteins were determined as described in Fig. 2.
Different Roles for Receptor Chains in an Activated LR Complex

To further define structural requirements for LR activation, different combinations of the two signaling-deficient mutants, and deletion variants thereof, were tested. In Fig. 5, we compared the combined expression of LR-F3 and LR-FFY-Δbox1 ΔCRH1,Δlg with the LR-F3 ΔCRH1,Δlg and LR-FFY-Δbox1 combination. The data showed that leptin-dependent STAT3 activation occurred only when a full-length LR-F3 mutant was expressed (Fig. 5A). It is of note that the drop in luciferase activity at very high leptin concentrations (10 μg/ml) was not reproducible. Combined deletion of the CRH1 and Ig-like domains in LR-F3 completely abolished signaling, even at leptin concentrations of 10 μg/ml (Fig. 5B). As with the wild-type receptor lacking both CRH1 and Ig-like domain, the lack of rPAP1 promoter activation can be explained by the total loss of JAK2 phosphorylation and activation. Taken together, these data confirm that the presence of an intact Ig-like module is necessary for signaling and, more specifically, for activation of the JAK kinases. Moreover, it appears that JAK2 activation is only possible when the Ig domain and the box 1 region are on the same receptor.

DISCUSSION

The receptor for leptin, an adipose tissue-derived cytokine involved in body weight homeostasis, was cloned in 1995 (14). In contrast to other well-studied cytokine receptors, i.e. the homodimeric erythropoietin and GH receptors, the granulocyte-colony stimulating factor receptor, or the more complex glycoprotein 130 (gp130)-based receptor family, mechanisms of LR clustering and activation remain largely unknown. In this study, we examined the structural requirements for LR activation using a novel complementation strategy based on two crucial, distinct steps in LR signaling, i.e. JAK2 activation and STAT3 recruitment. Two LR mutants, LR-F3, unable to bind...
and activate STAT factors, and LR-FFY-Δbox 1, deficient in JAK activation, only generate a STAT-dependent signal when coexpressed (Fig. 2). It could not be excluded a priori that the observed effect is due to mobility of the JAK kinases. This model would imply that upon stimulation of the LR-F3 mutant, the activated JAK kinases might dissociate from the receptor and phosphorylate in trans the tyrosine 1138 in the LR-FFY-Δbox 1 receptor, leading to subsequent STAT3 activation. To exclude this possibility, we analyzed the behavior of Epo-LR mutants. Several lines of evidence argue for the active state of the EpoR being a dimer: 1) the constitutively active R129C EpoR mutant was found to exist as Epo-independent disulfide-linked dimers when expressed in cells (39); 2) biophysical studies identified two interaction sites on Epo for EpoR extracellular domain monomers (40); and 3) crystal structures of the EpoR extracellular domain in complex with Epo (41), with an Epo-mimetic (42), or with an Epo-antagonistic peptide (43), all showed a 1:2 stoichiometry. Substitution of the extracellular part of the mutant LRs by that of the EpoR led to a complete loss of complementation (Fig. 3). Taking into account the inherent requirements for JAK/STAT signaling, i.e., two JAK activating receptors and at least one STAT recruitment site, and the differential behavior of the EpoR-LR mutant chimeras, the observed complementation can be explained only by assuming a higher order LR clustering. It is noteworthy that the EpoR-LR chimeras with intact JAK/STAT activation motifs generate a clear STAT3-dependent signal (Fig. 3), implying that Epo binding leads to correct positioning of JAKs, allowing their activation and subsequent STAT3 recruitment.

Leptin-dependent signaling by functional complementation of LR mutants is less efficient when compared with LR-FFY signaling (Fig. 2C). This is most likely due to the formation of noncomplementing and therefore inactive mutant LR complexes. Compared with the wild-type situation, here mimicked by the LR-FFY mutant, the decrease in sensitivity is rather moderate. Because physiological leptin concentrations can be used (leptin levels in healthy people vary around 5 ng/ml), data obtained from the complementation assays reflect normal leptin-LR interactions.

Higher order clustering of the LR has been suggested previously by White et al. (34). This may help explain why the LRio is only moderately sensitive to the presence of excess LRsh. Despite the presence of a box 1 motif, this short isoform is unable to activate JAK2 at physiological intracellular kinase levels (44) and is hence signaling deficient. Two groups showed independently that, next to the box 1 motif, the intracellular residues 31–36 in the long form are critical for mediating JAK2 activation (44, 45). These residues are absent in the LR short form. LRsh is often coexpressed with LRio, accounting for up to 95% of LR mRNA in many tissues (7) and may function in leptin clearance or transport through the blood-brain barrier (14). Our findings support oligomeric clustering (i.e., dimerization of dimers) of the LR and, given the presence of the additional N-terminal CRH1, even higher order clustering may occur. Obviously, in more complex receptor configuration, the presence of LRsh variants is less likely to completely block signaling. Alternatively, differential sorting of the LR short and long isoforms, perhaps due to their different ability to bind JAK2, could also explain this lack of interference (34).

Given the structural complexity of the extracellular domain of the LR, very few data related to the function of individual structural modules are available to date. A rather unique feature of the LR within the family of cytokine receptors is the presence of two distinct CRH modules. Other receptors containing two CRH modules are the β2 chain, shared in the receptors for IL-3, IL-5, and granulocyte-macrophage colony stimulating factor, the leukemia inhibitory factor receptor (LIFR), and thrombopoietin receptor. Crystallographic structure analysis of the β2 extracellular domain, consisting of two adjacent CRH modules, provides evidence that the receptor is expressed at the cell surface as an interlocked homodimer, even in the absence of ligand (46). Here, the G strand of the N-terminal domain of the membrane-distal CRH module interacts with the N-terminal domain of the membrane-proximal CRH of the other partner in the dimer and vice versa. The role for the CRH modules in the LIFR, now separated by an Ig-like domain, is less well defined. Both CRH modules are involved in ligand binding, while the membrane-proximal CRH is also involved in receptor dimerization (47). He et al. (48) showed that membrane-distal CRH of LIFR associates in vitro with the soluble ciliary neurotrophic factor receptor, even in the absence of ciliary neurotrophic factor. Here we show that the membrane distal CRH1 domain of the LR is not strictly required for wild-type LR signaling or for higher order clustering of the receptor, because LR-F3 and LR-FFY-Δbox 1 mutants lacking this domain are still able to generate a STAT3-dependent signal in a complementation setup (Fig. 4A). However, a clearly reduced signal and a marked decrease in JAK2 and STAT3 phosphorylation are observed upon deletion of CRH1 when compared with the full-length receptor (Fig. 4, B, E, and F). This effect can be explained by both lowered cell surface expression (Fig. 1) and reduced signaling capacity (Fig. 4, C and D). Two possible roles for the CRH1 can be put forward: 1) CRH1 provides further clustering of activated core LR complexes (see below), thereby enhancing the local concentrations of signaling components. We recently showed that antibody-induced formation of higher order clustering of the IL-5R potentiates signaling (49). 2) Alternatively, the CRH1 domain may also play a role in sorting or anchoring the LR to distinct membrane regions, such as lipid rafts or caveolae, thereby enhancing leptin responsiveness. So far, however, no evidence for expression of the LR in specialized membrane subdomains has been obtained. Therefore, additional studies are required to establish the precise role of the CRH1 module.
Combined deletion of both the CRH1 and the Ig-like domains in the wild-type LR led to a complete loss of JAK2 phosphorylation and concomitant STAT3-dependent signaling (Fig. 4E). Expression and membrane anchoring of the resulting LR mutant is comparable, however, to that of the full-length receptor (Fig. 1). A role for the Ig-like domain related to ligand binding and receptor activation is well established in the granulocyte colony stimulating factor receptor (50, 51), the LIFR (52, 53), and gp130 (54, 55), but was not yet shown for the LR.

Based on 1) the observed complementation data, 2) the postulated involvement of the Ig-like domain, and 3) the lack of a strict requirement for CRH1 in signaling, we propose a model wherein leptin functions as a trivalent ligand (Fig. 6B). Two ligand binding sites (sites I and II) interact with two membrane-proximal CRH2 modules of two distinct LR subunits, and residues belonging to a third site (site III) interact with the Ig-like domain of a third LR chain (Fig. 6B).

This model strongly resembles that of receptor complexes in the gp130-binding cytokine family. Mutagenesis and binding studies have indicated that these cytokines interact via three distinct binding epitopes with their receptor subunits (56–61). This model was recently supported by data from the crystal structure of Kaposi’s sarcoma-associated Herpes virus IL-6 in a complex with the extracellular part of gp130 (54). Two vIL-6 molecules bind to two gp130 CRH2 modules via their site I and to two gp130 Ig-like domains via their site III, leaving the sites II available for interaction with the specific IL-6Rα chains (Fig. 6A).

