

Molecular and Structural Basis of Cytokine Receptor Pleiotropy in the Interleukin-4/13 System

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SUMMARY

Interleukin-4 and Interleukin-13 are cytokines critical to the development of T cell-mediated humoral immune responses, which are associated with allergy and asthma, and exert their actions through three different combinations of shared receptors. Here we present the crystal structures of the complete set of type I (IL-4R α / γ_c /IL-4) and type II (IL-4R α /IL-13R α 1/IL-4, IL-4R α /IL-13R α 1/IL-13) ternary signaling complexes. The type I complex reveals a structural basis for γ_c 's ability to recognize six different γ_c -cytokines. The two type II complexes utilize an unusual top-mounted Ig-like domain on IL-13R α 1 for a novel mode of cytokine engagement that contributes to a reversal in the IL-4 versus IL-13 ternary complex assembly sequences, which are mediated through substantially different recognition chemistries. We also show that the type II receptor heterodimer signals with different potencies in response to IL-4 versus IL-13 and suggest that the extracellular cytokine-receptor interactions are modulating intracellular membrane-proximal signaling events.

INTRODUCTION

T cell-mediated immunity occurs in successive antigen-driven and cytokine-driven steps. In the first step, T cell receptors are activated by specific antigens presented by peptide-MHC complexes on the surface of antigen-presenting cells. In a second step, these activated T cells are induced to clonally expand by the cytokine Interleukin-2 (IL-2) and subsequently differentiate into CD4⁺ Th1 or Th2 cells as a result of a specific subset of cytokines engaging and activating their respective cytokine receptors (Leonard, 1999). IL-4 is the primary cytokine implicated in the development of Th2-mediated responses (Seder and Paul, 1994), which are also associated with allergy and asthma (Barnes, 2002). The cytokines IL-4 and IL-13 are produced by

Th2 cells, recruit and activate IgE-producing B cells, and enhance IgE-mediated responses. The strong association of Th2 cytokines with allergic disease has generated interest in targeting their receptors for therapeutic intervention (Foster et al., 2002; Mueller et al., 2002).

IL-4 alpha receptor (IL-4R α) is a unique member of the common-gamma chain (γ_c) family of receptors (Nelms et al., 1999), with the ability to signal within three different receptor complexes, known as type I and type II receptors (Andrews et al., 2006; Kelly-Welch et al., 2003; Mueller et al., 2002). On cells of hematopoietic stem cell origin, the type I receptor comprises IL-4R α and the common gamma-chain (γ_c), which is also shared by the cytokines IL-2, -7, -9, -15, and -21 (Nelms et al., 1999). On cells of nonhematopoietic stem cell origin, IL-4 can use the type II complex, comprising IL-4R α and IL-13R α 1 (Hilton et al., 1996; Obiri et al., 1995). This receptor complex is also a functional receptor for IL-13 (Aman et al., 1996), which shares only ~25% homology with IL-4, and this largely explains the overlap of the biological effects of IL-4 and IL-13 (Wills-Karp, 2004). Type I receptor complexes can only be formed by IL-4 and are more active in regulating Th2 development. In contrast, the type II receptor complex formed by either IL-4 or IL-13 is not found on T cells and is more active in regulating cells that mediate airway hypersensitivity and mucus secretion (Andrews et al., 2006). In fact, IL-13 appears to have its own distinct role in allergic inflammation, acting as a key regulator of allergen-induced airway inflammation and goblet cell metaplasia (Wills-Karp, 2004). The molecular basis for the cytokines' differing functional responses is unclear. Both type I and type II receptor complexes signal through the Jak/STAT cascade, with IL-4R α associating with Jak1, γ_c with Jak3, and IL-13R α 1 with Tyk2. A second mechanism of signal transduction activated by IL-4 and IL-13 leads to the insulin receptor substrate (IRS) family (Kelly-Welch et al., 2003).

IL-4 and IL-13 are prototypical four-helix bundle short-chain cytokines (Moy et al., 2001; Mueller et al., 2002; Walter et al., 1992; Wlodawer et al., 1992). The IL-4/IL-13 receptors γ_c , IL-4R α , and IL-13R α 1 contain the tandem Fibronectin-III domains and the WSXWS box that form the classical elbow-shaped "cytokine binding homology region (CHR)" that binds to the

helical faces of the cytokine (Bazan, 1990), as originally defined in the human Growth Hormone (hGH) system (de Vos et al., 1992). A previous structure of a nonsignaling binary complex between IL-4 and IL-4R α indicated a similarity to the hGH-R site I paradigm (Hage et al., 1999). While the structure of a complete short-chain cytokine receptor heterodimeric signaling complex was reported for IL-2 (Wang et al., 2005), the type I and type II IL-4/13 receptors have several novelties that pose interesting structural questions (Mueller et al., 2002). First, in the type II complex, the IL-13R α 1 contains an extra N-terminal Ig-like domain (D1) not found in other receptors of the γ_c subfamily that is required for IL-13 signaling but not for IL-4 (Arima et al., 2005). It is unknown how this top-mounted Ig-like domain participates in cytokine recognition.

A second novelty pertains to cytokine receptor “sharing” and the evolutionary relationships between IL-13R α 1, γ_c , and IL-4R α (Boulay et al., 2003; Leonard et al., 1994; Ozaki and Leonard, 2002). While γ_c is the primary short-chain cytokine shared receptor, IL-4R α is also shared in three signaling complexes. IL-13R α 1 bears an evolutionary relationship to γ_c (Boulay et al., 2003), but its ligand specificity is narrower and it has diverged from a common ancestral family with the acquisition of the N-terminal Ig-like domain. It will be interesting to determine if these receptors possess structural properties conducive to degenerate recognition and signaling in comparison to their highly specific alpha receptor counterparts. In addition, as only one γ_c -family complex has been solved (IL-2) (Wang et al., 2005), the type I complex could reveal a molecular basis for γ_c crossreactivity.

Finally, IL-4 and IL-13 can induce distinct functional responses through the identical type II receptor heterodimers (Kelly-Welch et al., 2003), raising the question of how ligand specificity is conferred by shared receptors and can influence signaling. Thus, the IL-4/13 system presents an opportunity to investigate whether downstream signaling differences through common receptors have origins in the structural/biophysical properties of the extracellular receptor-cytokine complexes. In order to probe these questions, we present structural, biochemical, and signaling studies on the complete set of type I and type II ternary receptor complexes, IL-4R α / γ_c /IL-4, IL-4R α /IL-13R α 1/IL-4, IL-4R α /IL-13R α 1/IL-13.

RESULTS

To obtain diffraction-quality crystals, we engineered glycan-minimized recombinant molecules (see [Experimental Procedures](#)) that behaved similarly to wild-type glycosylated proteins and determined the complex structures to resolutions between 2.9 and 3.0 Å (Table S1 and Figures S1 and S2 available online). All three ternary complexes (Figure 1) globally resemble the canonical cytokine-receptor complex architecture seen in several other cytokine receptor complexes (Stroud and Wells, 2004), albeit with substantial deviations apparent in the two type II complexes (Figures 1B, 1C, and S4) (discussed below). In the three complex structures, we refer to the cytokine/IL-4R α interfaces as site I, and the γ_c and IL-13R α 1 interfaces will be referred to as sites IIa, IIb, and III (Figure 1). The cytokines bridge the receptor heterodimers, resting in Y-shaped forks (sites I and IIa) that are buttressed underneath by extensive receptor-receptor contact

forming the “stems” of the Y’s (site IIb) between the D2-D2 (type I complex) and D2-D3 domains (type II complexes). In each complex extensive amounts of surface are buried within the cytokine-receptor and receptor-receptor interfaces (Table S2 and Figure S4) (3980 Å² for IL-4 type I, 4400 Å² for IL-4 type II, and 4030 Å² for IL-13 type II).

In both type II complexes there is a novel structural feature that we term site III (Figures 1B and 1C). The extra N-terminal IL-13R α 1 D1 “Ig-like” domain contacts the dorsal surfaces of both IL-4 and IL-13. The structure of the IL-13R α 1 D1 domain is an “s-type” Ig-like fold (Figure S3) (Bork et al., 1994), where the conventional *d* strand in a top sheet of an Ig-fold now becomes a *c*’ strand of the bottom sheet due to a switch in pairing (Figure S3). This has important implications for the type II complexes in that, by virtue of the strand swap that occurs in s-type Ig folds, the *c*’ strand of IL-13R α 1 is positioned to contact the C-D loop of the cytokine through a β sheet-like interaction.

IL-13R α 1 is derived from a common ancestral subgroup as γ_c ; the divergence of IL-13R α 1 is linked to the acquisition of the Ig-like domain (Boulay et al., 2003). The γ_c -cytokines IL-2 and IL-15 require unusual “sushi” domain alpha receptors for formation of the “high-affinity” IL-2/IL-15R α β γ_c complexes (Figure 2A) (Waldmann, 2006). The IL-2R α and IL-15R α bind to the dorsal surfaces of the cytokines (Figures 2A and 2C) (Chirifu et al., 2007; Rickert et al., 2005), at locations overlapping with the IL-13R α 1 D1 binding sites on IL-4 and IL-13 (Figures 2B and 2C). From the ancestral relationship of IL-13R α 1 and γ_c , it is tempting to speculate that the top-mounted D1 domain of IL-13R α 1 has structural and functional analogies to IL-2R α /IL-15R α . Possibly the divergence of three-domain IL-13R α 1 from the two-domain γ_c subgroup is the result of a gene fusion event between a separate Ig-like domain receptor (analogous to IL-2R α /IL-15R α) and the IL-13R α 1 D2D3 module (Figure 2B), consolidating both receptors on a single polypeptide chain.

