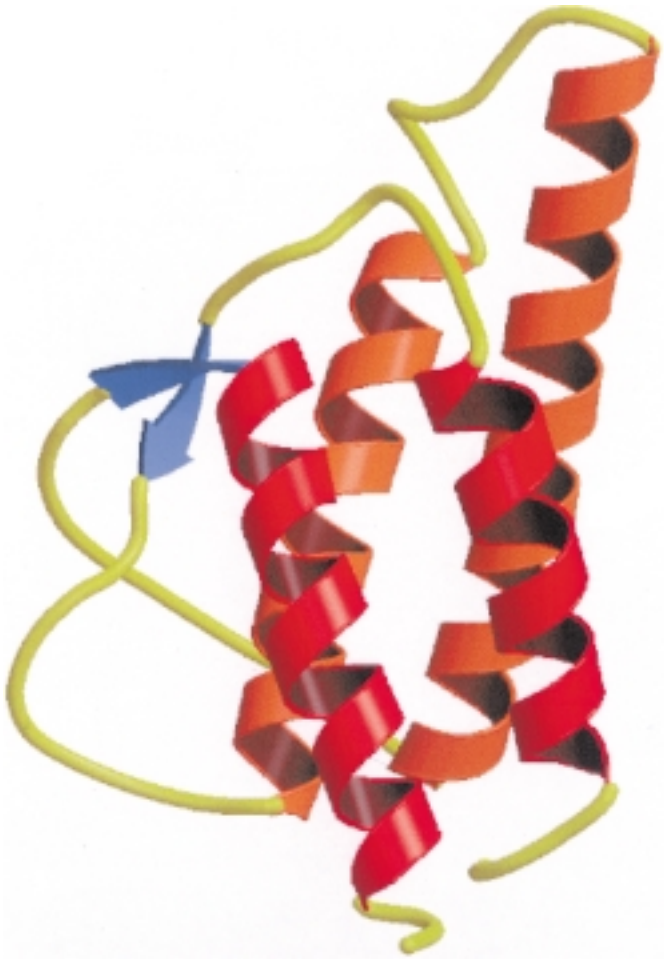
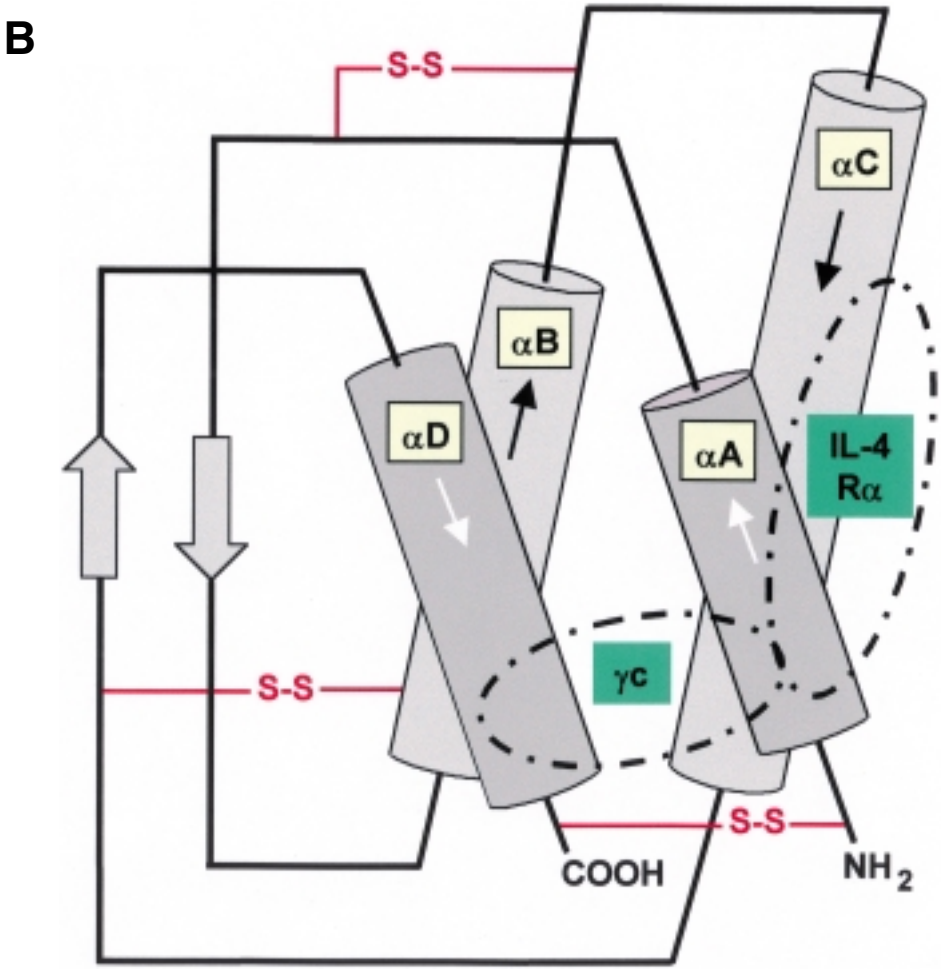


The structure of the pro-allergic protein hormone interleukin-4 has been known since the early 1990s (A). Through the analysis of the interaction of its receptor chains (B) it became possible to modify the molecule into an antiallergic mediator.



The Interleukin-4-Receptor: From Recognition Mechanism to Pharmacological Target Structure

Peter Reinemer, Walter Sebald, and Albert Duschl*

Organic synthesis of hormone derivatives is an established route to yield pharmacologically active agents. Until recently this has only been feasible for small organic compounds, but nowadays it is also possible to produce antagonists for larger protein hormones. In particular, the interleukin-4-receptor was a well-suited target for this approach since it plays a pivotal role in the release and progression of allergic diseases. Accordingly, a strong interest and a high medical need is associated with the development of inhibitors. The structural elucidation of the ligand/receptor complex and an improved understanding of the mechanisms concerning receptor binding and activation allow for the rational design of variants that inhibit interleukin-4. Since it is possible to specifically inhibit the interleukin-4-receptor system in this way, a completely new approach to the development of new drugs against allergy and asthma has been established.

Keywords: allergy · drug research · gene technology · hormones · interleukin-4

1. Emasculated Hormones

In 1988 James Black presented the concept of "emasculated hormones" in his Nobel prize lecture: hormone derivatives, that retain binding affinity to their receptors, but which are unable to activate them.[1] From this idea he developed beta blockers and antihistamines which are particularly impressive examples for the realization of this concept.

As a consequence of the spectrum of methods in genetic engineering and biotechnology that is available today the same principle can now be transferred to protein hormones. Here we present how directed mutagenesis of interleukin-4 (IL-4) leads to the development of a specific antagonist. The IL-4-receptor system represents a particularly lucky example of such an approach since the signaling of two protein hormones which are essential for the development of allergic diseases and asthma is triggered by using the IL-4 receptor (see Sections 2 and 3). Moreover, an understanding of the structure and recognition mechanism of this receptor has led in a direct way to the development of an "emasculated" IL-4-hormone.

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The IL-4-receptor is a prototype for receptors being activated by oligomerization—mostly dimerization—of receptor chains in the plasma membrane of the cell. As a consequence, the signal of the hormone is transmitted into the cell.[2, 3] The attributes of the IL-4-receptor—heterodimeric structure, binding with high affinity only to the α chain, little coupling between the receptor chains—offer a direct way to construct antagonistic IL-4 variants which retain the high-affinity binding, but no longer mediate the dimerization of the receptor chains. A refined analysis of the mutants results in selective antagonists, which only affect special receptor subtypes and therefore only particular cells, as well as super-antagonists, which show effects at decreased concentrations.

Antagonistic IL-4 variants are of pivotal interest in proof-of-principle studies to evaluate the IL-4 system as a fundamental new target in antiallergic therapy. Furthermore, it seems to be a realistic concept that these antagonistic proteins themselves could be developed as therapeutic agents. It will be a scientific challenge for the future to develop specific low molecular weight α-chain blockers based on the acquired knowledge of the recognition mechanism in this receptor system.