At this moment, we cannot exclude the possibility that additional ligand-independent receptor-receptor interactions can contribute to higher order clustering and that one of the binding sites (I, II, or III) may not be present. For example, homotypic CRH2-CRH2 interactions could functionally replace interaction sites I or II in the complex. To date, very few data concerning the structure-function correlation of the LR are available. However, this model can help explain the unexpected finding that signaling strongly differs when the combinations LR-F3 + LR-FFY-Δbox 1 ΔCRH1,Δlg and LR-F3 ΔCRH1,Δlg + LR-FFY-Δbox 1 are compared (Fig. 5). Leptin-induced complex formation in both combinations is most likely identical because the same extracellular receptor parts (full length + ΔCRH1,Δlg) and similar cytosolic domains are used. According to the model, simultaneous binding of leptin to one LR membrane-proximal CRH2 and to the Ig-like domain of a second LR chain leads to clustering of both receptors in such a spatial configuration that the JAKs become properly oriented allowing their cross-phosphorylation and activation (illustrated by the arrow in Fig. 6C). Much in contrast, recruitment of a JAK-associated receptor (in this case, LR-F3 or LR-F3 ΔCRH1,Δlg) by the putative site II, does not lead to JAK activation (Fig. 6D). This underscores the critical

**Fig. 6. Model for the LR Complex**
A schematic representation of the IL-6 receptor (A) and of the LR complexes (B) is shown. Panels C and D show receptor complexes composed of the LR-F3 + LR-FFY-Δbox 1 ΔCRH1,Δlg (C) and LR-F3 ΔCRH1,Δlg + LR-FFY-Δbox 1 (D) receptor combination, illustrating the functional asymmetry within the LR complex (see arrow). IL-6Rα, IL-6 receptor α-chain; Ig, Ig-like domain; I, II, and III, putative interaction sites on the ligand.
role for the Ig-like domain in JAK activation and explains why a LR lacking both CRH1 and Ig-like modules is not able to generate a STAT3-dependent signal. This strict requirement for JAK orientation is in marked contrast to STAT activation, because deletion of the CRH1 and Ig-like domains in LR-FFYΔbox 1 has no major effect on signaling. That precise JAK positioning is a prerequisite for efficient signaling is in line with recent reports showing that introduction of one to four alanine residues in the α-helical transmembrane domain or juxtamembrane intracellular region dramatically affects JAK activation (62, 63). The non-redundant use of individual receptor subunits within a homomer receptor complex, however, is a novel and unexpected finding and could only be revealed thus far using this complementation strategy. It will be of interest to apply this approach to other receptor systems, including heteromeric cytokine receptors.

In summary, we have demonstrated the use of a novel JAK/STAT complementation assay in unravelling structural requirements for LR complex formation and activation. We have provided evidence for 1) higher order LR complex formation, 2) a role for the CRH1 in optimal leptin receptor activation, 3) a critical role for the Ig-like domain in leptin binding and JAK activation, and 4) a functional nonredundant role for individual LR chains within the homomeric LR complex.

MATERIALS AND METHODS

Growth Factors and Antibodies

Recombinant mouse leptin and human erythropoietin (Epo) were purchased from R&D Systems (Minneapolis, MN). Typical stimulations were performed with 100 ng/ml leptin (= 6.25 nm), or 50 ng/ml Epo (= 2.5 nm), unless indicated otherwise.

A peptide was synthesized according to the primary sequence of the mouse LR at position aa 942–953. The KLH-coupled peptides were emulsified in TiterMax Gold Adjuvant (cytRx Corp., Los Angeles, CA) and injected intradermally into New Zealand rabbits. Animals were boosted several times as and if. The final antisera were passed over Protein-A Sepharose (Amersham Pharmacia Biotech, Arlington Heights, IL) and eluted with 100 mm glycine, 50 mm NaCl, pH 3.0. The IgG fraction was further affinity purified by binding to the corresponding peptide coupled to EAH-Sepharose (Amersham Pharmacia Biotech). Final elution was performed with 50 mm diethyldiamine, 10% glycerol, 50 mm NaCl (pH 11.7), and neutralization was achieved by addition of 1.5 M Tris–HCl (pH 6.8).

Vectors

The pMETS mouse LR (National Center for Biotechnology Information accession no. U46135) long form (pMETS-mlR) was kindly provided by L. Tartaglia. The mutant leptin receptor LR-F3 was generated as described previously (31). In brief, the three cysteine-rich tyrosine residues (Y985, Y1077, and Y1139) were mutated to phenylalanines using the QuikChange site-directed mutagenesis procedure (Stratagene, La Jolla, CA). In the second LR variant, LR-FFYΔbox 1, the tyrosines on positions 985 and 1077 were mutated to phenylalanines to reduce the negative feedback of the signal to a similar level as for the LR-F3. To eliminate JAK activation, two proline residues on positions 876 and 878 in the box 1 motif, were mutated to serines using the mutagenic oligonucleotides 5′-GGACATCTCTCTCCTGCAAGGAGC-3′ and 5′-GGACATCTCTCTCCTGCAAGGAGC-3′. The LR-FFYΔbox 1 variant was constructed as described previously (31).

Generation of the EpoR-mlR chimera, containing the extracellular part of EpoR, and the transmembrane and intracellular parts of the mlR, as well as generation of the derived EpoR-LR-F3, has been described previously (64). The EpoR-LR-FFYΔbox 1 was constructed in a way similar to the LR-FFYΔbox 1 mentioned above. LR variants lacking the membrane distal CRH module (aa 1–308) were generated using a site-directed mutagenesis strategy. Using the mutagenic oligonucleotides, 5′-GGCAATTCTTCCCTCCCTGCAAGTTAATGTGTTTCTGGG-3′ and 5′-CGACCAAAACCATCAAATTCCTCGAGGCGAGAAGTGGGATATGCGC-3′, a XhoI site was introduced at position 90, just after the signal peptide-encoding sequence. A second XhoI site was generated at position 966 (nucleotides 5′-CGCTGGAATTCCTCCTGCGGCAAGATTACCATGCTCGG-3′ and 5′-CTTCTGTGTTTAAGAAGCTGAGCTAGCTCCCCGTC-3′). The resulting construct was digested with XhoI, and the smallest fragment (228 bp) was ligated back into the opened vector. Deletion of the membrane-distal CRH and the neighboring Ig-like domain (aa 309–447) was performed along a similar strategy. A third XhoI restriction site was introduced at position 1275 (nucleotides 5′-GGCTGGAATTCCTCCTGCGGCAAGATTACCATGCTCGG-3′ and 5′-CTTCTGTGTTTAAGAAGCTGAGCTAGCTCCCCGTC-3′). The vector was circularized after digestion with XhoI. All constructs were verified by DNA sequence analysis.

Generation of the pXP2d2-αPAP1 (rat pancreas associated protein 1)-luciferase reporter was described previously (31). Activation of this reporter is dependent on STAT3, because overexpression of dominant-negative STAT3, but not of dominant-negative STAT1, completely blocks transcriptional activation (65).

Cell Lines and Transfection Procedures

HEK293T cells were cultured in 10% CO2 humidified atmosphere at 37 C, and grown using DMEM with 4500 mg/liter glucose, 10% fetal bovine serum, and 50 μg/ml gentamicin (all from Invitrogen, San Diego, CA).

For transfection experiments, HEK293T cells were freshly seeded and cultured overnight. Cells were transfected overnight with approximately 2 μg (unless stated otherwise) plasmid DNA using a standard calcium phosphate precipitation procedure. One day after transfection, cells were washed with PBS-A (PBS without calcium, magnesium and sodium bicarbonate), and cultured until further use.

Reporter Assay, Leptin Binding Assay, Western Blot Analysis, and Immuno precipitation

Cells were resuspended 48 h after transfection with cell dissociation agent (Invitrogen) and seeded in a 96-well plate (Costar). Cells were stimulated overnight with the appropriate cytokine, and luciferase activity was measured by chemiluminescence. Lysates were prepared (lys buffer: 25 mm Tris, pH 7.8; 2 mm EDTA: 2 mm dithiothreitol; 10% glycerol; 1% Triton X-100), and 3 μl luciferase substrate buffer (20 mm Tricine; 1.67 mm (MgCO3)2 Mg(OH)2; 5 H2O; 2.67 mm MgSO4·7 H2O; 0.1 mm EDTA: 33.3 mm dithiothreitol: 270 μg/ml coenzyme A; 470 μM Luciferin; 530 μM ATP; final pH 7.8) was added per 50 μl lysate. Light emission was measured for 5 sec in a TopCount Chemiluminescence Counter (Packard Instruments, Meriden, CT).
Cell surface expression of wild-type LR or LR mutants was measured using a binding assay with a mouse leptin-sea chimERIC protein. Generation of this leptin-sea chimERIC chimera has been described previously (14). Cells were washed (wash buffer: DMEM, 0.1% NaNO₃, 20 mM HEPES, pH 7.0, 0.01% Tween 20) 2 x 10⁵ cells and incubated for 90 min at room temperature with a 1:50 dilution of a Cos-conditioned medium containing the leptin-sea chimERIC chimera (final concentration, 10 ng/µL). After three successive washing steps, cells were lysed (lysis buffer: Triton X-100, 10 mM Tris-HCl, pH 7.4). Endogenous phosphatases in the lysates were inactivated (65°C, 30 min), and secreted alkaline phosphatase activity was measured using the 1% chemiluminescent AP substrate disodium 3-[4-methoxy-spiro[1,2-dioxetane-3,2'-5'-chloro]tricyclo[3.3.1.1³⁸]decan]-4'-yl]phenyl phosphate (CSPD) substrate method (PhosphoLight, Tropix, Inc., Bedford, MA) in a TopCount Chemiluminescence Counter (Packard Instruments).

