Conformational Changes Mediate Interleukin-10 Receptor 2 (IL-10R2) Binding to IL-10 and Assembly of the Signaling Complex

Interleukin-10 receptor 2 (IL-10R2) is a critical component of the IL-10-IL-10R1-IL-10R2 complex which regulates IL-10-mediated immunomodulatory responses. The ternary IL-10 signaling complex is assembled in a sequential order with the IL-10-IL-10R1 interaction occurring first followed by engagement of the IL-10R2 chain. In this study we map the IL-10R2 binding site on IL-10 using surface plasmon resonance and cell-based assays. Critical IL-10R2 binding residues are located in helix A adjacent to the previously identified IL-10R1 recognition surface. Interestingly, IL-10R2 binding residues located in the N-terminal end of helix A exhibit large structural differences between unbound cIL-10 and cIL-10-IL-10R1 crystal structures. This suggests IL-10R1-induced conformational changes regulate IL-10R2 binding and assembly of the ternary IL-10-IL-10R1-IL-10R2 complex. The basic mechanistic features of the assembly process are likely shared by six additional class-2 cytokines (viral IL-10s, IL-22, IL-26, IL-28A, IL-28B, and IL-29) to promote IL-10R2 binding to six additional receptor complexes. These studies highlight the importance of structure in regulating low affinity protein-protein interactions and IL-10 signal transduction.

Cellular IL-10 (cIL-10) is a multifunctional cytokine that regulates both innate and cell-mediated immunity (1). IL-10 dampens inflammatory responses by inhibiting the production of proinflammatory cytokines and chemokines as well as major histocompatibility complex and B7 on a variety of cell types (2, 3). However, IL-10 also exhibits immunostimulatory properties that include the proliferation and differentiation of thymocytes, mast cells, and B cells (4–6). Preliminary clinical data suggest antagonists that block IL-10 immunostimulatory properties may have therapeutic potential in the treatment of lupus and other inflammatory diseases (7).

cIL-10 biological activities are mediated by receptor engagement and assembly of the cIL-10-IL-10R1-IL-10R2 complex, which activates the JAK/STAT signaling pathway (8, 9). Based on a variety of cell surface and solution binding studies, the ternary signaling complex is assembled in a sequential manner, with IL-10R1 binding to cIL-10 first with high affinity (\(1 \times 10^{-10}\) M) followed by low affinity interactions between IL-10R2 and the cIL-10-IL-10R1 binary complex (Fig. 1). Surface plasmon resonance (SPR) studies show IL-10R2 exhibits at least a 10-fold increase in affinity for the cIL-10-IL-10R1 binary complex (\(K_d = 234 \mu M\)) compared with IL-10 alone (\(K_d \sim 2 \mu M\) (10, 11)). The cooperative binding mechanism by which IL-10R2 recognition is modulated by IL-10R1 has not been determined. Furthermore, the data suggest cIL-10 antagonists that bind tightly to IL-10R1 but do not engage the low affinity IL-10R2 chain could be designed if the mechanisms governing IL-10R1 and IL-10R2 binding are identified.

Several herpes viruses express cIL-10 mimics that aid viruses in establishing persistent infections in their hosts (12). In particular, cytomegalovirus and Epstein-Barr virus encode biologically functional IL-10 homologues cmvIL-10 and ebvIL-10, respectively (13, 14). With sequence identities of 83% (ebvIL-10) and 27% (cmvIL-10), both viral IL-10s bind and signal through the IL-10 receptor complex inducing overlapping biological activities with cIL-10 (15). Interestingly, sequence identity is not correlated with IL-10R1 or IL-10R2 affinity, suggesting the mechanisms governing receptor recognition and assembly are more complex than simple amino acid changes on a common ligand scaffold.

In addition to the importance of IL-10R2 in modulating cIL-10 signaling, the IL-10R2 chain also participates in a broad array of protein-protein interactions with at least five additional class-2 cytokines (IL-22, IL-26, IL-28A, IL-28B, and IL-29) that share ~10–20% sequence identity with cIL-10 and exhibit a diverse array of biological activities (16–18). Recently, ala-scanning mutagenesis identified five hotspot residues on helices A and D of IL-22 that are important for IL-10R2 binding (19). However, because helices A and D in cIL-10 and...
IL-22 exhibit extensive structural and sequence diversity, it is unclear if each molecule shares a common structural or energetic IL-10R2 binding site (19). Analysis of IL-10R2 binding to a series of overlapping 15-mer peptides, derived from cIL-10 and IL-22, provides initial evidence the sites are quite different (20).