2. Allergy Arises from a Misguided Immune System

Allergy is a hyperreaction of the immune system against compounds that are tolerated by the immune system of most individuals. Nonimmunological hyperreactions caused, for

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example, by heat or UV light are not counted as allergic reactions. As allergy is restricted to the hyperresponsiveness of the immune system it can be separated from toxicity. Particular allergens, such as bee venom, can be quite toxic, but this quality is not causal for their allergic potential. Accordingly, the allergic reaction can be considered as a misguided defense mechanism against compounds that have no harmful potential without this reaction.

Atopy is a genetic predisposition to develop allergic sensitivity against suitable stimuli. Up to 5% of the population have the potential to develop atopy. They exhibit an increased risk for developing allergies, often suffer from multiple allergies, and are extremely susceptible to develop allergic asthma.[4] Atopy originates from mutations in genes associated with allergies. They have been intensively searched in connection with asthma, and it has been found that a variety of genes can be responsible for the development of asthma.[5] The Asthma Gene Database, established and maintained by the GSF-Research Center for Environment and Health in Munich, (http://cooke.gsf.de/asthmagen/main.cfm) offers a continously updated list of the current status of research in this field. Interestingly, IL-4 and the IL-4 receptor α chain are found among the genes at risk from asthma.

IL-4 is specifically responsible for the development of immediate-type allergic reactions.[6–8] This type is characterized by a prompt occurrence of symptoms against the allergen after exposition. Pre-sensitized individuals can develop edemas within minutes. In addition, symptoms such as widening of capillaries, stimulation of pain receptors, and—in particular in asthma—contraction of bronchial smooth muscles are developed. The immediate-type allergic reactions comprise allergic rhinitis (hay fever), allergies against house dust, animal hair, and insect venoms, as well as allergic asthma. Allergies with a delayed development of symptoms, such as allergy against nickel, do not belong to the immediate-type allergic reactions and can therefore not be attributed to IL-4. Allergies that depend on IL-4 use the same set of molecules and cells as used for the defense of helminthic macroparasites. Accordingly, immediate-type allergic reactions are considered as a misguided activation of the cellular parasite defense system.

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Walter Sebald studied Chemistry at the University of Munich from 1959 to 1965. From 1965 to 1969 he carried out research for his Dissertation in the department of Prof. T. Buecher at the Institute for Physiological Chemistry. In 1974 he completed his Habilitation on the "Identification and Characterization of Mitochondrially Synthesized Protein" at the Medical Faculty of the University of Munich. From 1978 to 1986 he was at the GBF in Braunschweig and in 1983 he was promoted to Professor. In 1986 he was appointed to a professorship at the Institute for Physiological Chemistry II at the Theodor-Boveri-Institute for Biosciences at the University of Wuerzburg. The focus of his research since 1986 has been on the structure and function of cytokine receptors with particular emphasis on interleukins and bone morphogenetic proteins.

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The progression of the allergic disease is shown schematically in Figure 1. An antigen is absorbed by antigen-presenting cells, subsequently processed, and finally presented by the MHC-II complex to T helper cells. When this stimulus triggers differentiation to a Th2 cell (type 2 T helper cells), the antigen will be treated as an allergen. For Th2 differentiation to occur it is necessary that the T cell is stimulated with IL-4 during its activation. Mast cells and subsets of T cells are considered as a potential source for early IL-4.[9] Since Th2 cells and other cytokines also produce IL-4, autocrine amplification is triggered.

Two of the Th2 cytokines, namely IL-4 and IL-13, induce class switching of the antibodies in activated B cells to the IgE-type.[10, 11] The resulting allergen-specific IgE antibodies bind to surface receptors of mast cells, basophiles, and eosinophiles and thereby induce their sensitization to the antigen. Any new contact with the allergen will trigger an immediate allergic reaction: the allergen binds to cell-associated IgE, and leads to degranulation of the effector cells. In the case of hay fever it is mainly the mast cells in the mucous membranes of the eyes and the respiratory tract that degranulate. Compounds released thereby, such as histamine, serotonine, and prostaglandines finally cause the typical symptoms. A variety of further pathological conditions can be observed in asthma: increased production of mucus in the bronchi, increased reactivity of bronchial muscles, and production of an inflammatory infiltrate in the tissue that is dominated by eosinophiles.[12]

IL-4 and IL-13 are not restricted to the sensitization phase, but they also contribute to reactions during the effector phase. They increase the activity of mast cells[13], basophiles,[14] and eosinophiles[15], and they lead to differentiation and hypertrophy of mucus-producing goblet cells.[16, 17] Furthermore, they are involved in the production of an eosinophilic infiltrate by induction of specific adhesion molecules[18] and chemokines.[19]

The Th2 differentiation taking place at the beginning of the reaction cascade is the one and only reaction that is solely triggered by IL-4. It is particularly important to note that IL-13 is unable to induce Th2 differentiation.[20] However, IL-13 seems to be the dominating cytokine during the subsequent progression of the disease, since it is produced for longer periods and in higher amounts than IL-4. IL-13 can be found in large quantities in the asthmatic lung, while IL-4 is hardly detectable. Hence, IL-13 is considered as the more important cytokine during the effector phase.[21–24]

3. IL-4Rα—The Molecular Switch that Triggers Allergy

The reason for the large similarity in the effects of IL-4 and IL-13 is the use of a common receptor chain, IL-4Rα. Transgenic knock-out mice without an IL-4Rα gene do not show any measurable reaction upon stimulation of IL-4 or IL-13; hence both cytokines are dependent on IL-4Rα.[17, 25–28] It is known from binding studies that IL-4 can directly bind to IL-4Rα.[29–31] Subsequently, depending on the cell type, a second receptor chain will be recruited to initiate signaling: either γ_c[32, 33] or IL-13Rα1[34–37] (Figure 2). In contrast, IL-13

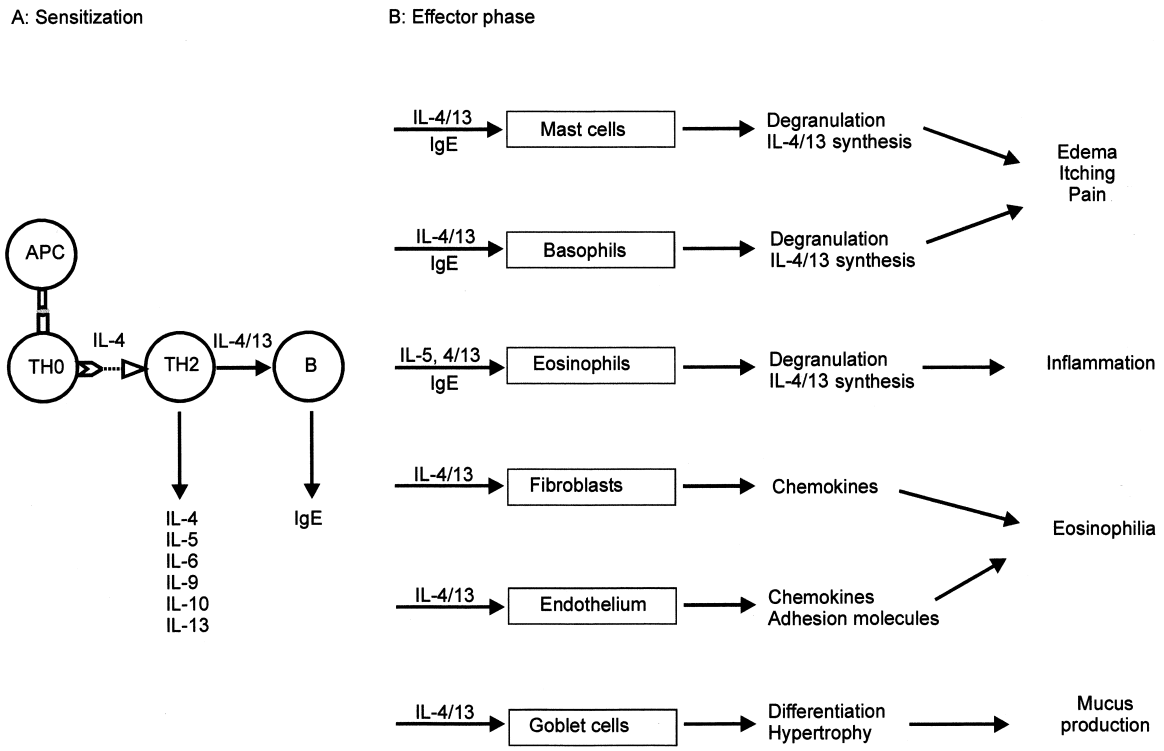

Figure 1. Schematic representation of sensitization (A) and effector phase (B) of the immediate-type allergic reaction. IL-4 and IL-13 are involved in both processes, since they have activating and/or differentiating effects on the involved cell types. APC = antigen presenting cell, TH0 = T helper cell type 0, TH2 = T helper cell type 2, B = B cell, IgE = immunoglobulin E.