Expression of wild-type LR or LR mutants was also demonstrated using Western blot analysis. Briefly, 4 x 10⁶ HK293T cells in a six-well were transfected with plasmids encoding the LR variants. Cells were directly lysed 60 h post transfection in 300 µL loading buffer. After sonication, 40 µL of the lysates were loaded on a 7.5% polyacrylamide gel. After overnight blotting onto nitrocellulose sheets, LR constructs were revealed using an h-ras-murine polyclonal antibody directed against the intracellular part of the receptor (see above), and a donkey-anti-rabbit horseradish peroxidase-coupled antibody.

For STAT3 and Jak2 phosphorylation, HK293T cells were transfected with the appropriate LR variants. Cells were starved 65 h later in serum-free medium for 5 h and were left untreated or stimulated with 100 ng/ml leptin for 15 min. Gel electrophoresis and blotting were as described above. STAT3 phosphorylation was checked using the phospho-STAT3-Tyr705 antibody (Cell Signaling), according to the manufacturer’s guidelines. STAT3 expression levels were verified using an anti-STAT3 antibody (Transduction Laboratories, Inc., Lexington, KY). To detect Jak2 phosphorylation and expression, 0.01 µg rJK5-Jak2 was cotransfected and Jak2 was revealed using an antiphospho-Jak2 (Y1007, Y1008) antibody (Upstate Biotechnology, Inc., Lake Placid, NY), or an anti-Jak2 antibody (Upstate Biotechnology, Inc.).

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Address all correspondence and requests for reprints to: Jan Tavernier Ph.D., Flanders Interuniversity Institute for Biotechnology, VIB09, Department of Medical Protein Research, Ghent University, Faculty of Medicine and Health Sciences, Baertsekenlaan 3, B-9000 Ghent, Belgium. E-mail: Jan.Tavernier@rug.ac.be.

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Chapter 11

Mechanisms Underlying Leptin Receptor Oligomerization and Activation

11.1. Introduction

The 16 kDa cytokine-like hormone leptin has been identified as one of the key players in the control of body weight and energy expenditure. The product of the ob gene is mainly produced and secreted by adipocytes (Frederich et al., 1995), and protein levels correlate with energy stores in the body fat mass. Leptin can also be expressed in the fundus of the stomach (Bado et al., 1998) and in skeletal muscle (Wang et al., 1999). Leptin reduces food intake and stimulates energy consumption by binding and activating its receptor in specific hypothalamic nuclei (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Spontaneous mutations that lead to a functional defect in either leptin (Zhang et al., 1994; Montague et al., 1997) or its receptor (Chen et al., 1996; Lee et al., 1996; Clement et al., 1998) result in a complex syndrome including morbid obesity, hypothermia, infertility, hyperglycemia, decreased insulin sensitivity, and hyperlipidemia. Administration of exogenous leptin to leptin deficient ob⁻/⁻ mice reverts these defects (Lord et al., 1998; Cunningham et al., 1999; Ahima and Flier, 2000; Matarese et al., 2002). In men, loss-of-function mutations in leptin or its receptor are very rare and most obese patients have elevated leptin levels (Maffei et al., 1995). This so-called leptin resistance could result from an impaired transport of leptin through the blood-brain barrier, defects in receptor activation, or from abnormalities in signaling. Besides the weight regulating function, leptin is also believed to play a role in basal metabolism, bone formation, reproduction and immunity (Mikhail et al., 1997; Muller et al., 1997; Shimabukuro et al., 1997; Zachow and Magoffin, 1997).

The leptin receptor (LR) was first cloned using an expression cloning strategy and was
found to be encoded by the db gene (Tartaglia et al., 1995). Db<sup>−/−</sup> mice exhibit a phenotype resembling that of the ob<sup>−/−</sup> strain (Chen et al., 1996; Lee et al., 1996; Clement et al., 1998). The receptor has a structure reminiscent of the class I cytokine receptor family. The extracellular domain is composed of two so-called CRH (cytokine receptor homology) domains, a membrane distal CRH1 and a membrane proximal CRH2. Both domains are separated by an Ig (immunoglobulin) like domain and are followed by two FNIII (fibronectin type III) domains close to the membrane. Fong et al. showed that CRH2 is necessary and sufficient for leptin binding, whereas the two FNIII, despite the lack of any affinity for the ligand, are needed for receptor activation (Fong et al., 1998). The LR can exist as six alternative spliced isoforms of the receptor. The LR long form (LRlo) contains the full cytosolic domain and is the only isoform capable of signaling, and is highly expressed in hypothalamic neurons. Expression at functional levels of this LRlo could also be shown in several other cell types including hepatocytes, lung, testis, immune cells, etc. thereby forming the basis for the peripheral biological functions of leptin. The four short forms (LRa, L Rc, LRd and LRf) have only a short intracellular tail, and cannot signal. High levels of LRa and L Rc have been detected in the choroid plexus and brain microvessels (Tartaglia et al., 1995), which might suggest that these receptors play a role in the transport of leptin through the blood-brain barrier. An alternative role for these short receptors is the clearance of leptin from the blood. Finally, a soluble isoform can be generated by alternative splicing (LR<sub>e</sub>) or by ectodomain shedding.

A common pathway in cytokine signaling is the JAK/STAT (Janus kinase/Signal transducer and activator of transcription) pathway. JAK kinases bind constitutively to the proline-rich box1 motif in the cytoplasmic tail of the receptor. Upon ligand-induced receptor activation, these kinases are brought in close proximity, and activate each other by cross-phosphorylation. LRlo predominantly activates JAK2 (White and Tartaglia, 1999). Activated JAK kinases phosphorylate receptor tyrosines, hereby providing docking-sites for the STAT factors. Upon phosphorylation, STATs translocate as dimers to the nucleus, and induce specific gene transcription. The predominant STAT activated by leptin is STAT3 (Vaisse et al., 1996).
Based on co-immunoprecipitation experiments it could be shown that the LR forms dimers in the absence of ligand in solution and on the cell surface (Devos et al., 1997; White and Tartaglia, 1999). More recently, Couturier et al. showed that approximately 60% of the expressed receptors exist as constitutive dimers (Couturier and Jockers, 2003). Here we report, using a co-precipitation strategy and classical chemical cross-linking, that both CRH2 and FNIII domains homotypically interact. These interactions result in CRH2 dimers, while di-, tri-, tetramers and even higher order FNIII clusters were observed. FNIII domains are covalently cysteine-linked in solution, but this is prevented in the presence of the CRH2 domain. Mutation of the two free cysteine residues in the LR completely abolishes signaling, without affecting receptor expression and leptin binding. We use these data to propose a model for LR activation.

11.2. Materials and Methods

11.2.1. Construction of LR domains and LR deletion variants

Generation of the LR mutant receptor mLR-F3, in which all three cytoplasmic tyrosine residues were mutated to phenylalanes, has been described previously (Eyckerman et al., 2000). Receptor deletion variants mLR ΔCRH1, and mLR ΔCRH1,ΔIg (and the F3 variants thereof) have been constructed using a mutagenesis strategy (Zabeau et al., 2004). In brief, a Xho I site was introduced immediately following the signal-peptide encoding sequence. A second Xho I site was inserted following the sequence coding for the membrane distal CRH1 domain, or for the Ig-like domain. Resulting vectors were Xho I digested and circularized. Deletion variant mLR ΔCRH1,ΔIg,ΔCRH2 was made by PCR amplification with oligo-1 and -2 (see table 1). The resulting amplicon was Xho I - Kpn I digested and ligated into the opened pMET7 mLR ΔCRH1,ΔIg vector. The vector pMET7 V5-mLR ΔEC encodes a LR variant in which the extracellular domain is replaced by the sequence encoding the V5 epitope. The LR transmembrane and cytoplasmic domains were amplified using the oligonucleotides oligo-3 and -4. The forward primer inserts an Xho I site and the V5 tag at the 5’ end. Also here, after Xho I - Kpn I digestion, the amplicon was inserted into the opened pMET7 mLR ΔCRH1,ΔIg vector.
In the full length receptor and in the deletion variants, the myc tag was replaced by the sequence coding for the FLAG tag. Therefore, the LR sequence between nucleotides 2161 to 2684 was amplified with oligonucleotides oligo-5 and -6. The amplicon was Dra III - Xba I digested and ligated in the appropriate expression vectors.