Diverse Ternary Complex Assembly Energetics

The three complexes form by the sequential, and cooperative, assembly of composite cytokine-receptor binding surfaces that exhibit striking differences in the extent and distribution of polar and apolar surfaces (Figure S4, Table S2, and contact Tables S4–S7). In order to determine whether this structural diversity is reflected in diverse binding chemistries, we investigated the complex assembly thermodynamics using isothermal titration calorimetry (ITC) with soluble receptor extracellular domains (ECDs) (Figure 3; Table S3). Consistent with previous studies (Andrews et al., 2002; Obiri et al., 1995; Russell et al., 1993), we find in the type I complex that IL-4 first binds to IL-4R α , followed by recruitment of γ_c to form a ternary complex (Figure 3A). For the type II IL-4 complex, IL-4 again first binds to IL-4R α followed by recruitment of IL-13R α 1 (Figure 3B). In contrast, for the type II IL-13 complex, IL-13 first binds IL-13R α 1 with an affinity of $K_d = 30$ nM (similar to previous BIAcore studies [$K_d = 34$ nM]; Andrews et al., 2002) and this binary complex then recruits IL-4R α (Figure 3C). Strikingly, in the type I and II IL-4 complexes, the same IL-4/IL-4R α binary complex engages γ_c and IL-13R α 1 through opposite thermodynamics (Figures 3A and 3B, far right; Table S3), in which the type I complex shows a strongly favored

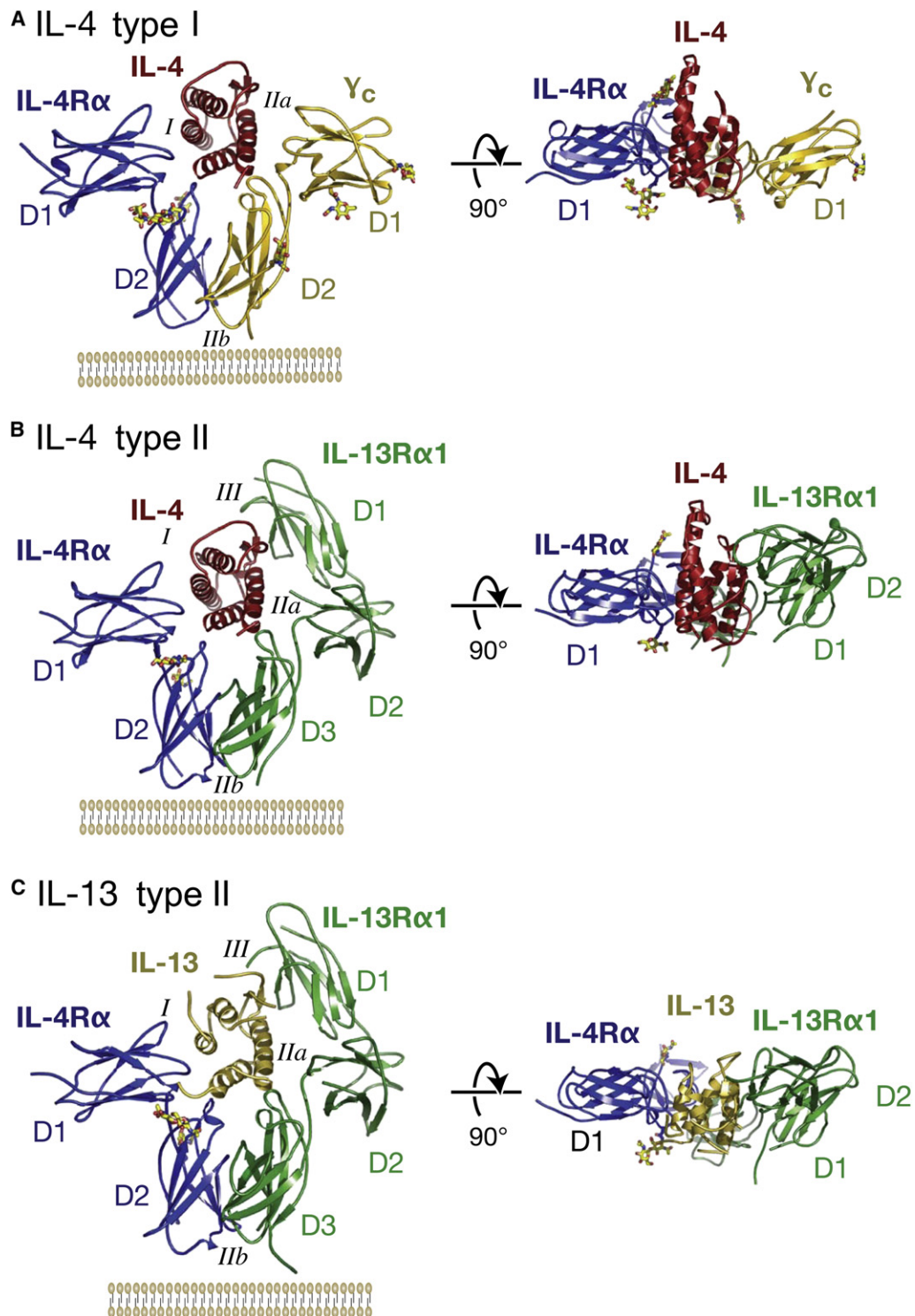


Figure 1. Structures of the Type I IL-4, Type II IL-4, and Type II IL-13 Ternary Complexes

(A) The type I complex with IL-4R α (blue), IL-4 (red), and γ_c (gold).

(B) The type II IL-4 complex with IL-4R α (blue), IL-4 (red), and IL-13R α 1 (green).

(C) The type II IL-13 complex with IL-4R α (blue), IL-13 (yellow-orange), and IL-13R α 1 (green).

The complexes are shown from the side with a cartoon of a membrane underneath (left), and as viewed from the “top” (right). Glycan moieties on Asn residues are shown in ball-and-stick representation. All figures were generated with PyMol (DeLano, 2002).

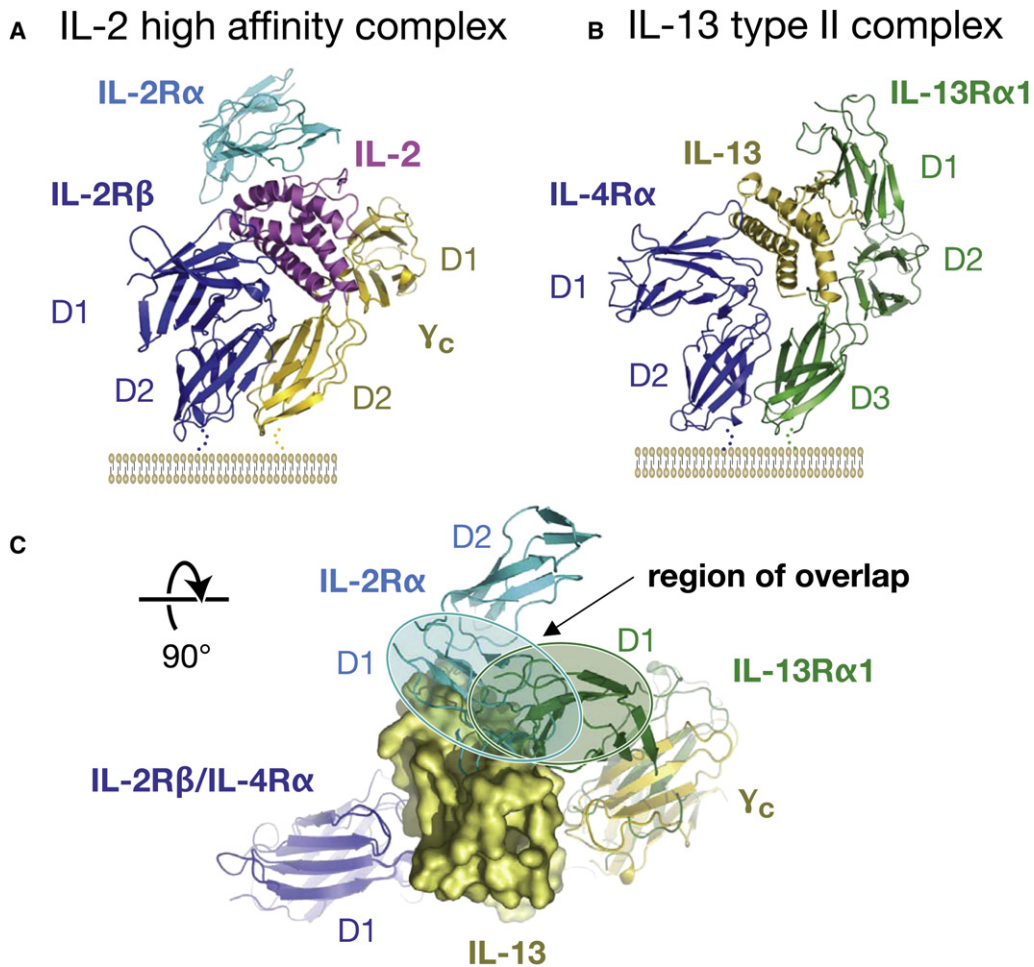


Figure 2. Structural Similarity between IL-2R α and IL-13R α 1 Cytokine Engagement

(A) The quaternary complex of IL-2 (pink), IL-2R α (cyan), IL-2R β (blue), and γ_c (gold). IL-2R α is bound to the top surface of IL-2.