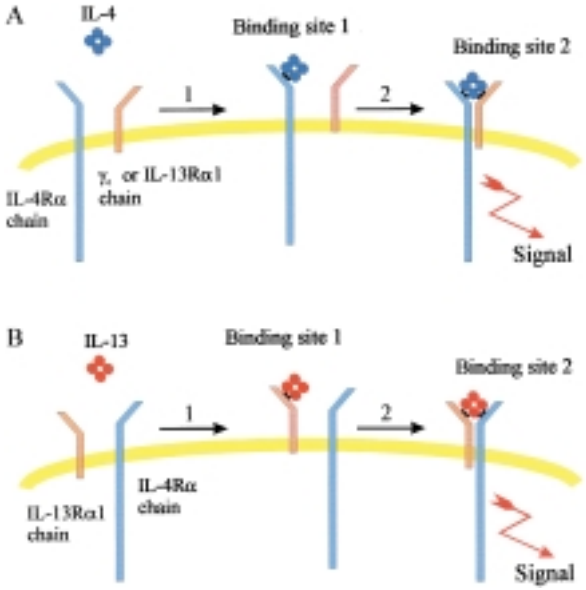

Figure 2. An identical heterodimeric receptor comprising IL-4Rα and IL-13Rα1 is used by both IL-4 (A) and IL-13 (B). The sequence of dimerization however is different (see text). The complex of IL-4 and IL-4Rα within the membrane can either recruit γ_c or IL-13Rα1. As far as is known, the resulting signal is identical.

binds first to IL-13Rα1 and subsequently recruits IL-4Rα. Despite the differing sequence of association to the receptor chain IL-4 and IL-13 both use the same IL-4Rα/IL-13Rα1 heterodimeric receptor complex.[20, 38]

Within the allergic process IL-4 can be regarded as the master switch, which triggers all following processes. Therefore, the IL-4 system is regarded as an ideal target for the prevention of allergy. Transgenic mice without a functional gene for IL-4 are not able to develop allergic reactions and the above-mentioned transgenic mice without a functional IL-4Rα gene can also not develop allergic reactions.[39] In contrast, transgenic mice with elevated IL-4 expression develop symptoms similar to allergic reactions even in the absence of allergens.[40–43] The administration of IL-4 into the lungs of asthmatic patients induced an asthma attack,[44] while corresponding experiments using IL-13 in mice led to a very high production of mucus and other asthmatic symptoms.[17, 21, 22, 24] This result demonstrates the pivotal importance of IL-4 and IL-13 for the induction and progression of allergic reactions and accordingly reveals that inhibition of the IL-4/IL-13-system may be highly beneficial in antiallergic and antiasthmatic therapy.

3.1. Selecting the Target Molecule

A particularly suitable target for IL-4/IL-13 inhibition seems to be IL-4Rα. The receptor is essential for both cytokines and as a consequence both cytokines will be inhibited. Inhibiting the ligands directly, however, would only affect one of the two cytokines. Moreover, as the sequences of IL-4 and IL-13 only exhibit 25% sequence homology there is almost no chance of developing bispecific inhibitors. Soluble variants of the receptors merely recognize one of the cytokines: separated IL-4Rα solely binds IL-4, separated IL-13Rα1 solely binds IL-13, and separated γ_c does not bind to either of the ligands.

Of the receptor chains both IL-13Rα1 and γ_c are unattractive targets, since their disengagement would not result in complete and simultaneous neutralization of both ligands. In particular, γ_c is not regarded as a pharmaceutical target at all. This receptor chain is shared between IL-2, IL-7, IL-9, and IL-15, and accordingly inhibition of γ_c will create interference with a variety of different regulation systems.[45] The genetic loss of γ_c leads to the severe immune deficiency syndrome X-SCID (X-chromosome-linked severe combined immunodeficiency), where the afflicted individual suffers from a severe breakdown of the immune system as a result of the inability to develop T cells in the absence of IL-7 signals.[45–48] In contrast, transgenic mice who lack IL-4Rα seem to cope well with the situation: these animals exhibit normal immune competency and have no difficulties in bacterial and viral defense.[25–28] Thus IL-4Rα can be identified as a key protein in the allergic reaction cascade and therefore as a preferential target for pharmaceutical intervention.

IL-4- and IL-13-dependent signaling is primarily arranged by IL-4Rα. A variety of signal-transducing factors can bind to IL-4Rα to mediate the downstream signal.[8] A large protein complex comprising IRS-2 (insulin receptor substrate-2) and proteins coupling to IRS-2 seem to be responsible for cell proliferation and inhibition of apoptosis.[49] A second signaling pathway involves the activation of kinases belonging to the Jak family and subsequent phosphorylation of Stat6.[50] The transcription factor Stat6 forms a homodimer after phosphorylation and, together with other transcription factors, regulates gene expression during cell differentiation. This process includes amplification of the IL-4 production during Th2 differentiation[51], class switching of B cells to IgE[52–54], expression of the IgE receptor CD23 on B cells and monocytes[55, 56], and induction of the antiinflammatory protein IL-1RA in macrophages.[57]

If these signaling pathways are considered in the search for attractive pharmaceutical targets, the pathway using IRS-2 is likely to be dismissed, since the control of cell proliferation and apoptosis by IL-4/IL-13 induction can be neglected in the context of allergy. In contrast, cell differentiation is of considerable importance. However, within the Jak/Stat pathway the Jak kinases seem to be less promising, since they are activated by numerous other receptors as well. Therefore, it seems to be almost impossible to achieve inhibition without serious side effects.[58, 59] In contrast to the Jak family, Stat6 is—at least as far as cytokine signaling is considered—specific for the signal transmission of IL-4 and IL-13, and therefore can be regarded as a promising target molecule.[58, 59]

Inhibition of IL-4Rα or Stat6 opens up a new perspective for the therapy of allergic diseases. Until now it has only been possible to either try to achieve a desensitization against a defined allergen by using a specific immunotherapy or to influence the course of the effector phase during an acute state of the disease by application of certain compounds such as antihistamines, cromoglicinic acid, nedocromil, or corticosteroids. The neutralization of the effects of IL-4/IL-13, however, would cause a specific immunosuppression of the allergic system.

4. Structure Elucidation of the IL-4/IL-4Rα Binding Interface

A rational understanding of the inhibition in the IL-4/IL-13 system requires a structural understanding of its components. From the structural biologists point of view the cytokine-mediated, sequential association of different receptor chains requires a sharply tuned coordination of molecular recognition events between the cytokine and the extracellular domains of the interacting receptor chains. The binding affinity of the IL-4Rα chain is of pivotal importance in regard to its intervention in the IL-4 system. A loss of its binding affinity would result in an effective inhibition of IL-4 and IL-13.