(a) pMET7 CRH1,Ig,CRH2; (b) pMET7 Ig,CRH2; (c) pMET7 CRH2; (d) pMET7 CRH1; and (e) pMET7 CRH1,Ig coupled to the FLAG-His tags (i) or SEAP-FLAG (ii) expression vectors were constructed by inserting a Bgl II immediately following the sequence encoding the CRH2 domain (a, b, and c; primers: oligo-7 and -8), the CRH1 domain (d; primers: oligo-9 and -10), or the Ig-like domain (e; primers: oligo-11 and -12 and). Resulting vectors were cut with the enzymes Bgl II and Xba I. (i) Oligonucleotides oligo-13 and -14, encode both FLAG and His tags, and 5’ and 3’ ends are complementary to cut Bgl II and Xba I sites. Oligo’s are annealed and ligated into the opened vectors, resulting in FLAG-His tagged proteins. (ii) Alternatively, oligonucleotides oligo-15 and -16 were used to amplify the sequence coding for the secreted alkaline phosphatase (SEAP). The amplicon was digested with the enzymes Bgl II and Xba I, and ligated into the opened vectors.

Expression vectors pMET7 mLR FNIII-FLAG-His and pMET7 mLR FNIII-SEAP-FLAG were constructed as follows; cDNA for FNIII domains was amplified using the primers oligo-17 and -18. CRH2 domain in pMET7 mLR CRH2-FLAG-His and pMET7 mLR CRH2-SEAP-FLAG was replaced by the cDNA for the FNIII domains by a Xho I – Bgl II digestion of the amplicon and the vectors.

Free cysteine residues in the wild type LR were mutated to serines. Primers used were oligo-19 and -20 for cys672ser, and oligo-21 and -22 for the cys751ser mutation. The double mutant LR cys672,751ser was constructed by digestion of pMET7 mLR cys751ser with enzymes Dra III and Sac I. The resulting insert of 1507 bp was ligated in the Dra III – Sac I opened pMET7 mLR cys672ser.
Mechanisms of Leptin Receptor Activation

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<td>5'-GGCTGCGAAAAATGACGAGTACGTTCCATCA-3'</td>
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<td>oligo-19</td>
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<td>oligo-20</td>
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<td>oligo-21</td>
<td>5'-GGCTGCGAAAAATGACGAGTACGTTCCATCA-3'</td>
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<td>Table 1: Oligonucleotides used in this study.</td>
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Generation of the pXP2d2-rPAP1 (rat pancreatitis associated protein 1)-luciferase reporter was described before (Eyckerman et al., 2000). Activation of this reporter is dependent on STAT3. Over-expression of dominant-negative STAT3, but not of dominant-negative STAT1, completely blocks rPAP-luciferase reporter activation (Broekaert et al., 2002).

11.2.2. Cell-line and transfection procedure

Human embryonic kidney Hek293T cells were grown in DMEM medium with 4500 mg/l glucose supplemented with 10% fetal bovine serum (all from Invitrogen) in 10% CO₂ humidified atmosphere at 37°C. For transfection experiments, 4.10^5 cells per 10 cm² well were freshly seeded and cultured overnight. Transfections were done overnight with a standard calcium phosphate precipitation procedure. One day after transfection, cells were washed with PBS-A, and cultured overnight until further use (western blot, co-precipitation, chemical cross-linking, reporter assay or leptin-SEAP binding).

11.2.3. Western blot analysis

Expression of LR domains or LR deletion mutants was checked using Western blot analysis. Therefore, an equal volume of loading buffer was added to supernatants of cells transfected with the different domains. Alternatively, cells expressing the LR variants were lysed in 300 µl loading buffer and sonicated. Samples were loaded on a
polyacrylamide gel, and blotted onto a nitrocellulose membrane. Proteins were revealed with a monoclonal antibody directed against the FLAG-tag (Sigma) and sheep anti-mouse horseradish peroxidase coupled secondary antibody (Amersham Bioscience).

11.2.4. Co-precipitation
Hek293T cells were transiently transfected overnight with SEAP-FLAG and FLAG-His (or empty vector as a negative control). Three days after transfection, supernatants were collected and subjected to a precipitation with the talon metal affinity resin (BD Bioscience). 50 µl bed-volume resin per precipitation was washed three times with wash-buffer (50 mM NaPO₄, 300 mM NaCl, 0.5% NP40, pH 7.0). Supernatants were incubated with the resin for one hour at 4°C. After three washes with wash-buffer, precipitated complexes were eluted with an acidic elution buffer (50 mM sodium acetate, 300 mM NaCl, pH 5.0). Co-precipitated SEAP activity was measured using the chemiluminescent CSPD substrate (PhosphaLight, Tropix) in a TopCount Chemiluminescence Counter (Packard).

11.2.5. Chemical cross-linking
To determine the oligomerization state of the LR subdomains, supernatants of transfected cells were incubated with a serial dilution of BS₃ cross-linker (Pierce) as indicated for six hours at 4°C. The reaction was stopped by the addition of glycine to an end-concentration of 50 mM. Samples were analyzed by western blot using an anti-FLAG antibody. Cross-linking on cells expressing the different deletion variants of the LR was performed in a similar way. Cells were incubated with 1 mM BS₃ for two hours, washed two times with PBS-A, and lysed in loading buffer.

11.2.6. Reporter assays
Two days after transfection, cells expressing different combinations of LR variants were resuspended with cell dissociation agent (Invitrogen) and seeded in a 96 black 96-well plate (Costar). Cells were stimulated overnight with a serial dilution of leptin (R&D systems) as indicated, or were left unstimulated. Lysates were prepared (lysis buffer: 25 mM Tris, pH 7.8; 2 mM EDTA; 2 mM DTT; 10% glycerol; 1% Triton X-100), and 35
µl luciferase substrate buffer (20 mM Tricine; 1.07 mM \(\text{MgCO}_3\)_4\(\text{Mg(OH)}_2\cdot5\text{H}_2\text{O}\); 2.67 mM \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\); 0.1 mM EDTA; 33.3 mM DDT; 270 µM Coenzyme A; 470 µM Luciferin; 530 µM ATP; final pH 7.8) was added per 50 µl lysate. Light emission was measured for 5 seconds in a TopCount Chemiluminescence Counter (Packard).

11.2.7. Leptin-SEAP binding

Cell surface expression of wild type LR or LR mutants was measured using a binding assay with a mouse leptin-SEAP chimeric protein. Generation of this leptin-SEAP chimera has been described before (Tartaglia et al., 1995). Two days after transfection, cells were washed (wash buffer: DMEM, 0.1% NaN\(_3\), 20 mM Hepes pH 7.0, 0.01% Tween 20) and incubated for 90 minutes at room temperature with a 1/50 dilution of a COS1 conditioned medium containing the leptin-SEAP chimera (final concentration: 10 ng/ml). After 3 successive washing steps, cells were lysed (lysis buffer: 1% TritonX-100, 10 mM Tris-HCl pH 7.4). Endogenous phosphatases in the lysates were inactivated (65°C, 30 min), and secreted alkaline phosphatase activity was measured as described above.

11.3. Results

11.3.1. Expression of LR subdomains

We first analyzed the ligand-independent clustering of the LR in more detail. We therefore expressed the different LR subdomains, and combinations thereof, as either a FLAG-His, or SEAP-FLAG fusion proteins (figure 1). The His-tag allows specific enrichment of the expressed fusion proteins, while to the presence of SEAP-fusion proteins can be quantified using the chemiluminescent CSPD substrate. The additional FLAG-tag can be used to monitor expression levels of the different proteins. The resulting recombinant proteins were transiently expressed in the human embryonic Hek293T cell-line and analyzed using Western blot. Results, as well as a schematic representation of the constructs used, are shown in figure 1. No expression was observed for the separate Ig-like domain as either FLAG-His or SEAP-FLAG fusion. Nevertheless, characteristics of this domain can, at least in part, be deduced from the behavior of CRH1-Ig and Ig-CRH2 fusion proteins.
Figure 1: Schematic presentation and expression of LR domains used in this study. A schematic presentation of the full length mLR (A), FLAG-His (B) and SEAP-FLAG (C) tagged LR domains is given. CRH: cytokine receptor homology domain; Ig: Immunoglobulin-like domain; FNIII: Fibronectin type III domain; TM: transmembrane domain; cyto: cytoplasmic tail. Western blot analysis of LR domain expression. cDNA encoding the different FLAG-His (B) and SEAP-FLAG (C) tagged domains was transiently expressed in Hek293T. Supernatants of FLAG-His transfected cells were subjected to a precipitation with Ni²⁺ metal affinity resin. Precipitated proteins were eluted from the resin with loading buffer and separated using 10% SDS-PAGE, blotted onto a nitrocellulose membrane and revealed with an anti-FLAG antibody. Expression of SEAP-FLAG proteins was detected in a similar way, but here supernatants from transfected cells were directly loaded onto a 7.5% SDS-PAGE gel.