(B) Ternary complex of IL-13 (yellow-orange), IL-4R α (blue), and IL-13R α 1 (green).

(C) As viewed from the top, the overlapping cytokine binding sites by IL-2R α and IL-13R α 1 D1 domains are apparent. Semitransparent ovals have been drawn around the D1 domain of IL-2R α and IL-13R α 1 so that regions of overlap are clear.

enthalpy $\Delta H = -11.7$ kcal/mol and a disfavored entropy $\Delta S = -10.5$ cal/molK $^{-1}$, compared to the type II complex showing a weakly favored enthalpy $\Delta H = -4.8$ kcal/mol and strongly favorable entropy $\Delta S = 13.0$ cal/molK $^{-1}$. Further, even in a case where several key interfacial residues persist, such as the site I interface of IL-4R α with IL-4 ($\Delta H = -11.2$ kcal/mol, $\Delta S = +3.5$ cal/molK $^{-1}$) versus IL-13 ($\Delta H = -5.8$ kcal/mol, $\Delta S = +16.0$ cal/molK $^{-1}$), distinct thermodynamic binding signatures are observed (Figures 3A–3C; Table S3). Although in our structures it is difficult to visualize trapped water molecules potentially impacting the entropy of the interactions, our results show that these shared receptors utilize diverse binding thermodynamics tailored to each ligand, in contrast to the basis of cross-reactivity in the gp130 system, which has converged on a desolvation-driven mechanism for all of its ligands (Boulanger et al., 2003).

With respect to formation of the ternary signaling complexes, the IL-13 type II complex appears to be the most stable by virtue of a higher affinity of the IL-13/IL-13R α 1 binary complex for

IL-4R α ($K_d = 20$ nM) (Figure 3C, far right; Table S3). The IL-4 type I and type II complexes both have very low affinity interactions with γ_c ($K_d = 559$ nM), similar to BIAcore measurements (Andrews et al., 2006; Zhang et al., 2002a), and IL-13R α 1 ($K_d = 487$ nM), respectively, resulting in lower overall complex stabilities (Figures 3A and 3B, far right). Thus, the recruitment of the second “trigger” receptors appears energetically limiting for the IL-4 complexes and could play a role in the differential signaling properties of the IL-4 versus IL-13 complexes (discussed later).

The Site I Interactions of IL-4R α with IL-4 versus IL-13

The interactions seen in the type I and type II IL-4/IL-4R α interfaces are similar to the previously reported IL-4/IL-4R α binary complex (Hage et al., 1999) (Figure 4A), except that in the ternary complexes the IL-4R α FN-III domains are rotated several degrees counterclockwise around the IL-4 helical bundle, likely due to the interactions formed between the bases of the

receptors (i.e., the stem of the Y). The principal feature of the large IL-4/IL-4R α interface (Figures 4A and S4) remains a charge complementarity between patches centered on “hotspot” residues Glu9 and Arg88 on the IL-4 A and C helices (Figure 4A) (Zhang et al., 2002b), respectively, which form polar contacts with Tyr13, Tyr183, Ser70, and Asp72 on IL-4R α .

The site I interface of IL-13 with IL-4R α (Figure 4B) is presented on a more compact IL-13 helical scaffold (see Figures 1B and 1C, top view) (two helical turns shorter in both A and C helices than IL-4). Although the binding surface and amino acids used on IL-4R α to bind IL-13 are nearly identical to that used to contact IL-4 (Figure 4A), their four-helix bundles superimpose poorly when the complexes are aligned on IL-4R α (Figure 4C). Yet, IL-13 exactly recapitulates two critical charged residues by presenting Glu12 and Arg65 to IL-4R α in the same positions to form similar receptor contacts as seen for IL-4 residues Glu9 and Arg88 (Figures 4B–4D), which have been shown by mutagenesis to be energetically important (Kraich et al., 2006). However, the composition of remaining IL-13 contact residues with IL-4R α is different (Figures 4C and 4D), in accord with the distinct binding thermodynamics the two cytokines use to bind IL-4R α (Figures 3B and 3C; Table S3). In the IL-13/IL-4R α site I, the interface lacks interactions ringing the charged hotspots observed in the IL-4/IL-4R α interface, such as the loss of IL-4 residue Trp91 on the C helix (Figure 4C). The number of polar bonds per 100 Å² of surface buried in the IL-13/IL-4R α interface is 1.0, denoting a substantial loss in specificity relative to the extraordinarily specific IL-4/IL-4R α complex (2 polar bonds per 100 Å²). Given that IL-4 binds to IL-4R α with subnanomolar affinity, and IL-13 has no measurable affinity for IL-4R α alone, the energetic value of the two “mimicked” charged contacts on IL-13 (Kraich et al., 2006) is apparently manifested within the context of the “composite” interface formed together with the receptor-receptor contacts (site IIb). In this fashion, the IL-4R α /IL-13 site I works in concert with site IIb to engage IL-4R α through tandem, multi-point attachment of two weak interfaces.

The Site IIa and Site III Interactions

The formation of the ternary signaling complex is completed by the recruitment of a second receptor, either γ_c or IL-13R α_1 , through structurally separated binding contacts designated sites IIa (fork of the Y) (Table S5), IIb (stem of the Y) (Table S6), and, in the cases of the type II complexes, site III (Table S5).

The three site IIa interfaces are uniformly characterized by the convex elbow of the receptors occupying a canyon on the A and D helices of the cytokines (Figures 5A–5C), exhibiting excellent knob-in-holes shape complementarity (Table S2) ($S_c = 0.82, 0.73, \text{ and } 0.65$ for the IL-4 type I and IL-4 and IL-13 type II complexes, respectively). Several hydrogen bonds flank the sides of the apolar canyons in each complex and likely stabilize the respective docking orientations. Consistent with their ancestral relationship, IL-13R α_1 and γ_c present binding sites with convergent structural features (Figures 5A and 5B). The site IIa contacts in each of the receptor complexes are primarily formed by the FG2 loop of the membrane-proximal FN-III domains of γ_c and IL-13R α_1 (D2 and D3, respectively), but γ_c also uses the EF1 loop in its D1. The receptor binding surfaces present a somewhat chemically inert main chain surface that inserts into a cleft in the

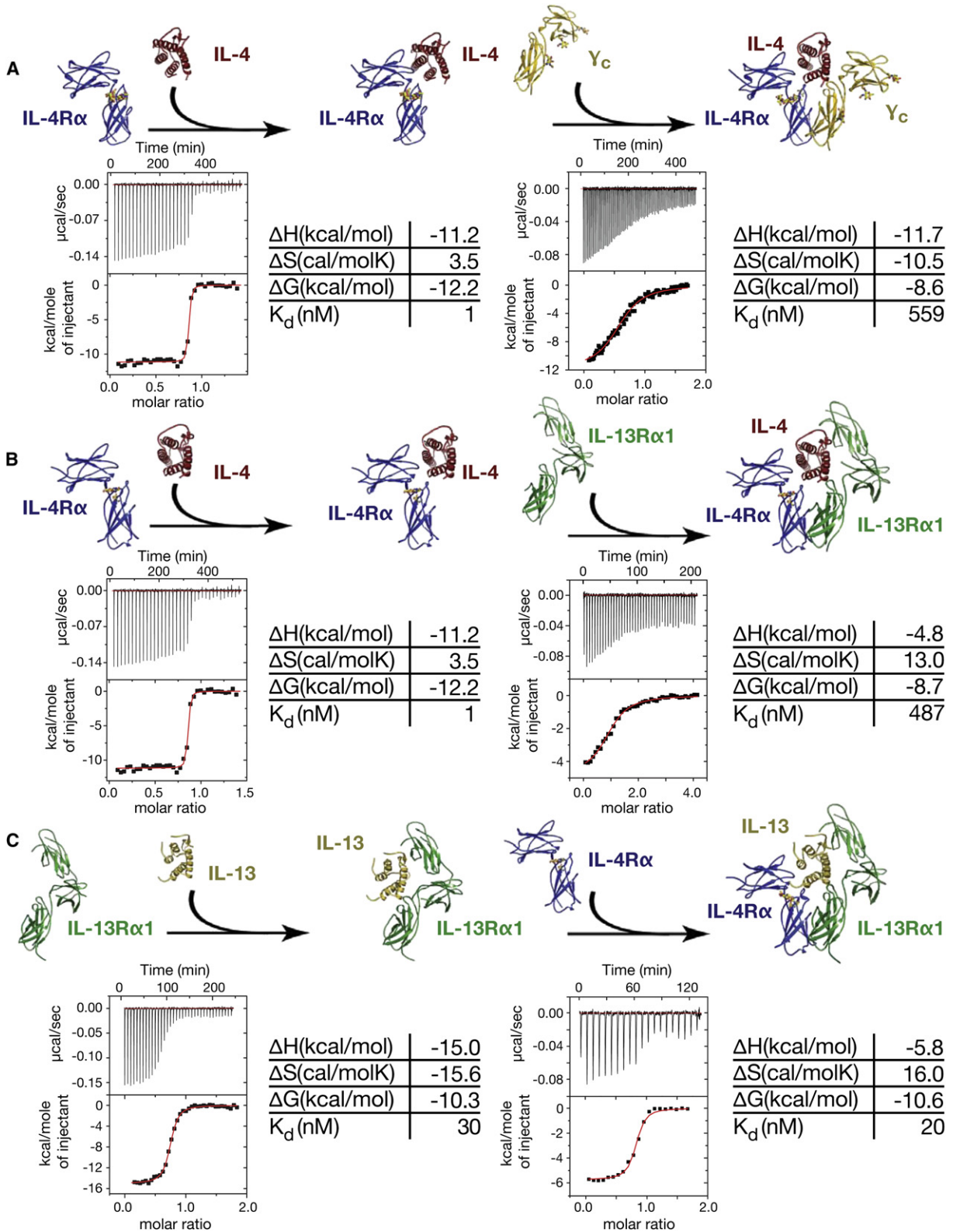
bases of cytokines. Both γ_c and IL-13R α_1 insert an unusual surface-exposed disulfide bond linking the FG2 and BC2 loops, together with a neighboring side chain (Leu208 for γ_c , Leu319 for IL-13R α_1), into the cavity on the cytokine surface (Figures 5A–5C). One substantial deviation between γ_c and IL-13R α_1 is that γ_c also forms extensive van der Waals contacts with IL-4 through Tyr103 on the EF1 loop of the D1 domain, resulting in a two-point site IIa attachment to IL-4 (Figure 5A). This EF-loop interaction is missing in both type II complexes (Figures 5B and 5C), consistent with mutational data on the IL-13R α_1 EF loop showing minor effects on binding (Arima et al., 2005).