Molecular recognition of the ligand within the IL-4Rα chain is solely mediated by its 207 amino acid residues that comprise the extracellular domain. This domain, hereafter referred to as the IL-4 binding protein (IL4-BP), can be expressed as a separate protein and exhibits the same binding affinity, specificity, and association constant of IL-4 binding as the entire receptor α chain ($K_d \approx 150$ pM, $K_{on} \approx 10^7\,M^{-1}s^{-1}$).[60, 61] The X-ray crystal structure of the binary complex of IL4-BP and IL-4 has recently been determined.[62] Prerequisite was the establishment of IL4-BP expression in *E. coli* and subsequent refolding of the protein into its functional structure.[63] The knowledge of the structure and spatial arrangement of the binding partners and their molecular interactions in the binary complex together with the mutation and quantitative interaction analysis[64] enables a better understanding of the molecular recognition between IL-4 and IL-4Rα. This knowledge sheds further light on receptor/ligand interactions in the cytokine superfamily and promotes the development of suitable inhibitors.

4.1. The Cytokine Receptor Superfamily

The cytokine receptors are pooled in a superfamily, since they possess conserved sequence motifs—the so called "cytokine receptor homology (CRH) region"—in their extracellular domains.[65] The CRH, which comprises approximately 200 amino acid residues, contains the ligand-binding determinants of the receptor and is organized in two protein domains, which are covalently connected by a short linker segment. In addition, the CRH in most receptors is characterized by four conserved cysteine residues in the N-terminal domain and a strictly conserved Trp-Ser-Xaa-Trp-Ser sequence motif in the C-terminal domain. Receptors with these characteristics, such as IL-4Rα, are referred to class I cytokine receptors. The closely related class II receptors are characterized by a CRH with four different conserved cysteine residues and the lack of the Trp-Ser-Xaa-Trp-Ser motive.[65]

4.2. The Structure of IL4-BP

The interleukin-4 binding protein exhibits an L-shaped structure and consists of domains D1 (amino acid residues 1–91) and D2 (amino acid residues 97–197), which are covalently connected by a short helical segment (Figure 3). Both domains belong to the immunoglobulin superfamily and show structural similarity to the fibronectin type III module.[66, 67] Their folding is characterized by a sandwichlike structure. It comprises seven antiparallel β strands organized in a three-stranded (strand βA, βB, βE) and a four-stranded β-pleated sheet (strand βG, βF, βC, βC′) and are twisted by approximately 40° relative to each other. The folding topology of domain D1 belongs to subtype "h"[66] of the immunoglobuline family where the β-strand C′ first interacts with β-strand C and then its direct continuation—now designated β-strand D-–interacts with β-strand E of the other sheet. The domain contains six cysteine residues, which are engaged in three disulfide bridges, namely Cys^9–Cys^{19}, Cys^{49}–Cys^{61}, and Cys^{29}–Cys^{59}. The first two are both conserved in class I CRHs[65],

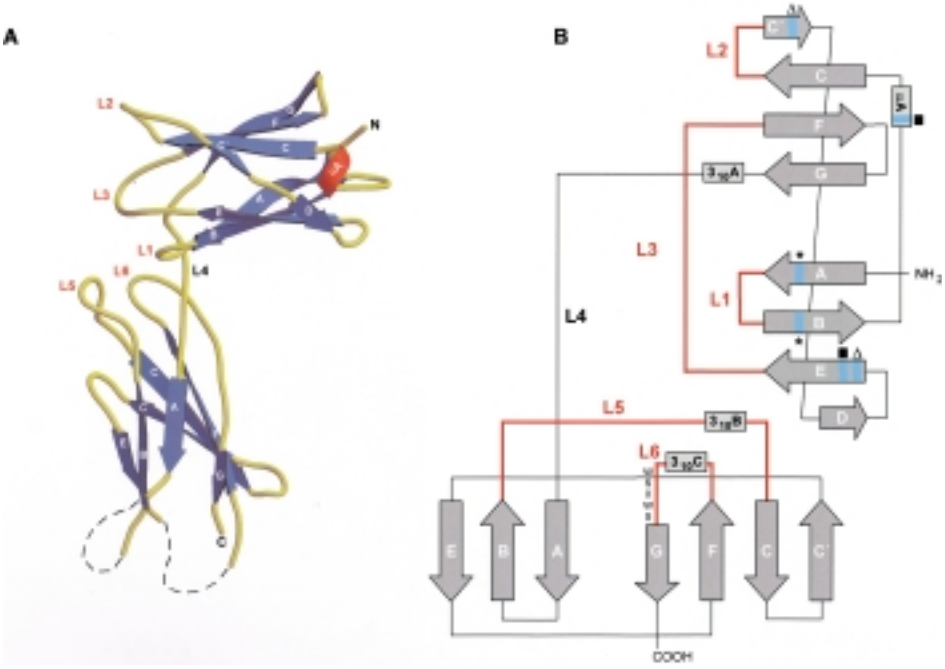

Figure 3. Structure of the extracellular domain of IL-4Rα (IL4-BP). A) Ribbon-type representation of IL4-BP. α helices are depicted in red, β strands in blue, and connecting loops in yellow. Amino and carboxy termini are marked with N and C, respectively. The β strands are labeled according to ref. [66] and the loops orientated towards the ligand labeled according to ref. [76]. B) Topologic diagram of the structure of IL4-BP. The folding topology of both domains belongs to the immunoglobulin family and both exhibit structural similarity to the fibronectin type III module.[79] Secondary structural elements are depicted as arrows (β strands) and boxes ($\alpha/3_{10}$ helices). The positions of the six cysteine residues engaged in the three disulfide bridges (Cys^9–Cys^{19}, Cys^{29}–Cys^{59}, and Cys^{49}–Cys^{61}) are marked as blue bars. The position of the conserved WSXWS motif in D2, which preceeds strand βG, is also indicated. The five loops, which contribute to ligand binding (L1–L3, L5, and L6) are labeled in red.

while the third only occurs in IL-4Rα. Domain D2 exhibits standard fibronectin type III topology ("s"-type of the immunoglobulin superfamily).[66] It does not contain any disulfide bridges and is further characterized by the conserved Trp-Ser-Xaa-Trp-Ser motif, which is situated in a bulge that preceeds strand βG (Figure 3). The orientation of the domains relative to each other within the binding protein is characterized by a relatively open total structure. The C-terminal edge of domain D1 and the N-terminal edge of domain D2 are both oriented towards bulk solvent and not to each other. This arrangement creates a domain – domain angle of about 90° and a comparably small domain – domain interface, which has important consequences for ligand binding since the loops AB_{D1} (marked as L1 in Figure 3), CC'_{D1} (=L2), DF_{D1} (=L3), BC_{D2} (=L5), $C'E_{D2}$ (not marked in Figure 3), and FG_{D2} (=L6) are thereby situated in the vicinity of the linking segment and are largely accessible to bulk solvent.

4.3. The Structure of IL-4

Mature human IL-4 is a monomeric protein comprising 129 amino acid residues, whose structure has been independently elucidated by NMR spectroscopy and X-ray crystallography.[68–71] The molecule consists of four α helices, hereafter referred to as αA, αB, αC, and αD, which are arranged in a bundlelike fashion (Figure 4). Helices αA and αB are oriented upwards within this arrangement, while helices αC and αD are oriented downwards. This unusual arrangement is referred to as an "up-up-down-down" topology. In this arrangement each helix is in an antiparallel orientation to both neighboring helices and there are two long connecting segments situated after helices αA and αC that both cover the whole molecule. In addition, there is a short connecting segment situated after helix αB which joins two sequential helices. The two long connecting segments furthermore contain two short β strands. These build a small antiparallel β sheet, which is packed against helices αB and αD. The molecule contains six cysteine residues which are engaged in three disulfide bridges, namely Cys^3–Cys^{127}, Cys^{24}–Cys^{65}, and Cys^{46}–Cys^{99}. Such a structure, hereafter referred to as a "four-helix bundle", can also be found in other cytokines. Two variants can be discriminated: the short-chain four-helix-bundle cytokine family, which comprises IL-4 as well as IL-2, -3, -5, -7, -9, -13, -15, and GM-CSF, and the long-chain four-helix-bundle cytokine family with growth hormone, prolactin, G-CSF, erythropoietin, IL-6, IL-11, oncostatin M, LIF, and CNTF.[72] Members of the short-chain family can be characterized by chain lengths of 105 – 145 amino acid residues, short α helices (about 15 amino acid residues) and an angle of about 35° between the helix pairs A/D and B/C. For members of the long-chain family it is typical to have between 160 and 200 amino acid residues, long α helices (about 25 amino acid residues) and a stronger alignment of the helical axes (angle of about 20° between helix pairs A/D and B/C).