We first determined the leptin binding properties of these receptor fragments. Supernatants of cells transfected with the cDNA for the FLAG-His fusion proteins were incubated with leptin-SEAP, subjected to a precipitation with Ni²⁺ metal affinity resin and the co-precipitated SEAP activity was measured (figure 2). Only proteins containing the CRH2 domain (CRH1-Ig-CRH2, Ig-CRH2 and CRH2) were able to selectively bind the leptin-SEAP ligand (panel A). We next determined the binding constants (K_D) for this interaction. The three CRH2 containing FLAG-His fusion proteins were coated on a 96-well plate and incubated with a serial dilution of leptin-SEAP. K_D values were determined by plotting the bound leptin to the leptin concentration (figure 2, panel B). The binding constants, calculated by a curve fitting using the Graph Path software,
were respectively $0.45 \pm 0.16 \text{ nM}$, $0.40 \pm 0.04 \text{ nM}$, and $0.22 \pm 0.02 \text{ nM}$ for CRH1-Ig-CRH2, Ig-CRH2 and CRH2. Given the lack of significant differences between these values, it seems reasonable to assume that CRH2 is the main and only determinant for leptin binding. This is in line with previously published work using membrane-anchored receptor variants (Fong et al., 1998; Zabeau et al., 2004). These data indicate that the different LR domains can be separately expressed as functional fusion proteins.

**Figure 2: Leptin binding properties of the LR domains.**

Binding of leptin to the different domains is determined by co-precipitation (A) or by binding to immobilized domains on plates (B). (A) Hek293T cells were transiently transfected with LR domains as indicated, or with empty vector (MOCK). Supernatants of these cells was incubated with leptin-SEAP for two hours. LR domains were selectively precipitated with a Ni$^{2+}$ metal affinity resin. After elution, co-precipitated alkaline phosphatase activity was measured. Mean phosphatase values and error bars of triplicate measurements are plotted. (B) MaxiSorp plates coated with anti-His antibody were incubated overnight with supernatants of Hek293T cells transfected with the domains CRH1-Ig-CRH2 (open circle), Ig-CRH2 (open square) and CRH2 (open triangle). Plates were washed and incubated with a serial dilution of leptin-SEAP as indicated. After four successive washing steps, bound SEAP activity was determined with the CSPD substrate. Error bars represent SD of triplicate samples.
11.3.2. Ligand-independent clustering of the LR domains

In order to determine which LR domains are involved in receptor clustering, we used a His/SEAP co-precipitation assay. In this test, we co-transfected different FLAG-His domains together with the CRH1-Ig-CRH2-SEAP-FLAG fusion protein. Supernatants were collected 72 hours after transfection and precipitated using the His binding Ni\(^{2+}\) metal affinity resin. The precipitated protein complexes were eluted and the co-precipitated SEAP activity was measured using the CSPD substrate. Clustering of the membrane proximal FNIII domains was tested in a similar fashion by co-transfection of expression vectors pMET7 FNIII-FLAG-His and pMET7 FNIII-SEAP-FLAG. Results in figure 3 show that both CRH2 and FNIII domains form homotypic clusters in the absence of leptin. No significant co-precipitation was observed with FLAG-His proteins consisting of only CRH1 and Ig, thereby excluding a possible role of these domains in the ligand-independent clustering of the LR.

![Figure 3: Domains involved in LR homodimerization identified with a His/SEAP co-precipitation assay.](image)

(A) Different FLAG-His tagged domains (as indicated) or empty vector (MOCK) were co-transfected with the cDNA encoding CRH1-Ig-CRH2-SEAP-FLAG in Hek293T cells. Three days after transfection, supernatants were collected and subjected to precipitation with the Ni\(^{2+}\) metal affinity resin. After three successive washes, elution, co-precipitated alkaline phosphatase activity was measured using the CSPD substrate. Bars shown represent mean values, and S.D. values of triplicate measurements. (B) FNIII oligomerization was studied in a similar way. Hek293T cells were co-transfected with cDNA encoding FNIII-SEAP-FLAG in combination with FNIII-FLAG-His or empty vector (MOCK). Co-precipitation was determined as described above.
11.3.3. Characteristics of CRH2 and FNIII clustering

Using the chemical cross-linker BS\(^3\) we wanted to confirm the ligand-independent clustering of the CRH2 or FNIII domains. Supernatants of cells expressing LR domains CRH1, CRH2 and FNIII with FLAG-His tag were treated with the cross-linker, and analyzed by Western blot using an anti-FLAG antibody (figure 4, panel A). Cross-linking of CRH2 indicated the presence of dimers, while next to dimers also oligomers of the FNIII-FLAG-His protein can be observed. No complex formation of CRH1 resulted from the BS\(^3\) treatment. These cross-linking data confirmed the results of the His/SEAP co-precipitation assays.

**Figure 4: Characteristics of CRH2 and FNIII oligomerization.**

(A) Chemical cross-linking. Supernatants of cells expressing domains CRH1-FLAG-His, CRH2-FLAG-His or FNIII-FLAG-His was incubated with BS\(^3\) for six hours. Protein complexes were analyzed using an anti-FLAG antibody as described above. (B) The necessity of co-expression of FLAG-His and SEAP-FLAG proteins for co-precipitation was assayed. Hek293T cells were transfected individually with CRH2-FLAG-His, CRH2-SEAP-FLAG, or empty vector. The next day, cells were resuspended and equal amounts of cells expressing CRH2-FLAG-His and CRH2-SEAP-FLAG (filled bars), or cells transfected with CRH2-SEAP-FLAG and empty vector (open bars) were mixed. As a positive control, cells were transfected with a combination of FLAG-His and SEAP-FLAG fusion, or SEAP-FLAG and empty vector. These transfected cells were also resuspended but not mixed. Two days later, the formation of oligomers was determined by subjecting supernatants of cell mixtures to co-precipitation with the metal affinity resin. Bars shown represent mean alkaline phosphatase values, and S.D. values of triplicate measurements.
In the co-precipitation assay described thus far (figure 3), the FLAG-His and SEAP-FLAG fusion proteins were co-transfected. We next determined what the effect of co-expression on the observed homotypic interactions of the CRH2 and FNIII domains. Therefore, the FLAG-His proteins (CRH2-FLAG-His or FNIII-FLAG-His) and SEAP-FLAG fusions (CRH2-SEAP-FLAG or FNIII-SEAP-FLAG) were separately transfected. One day after transfection, transfected cells were resuspended and mixed. 48 Hours later, clustering of the CRH2 and FNIII domains was determined by a His/SEAP co-precipitation assay (figure 4, panel B). Results illustrated that in both cases co-precipitation can only be observed when His and SEAP fusion proteins were co-expressed.

### 11.3.4. LR clustering on the cell surface

We next studied oligomerization of the LR on the cell surface. In a first approach we tried to inhibit leptin signaling with LR-F3 mutants. In these receptors the three cytoplasmic tyrosine residues are mutated to phenylalanines, thereby eliminating the recruitment and activation of STAT3. We recently showed that a LR variant lacking the membrane distal CRH1 domain is the minimal signaling-competent LR variant, while further deletion of the Ig-like domain results in complete loss of signaling (Zabeau et al., 2004). LR ΔCRH1 was transiently transfected with increasing amounts of cDNA, as indicated, encoding mLR-F3 ΔCRH1, ΔIg (figure 5, panel A), mLR-F3 ΔCRH1, ΔIg, ΔCRH2 (panel B), or V5-mLR-F3 ΔCRH1, ΔIg, ΔCRH2, ΔFNIII (a LR variant in which the complete extracellular domain is replaced by the V5 epitope, panel C). The STAT3 responsive rPAP1-luciferase reporter was co-transfected and used as a read-out for JAK/STAT signaling. Results illustrated that the LR-F3 variants with an extracellular domain composed of CRH2 and FNIII or FNIII alone could inhibit LR signaling. In contrast, when the complete extracellular domain was deleted, no such inhibition was observed. These data suggest that in the absence of ligand the combination CRH2-FNIII or FNIII in the extracellular part of the LR-F3 receptors allows pre-clustering with LR ΔCRH1 receptor on the cellular surface, thereby reducing the number of signaling-competent receptor complexes.
Figure 5: Inhibition of LR signalling by truncated mutants.

The role of the different LR domains in the formation of an activated receptor complex was tested using a panel of deletion mutants. Therefore, 0.1 µg plasmid encoding the mLR ΔCRH1 was co-transfected with different amounts of (A) mLR-F3 ΔCRH1,ΔIg, (B) mLR-F3 ΔCRH1,ΔIg,ΔCRH2 and (C) V5-mLR-F3 ΔEC as indicated. The pXP2d2-rPAP1-luci reporter construct was also transfected to measure STAT3 activation. Cells were stimulated overnight with a serial dilution of leptin as indicated. Luciferase measurements were performed in triplicate, and bars represent mean values.