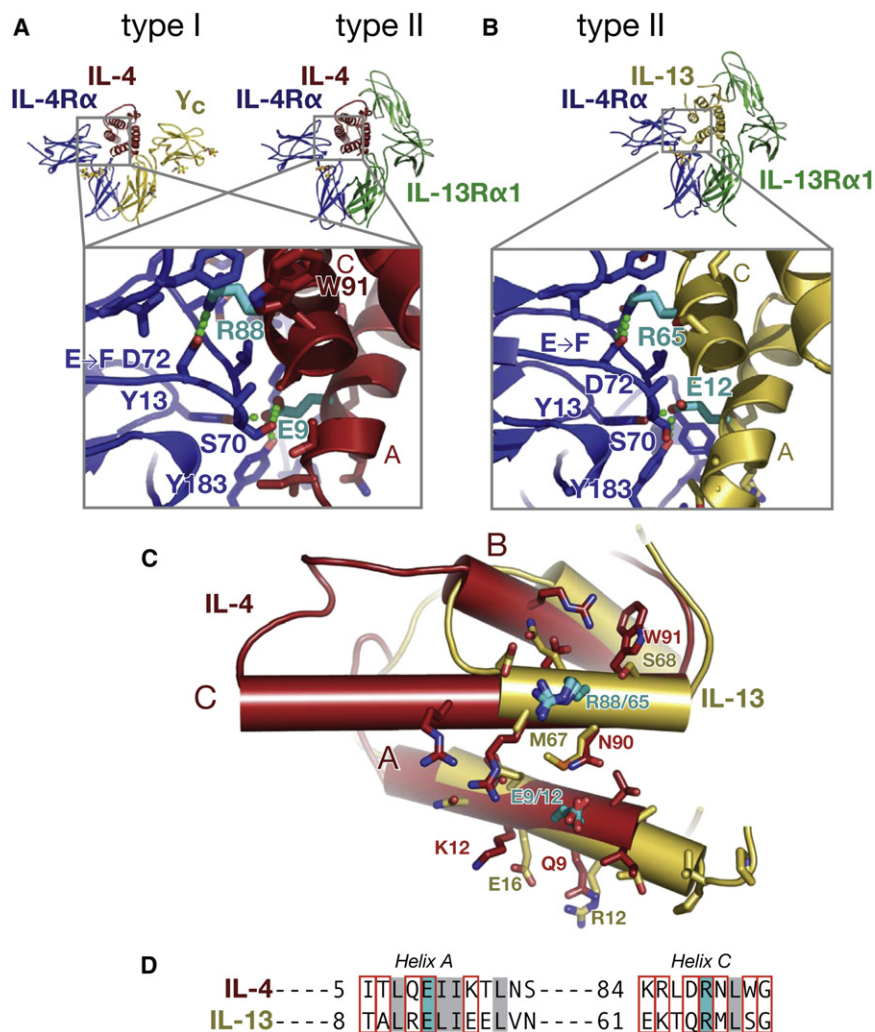
A stripe of amino acids on the IL-4 and IL-13 A and D helices demarcates the hydrophobic canyon lined by the alkyl moieties of both polar and nonpolar amino acids (Figures 5D and 5E). These side chains part to both sides to form clefts into which the narrow receptors insert to contact the main chains of the cytokine helices, facilitating degenerate recognition (Figures 5B and 5C). The canyon on IL-4 engages nonidentical amino acids on γ_c versus IL-13R α_1 in structurally similar fashions (Figures 3A and 3B). For example, IL-4 residues Gln8, Ile11, and Asn15 interact with γ_c Pro207 and Leu208, which appear to serve as structural equivalents to Lys318 and Leu319 of IL-13R α_1 (Figures 5A and 5B). Although the binding canyons on IL-4 and IL-13 utilize different amino acids to engage their receptors, they retain similar surface and shape properties, suggesting a manner of convergent structural evolution to enable crossreactivity.

The most unique structural feature in the type II complexes is site III (Figures 5F and 5G). The principal structural characteristic of site III is an antiparallel beta sheet between the c' strand of IL-13R α_1 and the C-D strand of IL-4 and IL-13. Similar sheet-like contacts form between IL-4 strand residues Asn105 to Thr108 or IL-13 residues Thr88 to Glu91 and the c' strand residues of IL-13R α_1 Lys76 to Ile78. However, there is an additional source of contact in the IL-13 site III that may explain the requirement of the IL-13R α_1 D1 for signaling in the IL-13 type II complex, in contrast to IL-4 (Arima et al., 2005). We also found in mutational studies that after deletion of IL-13R α_1 D1, the D2D3 CHR module alone does not detectably bind to IL-13, whereas IL-13R α_1 D2D3 does form a ternary complex with IL-4/IL-4R α (Figure S5). In the IL-13 complex site III (Figure 5G), a prominent saucer-shaped, hydrophobic patch on the IL-13 surface is formed by the alkyl sides chain of Met 33, Asp87, Lys89, and Trp35, creating a surface apposing a hydrophobic patch on the tip of IL-13R α_1 D1 composed of Trp65 and Ile78 on the c' strand (Table S2; Figure S4). By contrast, the analogous region of IL-4 lacking the complementary hydrophobic patch (Figures 5F and S4) is apparently relatively devoid of energetic value. However, as for the site IIa energetics, the energetic basis of the respective IL-13R α_1 D1 dependencies for IL-4 and IL-13 is not explained by the structure of site III alone but is likely highly energetically coupled to sites IIa (for IL-13) and IIa/IIb (for IL-4).

Receptor-Receptor Interactions (Site IIb) within the Ternary Complexes

In all three complexes, extensive contacts ($\sim 1200\text{--}1300$ Å² BSA) are formed between the membrane-proximal domains of the receptors (Figure S4 and Table S2). Although the sequences of γ_c and IL-13R α_1 are highly divergent (26% identity), the site IIb





interactions appear to utilize a limited degree of molecular mimicry to engage the shared IL-4R α in that several pairwise amino acid contacts are replicated. For example, both γ_c and IL-13R α 1 use structurally equivalent Phe186 and Phe297, and Pro189 and Pro300, respectively, to interact with a hydrophobic stretch of IL-4R α d strand residues Tyr150 through Tyr159 (Table S6). The receptor-receptor interfaces have very poor shape complementarity values of 0.49, 0.52, and 0.57 for the three complexes. By comparison, the excellent shape complementarity of the site IIa interfaces would appear to suggest that the cytokine-receptor contact is the primary driving force for site II

formation. Collectively, the three weak interactions alone (sites IIa, IIb, and III) cooperatively form a tripartite binding site where productive affinity is achieved through multipoint attachment.