In this report, we identify the IL-10R2 binding site on cIL-10 and show that cIL-10/IL-10R2 interactions are dependent on IL-10R1-mediated conformational changes in IL-10. Residues important for IL-10R2 binding are located at the N- and C-terminal ends of helix A and form two distinct interaction surfaces (site 2a and 2b) located adjacent to the previously identified site 1a and site 1b IL-10R1 binding sites (21). The overall location of the IL-10R2 binding sites on cIL-10 and IL-22 are conserved. However, the most important residues for IL-10R2 binding are located in site 2b for cIL-10 and site 2a for IL-22. The importance of structure in IL-10R2 recognition is emphasized by the lack of sequence conservation across the IL-10R2 binding cyto- kines. However, each molecule conserves key structural features, including an N-terminal disulfide bond and a hydrophobic sequence after the AB loop, which allow the proteins to access significant conformational changes required for their biological function. These studies further define the molecular mechanisms governing IL-10R2 receptor sharing and provide insights into the design of cIL-10 antagonists that might be useful in a variety of inflammatory diseases.

EXPERIMENTAL PROCEDURES

Mutagenesis—Mutations to generate sIL-10R1T213C, and all variants of cIL-10 were carried out using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing.

Expression and Purification of cIL-10 Variants and IL-10 Receptors—Wild-type and variant cIL-10 proteins were overexpressed in Escherichia coli BL21 (DE3) strain, refolded, and purified as previously described (22). The extracellular domains of IL-10R1 (sIL-10R1), sIL-10R1T213C, and IL-10R2 (sIL-10R2) were expressed in Drosophila S2 cells and purified by using affinity chromatography as previously described (10, 21, 23). Purified sIL-10R1T213C was reduced with 2 mM β-mercaptoethanol in 50 mM phosphate buffer (pH 7.0) containing 200 mM NaCl and 5 mM EDTA. β-Mercaptoethanol was removed by a desalting column (GE Healthcare) and biotinylated (sIL-10R1T213C-Bt) with 1 μM PEO-maleimide activated biotin (Pierce) followed by exhaustive dialysis against 10 mM Hepes (pH 7.0).

SPR Experiments—All SPR experiments were performed on a BIAcore 2000 system (BIAcore) at 20 °C using HBS running buffer (10 mM Hepes (pH 7.4), 0.15 M NaCl, and 0.005% Surfac- tant P20). Immobilization of all proteins on the chip surfaces was performed at a flow rate of 5 μl/min. Surfaces were regenerated by injecting 2 μl MgCl2 (pH 7.4) for 1 min followed by HBS running buffer containing 20 mM EDTA for 1 min at the flow rate of 50 μl/min.

Streptavidin (Pierce) in 10 mM sodium acetate (pH 5.0) was immobilized at the level of 1900–3600 RU by amine-coupled biotin (sIL-10R1T213C) that diffract to 2.5Å resolution, was immobilized at the level of 1900–3600 RU by amine-coupled biotin (sIL-10R1T213C) that diffract to 2.5Å resolution. Surfaces were captured on streptavidin surfaces at final densities of 80–240 RU for CM5 chips. Control surfaces of free streptavidin or streptavidin containing the biotinylated extracellular domain of IL-22R1 were used and gave identical results.

Kinetic interaction experiments were performed by injecting serially diluted monomeric cIL-10s (cIL-10M1s) over sIL-10R1T213C-Bt surfaces in random order at 50 μl/min. The data were prepared by the method of double referencing as described by Rich and Myszka (24). Using BIAevaluation software version 3.2 (BIAcore), the response curves were globally fit to a 1:1 Langmuir binding model. For IL-10-sIL-10R1 interactions, cIL-10 was amine coupled to CM5 chips. Amine coupled IFN-γ was used as the control surface. sIL-10R1 was serially diluted and injected over the amine-coupled cil-10 surfaces in random order at 50 μl/min.