IL-4 exhibits one binding site for each of its interacting receptor chains. The functional epitope of IL-4 that determines its high-affinity binding to the receptor α chain is localized on the surface built up by helices αA and αC (hereafter refered to as "helix AC-face", Figure 4).[64, 73] The epitope responsible for the binding of γ_c is located on the surface created by helices αA and αD ("helix AD-face").[73, 74]

Investigations with IL-4 mutants on cells which express the type-II IL-4 receptor have led to the conclusion that the binding interfaces of IL-13Rα1 and γ_c overlap on the IL-4 molecule.[75, 76] IL4-BP binds through the above-mentioned loops to the helix AC-surface of IL-4. The quarternary structure of the complex is characterized by an almost perpendicular arrangement of the molecular main axes. The binding loops of IL4-BP are arranged in a stacklike fashion and are approximately colinear to the helical axes of the IL-4 helices (Figure 5).

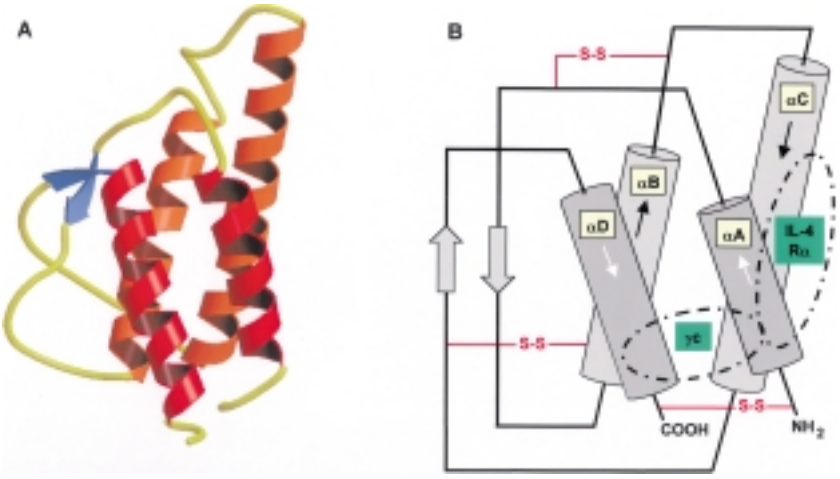

Figure 4. Structure of interleukin-4 (IL-4). A) Ribbon-type representation of IL-4. α helices are depicted in red (α helices αA and αD in the foreground) and orange (α helices αB and αC in the background), β strands in blue, and connecting loops in yellow. B) Topologic diagram of the structure of IL-4. Secondary structural elements are depicted as cylinders (α helices) and arrows (β strands). The positions of the six cysteine residues engaged in the three disulfide bridges (Cys^3–Cys^{127}, Cys^{24}–Cys^{65}, and Cys^{46}–Cys^{99}) are labeled in red. The positions of the binding sites on both receptor chains are indicated as dashed lines: the binding epitope of the receptor α chain on the helix AC-face and the binding epitope of the γ_c chain on the helix AD-face.

4.4. The IL-4/IL4-BP Complex

The structure of receptor-bound IL-4 is very similar to that of free IL-4. The crystallographic analysis[62] however shows a slightly changed relative conformation of the IL-4 helices. Whereas the arrangement of the helical axes of helices αB and αC remains unchanged, the axes of helices αA and αD are twisted towards each other. The most significant structural changes occur in the region of the functional binding epitope of γ_c[74], where reduced intramolecular Cα–Cα distances of the determining amino acid residues (Thr^{11}, Asn^{15}, and Tyr^{124}) are observed. The distances between the pairs Ile^{11}–Tyr^{124} and Asn^{15}–Tyr^{124} are decreased by 1.0 and 1.5 Å, respectively. Since γ_c does not bind free IL-4 at submicromolar concentrations, the conformational changes of IL-4

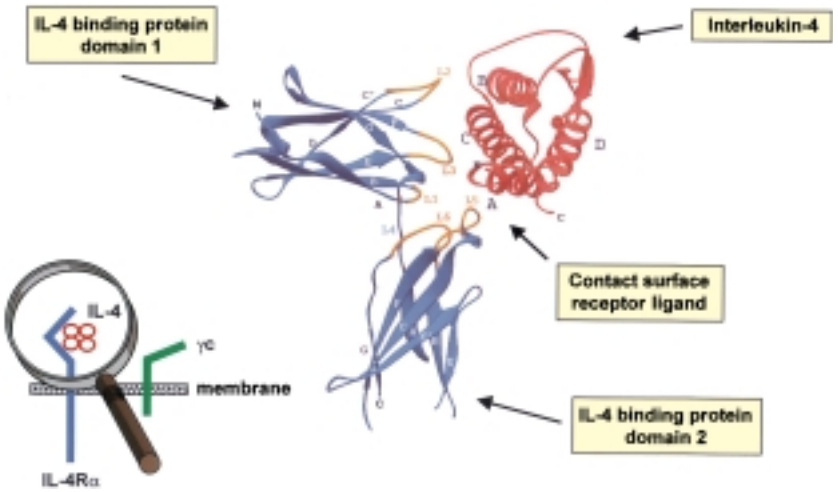

Figure 5. Structure of the IL-4/IL4-BP complex. Ribbon-type representation of the IL-4/IL4-BP-complex (IL4: red, IL4-BP: blue/orange, see also the small scheme). Amino and carboxy termini are labeled with N and C, respectively; the helices and strands are labeled according to ref. [66]. The five loops of IL4-BP which are involved in interactions with IL-4 are highlighted in yellow and are labeled according to ref. [76]. They are arranged in a stacklike fashion comprising four levels in the sequence L2, L3, L1, and L5/L6. Loop L4, which is depicted in blue, connects both domains and has no interaction with IL-4 but is included for consistency.

after binding to IL-4Rα can be of functional importance.[74] Conformational changes in IL-4 triggered by binding to IL-4Rα can therefore be prerequisite for an association of γ_c to the binary complex and therefore for signal transduction.

The structural binding epitope comprises 18 amino acid residues on each of the binding partners, which are distributed over multiple sequence segments. The contact areas deviate strikingly from planarity and it is important to note the polar character of the complementary binding surfaces (Figure 6A). Amino acid residues that contribute to IL-4 binding are dispersed over three helices and mainly comprise polar and charged amino acids. The receptor epitope, however, consists of amino acids from five loops and has a midline of hydrophobic side chains with patches of tyrosine and serine residues on one side and a patch of aspartic residues on the other side (Figure 6A). The structural binding epitope as a whole has a surface area of about 800 Å^2 on each binding partner (measured as the decrease in the solvent-accessible surface[77] upon complex formation). The covered surface is similar in size to that buried in other cytokine/receptor interactions, such as the topologically equivalent low-affinity interaction between the growth hormone and its binding protein.[78] This observation indicates that a high-affinity interaction can also be generated from a likewise small contact area and that its size is not directly related to binding affinity.