Figure 4. The Interactions of IL-4R α with IL-4 and IL-13

For clarity the overall complexes are shown above blowups of the site I IL-4 (A) and IL-13 (B) interfaces, with color scheme retained from Figure 1. Zoomed up region of the site I interfaces focuses on the IL-4R α EF loop interactions with cytokine A and C helices (labeled). Key interacting residues are shown in stick representation, and the “mimicked” Arg and Glu hotspot residues found in both IL-4 and IL-13 are thicker sticks and colored cyan, with polar contacts drawn as dashed green lines. (C) “Open book” view of binding epitopes presented by IL-4 and IL-13 to IL-4R α . The resulting IL-4 and IL-13 cytokine (shown as cylinders) positions after their respective IL-4R α were superimposed show the relative structural overlap of the cytokine site I binding sites. (D) Structure-based sequence alignment between the interacting portions of helices A and C of IL-4 and IL-13 with IL-4R α , where contact positions are in red boxes, common hydrophobic core residues are highlighted in gray, and “hot spot” residues are highlighted in cyan to coincide with panels (A), (B), and (C).

How Does γ_c Crossreact with Six Different Cytokines?

γ_c is a signaling receptor for at least six different short-chain cytokines, including IL-2, -4, -7, -9, -15, and -21 (Ozaki and Leonard, 2002). Previously we determined the structure of the quaternary complex of IL-2 with IL-2R α , IL-2R β , and γ_c (Wang et al., 2005). With a second γ_c ternary complex, we analyzed how γ_c can engage six cytokines with less than 15% sequence identity (Figure 6). The IL-2 and IL-4 interfaces with γ_c are strikingly similar, both burying $\sim 1000 \text{ \AA}^2$ and having similar ratios of polar/apolar surface and low specificity indices (0.7–1.0 polar bonds/100 \AA^2). The γ_c binding sites on IL-2 and IL-4 exhibit apolar “canyons” that receive the protruding elbow of γ_c through near-ideal shape complementarity (Figure 6B) (Sc for IL-4 site IIa is 0.82, for IL-2 is 0.84). γ_c residues Tyr103 and the disulfide Cys160-Cys209 engage the IL-4 canyon

Figure 3. Contrasting Assembly Sequences, Binding Thermodynamics, and Stabilities of the Three Ternary Complexes

Each row illustrates the reaction in the order in which the binary and ternary complexes assemble (color scheme is maintained from Figure 1). Inset tables list the thermodynamic parameters calculated based upon the isothermal titration calorimetry (ITC) binding isotherm shown below each reaction diagram. Data are also summarized in Table S3.

(A) Type I complex: reaction diagram for the interaction of IL-4R α and IL-4 followed by recruitment of the second receptor, γ_c . (B) Type II IL-4 complex: the interaction of IL-4R α and IL-4 followed by the recruitment of the second receptor, IL-13R α 1. In both (A) and (B), the extremely high affinity of IL-4/IL-4R α ($K_d < 1 \text{ nM}$) is an estimate due to the steepness of the ITC trace. (C) Type II IL-13 complex: the interaction of IL-13R α 1 and IL-13 followed by the recruitment of the second receptor, IL-4R α .

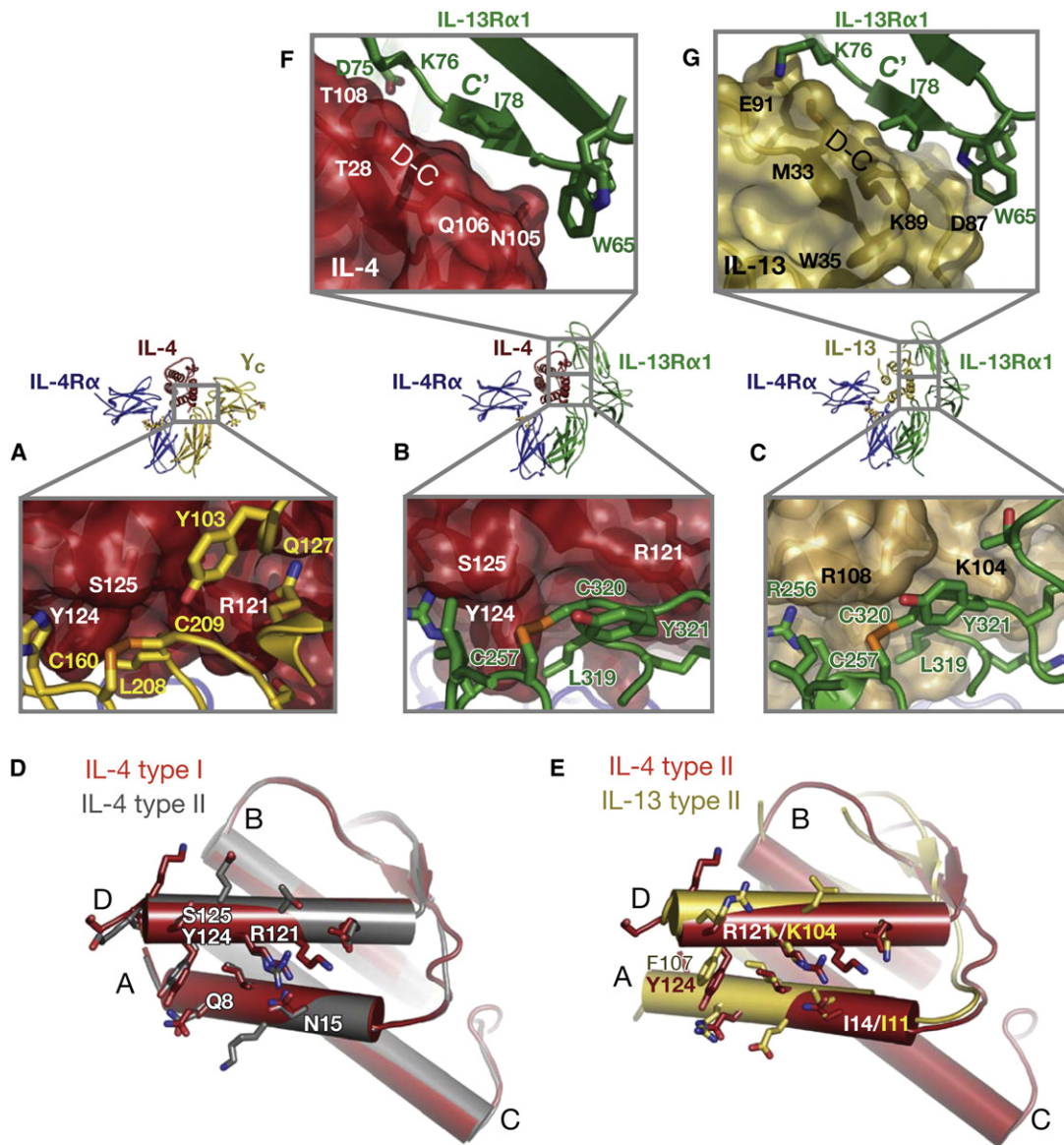


Figure 5. The Interactions of IL-4 and IL-13 with γ_c and IL-13R α 1

For clarity the overall complexes are shown in between blowups of the site IIa (underneath) and III (above) interfaces using the same color scheme as previous figures. Panels (A), (B), and (C) show zoomed views of the site IIa interfaces of the IL-4 type I, IL-4 type II, and IL-13 type II complexes, respectively, where the receptor residues are projected on semitransparent cytokine surfaces. IL-4 (red) and IL-13 (yellow-orange) residues underneath the surfaces are labeled in white and black, respectively, while γ_c (gold) and IL-13 R α 1 (green) residues are labeled in yellow and green, respectively.

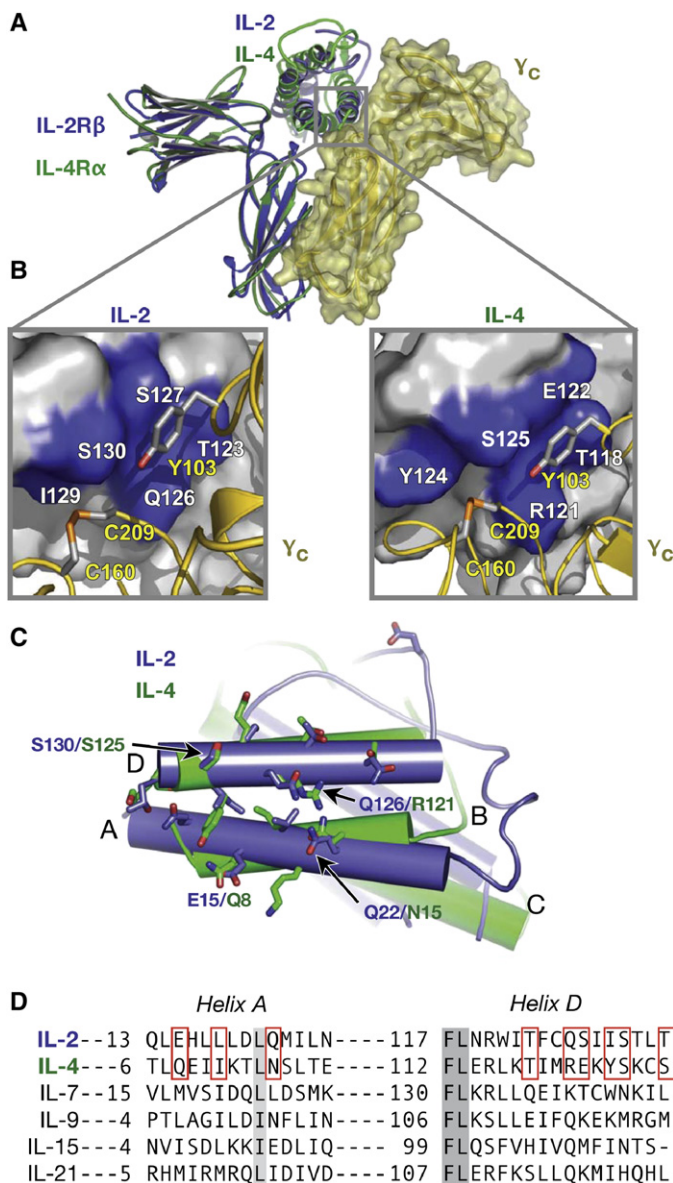
(D and E) "Open book" views of the site IIa binding sites. Panel (D) shows the resulting structural positions of IL-4 on IL-4 after superposition of IL-4R α from the type I and type II complexes, and panel (E) shows the resulting structural positions of IL-4 on IL-13 after superposition of IL-4R α from the two different type II complexes. All interacting residues in the site IIa interfaces are shown as sticks.