IL-10R2 binding was evaluated using cIL-10 or cIL-10 alan- nine mutants amine coupled to CM-5 chips at high levels (1020–1230 RU). The binary cIL-10-sIL-10R1 complex was formed by injecting 500 nm of sIL-10R1 to saturate the cIL-10 surface. This was followed by a second injection of 700 μm sIL-10R1 and 500 nm sIL-10R1. The contribution of sIL-10R2 to the binding response was calculated by normalizing sensorgrams with the responses of the first injection and then by subtracting a baseline response obtained by the injection of 500 nm sIL-10R1 only. Equilibrium responses were obtained by averaging the responses obtained in the final 10 s of the latter injection.

Structure Comparisons of Unbound cIL-10 and cIL-10-sIL-10R1 Complexes—To ensure the structures used to interpret the mutagenesis data were of the highest quality possible, we completed the refinement of unbound cIL-10, 1INR, to give a final structure with PDB code 2H24. In addition, crystals of the cIL-10-sIL-10R1 complex that diffract to 2.5Å resolution, compared with 2.9 Å for the previous 1H2V structure, were used to refine the cIL-10-sIL-10R1 model (1Y6K) for the analysis. No significant changes were observed in the new structures, and the interpretation of the data is the same regardless of the structure used. Details of the structure refections are described in the supplemental material. Structures used in the analyses were cIL-10 (2H24, 2H2L), ebvIL-10 (1VLK), cIL-10-sIL-10R1 (1Y6K), ebvIL-10-sIL-10R1 (1Y6M), cmvIL-10-sIL-10R1 (1LQ5), and IL-22 (1M4R, 1YKB). Figs. 4 and 5 were generated using Ribbons (25).

Cell Proliferation Assay—TF-1 cells, transfected with the human IL-10R1 gene (TF-1/cIL-10R1 (15)), were maintained in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (BioWhittaker), 2 mM L-glutamine, 100 units/ml penicil- lin, 100 μg/ml streptomycin, 50 μg/ml β-mercaptoethanol, 2 μg/ml puromycin (Sigma), and 2 ng/ml recombinant human granulo- cyte-macrophage colony-stimulating factor (R&D Systems). cIL-10s were dispensed into 96-well microplates (BD Biosciences) in duplicate wells and serially diluted 3-fold across the plates. 5000 TF-1/cIL-10R1 cells were added to each well and incubated for 2 days at 37 °C with 5% CO2. Viable cell density was assayed using alamar blue. Fluorescence intensity was measured at room temperature using a POLARstar plate reader.
STAT3 Activation Assays—Human peripheral blood monocytes were isolated by elutriation in a Beckman JE-6B centrifugal elutriator as described previously (26). Monocytes were incubated with wild-type cIL-10 or cIL-10 mutants for 30 min at 37 °C. After cytokine treatment, whole cell lysates were prepared as described previously (27). Immunoblot analysis was performed using rabbit anti-phospho-STAT3 (Tyr705, Cell Signaling Technology) or anti-STAT3 (Santa Cruz Biotechnologies) antibodies.

Statistical Methods—The Kolmogorov-Smirnov method was used to confirm the receptor binding, and biological activity datasets were normally distributed. Analysis of variance was performed to confirm each dataset contained significant overall differences ($p < 0.05$). A 2-sided Dunnett’s test, which controls the type I experiment-wise error, was utilized to determine significant differences ($p < 0.05$) between cIL-10M1 or cIL-10 and their respective mutants.

RESULTS AND DISCUSSION

Experimental Strategy to Identify the IL-10R2 Binding Site—The dissociation constant of the soluble IL-10R2 chain for the soluble cIL-10/sIL-10R1 binary complex is approximately $230 \mu M$ (11). In this study we sought to define specific cIL-10 residues that mediate IL-10R2 binding and signal transduction. Regions of cIL-10 targeted for mutagenesis were identified based on previous studies that defined the IL-10R2 binding site on IL-22, a structural homolog of cIL-10 (19). To map the IL-10R2 binding site, cIL-10 mutants were initially screened for IL-10R1 affinity using SPR, and their ability to induce the proliferation of human TF-1 cells transfected with the IL-10R1 chain (Fig. 1). Mutation of cIL-10 residues that bind IL-10R2 are expected to display essentially wild-type affinity for sIL-10R1 but exhibit a reduced capacity to induce TF-1/cIL-10R1 cell proliferation.