4.5. High Resolution Interaction Analysis

The analysis of the binding of IL-4 to the receptor on whole cells or to soluble IL-4BP results in a dissociation constant K_d of about 100 pM (Figure 7). This high affinity is the result of a quite fast association ($k_a = 1-2\times10^7$) and a rather slow dissociation ($k_d = 2\times10^{-3}$) process. The analysis of the binding of mutations together with quantitative interaction analysis allows the contribution of each contact residue to the free energy of binding to be estimated. Therefore, an alanine-substituted variant of each contact residue in IL-4 and IL4-BP was created by site-directed mutagenesis and corresponds to the replacement of the original amino acid side chain with a methyl group. Most of the alanine variants of the contact residues in IL-4 and IL4-BP could be obtained with $>96\%$ yield and their binding affinity analyzed after expression in *E. coli*, renaturation, and purification

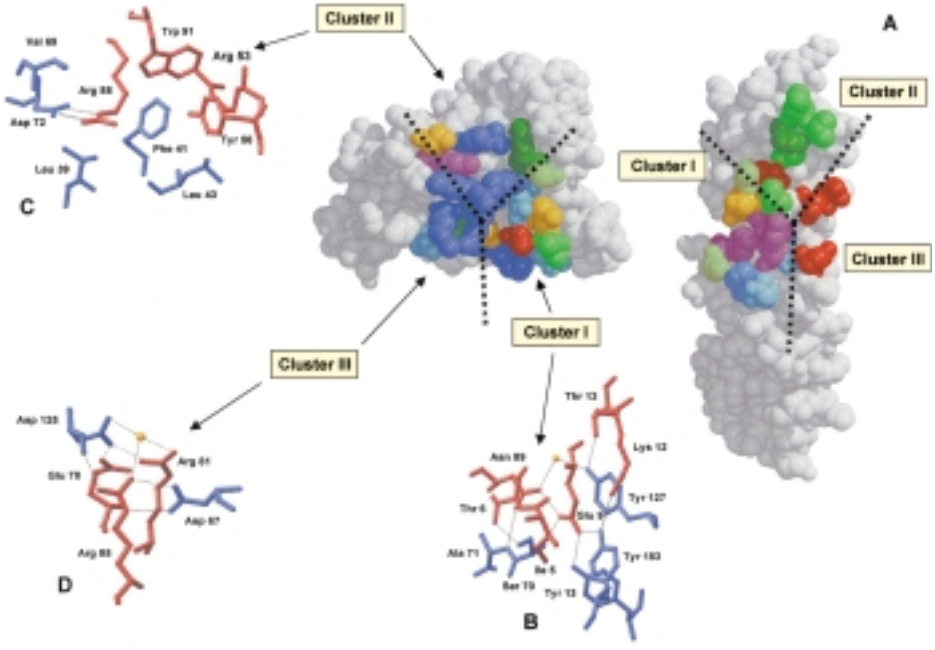

Figure 6. The structural epitope of the IL-4/IL4-BP complex. A) Open-book view of the complementary structural epitopes of IL-4 (left) and IL4-BP (right). Amino acid residues that form interactions between the receptor and ligand are highlighted and colored according to their physicochemical properties: red: negatively charged (Asp, Glu); dark blue: positively charged (Arg, Lys); light blue: His; cyan: Gln/Asn; magenta: Tyr; orange: Ser/Thr; and green: hydrophobic. B)–D) Detailed views of the IL-4/IL4-BP contact. IL-4-Glu^9 forms the center of a network of hydrogen bonds and is surrounded by hydrophobic side chains ("cluster I", B). IL-4-Arg^{88} and IL4-BP-Asp^{72} build a central salt bridge and are surrounded by aromatic and aliphatic moieties ("cluster II", C). Electrostatic interactions dominate in "cluster III" (D).

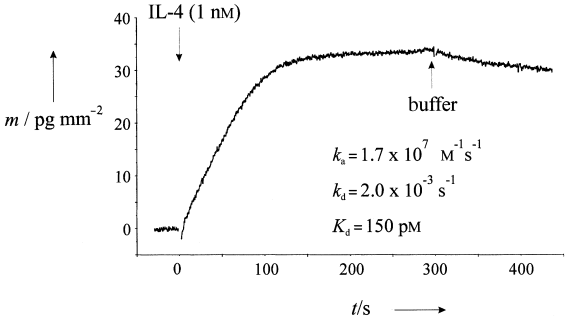

Figure 7. The binding of IL-4 to IL4-BP is extraordinarily fast. In contrast, the dissociation of the complex is rather slow. The graph obtained by analysis of a biosensor shows the kinetics for the association of 1 nM of IL-4 to IL4-BP, which is immobilized on the biosensor.[61] In the presence of buffer the complex decomposes at a half-life rate $\tau_{1/2}$ of about 6 min. The rate constants of association (k_a) and dissociation of the complex (k_d) as well as the calculated dissociation constant K_d are also indicated.

Substitution of the IL-4 side chains Glu^9 and Arg^{88} leads to a loss of affinity (K_a) by a factor of 300 to 1000. This loss corresponds to a loss of free binding energy ($\Delta\Delta G_o$) by 3–4.5 kcal mol^{-1}. Clearly, Glu^9 and Arg^{88} are the main determinants of binding. Five additional side chains, namely Ile^5, Thr^{13}, Asn^{89}, Trp^{91}, and Arg^{53} are secondary determinants of binding, whose substitution by alanine results in a loss of binding affinity by a factor of 3 to 10 ($\Delta\Delta G_o = 0.4$–1 kcal mol^{-1}). The other contact residues of IL-4 do not directly contribute to binding. A smaller contribution to the association constant (K_a), summing to a factor of 10 in total, is conveyed by the positively charged amino acid side chains of IL-4. This global charge contribution from six positively charged side chains results in a preliminary orientation of the complementary charged proteins during association (electrostatic steering).[79]

Mutagenesis of the receptor α chain completes the energy profile of the IL-4 binding epitope. The highest loss in binding affinity is obtained after substitution of Asp^{72}, Tyr^{13}, and Tyr^{183} with alanine. The negatively charged residue Asp^{72} seems to form an electrostatic interaction with the side chain of Arg^{88} in IL-4. The structure reveals that a perfect spatial orientation of their carboxylate and guanidino groups is adopted. The residues Tyr^{13} and Tyr^{183} of the receptor employ their phenolic hydroxyl groups to form hydrogen bonds to the side chain of IL-4-Glu^9. Additional interactions are contributed from the carbonyl moiety of Ser^{70} in the receptor and the carbamide group of residue Asn^{89} in IL-4. The structure reveals the participating atoms have a perfect "sp^2 geometry". Interestingly, secondary determinants of the receptor, which lead to a loss of binding affinity by a factor of 10 to 50, are hydrophobic residues, such as Tyr^{125}, Val^{90}, Phe^{45}, and others. Presumably, this outer mantle of hydrophobic side chains ("avocado cluster") leads to an intensified binding of the polar, main determinants. Thus, the arrangement of these binding epitopes exhibits a mosaic structure and is mainly polar. This observation is contrary to individual hydrophobic interactions ("hot spot of hydrophobic binding energy") that have been described for the high-affinity binding in the growth hormone/receptor complex.[78]

4.6. The Mechanism of Protein/Protein Interaction: The Three-Cluster Model

The contact surface between IL-4 and the binding protein exhibits a mosaiclike structure comprising three distinct groups of interacting amino acid residues (hereafter referred to as "cluster"; Figure 6A). Two of these clusters are arranged as "avocado clusters", which means that they reveal an eye-catching amphiphilic structure with an outer mantle of hydrophobic side chains arranged around an inner core of polar groups. Cluster I is arranged around Glu^9 of IL-4. Its carboxylate group accepts three bonds from residues Tyr^{13}, Tyr^{183}, and Ser^{70} of the binding protein (Figure 6A and 6B). The geometrically perfect arrangement seems to be preorientated by an intermolecular bond to IL-4 Asn^{89} and is further completed by interactions between Thr^6, Lys^{12}, Thr^{13}, and Asn^{89} of IL-4 and Ser^{70}, Tyr^{183}, Tyr^{127}, and Ala^{71} of IL4-BP. A single water molecule is locked in the contact area and forms a hydrogen-bonding interaction between IL-4-Asn^{89} and IL4-BP-Tyr^{127}. This network of polar interactions is surrounded by aromatic and aliphatic side chains: IL-4-Glu^9 has van der Waals contacts with Ile^5, Lys^{12}, and Thr^{13} of IL-4, packs flat against the side chain of IL-4-BP-Tyr^{127}, and its carboxylate group is encased by the side chains of Tyr^{13}, Tyr^{183}, Val^{69}, and Ala^{70} of the binding protein. Cluster II (Figure 6A and 6C) is assembled around the central salt bridge that links IL-4-Arg^{88} and IL4-BP-Asp^{72}. The guanidino and the carboxylate group are orientated in the same plane and align edge to edge through two hydrogen bonds. Asp^{72} seems to be completely buried at the base of a cleft in the surface of the binding protein. IL-4 Arg^{88}, however, completely stands out from the surface of IL-4 and is situated between the side chains of Val^{69} and Phe^{41} in the complex. Cluster III deviates from the previous design principle and exhibits a completely different architecture that is dominated by electrostatic interactions (Figure 6A and 6D). However, the interacting residues Arg^{81}, Arg^{85}, and Gln^{78} of IL-4 and Asp^{66}, Asp^{67}, and Asp^{125} of the binding protein cannot establish strong interactions, since their binding geometries deviate significantly from standard sp^2-type interactions.