Panels (F) and (G) illustrate the site III interfaces in IL-4 and IL-13 type II complexes, respectively, using a similar coloring and labeling scheme as above. The antiparallel beta sheet between the IL-13R α 1 c' strand and the cytokine C-D loop is labeled.

in a coupled manner as seen in the IL-2- γ_c interface (Figure 6B). Cytokine contact residues on γ_c loops BC2 and FG2 maintain similar conformations in both IL-2 and IL-4 complexes, suggesting that crossreactivity is not occurring through structural plasticity of the receptor, but rather a rigid γ_c surface encounters extraordinarily complementary shape features on γ_c -cytokines,

which are further enhanced by specific hydrogen bonds peripheral to the canyon.

When IL-2 and IL-4 carbon- α (abbreviation C α) positions are compared in the complexes (Figure 6C), five residues each on helices A and D overlap with very close rmsds of 0.2 Å and 0.3 Å, respectively, as compared with the overall IL-2/IL-4 rmsd of



1.7 Å. The structurally equivalent side chains of IL-2 residues Glu15, Leu19, Gln126, and Ser130 and IL-4 residues Gln8, Lys12, Arg121, and Ser125 collectively line the complementary canyon, making analogous interactions with γ_c (Figure 6C). Several key hydrophobic residues within the helical core, which dictate the resultant positions of “external” interacting side chains on the helices, are conserved (Figure 6D). We propose these cytokine residue positions constitute a γ_c “recognition motif” that likely demarcates residues serving similar structural roles in other γ_c -cytokine complexes.

Signaling by the Type I and Type II Receptor Complexes on Cells

Given the shared use of receptors in the IL-4/13 system, we asked what the relative potencies of receptor activation were

Figure 6. Basis of Crossreactive Cytokine Recognition by γ_c

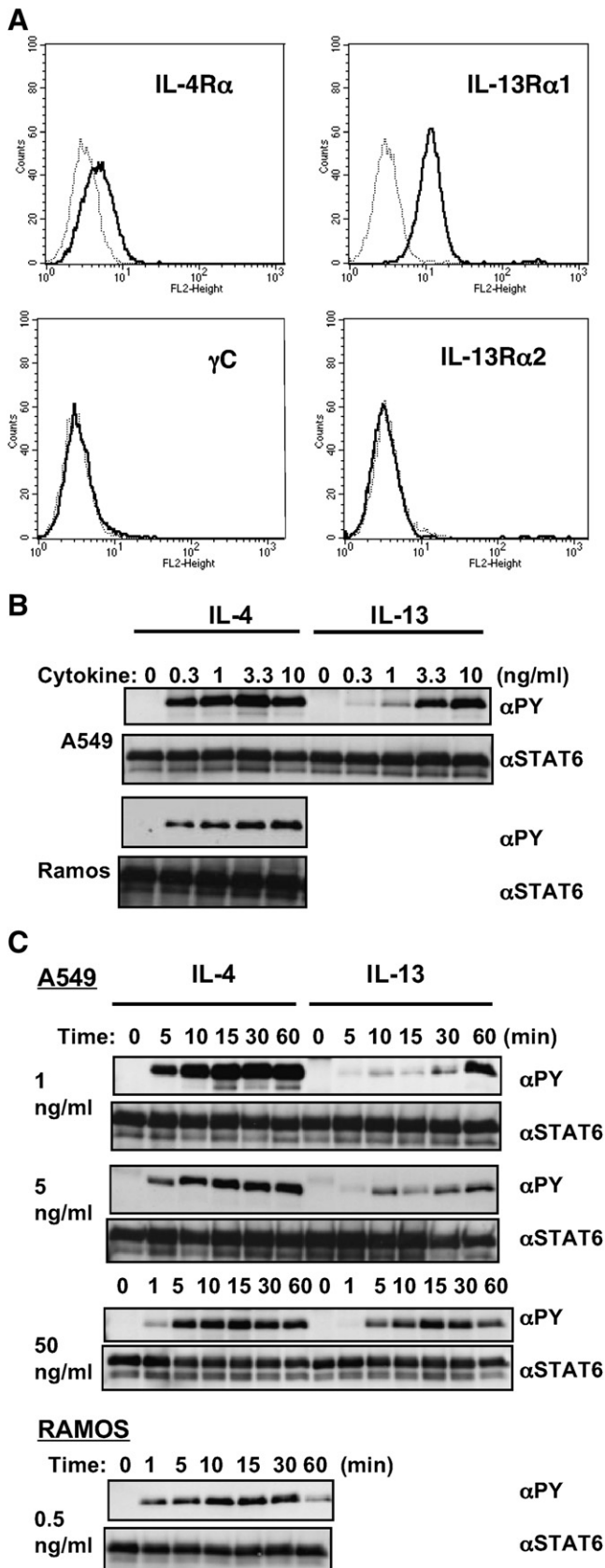
(A) Structural alignment of the IL-2 quaternary (IL-2R α not shown) and IL-4 type I ternary complexes after superposition on γ_c (gold). The IL-2 complex is blue, and the IL-4 complex is green.

(B) The conserved apolar “canyon” on the cytokine surfaces (highlighted in blue) accommodates the protruding γ_c binding loops (gold and silver residues).

(C) “Open book” views of the overlap of IL-2 versus IL-4 site IIa binding sites after superposition of γ_c .

(D) Structure-based sequence alignment of all known γ_c cytokines for helices A and D based on γ_c contact positions by IL-2 and IL-4. Residue numbers are included and contact residues from IL-2 and IL-4 are marked with red boxes. Hydrophobic residues are shaded in gray and correspond predominantly with buried core residues.

for IL-4 versus IL-13 through the type I and II receptors on cell lines (Figure 7). Since engagement of the type I and type II receptor complexes leads to the activation of STAT6 (Kelly-Welch et al., 2003), we compared the IL-4- and IL-13-induced phosphorylation of STAT6 as a measure of receptor complex activation (Figure 7). In addition, we analyzed the phosphorylation of STAT3 (Figure S6). We analyzed signaling by the type I IL-4 receptor in the human B cell line Ramos, which express γ_c and IL-4R α . We analyzed signaling by the type II receptors in the human epithelial carcinoma cell line A549, which express IL-4R α and IL-13R α 1 but do not express the γ_c or the decoy receptor IL-13R α 2 (Figure 7A). Treatment of A549 cells with either IL-4 or IL-13 for 30 min stimulated the potent tyrosine phosphorylation of STAT6 and the relatively weak phosphorylation of STAT3 (Figures 7B and S6). IL-4 was able to stimulate phosphorylation at significantly lower doses (five- to ten-fold) than IL-13. Treatment of Ramos cells with IL-4 stimulated the tyrosine phosphorylation of STAT6 with a dose response similar to that observed on A549. We also examined the kinetics of STAT6 and STAT3 phosphorylation at several cytokine concentrations (Figures 7C, S6, and S7). At all concentrations tested (1, 5, 50 ng/ml), IL-4 rapidly induced the tyrosine phosphorylation of STAT6 in A549 reaching plateau levels between 10 and 15 min of stimulation. However, the response to IL-13 was substantially slower. Treatment of A549 cells with 1 ng/ml, 5 ng/ml, or 50 ng/ml stimulated the tyrosine phosphorylation of STAT6 to plateau levels at >60, 30, and 15 min, respectively. The relative delay in STAT6 phosphorylation induced by IL-13 via the type II receptor complex was most apparent at lower concentrations of cytokine (1 ng/ml) (Figures S6 and S7). The stimulation of STAT3 phosphorylation by either IL-4 or IL-13 was quite weak in these cells; however, the pattern of responsiveness was similar to what we observed for STAT6 (Figure S6). Treatment of Ramos cells with IL-4 stimulated the tyrosine phosphorylation of STAT6 with kinetics similar to that observed on A549; IL-4 achieved maximal phosphorylation between 10 and 15 min of stimulation (Figures 7C and S7). We did not observe the induction of STAT3 phosphorylation by IL-4 in these cells (Figure S6). The implications of these signaling data in the context of the structural and thermodynamic data are discussed further below.