IL-10-IL-10R1 SPR Assay—A new SPR assay was developed to accurately evaluate the binding kinetics of cIL-10 alanine mutants for IL-10R1 (Fig. 1B). Threonine-213 in sIL-10R1 was mutated to a cysteine (sIL-10R1*T213C) and biotinylated (sIL-10R1*T213C-Bt) with maleimide biotin for oriented and homogeneous capture on streptavidin CM5 chips. The T213C mutation was made in the stalk region of sIL-10R1 just before where IL-10R1 enters the membrane to simulate its orientation on the cell surface (Fig. 1B). Attempts to accurately fit sensorgram data collected by injecting dimeric cIL-10 over the sIL-10R1*T213C-Bt surface were not successful because cIL-10 dimers
The IL-10R2 Binding Site on IL-10

formed mixtures of 1:1 and 1:2 cIL-10 dimer-sIL-10R1\textsuperscript{T213C-Bt} complexes (data not shown). This mixture occurs because streptavidin-labeled sIL-10R1\textsuperscript{T213C-Bt} molecules do not form 2-fold-related receptor pairs on the sensorgram surfaces as required for bivalent IL-10R1 binding (11, 21).

In contrast to SPR studies with cIL-10, injecting a monomeric form of cIL-10 (cIL-10M1) over the sIL-10R1\textsuperscript{T213C-Bt} surface resulted in sensorgrams that could be globally fit to a simple 1:1 Langmuir interaction model (Fig. 1C). As previously described, cIL-10M1 is biologically active, and its structure is identical to one subunit of the cIL-10 dimer (10, 22, 29). Thus, cIL-10M1 provides an important tool for determining accurate binding parameters for the cIL-10-IL-10R1 site 1 interface and for mapping the IL-10R2 binding site. Based on the global fit of the data, the association (\(k_+\)) and dissociation rates (\(k_-\)) are 5.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1} and 2.6 \times 10^{-4} \text{s}^{-1}, respectively. Using these kinetic constants, the equilibrium dissociation constant (\(K_d = k_-/k_+\)) for the interaction between cIL-10M1 and sIL-10R1\textsuperscript{T213C-Bt} is 0.47 nM. The kinetic and equilibrium parameters are in good agreement with those from previous SPR analysis using cIL-10M1-immobilized surfaces and isothermal titration calorimetry data (10, 11).

Identification of the IL-10R2 Binding Site on cIL-10M1—A total of 13 cIL-10M1 mutants, located on \(\alpha\)-helices A and D, were evaluated for IL-10R1 binding on sIL-10R1\textsuperscript{T213C-Bt} surfaces, biological activity in TF-1/cIL-10R1 cell proliferation assays, and STAT3 activation in human monocyes (Fig. 2 and supplemental Table I). Twelve of the 13 cIL-10M1 mutants exhibited sIL-10R1 binding affinities similar to cIL-10M1 (Fig. 2 and supplemental Table I). In contrast, cIL-10M1\textsuperscript{R24A} displayed a 10-fold lower affinity for sIL-10R1\textsuperscript{T213C-Bt} (\(K_d = 4.7\) nM). Thus, with the exception of cIL-10M1\textsuperscript{R24A}, mutants that exhibit reduced biological activity in the proliferation and STAT3 assays are likely involved in IL-10R2 binding.

To quantify the potency of each mutant in the proliferation assay, dose-response curves (Fig. 1D) were generated for each cIL-10M1 mutant, and the concentration required to induce 50% maximal proliferation (EC\(_{50}\)) was plotted in Fig. 2A along with the IL-10R1 affinity of each mutant (supplemental Table I). These experiments identified two cIL-10M1 mutants (M22A and R24A) that displayed substantially higher EC\(_{50}\) values than cIL-10M1. cIL-10M1\textsuperscript{M22A} exhibited the largest increase in EC\(_{50}\) (EC\(_{50}\) = 23 ng/ml) and could only reach 80% of the maximal cIL-10M1 activity at concentrations as high as 100 \(\mu\)g/ml (Fig. 1D). This was the only mutant that could not achieve maximal proliferation by increasing its concentration in the assay. cIL-10M1\textsuperscript{R24A} exhibited a similar increase in EC\(_{50}\) value as cIL-10M1\textsuperscript{M22A} (EC\(_{50}\) = 14 ng/ml). After cIL-10M1\textsuperscript{M22A} and cIL-10M1\textsuperscript{R24A}, cIL-10M1\textsuperscript{N21A} and cIL-10M1\textsuperscript{R32A} exhibited the next highest EC\(_{50}\) values (EC\(_{50}\) \~ 1 ng/ml), although they were not statistically different from cIL-10M1 (\(p < 0.05\)).