11.3.5 Ligand-independent signaling by LR ΔCRH1, ΔIg, ΔCRH2

We also observed that spontaneous (i.e. ligand-independent) rPAP1-luciferase activation was significant higher in cells expressing the mLR ΔCRH1,ΔIg,ΔCRH2 variant, compared to other LR deletion variants (figure 6, panel A). We analyzed the effect of simultaneous expression of the F3 form of the same receptors on this spontaneous signaling. To test this, mLR ΔCRH1,ΔIg; mLR ΔCRH1,ΔIg,ΔCRH2 and V5-mLR ΔEC and
an increasing amount of cDNA (as indicated) encoding their F3 counterpart were transiently co-transfected in Hek293T cells (figure 6, panel B). Clearly, the spontaneous leptin-independent activity of the mLR ΔCRH1,ΔIg,ΔCRH2 receptor could be completely suppressed by expression of the F3 form of this receptor. Over-expression of the other F3 deletion variants has no effect on the ligand-independent signaling. It is of note that differences in ligand-independent activity could not be explained by differences in expression levels of the different LR deletion variants, as measured by Western blot analysis (data not shown).

Figure 6: Ligand independent signaling of mLR deletion variants
(A) several mLR deletion variants were expressed in Hek293T cells along with the rPAP1-luciferase reporter. Four days later, luciferase activity was measured as described above. (B) Deletion variants mLR ΔCRH1,ΔIg; mLR ΔCRH1,ΔIg,ΔCRH2; and mLR ΔEC were transfected with increasing amounts of cDNA encoding their F3 counterpart. Bars shown represent mean luciferase values, and S.D. values of triplicate measurements.

11.3.6. Involvement of free FNIII cysteine residues in LR activation
We observed that in the absence of a reducing agent, LR FNIII domains were covalently linked in solution (figure 7, panel A). Addition of β-mercaptoethanol completely abolished this oligomerization. However, this disulphide-linkage is only observed when FNIII domains were expressed alone. When the FNIII domains were expressed fused to the CRH2 domain (and Ig, and CRH1 domains), no such covalently clustering was observed (figure 7, panel B). These data indicate that the CRH2 domain prevents ligand-independent disulphide-bridge formation between the FNIII domains.
Figure 7: Covalent LR-LR interactions

(A): FNIII domains are covalently linked in solution. Supernatants of cells expressing the FNIII domains were subjected to SDS-PAGE in the presence and absence of β-mercaptoethanol, as indicated. Protein complexes were revealed with Western blot using an anti-FLAG antibody as described above. (B): CRH2 prevents disulphide-linkage of FNIII domains. Hek293T cells were transiently transfected with cDNA encoding LR domains FNIII, CRH2-FNIII, Ig-CRH2-FNIII and CRH1-Ig-CRH2-FNIII. Protein complexes were analysed under non-reducing conditions using an anti-FLAG antibody as described above.

The mLR FNIII domains each contain one free cysteine residue at positions 672 and 751 (Haniu et al., 1998). To examine their role in the activation of the LR, these residues were mutated to serines in the wild type LR. The resulting single, and double mutants were tested for their signaling capacity in the Hek293T cell-line. Results are shown in panel A of figure 8. Mutation of cys672 had the most pronounced effect on the activation of the rPAP1-luciferase reporter. Additional mutation of cys751 resulted in a receptor almost completely devoid of biological activity. To test the effect of these mutations on cell surface expression and ligand binding, transfected cells were subjected to a leptin-SEAP binding assay (figure 8, panel B). As shown, these mutations had no major effect on LR expression.
Figure 8: Role of free FNIII cysteine residues in LR activation, expression and leptin binding
(A): pMET7 expression plasmids with mLR, mLR cys672ser, mLR cys751ser, or mLR cys672,751ser were transfected in Hek293T cells. The pXP2d2-rPAP1-luci reporter construct was also transfected to measure STAT3 activation. Transfected cells were stimulated overnight with a serial dilution of leptin as indicated. Luciferase measurements were as described above. (B) Effect of cysteine mutations on leptin binding. Transfections were as in panel A. Cells were incubated with leptine-SEAP, with or without an excess unlabeled leptin for two hours. After four successive washing steps, bound alkaline phosphatase activity was measured using the CSPD substrate. Bars represent mean values of triplicate measurements.

11.4. Discussion
Initially it was accepted that cytokine receptors become activated upon ligand induced di- or oligomerization. According to this model, receptor clustering brings the cytoplasmic associated JAKs in close proximity, which allows them to activate each other by cross-phosphorylation and intracellular signaling. However, recent data have indicated that the situation appears to be more complex. Several cytokine receptors are pre-assembled at the plasma membrane. This has been demonstrated for the receptors for erythropoietin (EpoR) (Livnah et al., 1999; Remy et al., 1999) growth hormone
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(GHR) (Frank, 2002), interferon-γ (Krause et al., 2002), the interleukin-6 receptor α-chain (Schuster et al., 2003), and the βc signaling component in the receptors for interleukin-3 (IL-3), IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) (Carr et al., 2001). These observations of pre-assembled complexes suggest that ligand binding induces a spatial reorganization of the receptor chains, thereby triggering intracellular signaling.

Ligand-independent clustering of the LR could also be demonstrated in solution (Devos et al., 1997) and at the cellular surface (White and Tartaglia, 1999; Couturier and Jockers, 2003). More recently, Couturier et al., showed that a correct conformational change is also the basis for activation of the LR (Couturier and Jockers, 2003). In this study, LR short and long forms were coupled to the luciferase enzyme and YFP (yellow variant of the green fluorescent protein) and tested in a bioluminescence resonance energy transfer (BRET) assay. In the case of the short isoforms, leptin stimulation resulted in a marked increase in energy transfer signals, probably reflecting a conformational reorganization in the pre-formed complex (Couturier and Jockers, 2003).

We here studied the interactions between the extracellular LR domains in more detail. We show that both CRH2 and FNIII domains homotypically interact in solution (figure 3 and 4). CRH2 forms only dimers, while FNIII oligomers are found. We could also show that both CRH2 and FNIII oligomerization occurs inside the cell, since mixture of FLAG-His and SEAP-FLAG expressing cells does not result in a significant co-precipitation of both proteins. This implies that LR’s are expressed as dimers, and argues against exchange of subunits between the pre-formed complexes. Both domains are also involved in ligand-independent clustering of the LR at the cellular surface (figure 5). Leptin-dependent signaling by mLR ΔCRH1,ΔIg can be abolished by overexpression of mLR-F3 ΔCRH1,ΔIg and LR-F3 ΔCRH1,ΔIg,ΔCRH2. At this point we cannot exclude the possibility that the inhibition by the LR-F3 ΔCRH1,ΔIg (figure 5) variant is mediated by the interaction between the FNIII domains, more than by CRH2 dimerization. However, this would be in contrast with the observation that interactions between the FNIII domains only occur in the absence of CRH2 (figure 7). This indicates that the CRH2

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dimers keep the FNIII domains spatially apart. This hypothesis is further supported by the observation that the presence of CRH2 prevents constitutively signaling mediated by FNIII clustering (figure 6).

We use these data to propose a model for LR activation (figure 9). Herein the receptor appears as preformed dimers mainly based on the homotypic interactions between the CRH2 domains. In this configuration, FNIII domains, and therefore also the cytoplasmic receptor tails, are spatially held apart, so that there is no JAK activation. Two leptin molecules cluster two such LR dimers. Leptin site II interacts with the LR CRH2, while residues of site III interact with the LR Ig-like domain. In a first hypothesis (panel A), FNIII domains of different pre-formed dimers are brought in close proximity, allowing S-S bridge formation and orientation of the cytoplasmic domains in a correct fashion. Alternatively, simultaneous binding of leptin via its sites II and III, induces a conformational change in the CRH2 domain (panel B). This re-organization allows interaction between the FNIII domains of dimer-related receptors, and signaling.

Figure 9: Model for LR activation

Two possible mechanisms for the activation of the LR. Arrows represent covalent interactions between the FNIII domains. II and III indicate the binding sites on the leptin ligand. For abbreviations, see text.

Both models can explain the necessity of the Ig-like domain in LR signaling. A LR deletion variant lacking both CRH1 and Ig-like domains retains leptin-binding properties comparable to wild type receptor, but lacks any signaling capacity (Zabeau et al.,
2004). In hypothesis A, interaction between leptin site III and LR Ig-like domain is needed for the clustering of two LR dimers. In hypothesis B, interaction of leptin via site III is necessary for the induced conformational change in the LR pre-formed dimer.

Our model might help to explain the observed higher order clustering (i.e. more than two receptors per activated complex) of the LR based on CRH2-CRH2 interactions. Recently we used two signaling deficient LR mutants (one deficient in JAK activation, the other unable to recruit STAT molecules to the receptor) in a complementation strategy (Zabeau et al., 2004). In this assay, both receptors are only able to generate a STAT3 dependent signal when they are co-expressed, and this complementation of signaling is completely lost when the extracellular part of the mutants are replaced by that of the homodimeric EpoR. This LR oligomerization can be explained by assuming that leptin has three receptor interaction sites. Two will be used to form a LR dimer, while a third is necessary to recruit a second LR dimer into the complex. Alternatively, one or more of these binding interfaces can be replaced by receptor-receptor interactions. Our model for the LR is reminiscent of findings reported for the EpoR. Crystal structure of the EpoR CRH illustrates that it is expressed as a dimer in the absence of ligand, and that the self-dimer interface consists mainly of residues which are also involved in Epo binding (Livnah et al., 1999). On the other hand, Constantinescu et al. argue that the transmembrane domain, more than the extracellular part of the receptor, is involved in the pre-formation of receptor complexes (Constantinescu et al., 2001).