DISCUSSION

The results presented here on the IL-4/13 system bear on several coupled issues in ligand-receptor interactions, namely (1) the structural basis of degeneracy versus specificity of shared receptors, (2) the assembly energetics and sequences of heterodimeric receptor complexes, and (3) whether differences in these *extracellular* parameters could qualitatively influence the coupling of ligand engagement to *intracellular* receptor activation. Surprisingly, we found that the identical type II receptor heterodimer, coupled to the same intracellular signaling molecules, responds to different ligands, IL-4 versus IL-13, with different signaling potencies and kinetics (Figures 7B, 7C, S6, and S7). One can consider the initial cytokine-receptor interaction as the “driver,” and the second receptor recruited for ternary complex signaling as the “trigger,” akin to terminology previously proposed for the assembly of γ C heterodimeric complexes (Lai et al., 1996). The IL-4/13 system has the unique property that IL-4 and IL-13 have swapped their trigger and driver. The evolutionary expansion of receptor usage by switching driver and trigger modules has also occurred in other receptors in the family, such as IL-7R α / γ C (Noguchi et al., 1993) and IL-7R α /TSLP-R (Pandey et al., 2000). In the IL-4/13 system this switch is mainly due to the acquisition of the energetically critical site III in IL-13 and the “hobbling” of its site I. From previous studies we know that the equilibrium binding affinities of IL-4 and IL-13 for their heterodimeric receptor complexes on cell surfaces are similar (K_d ranging from 30–400 pM) (Aman et al., 1996; Andrews et al., 2002; Hilton et al., 1996; Park et al., 1987), so the explanation for the potency differences is not simply receptor occupancy. The facts that IL-4 binds to the IL-4R α with very high affinity (K_d ~subnanomolar) (Andrews et al., 2006; Park et al., 1987) and that the presence of γ C or IL-13R α 1 provides little additional affinity (Murata et al., 1998; Russell et al., 1993; Zhang et al., 2002a) suggest that driver association is dictating IL-4 type II signaling potency. Yet, we find that the high-affinity driver complex (IL-4/IL-4R α) recruits the IL-13R α 1 trigger receptor with very low affinity (K_d ~487 nM), which would be energetically rate limiting for signaling. In contrast, IL-13 initially binds to its driver receptor (IL-13R α 1) on the surface of cells with a relatively slow on-rate but still with a moderately strong binding constant (K_d ~30 nM) (Aman et al., 1996; Hilton et al., 1996). However, the presence of its trigger IL-4R α increases the cell surface affinity for IL-13 from

Figure 7. Comparison of Signaling Activated by IL-4 and IL-13
 (A) A549 express IL-4R α and IL-13R α 1 but not IL-13R α 2 or γ C. Expression of receptor subunits on A549 was analyzed by FACS as described in Experimental Procedures. The heavy black histograms indicate staining with specific antibody and the dotted histograms indicate staining by the isotype-matched control.
 (B) A549 cells and Ramos cells were washed and treated with various concentrations of IL-4 or IL-13 as indicated for 30 min. Cell lysates were prepared and immunoprecipitated with anti-STAT6. Western blots were probed with anti-phosphotyrosine antibody. The blots were stripped and reprobed with anti-STAT6. STAT3 activation is analyzed in Figure S6.
 (C) A549 cells and Ramos cells were treated with various concentrations of IL-4 or IL-13 for various times; STAT6 phosphorylation was analyzed as described in (B). Data are quantitated in graphical format in Figure S7.

$K_d \sim 10$ nM to 30 pM (Miloux et al., 1997). Thus, the recruitment of the trigger receptor (IL-4R α) by the IL-13/IL-13R α 1 driver complex is more energetically favorable ($K_d \sim 20$ nM) than is the converse trigger receptor (IL-13R α 1) recruitment by the IL-4/IL-4R α driver complex ($K_d \sim 487$ nM). Yet, paradoxically, we find that IL-4 is more potent at stimulating the tyrosine phosphorylation of STAT6 in several cell lines.

From the aforementioned energetic results we would predict that if the trigger receptor becomes limiting, the sensitivity and kinetics of STAT6 phosphorylation may change. Based on estimates in cell lines, the numbers of IL-4R α chains (50–5000 molecules/cell) appear to be limiting as compared to the numbers of IL-13R α 1 chains (5000–150,000 molecules/cell) (Park et al., 1987; Obiri et al., 1995). In the two cell lines we analyzed herein, expression of the IL-4 trigger receptor (γ_c or IL-13R α 1) was greater than IL-4R α (Figure 7A). However, in cells where IL-13R α 1 is limiting, IL-13 can become more potent than IL-4 (W. Paul, I.S. Juntilla, K. Mizukami, H. Dickenstein, M. Meier-Schellersheim, R.P. Donnelly [NIH], personal communication). Thus, it appears that when the IL-4 trigger receptor (IL-13R α 1) is abundant, the high-affinity IL-4/IL-4R α driver complex determines the ultimate signaling potency. By comparison, the affinity of IL-13 for its driver IL-13R α 1 ($K_d \sim 30$ nM) results in efficient formation of the IL-13/IL-13R α 1 driver complex even when IL-13R α 1 is limiting, and the substantially higher affinity of the IL-13/IL-13R α 1 driver complex for its trigger receptor (IL-4R α) ($K_d \sim 20$ nM) could result in more potent IL-13 signaling versus IL-4. Thus, the potency of signal transduction can be influenced by a variety of physical-chemical properties that are communicated through the cytokine receptor ECD interactions, including the concentration of cytokine and expression levels of receptor, the order of assembly of the signaling complexes, and the affinities of trigger and driver receptor recruitment. In vivo, the potential for higher-order assemblies of receptors on the cell surface, soluble shed receptors in the extracellular milieu, and the expression of the nonsignaling IL-13R α 2 serving as a decoy could play unknown roles in IL-4/13 signaling regulation.

Although highly speculative, another possible contributing factor to the enhanced potency of IL-4 may lie in the relative orientations of the receptor heterodimers in the two complexes. It has been shown for gp130 and the Erythropoietin receptor (EPO-R) that perturbations in the rotational “pitch” of the membrane-proximal intracellular regions, which are proximal to the Jak binding sites, can modulate signaling potency (Constantinescu et al., 2001; Greiser et al., 2002). Additionally, one study examined structures of complexes of EPO-R ectodomains with synthetic peptide agonist and antagonist mimics of EPO (Livnah et al., 1998) and found that the antagonist complex was rotated approximately 14 degrees away from the two-fold axis compared to the agonist complex (Figure S8B). We find that, when the IL-4 and IL-13 type II complexes are superimposed on either IL-4R α or IL-13R α 1, the relative orientation of the membrane-distal domains of the “partner” receptor differs by ~ 7 – 8 degrees (Figure S8A). The origin of this difference appears to lie mainly in the IL-4 versus IL-13 site I interfaces, which have diverged more in their relative cytokine/receptor positioning than the membrane-proximal regions. This perturbation in orientation could (Figure S8), in principle, exert a modulatory role on signaling in

a fashion analogous to the EPO peptide mimetics, although this remains speculative.

Collectively, our structural, biochemical, and signaling results invite revision of the concept that extracellular ligand engagement of cytokine receptors does not relay “instructive” information intracellularly. Experiments in which the ECD of EPO-R was replaced with the Prolactin ECD, resulting in red blood cell formation, concluded that structurally indiscriminate dimerization was adequate to initiate cytokine receptor signaling (i.e., “permissive signaling”) (Socolovsky et al., 1998). While we concur that downstream signaling specificity is manifested intracellularly, our results appear to suggest that the potency of receptor signaling can be influenced by the biophysical and structural properties of the extracellular receptor-ligand interactions, which may, in this case, be utilized as a means for different ligands to induce divergent signaling responses through shared receptors in different cellular contexts.

EXPERIMENTAL PROCEDURES

Ternary Complex Protein Expression and Purification

Human IL-4 and IL-13 and the ectodomains of γ_c , IL-4R α , and IL-13R α 1 cDNA were subcloned into pAcGP67-A with C-terminal hexahistidine tags (BD Biosciences, San Diego, CA) and secreted from High-Five insect cells in Insect-Xpress medium (Lonza, Walkersville, MD). In order to reduce protein heterogeneity due to Asn-linked glycosylation, we expressed the molecules in various glycan-minimized forms. IL-4 was expressed in the presence of tunicamycin (2.5 mg/l) (Calbiochem, San Diego, CA) to suppress Asn-linked glycosylation. For IL-4R α we mutated Asn \rightarrow Gln in four, out of a possible six, N-linked glycosylation sites (N28Q, N73Q, N109Q, N151Q). IL-13 and IL-13R α 1 were coexpressed in the presence of tunicamycin (0.65 mg/l). For the type I complex, each component was separately purified by gel filtration FPLC Superdex 200 (GE Healthcare, Piscataway, NJ) after which a 1:1:1 molar ratio of IL-4, IL4R α , and γ_c was mixed, digested with carboxypeptidase A (EMD Biosciences, San Diego, CA) to remove C-terminal His-tags, and then repurified by gel filtration. The IL-4 type II complex was produced in a similar fashion for crystallization trials, using IL-13R α 1 expressed in the presence of tunicamycin. For the IL-13 type II complex, IL-13 and IL-13R α 1 coexpressed in the presence of tunicamycin was digested with carboxypeptidase A and purified as a binary complex by gel filtration. The IL-4R α tetra-glycan mutant was separately expressed and purified and added to the binary complex in a 1:1:1 ratio and purified by MonoQ FPLC. Ternary complex molecular masses were confirmed by MALS analysis.