Because the TF-1/cIL-10R1 cell line used in the proliferation assay is an artificial cell line transfected with the IL-10R1 chain (15), we also evaluated the ability of each cIL-10M1 mutant to activate STAT3 in primary human monocytes, which express endogenous IL-10 receptor levels (Fig. 2B). Consistent with the results obtained using the TF-1/cIL-10R1 proliferation assay, cIL-10M1\textsuperscript{M22A} and cIL-10M1\textsuperscript{R24A} were completely inactive in the STAT3 activation assay. In addition, cIL-10M1\textsuperscript{N21A} and cIL-10M1\textsuperscript{R32A} also exhibited essentially no detectable STAT3 phosphorylation activity on monocytes. Furthermore, the S31A and S93A mutants showed reductions in STAT3 activation. In summary, STAT3 activation studies using human monocytes indicated cIL-10M1 residues Asn-21, Met-22, and Arg-32 are most critical for the cIL-10M1-IL-10R2 interaction, and Ser-31 and Ser-93 may play a minor role in IL-10R2 engagement. The Arg-24 mutant is clearly important for biological activity, but its role in IL-10R2 binding, rather than IL-10R1 binding, remains to be determined.

Characterization of cIL-10 Dimer Mutants—Because the initial alanine scan was performed with monomeric cIL-10 rather than dimeric cIL-10, putative IL-10R2 binding residues (Asn-21, Met-22, Arg-24, and Arg-32) as well as His-90 and Ser-93 were mutated in the cIL-10 dimer and characterized for IL-10R1 and IL-10R2 binding and the ability to activate STAT3 in primary human monocyes. To characterize the cIL-10-sIL-10R1 interactions, cIL-10 and cIL-10 mutants were amine-coupled to CM-5 chips, and multiple concentrations of sIL-10R1 were injected over the cIL-10 surfaces (Fig. 3A). The \(K_f\) values obtained between amine-coupled cIL-10 or cIL-10 mutants and sIL-10R1 were slightly higher than when specific coupling of sIL-10R1\textsuperscript{T213C-Bt} to the chip surface was used (supplemental Table I). Nonetheless, the results of the cIL-10-sIL-10R1 binding studies were consistent with the cIL-10M1-sIL-10R1\textsuperscript{T213C-Bt} SPR analysis. Specifically, cIL-10M1\textsuperscript{R24A} exhibited the greatest decrease in sIL-10R1 affinity
The IL-10R2 Binding Site on IL-10

C. weak affinity of sIL-10R2 for cIL-10 alone (10R1 binary complexes was evaluated because of the extremely
kinetic SPR experiment used to obtain the values is shown to the
interactions are plotted as
black bars
JOURNAL OF BIOLOGICAL CHEMISTRY
35092

2-fold changes in affinity for sIL-10R1.

Because of the large quantity of protein required to characterize
signal transduction machinery, suggesting a possible strategy for
the design of cIL-10 antagonists.

Structure of the IL-10R2 Binding Site—Residues corresponding
to the IL-10R2 binding site were mapped onto the cIL-10sIL-10R1 binary complex (Fig. 4). Consistent with IL-10R2
low affinity for cIL-10, the IL-10R2 binding surface is composed

(5.4-fold), whereas the other cIL-10 mutants exhibited less than
2-fold changes in affinity for sIL-10R1.