In this study we also present evidence that LR FNIII domains cluster in the absence of ligand in a covalently manner. In contrast to CRH2, FNIII domains form di-, tri-, tetramers and even higher clusters are observed. This implies that there is more than one FNIII-FNIII interaction site. Possible candidates are the two free cysteine residues (on position 672 and 751) in the FNIII domains. Mutation of both residues in the wild type receptor had no significant effect on expression and ligand binding. However, signaling was completely blocked, implying that covalent oligomerization is strictly necessary for LR activation. Disulphide-linkage between receptor chains has been described for IL-3, -5 and GM-CSF (Stomski et al., 1996; Stomski et al., 1998). In these
receptors, cysteine residues in the N-terminal domain of the βc chain covalently bind to cysteines in the ligand specific α-receptors upon stimulation with the appropriate ligand. Disulphide linkage appears to be essential for phosphorylation of the βc receptor. Further supporting our model is the observation that a LR deletion variant with an extracellular domain consisting of only the FNIII domains is constitutively active. This implies that FNIII-FNIII interactions can bring the cytoplasmic receptor tails in close proximity and correct orientation so that JAK activation is possible.

These observations strongly suggest that these domains are involved in LR activation, by stabilization and/or by correctly orientating the receptors in the activated complex. This has also been suggested for the FNIII domains in gp130. (i) Pflanz et al. showed that the bacterial expressed membrane distal gp130 FNIII domain (D4) oligomerizes in solution (Pflanz et al., 2001). (ii) In isothermal titration calorimetry experiments, formation of the hexameric IL-6:IL-6Rα:gp130 complex with the gp130 FNIII domains present resulted in the generation of a lower overall free energy when compared to complex formation in the absence of these domains (Boulanger et al., 2003). (iii) A neutralizing antibody directed against the gp130 FNIII D4 domain is able to block IL-6 signaling, further supporting its role in receptor activation (Wijdenes et al., 1995).
11.5. References


Chapter 12

General Discussion and Prospects

The genes for leptin and its receptor have been cloned nearly ten years ago (Zhang et al., 1994; Tartaglia et al., 1995). The role of this cytokine in the regulation of body weight is well accepted. There is also a growing body of evidence that leptin has functions in other processes and responses, like the immune system, development, reproduction, hematopoiesis, and others. However, in strong contrast to other well-studied receptor systems (e.g. the receptors for GH, Epo, IL-6, G-CSF) relatively little is known about the mechanisms underlying the activation of the leptin receptor. More insights in these mechanisms might help to explain the observed leptin resistance observed in most obese patients.

In the work presented in this thesis, we provide evidence for the formation of a higher order LR complex, using a JAK/STAT complementation strategy. In this assay, two signaling deficient receptors, mLR-F3 and mLR Δbox1, are only able to generate a STAT3-dependent signal when they are co-expressed. This complementation of signaling is not observed when the extracellular parts of these mutants are replaced by those of the EpoR. We furthermore show that the membrane distal CRH1 domain is not strictly needed for signaling, but its presence enhances leptin-induced signaling. The LR Ig-like domain on the other hand is absolutely necessary for activation of the JAK kinases and hence also for signaling.

In the second part, we show that both the LR membrane proximal CRH2 and FNIII domains interact homotypically both in solution and at the cellular membrane. We have developed a His-SEAP co-precipitation assay, and also used classical chemical cross-linking. While only CRH2 dimers are observed, FNIII domains form di-, tri-, tetramers and even higher order clusters. For CRH2, as well as for FNIII, co-precipitation is only observed when His and SEAP domains are co-expressed. This most likely illustrates that oligomerization occurs inside the cell during biosynthesis of the receptor. We could also
show that FNIII domains are covalently linked in solution. In the presence of CRH2, no such FNIII S-S bridges could be detected, illustrating that the CRH2 domain keeps these FNIII domains apart. Further supporting this hypothesis is the observation that a LR deletion variant which extracellular part only consists of the FNIII domains is constitutively active. Mutation of two free cysteine residues in the wild type receptor has no significant effect on expression and ligand binding. However, signaling was completely blocked, implying that covalent oligomerization is strictly necessary for LR activation, by stabilization and/or correctly orienting the receptors in the activated complex.

Based on the data from the complementation experiments and from the homotypic LR domain interactions, we propose a model for activation of the LR. In this model, the LR is expressed as dimers on the cellular surface mainly based on interactions between the CRH2 domains. In this configuration, the FNIII domains are too far apart to interact. The binding of leptin clusters two such dimers, allowing S-S bridge formation between FNIII domains, thereby orienting the cytoplasmic receptor tails in such a way that JAKs become activated and signaling is initiated. Alternatively, leptin binding can also result in a conformational reorganization of the CRH2 domains, resulting in the interaction between FNIII domains of one dimer receptor subunit.

The formation of receptor disulphide bridges may represent a general mechanism of cytokine class I receptor activation. This mode has been described for the receptors for IL-3, IL-5 and GM-CSF (Stomski et al., 1996; Stomski et al., 1998). Of special interest is the gp130 chain in the IL-6 receptor, and the receptor for G-CSF. Like the LR, these receptor components have free cysteine residues in their FNIII domains (moritz 2001 276 8244). The role of these residues in receptor activation can be tested as was done for the LR.

It will be of interest to characterize the homotypic interaction between the CRH2 domains. Using a mutagenesis study and co-precipitation experiments, this should allow to identify the residues involved in this association. This approach would furthermore
help us to elucidate whether this dimerization is needed for: (i) receptor expression at
the cell surface, (ii) inhibition of constitutive receptor activation based on FNIII
clustering, (iii) ligand binding. It is not unlikely that the leptin binding epitope in CRH2
is composed of residues of both receptors in the dimer. This information will help us to
further refine our model for the LR.

The fact that additional LR FNIII receptor-receptor interactions are likely needed for the
formation of an activated receptor complex, opens the possibility to block leptin
signaling with high affinity molecules directed against the receptor (receptor domains,
peptides and antibodies), and without interfering with ligand binding. An interesting
possibility is the use of single-stranded camelid antibodies. These antibodies offer two
advantages: (i) they are much smaller than classical antibodies which allow a better
bio-distribution and tissue-penetration, and (ii) they have a relative long complementary
determining region (CDR). This long CDR allows binding to regions that lie deeper
inside proteins, such as an active site of an enzyme or receptor clefts. Such antibodies
may be targeted to the cleft in between the FNIII domains, perhaps even to the region
containing the free cysteine residues. Resulting antibodies can be tested for their
inhibition of leptin-mediated signaling.

As mentioned in this thesis, leptin likely may contribute to the generation and
progression of a set of autoimmune diseases (chapter 9). A major consideration in the
development of leptin-based therapeutic strategies for these diseases is that blocking of
the peripheral functions will also interfere with the central weight-regulating role of
leptin, resulting in an unwanted weight gain. Treatments with leptin antagonists will
thus inevitably lead to a marked increase in body weight. The BBB forms the
physiological border between the peripheral and central functions of leptin. The
molecular mechanisms underlying the transport of leptin across the BBB are still poorly
understood. Initially it was believed that the LR short form plays a central role herein,
but a more complex transport-system was recently put forward (Banks et al., 2002). In
this system the short form would only have a modulating role, e.g. transfer of leptin to
the functional transporter. It will be a challenge to generate a leptin or LR antagonist
that interferes with the peripheral, but not the central functions of leptin. Such antagonists must block peripheral LR activation, but, possibly, may not interfere with the function of the LR short form in BBB transport.

References


PART IV: Samenvatting
Hoofdstuk 13

Samenvatting

Het werk voorgesteld in deze thesis kadert in het onderzoek naar de activerings-mechanismen van klasse I cytokine receptoren, en meer bepaald van de interleukine-5 (IL-5) receptor en van de leptine receptor (LR). Deze structureel sterk verwante receptoren worden gekenmerkt door de aanwezigheid van een zogenaamd cytokine receptor homologie (CRH) domein. Deze receptoren hebben geen intrinsieke kinase activiteit, en gebruiken cytoplasmatisch geassocieerde Janus kinasen (JAKs) om hun signaal te propageren.

Het cytokine IL-5 speelt een centrale rol in de groei, overleving en differentiatie van eosinofilen (Sanderson et al., 1986). Daarnaast reguleert het ook de effector functies van deze cellen, zijdne vrijgave van granulaire eiwitten (Kita et al., 1992) en migratie (Sehmi et al., 1992). IL-5 oefent zijn effecten uit door binding en activering van de membraan-gebonden IL-5 receptor (IL-5R) (Tavernier et al., 1991). Deze receptor bestaat uit een ligand-specifieke IL-5Rα keten en de gemeenschappelijke βc-keten, die gedeeld wordt met de receptor complexen voor IL-3 en granulocyte-macrofaag kolonie stimulerende factor (GM-CSF). IL-5 en eosinofilen spelen een cruciale rol in allergische aandoeningen, zoals astma. Astma wordt gekenmerkt door obstructie en hyper-responsiveitie van de bovenste luchtwegen, waartoe onder meer de IL-5 gestuurde overmatige vrijstelling van granulaire eiwitten door eosinofilen bijdraagt. In dierlijke model systemen vermindert de toediening van antilichamen gericht tegen IL-5 of zijn receptor de allergische inflammatie van de luchtwegen (Coffman et al., 1989; Nagai et al., 1993; Egan et al., 1999).