Isothermal Titration Calorimetry

Calorimetric titrations were carried out on a VP-ITC calorimeter (MicroCal, Northampton, MA) at 27°C. Gel filtration purified samples were dialyzed into sterile filtered 10 mM HEPES, pH 7.2, 150 mM NaCl. Data were processed with OriginLab software. Protein concentrations were determined using the BCA method. The n , or stoichiometry values determined by ITC, vary by $\sim 25\%$ (e.g., n values between 0.7 and 1.3 for an n value of 1.0) and are consistent with gel filtration molecular weight estimation. In the titrations of binary complexes, the titrand was used in the cell at a concentration of 5 μ M (IL-13R α) or 8 μ M (IL-4R α), and the titrant in the syringe at a concentration of 81 μ M (IL-13) or 65 μ M (IL-4). In the titration of the ternary complexes two approaches were used in order to compare similar complexes. Preformed and purified 1:1 IL-4/IL-4R α binary complex (55–57 μ M) was titrated into either γ_c (8 μ M) or IL-13R α 1 (3 μ M). Conversely, preformed and purified 1:1 IL-13/IL-13R α 1 binary complex (3 μ M) was the titrand in the cell while IL-4R α (22 μ M) was the titrant in the syringe.

Ternary Complex Crystallization and X-Ray Data Collection

The IL-4R α / γ_c /IL-4 ternary (8–12 mg/ml) was screened for crystallization using sparse matrix crystallization reagent Wizard III (deCODE Biostructures,

Bainbridge Island, WA). Crystals grew in 8%–12% PEG 8000, 0.1 M HEPES pH 7.5, and 8% ethylene glycol. Data collection was performed at the beamline 11-1, Stanford Synchrotron Radiation Laboratory (SSRL). The crystals diffracted to 2.95 Å resolution in space group $P2_12_12_1$, with unit cell dimensions of $a = 52.58$ Å, $b = 86.65$ Å, $c = 175.67$ Å. The data set was indexed, integrated, and scaled with HKL2000 (Otwinski and Minor, 1997).

The IL-4R α /IL-13R α 1/IL-4 ternary complex grew in conditions containing 5%–10% PEG 8000, 0.1 M cacodylate pH 6.5, 0.16 M calcium acetate, and 20% glycerol. Data collection was performed at beamline 8.2.2, Advanced Light Source (ALS), University of California, Berkeley. The crystals diffracted to 3.0 Å resolution in the space group $P2_1$, with unit cell dimensions of $a = 61.64$ Å, $b = 62.84$ Å, $c = 115.13$ Å, and $\beta = 96.31^\circ$. The data set was indexed, integrated, and scaled with HKL2000 (Otwinski and Minor, 1997).

For the IL-4R α /IL-13R α 1/IL-13 ternary complex two crystal forms were obtained, using either the fully glycosylated IL-4R α (form I), or the quadruple glycosylation knockout version as described above (form II). The form I crystals grew in 10%–15% PEG 8000, 8% ethylene glycol, 0.1 M ammonium citrate pH 6.0 and were cryopreserved in 23% ethylene glycol. Data collection was performed at ALS beamline 8.2.1. The crystals diffracted to 3.5 Å resolution with anisotropic diffraction extending to 3.2 Å and belong to space group $P2_12_12_1$, with unit cell dimensions of $a = 91.98$ Å, $b = 125.10$ Å, $c = 148.27$ Å, with two copies of the complex per asymmetric unit. Data were indexed, integrated, and scaled with HKL2000 (Otwinski and Minor, 1997).

The higher-resolution, glycan-minimized form II crystals were obtained in 12% PEG 6000, 0.2 M ammonium citrate, and 20% glycerol and cryoprotected with 15% ethylene glycol. These crystals diffracted at SSRL BL 11-1 to 3.0 Å with anisotropic diffraction extending to 2.8 Å. They belong to the space group $C2$, with unit cell dimensions of $a = 211.51$ Å, $b = 58.17$ Å, $c = 64.24$ Å, with one copy of the complex per asymmetric unit. The data set was indexed, integrated, and scaled with Mosflm and SCALA as implemented in CCP4 (CCP4, 1994).

Structure Determination and Refinement

The IL-4R α / γ _c/IL-4 ternary complex structure was determined by molecular replacement (MR) with the program Phaser (McCoy, 2007) from the CCP4 program suite (CCP4, 1994), using the binary complex of IL-4/IL-4R α (Protein Data Bank accession code 1IAR) and the γ _c chain from the IL-2 quaternary complex (PDB accession code 2B5I) as search models. Rigid-body refinement on the molecular replacement solution, followed by simulated annealing and individual B-factor refinement, were performed using CNS. After model rebuilding with COOT based on composite omit maps and further refinement with CNS and Refmac5 (Brunger et al., 1998; Emsley and Cowtan, 2004; Murshudov et al., 1997), the final R_{cryst} and R_{free} were 22.6% and 29.7%, respectively. For this as well as the two structures described below, Procheck (Laskowski et al., 1993) indicates that Ramachandran statistics and the geometry of the protein models are compatible with structures at similar resolutions (Table S1).

For the IL-4R α /IL-13R α 1/IL-13 ternary complex, MR solutions were obtained using the diffraction data of form I crystals (with fully glycosylated IL-4R α), with IL-4R α as the search model (PDB accession code 1IAR). These solutions enabled the establishment of a noncrystallographic symmetry (NCS) operator relating molecules. Phaser also identified one MR solution for IL-13, by searching with the averaged IL-13 NMR model (PDB accession code 1IJZ), and the second copy of IL-13 was properly placed into the crystal lattice using the NCS operator. Rigid-body refinement was performed on the MR solutions of the IL-4R α /IL-13 binary complex. Subsequently, the D2D3 portion of IL-13R α 1 was obtained by Molrep (Vagin and Teplyakov, 2000) using a “culled” receptor model based on the D2D3 domains of gp130 (PDB accession code 1BQU). The second copy of the IL-13R α 1 D2D3 domains was then placed using the NCS operator, and the partial model was rigid-body refined using CNS. The diffraction data of higher resolution from the form II crystals of the IL-4R α /IL-13R α 1/IL-13 ternary complex were used for model rebuilding and refinement. After molecular replacement with Phaser to place the partially refined ternary complex model into the current crystal lattice, several rounds of multiscrystal averaging improved the density maps, allowing the rebuilding of the D2D3 domains of IL-13R α 1. The remaining D1 domain of 13R α 1 was placed into the complex with Phaser, using a fibronectin type III domain structure (PDB accession code 1FNA) as search model. Following rigid body refine-

ment, reiterations of model rebuilding with COOT, and structure refinement using CNS and Refmac5, the final R_{cryst} and R_{free} for this ternary complex were 25.6% and 31.2%, respectively (Table S1).

For the remaining IL-4R α /IL-13R α 1/IL-4 ternary complex, the MR search was performed with Phaser, using models from the other two complexes. Following rigid body refinement, model rebuilding using COOT, and structure refinement with CNS and Refmac5, the R_{cryst} and R_{free} were 23.8% and 30.4%, respectively (Table S1).

Analysis of Receptor Expression and STAT Phosphorylation

Expression of receptor subunits was analyzed by FACS. PE-conjugated antibodies to human IL-4R α , γ _c, and IL-13R α 1 were from BD Pharmingen (San Diego, CA); PE-conjugated anti-IL-13R α 2 was from Cell Sciences (Canton, MA). The signaling experiments were performed as described previously (Zamorano et al., 1996). Briefly, A549 or Ramos cells were washed three times and treated with various concentrations of IL-4 or IL-13 (R & D Systems Minneapolis, MN) for various times. Cells were harvested and lysed. Equal amounts of protein (~1 mg) were immunoprecipitated with rabbit anti-STAT6 (S-20, Santa Cruz Biotechnology, CA). The samples were separated on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were probed with an anti-phosphotyrosine antibody, RC20-H (Transduction Labs, Lexington, KY). The bound antibody was detected using enhanced chemiluminescence. Where indicated the blots were stripped and probed with anti-STAT6. In some cases, total cell lysate (30 μ g/lane) was analyzed using anti-phospho-specific STAT6 (Y641) or STAT3 (Y705) antibodies (Cell Signaling Technology, Danvers, MA). The films were scanned and analyzed using NIH Image. The density of signal from the phospho-STAT6 was normalized to the density of total STAT6 and expressed as phosphorylated STAT6/total STAT6 ratios. The calculated percent maximum from three independent experiments was averaged and graphed \pm SEM. The student's *t* test was used to determine the significance of differences between groups.

Supplemental Data

Supplemental Data include eight figures and seven tables and can be found with this article online at <http://www.cell.com/cgi/content/full/132/2/259/DC1>.

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Accession Numbers

The structure factors and coordinates for the IL-4 type I and IL-4 and IL-13 type II complexes have been deposited in the Protein Data Bank under accession codes 3BPL, 3BPN, and 3BPO, respectively.