Altogether the crystal structure analysis[62] and the mutational analysis of contact residues of IL-4[64] yields a mechanistic model of the interaction of IL-4 with the extracellular domain of its high-affinity receptor. The main determinants of the binding affinity of IL-4 are the residues Glu^9 and Arg^{88}, which are the central residues embedded in clusters I and II. Both residues are equally important for structure and function and together are responsible for a large portion of the whole binding affinity. Mutations of Glu^9 and Arg^{88} result in a loss of binding affinity of two to three orders of magnitude. Mutations of peripheral amino acid residues, however, only lead to a loss of binding affinity of one order of magnitude. This result is in accordance with their subordinate structural importance. In contrast, the structure suggests a different role for cluster III: this cluster seems to have only minor functional significance in the stabilization of the complex, but does, however, promote the extraordinarily fast formation of the complex by electrostatic steering. Hence, all mutations in cluster III of IL-4 show hardly any effect on binding, but influence their rate of association.

4.7. The Binding of the Second Receptor Chain (γ_c or IL-13Rα1)

The high affinity of the interaction of IL-4 with the receptor α chain, as described in the preceeding discussion, is now well understood. Both the structural epitope (contact surface) and the functional epitope (energy profile/map of the binding residues) have been characterized. The kinetic and thermodynamic parameters of binding resulting from IL-4 binding to the soluble receptor domain and from IL-4 binding to the receptor on whole cells are equal. This is not unexpected, since in both experiments IL-4 binds out of solution.

However, less well understood and much more difficult to analyze is the interaction with the second receptor chain, which may be either γ_c or IL-13Rα1. The second activation step of the IL-4 receptor—and as far as is known of other cytokine receptors—proceeds by the binding of the second receptor chain to the complex of the ligand and high-affinity receptor chain which is located in the plasma membrane. IL-4 does not bind to γ_c (or to IL-13Rα1) on whole cells up to concentrations of 100 nM if the α chain is not simultaneously present. However, IL-4Rα is already fully saturated at these IL-4 concentrations ($K_d \approx 100$ pM). For this reason, γ_c and IL-13Rα1 are called low-affinity binding partners of IL-4.

The formation of the ternary complex in the presence of IL-4 can be demonstrated on a biosensor by using soluble ectodomains of the α chain and the γ_c chain (Figure 7). The γ_c ectodomain binds to the complex of IL-4 and immobilized IL4-BP with an affinity constant of $3 \times 10^5\,M^{-1}$, while the affinity between free IL-4 and the γ_c ectodomain is only reduced by a factor of 50. The increased affinity to the IL-4/IL4-BP complex could originate from a small conformational change in the IL-4 complex or from rather weak interactions between both ectodomains.

The dissociation constant of 3.5 μM for the dissociation of the γ_c ectodomain from the ternary complex is far beyond the physiological concentration of IL-4. Nevertheless, IL-4 is already active at subnanomolar concentrations. It is therefore conclusive that an IL-4-dependent dimerization and transactivation of the chains takes place. Certain possibilities may be considered.

Direct interactions between the α and the γ_c ectodomain are clearly very low as reflected by a K_d value of 5 μM. However, direct interactions could occur between the receptor chains in the cytoplasmatic domains or in the transmembrane regions. Then IL-4 would only act as the necessary trigger for this receptor–receptor binding. In cells exhibiting high expression levels such ligand-independent receptor activation, which hints to direct interaction between the receptor chains, has been demonstrated for other receptors. The expression levels of IL-4 receptor chains are often low, but it cannot be excluded that additional concentration takes place in membrane microdomains such as the "rafts".[80]

The probability of productive collisions among membrane proteins is significantly higher than among dissolved molecules. A density of 1000 receptor α chains homogenously distributed on a cell surface area of 100 μm^2 exhibits molecular distances corresponding to a 50 nM solution. Therefore, IL-4 is concentrated by orders of magnitude upon receptor binding. Since the degrees of freedom in diffusion and in particular in rotation are restricted in the membrane productive collisions will happen more frequently. As a result of these membrane effects the binding of the ligand to the second chain is sufficient for receptor activation despite its low affinity for the second receptor chain.

4.8. From Theory to Practice: IL-4 Receptor Antagonists

The high-affinity binding to the receptor α chain is responsible for almost the entire binding affinity of the whole IL-4 receptor. Clearly, there are only limited interactions between both receptor chains, because otherwise γ_c affinity would make a stronger contribution to the binding of IL-4 to the entire receptor. Since the required interaction between IL-4 and γ_c is only weak, it is not sufficient to replace the involved interacting residues by the neutral amino acid alanine. Such variants will still keep a significant agonistic activity. Therefore, repulsive residues must be inserted into the γ_c-binding epitope of IL-4 to obtain complete antagonists effectively.

The substitution of Ile^{11}, Asn^{15}, and in particular of Tyr^{124} in IL-4 with alanine identified these residues as the main determinants of γ_c binding. Although the corresponding alanine variants exhibit a 5 to 100-fold decreased binding to the γ_c ectodomain, their biological activity is hardly impaired: their ED_{50} values—the dose at which the target cell is half maximally stimulated—are unchanged and their maximal signals are reduced to 50 % at best. Substitution of Tyr^{124} with a variety of amino acid residues yields partial agonists to which cells respond with different efficiencies (Figure 8). Individual variants yield between 0.5 and 90 % activity of

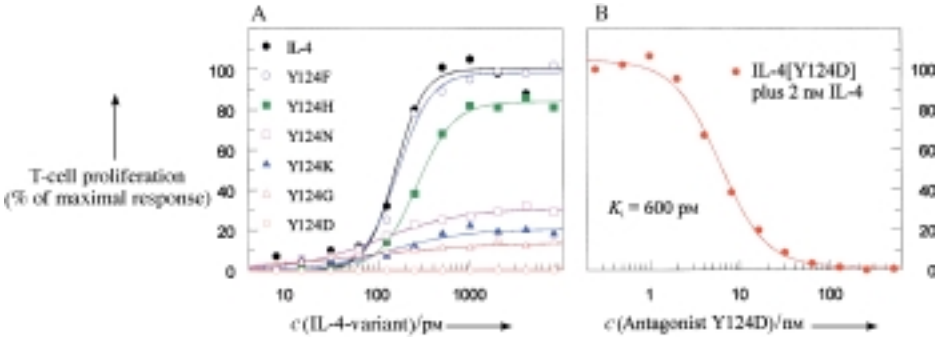

Figure 8. IL-4 mutant proteins are partial agonists or antagonists if the low-affinity binding epitope for γ_c or IL-13Rα1 is impaired or completely blocked.[73] A) The exchange of IL-4 Tyr^{124} for Phe, His, Asn, Lys, Gly, or Asp creates partial agonists. Their ED_{50} values remains almost unchanged relative to that of IL-4. B) The variant Tyr^{124}Asp is a competitive inhibitor of IL-4 in a T-cell proliferation test and exhibits an inhibiting constant K_i of 600 pM.[73]

the wild-type, but none of them really shows complete inactivity. However, a double substitution of Arg^{121} and Tyr^{124} by two aspartate residues forms a completely inactive variant of IL-4.[81] The K_d value for the binding of this variant to the α chain and its IC_{50} value are elevated relative to the wild-type by a factor of two to three to 200–300 pM. The aspartate residues in the γ_c-binding epitope of IL-4 prevent association with the γ_c chain and therefore inhibit dimerization of the receptor chain through electrostatic repulsion.