In het eerste luik van dit doctoraatsonderzoek hebben we het gedrag van drie anti-IL-5 antilichamen in meer detail bestudeerd (hoofdstuk 3); de sterk neutraliserende 5A5 en 1E1, en het zeer zwak neutraliserende H30 antilichaam (Zabeau et al., 2001). De
epitopen van antilichamen 5A5 en H30 overlappen met de interactie-plaats met de IL-5Rα receptor. Het 1E1 epitoop bevat het glutamine residu op positie 13, de determinant voor activering van de βc keten in het receptorcomplex. Hoge concentraties antilichaam blokkeren de proliferatie van IL-5 responsieve cellen, terwijl lage concentraties geen effect hebben. Bij intermediaire concentraties 5A5 en H30, en niet 1E1, vonden we dat de antilichamen de proliferatie kunnen versterken boven het niveau veroorzaakt door IL-5 alleen. Dit potentiërende effect kon waargenomen worden bij elke sub-optimale cytokine concentratie. 5A5 en H30 F\textsubscript{AB}-fragmenten waren niet meer in staat het IL-5 gestuurde signaal te potentiëren, hoewel het neutraliserende effect intact bleef. Ook kon geen verhoogde proliferatie waargenomen worden wanneer gebruik gemaakt werd van GM-1, een monomere IL-5 variant. Deze waarnemingen doen sterk vermoeden dat de waargenomen potentiatie kan verklaard worden door een antilichaam-gestuurde aggregatie van geactiveerde IL-5 receptor complexen. Onze bevindingen impliceren dat gehumaniseerde anti-IL-5 antilichamen, ook deze met een sterk neutralizerend karakter, strenge dienen geëvalueerd te worden voor dergelijk activiteitsverhogend effect, alvorens ze kunnen gebruikt worden in klinische toepassingen.

De IL-3, IL-5 en GM-CSF receptoren werden eveneens gebruikt om de in de onderzoeksgroep ontwikkelde MAPPIT (mammalian protein-protein interaction trap) technologie verder uit te bouwen (hoofdstuk 4). In deze techniek wordt een “bait”-eiwit gekoppeld aan een signalisatie-deficiënte (chimere) receptor, het “prey”-eiwit aan het cytoplasmatische deel van de gp130 receptor. De (fosforylerings-afhankelijke) bait-prey interactie leidt tot rekrutering van de gp130 staart ter hoogte van de geactiveerde receptor, en het genereren van een STAT3-afhankelijk signaal. Deze experimentele set-up kan gebruikt worden voor de detectie van JAK2 tyrosine fosforylering-afhankelijke interacties. Om de te testen interacties uit te breiden naar andere modificaties (serine/threonine fosforylering, acylatie, methylatie en andere) kan gebruik gemaakt worden van een heteromere receptor waaraan de bait gekoppeld wordt. Hierdoor kan het modificerende enzyme aan één keten gekoppeld worden, de bait aan een tweede receptor. Daartoe werden chimere receptoren op basis van de IL-3, IL-5 en GM-CSF
receptoren getest. Deze laatste werden gebruikt om de serine fosforylerings-afhankelijke interactie tussen componenten van de TGFβ/SMAD signaal transductieweg aan te tonen.

Een tweede luik van dit werk bestaat uit de studie van de leptine receptor (LR). Leptine speelt een centrale rol in de regulatie van het lichaamsgewicht door binding en activering van de LR in specifieke nuclei van de hypothalamus. Daarnaast speelt leptine ook een belangrijke rol in het sturen van immuun-responsen, reproductie, ontwikkeling, en andere processen. Hoewel de fysiologische functies van het cytokine goed bestudeerd zijn, is relatief weinig geweten over de mechanismen die aan de basis liggen van de activering van de LR.

Het model waarin de LR wordt geactiveerd als een “simpel” dimer werd reeds meermaals in de literatuur in vraag gesteld (White et al., 1997; Couturier and Jockers, 2003). In hoofdstuk 10 tonen we voor de eerste keer onomstotelijk aan dat de LR geactiveerd wordt als een hogere orde complex. Daartoe hebben we gebruik gemaakt van een JAK/STAT complementatie-test. Hierin zijn twee signalisatie-deficiënte receptoren, waarbij één niet meer in staat de JAKs te activeren, en de andere deficiënt is in het recruteren van de signaalmolecule STAT3, enkel in staat een signaal te genereren wanneer ze samen tot expressie gebracht worden. Wanneer het extracellulair domein van deze mutanten vervangen wordt door dat van de strikt homodimere erythropoietine receptor (EpoR), treedt geen complementatie van signalisatie meer op. Gezien de inherente vereisten voor JAK/STAT signalisatie (twee JAK activerende receptoren, en ten minste één STAT recruterende keten per geactiveerd complex), kunnen deze waarnemingen enkel verklaard worden door een hogere orde klustering van de LR. Deze experimentele set-up werd eveneens gebruikt om aan te tonen dat de verschillende ketens in het complex een niet-redundante rol spelen.
Daarnaast werd in dit luik eveneens aangetoond dat de LR membraan distale CRH1 niet absoluut noodzakelijk is voor het genereren van een signaal, maar wel voor een optimale signalisatie. Het immunoglobuline- (Ig-) achtige domein blijkt strikt noodzakelijk voor de activering van de JAK kinasen en dus ook voor signalisatie.

Co-immunoprecipitatie experimenten hebben aangetoond dat de LR voorkomt als dimer in oplossing en op de cellulaire membraan (Devos et al., 1997; White and Tartaglia, 1999). In deze studie (hoofdstuk 11) hebben we getracht de domeinen in de LR te identificeren die instaan voor deze ligand-onafhankelijke klustering. Daarvoor hebben we een His-SEAP co-precipitatie test ontwikkeld. Daarin worden LR domeinen tot expressie gebracht als een His-gemerkt eiwit of als een SEAP (gesecreteerd alkalisch fosfatase) fusie-eiwit. Complexen worden vervolgens aangerijkt met behulp van een Ni\(^{2+}\) chelaat hars, en de meegeprecipiteerde activiteit wordt gemeten. Daarnaast werd beroep gedaan op klassieke chemische cross-linking. Uit deze experimenten bleek dat zowel de membraan-proximale CRH2, als de FNIII domeinen homo-typisch interageren. Waar voor CRH2 enkel dimeren konden aangetoond worden, werden voor FNIII di-, tri-, tetrameren, en zelfs hogere orde aggregaten gevonden. Voor zowel CRH2 als FNIII domeinen kon enkel co-precipitatie van His en SEAP fusies aangetoond worden wanneer beide eiwitten samen tot expressie werden gebracht. Dit wijst erop dat de oligomerisatie wellicht tijdens de eiwit-synthese in de cel gebeurt. We konden eveneens aantonen dat zwavelbruggen worden gevormd tussen de FNIII domeinen in oplossing. Deze covalente koppeling treedt niet op wanneer de FNIII domeinen gekoppeld worden aan het CRH2 domein, wat erop wijst dat de CRH2 dimerisatie de FNIII domein uit elkaar houdt. In overeenstemming met deze hypothese is de waarneming dat een LR deletievariant waarvan het extracellulaire deel enkel bestaat uit de FNIII domeinen constitutief actief is. Mutatie van de twee vrije cysteines in de LR resulteert in een compleet signalisatie-deficiënte receptor. Mutaties blijken geen effect te hebben op expressie, noch op ligand binding.
Resultaten van de complementatie experimenten en van de homo-typische LR domein interacties werden gebruikt om een model voor het LR complex voorop te stellen. In dit model komt de receptor als dimer tot expressie op de cellulaire membraan, voornamelijk op basis van interacties tussen de CRH2 domeinen. In deze configuratie worden de FNIII domeinen uit elkaar gehouden zodat ze niet met elkaar kunnen binden. Leptine binding klustert twee zulke dimeren zodat zwavelbruggen kunnen gevormd worden tussen de FNIII domeinen van verschillende dimeren. Op die manier worden de cytoplasmatische staarten op correcte manier georiënteerd zodat JAK activering mogelijk wordt. Een tweede mogelijk is dat leptine-binding aanleiding kan geven tot een conformationele verandering binnen de CRH2 domeinen, zodat zwavelbrug vorming optreedt tussen FNIII domeinen binnen eenzelfde dimer. Dit mechanisme van receptor activering laat de ontwikkeling toe van componenten die LR signalisatie blokkeren door te interferen met de FNIII-FNIII interacties.

Referenties


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