Up until now the IL-4 binding epitope for IL-13α1 has not been determined. Investigations with different variants of IL-4 on cells, using either type 1 or type 2 receptors, show an overlap of the binding epitopes of IL-13Rα1 and γ_c.[75, 76] However, their repulsion epitopes are distinct from each other, so that selective agonists can be created. The IL-4 variants Y124E or Y124D hardly exhibit any activity with γ_c, but they show considerable activity with IL-13Rα1. Likewise, the variants R121D or R121E are still active with γ_c, but almost inactive with IL-13Rα1.

5. Preclinical and Clinical Application of IL-4 Antagonists

Antagonistic IL-4 mutants, in particular the complete antagonist R121D/Y124D, show their potential as inhibiting compounds in a variety of cell culture systems. These variants suppress cellular reactions to IL-4 and IL-13, such as phosphorylation of signal transduction proteins, regulation of gene expression, and cytokine-dependent cell proliferation. These experiments also demonstrate the specificity of these variants as inhibitors, since other cytokines are not impaired.

In order to evaluate their effectiveness in a mouse model an analogous variant of murine IL-4 had to be established, since IL-4 is specific to each species. This murine IL-4 variant, Q116D/Y119D, is a complete antagonist for murine IL-4 and IL-13 in vitro.[82] When applied in vivo in a murine allergy model against a protein antigen it demonstrated the suppression of allergic reactions.[39] Moreover, when the antigen and IL-4 antagonist were simultaneously applied the mice did not develop measurable levels of specific IgE and IgG1 antibodies, which are both associated with allergic reactions in the mouse. The intradermal or intravenous application of the antigen remains without any symptoms, although all animals which had not been treated with the IL-4 antagonist developed strong allergic reactions including lethal shocks. Thus, the murine IL-4 antagonist Q116D/Y119D protected treated animals from developing an allergy. The potential to suppress already established allergies by inhibition of IL-4Rα is currently under examination.

Monkeys (*Macaca fascicularis*) were used to evaluate IL-4 antagonists in asthma.[83] Here, the human IL-4 antagonistic variant could be used. Also in this case, the antagonist, which was applied before sensitization, prevented the development of symptoms. Moreover, a therapeutic effect could be established: the application of the IL-4 antagonist in asthmatic animals resulted in a decreased bronchial hyperreactivity and a lower rate of infiltration of eosinophiles into the respiratory tract. Neither in these experiments nor during the above mentioned mouse studies could toxicities or other harmful side effects of the IL-4 variants be observed.

The favorable in vitro and in vivo tests, and in particular the therapeutic effects observed in the asthmatic monkey model, resulted in clinical development of the IL-4 R121D/Y124D variant. Since spring 1999 the effectiveness of IL-4 R121D/Y124D in humans has been evaluated in phase 1 clinical trials conducted by Bayer AG; however, results from these studies are not yet available.

6. IL-4 Antagonists: Paradigma or Lucky Strike?

Cytokine receptors are activated by homo- or heterodimerization of receptor chains within the plasma membrane. Some receptors such as the IL-2 or the IL-6 receptor employ a third chain to increase the affinity of ligand binding. The ligand primarily binds to the first chain with high affinity in both homodimeric as well as heterodimeric receptors. Subsequently, the intermediate complex formed recruits the low-affinity chain. The question arises, whether it is feasible to create inhibiting variants for each cytokine receptor by following the principle established with the IL-4 receptor antagonist, namely to block the low-affinity binding epitope of the ligand by mutation.

Presumably this is not possible. Problems with the binding affinity of the corresponding antagonistic variants of other cytokines—even in structurally similar systems—can be predicted. Most cytokine receptors bind their ligands with $K_d = 100–500$ pM. However, this total affinity of the receptor complex is often not only provided by the high-affinity chain, as occurs within the IL-4 receptor complex. The high-affinity chain usually binds up to a 100 times weaker than the entire receptor. Only by association with the low-affinity chain is the whole binding strength achieved. This binding strength can even vary within receptor families which employ shared receptor chains (gp130, γ_c, or β_c).

GM-CSF binds to its α chain with $K_d = 2–12$ nM, IL-3 binds its α chain with $K_d = 20–100$ nM, and IL-5 binds its α chain with $K_d = 1.2–2$ nM. By addition of the common β chain to each of these receptors, the affinity for their ligands increases to 100–500 pM. The increase in K_d amounts to a factor of 3 for IL-5, to a factor of 100 for GM-CSF, and to almost 1000 for IL-3. Presumably, the increase for GM-CSF and IL-5 originates from a direct interaction of the corresponding α chain with β_c, where the extent of coupling is different.

Similar differences can be found within the γ_c family. Until now it has been impossible to develop useful antagonists for IL-2, since the binding affinity of IL-2 for IL-2Rβ and even for a complex of IL-2Rβ and γ_c is unsatisfactorily low. An antagonistic variant would exhibit a corrrespondingly low binding affinity and therefore would be rather ineffective in displacing the stronger binding wild-type ligand from its receptor. However, a possible solution to this dilemma may be the creation of superagonists, whose affinity for the first chain is increased. Mutagenesis of several cytokines such as hGH, IL-6, and IL-4 yielded variants that exhibit stronger binding affinity to the high-affinity receptor chain than the corresponding wild-type ligand. A combination of superagonistic

and antagonistic mutations may therefore allow the design of antagonistic cytokine variants that employ the inhibiting principle of the IL-4 antagonist.

7. Perspectives: Miniproteins, Pepidomimetica, and Low Molecular Weight Ligands

A rational approach for the production of an IL-4 "miniprotein" arises from the characterized binding epitopes. The high-affinity binding epitope of IL-4 for the α chain is located on two antiparallel α helices. The transfer of the IL-4 binding residues to the parallel α helices of a leucin zipper (Gal4) yielded synthetic peptides which bind to the α chain with a micromolar K_d value.[83]

From a pharmacological point of view small mimetica for IL-4 that bind IL-4Rα with high affinity and effectively compete with IL-4 and IL-13 are desirable. In principle, the production of synthetic analogues seems to be possible. A variety of different approaches has already yielded small ligands for other cytokine receptors that can be chemically synthesized. Dimeric peptides for erythropoeitin and thrombopoietin have already been identified from peptide libraries. They comprise approximately 20 amino acid residues, which dimerize by forming disulfide bridges and exhibit agonistic activity down to nanomolar concentrations. Agonistic ligands for G-CSF have been discovered by high throughput screening. These results show that small high-affinity ligands can be created for the large and relatively flat contact surfaces of the cytokine receptors.

Looking ahead there is a further possible approach for a structure-based ligand development arising from the structure of the IL-4/IL4-BP contact. In regard to the mosaic structure with two independently binding clusters ("avocado-cluster" I and II) it may be possible to find low-affinity ligands for each of the two clusters independently and subsequently link them to form a high-affinity ligand.

8. Summary and Outlook

The development of an antagonistic IL-4 variant shows that the idea of "emasculated hormones" can be successfully transferred to protein hormones. Decisive in the success was both the structural elucidation of the binding surface and the understanding of the mechanism of receptor activation. It is as yet unclear how far the results gained in the IL-4 system can be transferred to structurally similar hormone/receptor systems. The strong interaction between IL-4Rα and its ligand—independent of other binding partners—is quite unique among the cytokines. Presumably, it may be possible within ligand/receptor systems that are characterized by weaker binding interactions to create variants that combine superagonistic and antagonistic features in one molecule, that is, that bind to their high-affinity chains with increased affinity but do not trigger downstream signaling because of impaired low-affinity binding epitopes. Therefore, development of antagonistic protein ligands for other protein hormones that employ the IL-4-like mode of action may be possible in the future.

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