IL-10R2 Binding Assay—cIL-10 residues implicated in
IL-10R2 binding were further evaluated in a direct IL-10R2
binding assay (Fig. 3B). For this assay, cIL-10 mutants (N21A,
M22A, R24A, R32A, and H90A) were amine-coupled at high
surface densities (~1000 RU) onto CM-5 sensor chips. The
ability of sIL-10R2 to bind amine-coupled cIL-10 mutant-sIL-10R1 binary complexes was evaluated because of the extremely
weak affinity of sIL-10R2 for cIL-10 alone (Kd ~2 mM (10)).
Because of the large quantity of protein required to characterize IL-10R2 binding, only selected mutants were evaluated at a single
sIL-10R2 concentration of 700 µM. In this way, a relative
sIL-10R2 binding strength could be assigned for each cIL-10
mutant. Consistent with its low biological activity, the cIL-10M22A mutant exhibited the poorest binding to IL-10R2 (35%
A residues 18–22, which is accomplished almost exclusively by rotating the ϕ/ψ torsion angles of Leu-23. This rotation straightens and lengthens helix A and results in main chain movements of up to 5.5 Å. The conformational change in helix A is accompanied by a significant reorganization and ordering of cIL-10 residues 12–18, which connects helix A via the disulfide bond between Cys-12 and Cys-108 to helix D. The term “ordering” is used to describe the observation that residues 12–17 are not observed in the final electron density maps of cIL-10 (2H24) but are visible in the cIL-10sIL-10R1 complex (1Y6K). Comparison of ebvIL-10 and the ebvIL-10sIL-10R1 structures (1VLK versus 1Y6M) reveals ebvIL-10 undergoes essentially the same rotation of helix A despite having only one residue compared with four in cIL-10, linking helix A to helix D.

Structural analysis sheds considerable light on the critical nature of Met-22 for efficient IL-10R2 binding. In unbound cIL-10, the side chain of Met-22 is located near the position of Leu-19 in the cIL-10sIL-10R1 complex (Fig. 4). Upon IL-10R1 binding, the Met-22 side chain moves ~6 Å to pack against Leu-101 and Arg-104. Mutation of Met-22 to alanine is expected to significantly disrupt this packing interaction, which would subsequently alter the conformation of the entire N-terminal region. Thus, the role of Met-22 in the IL-10R2 interaction is likely 2-fold. First, it plays an important role in organizing/rigidifying the N-terminal region of cIL-10 around site 2b and possibly additional residues. Second, it can directly participate in interactions with the IL-10R2 chain.

Comparison of the IL-10R2 Binding Epitopes—A structural role for Met-22 is strengthened by comparing the putative IL-10R2 binding site of cmvIL-10 with cIL-10 (Fig. 5A). Met-22 in cmvIL-10 is replaced by an arginine in cmvIL-10 (Arg-22\textsuperscript{cmv}). As described for Met-22 in cIL-10, Arg-22\textsuperscript{cmv} participates in numerous interactions between helices A and D. In particular, the guanido group of Arg-22\textsuperscript{cmv} forms four specific hydrogen bond interactions with cmvIL-10 residues Asp-18\textsuperscript{cmv}, Ser-100\textsuperscript{cmv}, and Asp-104\textsuperscript{cmv} (Fig. 5A). In addition to their structural roles, the aliphatic segments of Met-22 and Arg-22\textsuperscript{cmv} can both participate directly in van der Waals/hydrophobic interactions with IL-10R2.

Arg-24 and Arg-32 also exhibited significant reductions in IL-10R2 binding levels when mutated to alanine. Interestingly, the Arg-24 to alanine mutant was the only molecule tested that exhibited reduced affinity for IL-10R1. The 10-fold increase in sIL-10R1 off-rate observed for the cIL-10Arg-24\textsuperscript{A} mutant can be rationalized by the loss of the interaction between the Ne atom...
The IL-10R2 Binding Site on IL-10

FIGURE 5. Comparison of sIL-10R2 binding sites. A, helices A and D from cIL-10 (magenta) and cmvIL-10 (blue) are shown. cIL-10 side-chain residues around the IL-10R2 binding site are gold. Structurally similar side-chain residues for cmvIL-10 are white. B, helices A and D from cIL-10 (magenta) and IL-22 (green) are shown. cIL-10 side-chain residues around the IL-10R2 binding site are gold as described for A. Side chains that contribute to the IL-22-IL-10R2 binding interface are shown in cyan. The labels of the most energetically important residues are circled.

FIGURE 6. Sequence alignment and conformational changes required for complex assembly. Structure-based sequence alignment of cellular IL-10 (cIL-10) residues 12–48 to Epstein-Barr virus (ebvIL-10) and cytomegalovirus (cmvIL-10) IL-10s and to IL-22 (IL22). Arrows denote key structural features (Cys-12 and the Leu-Leu-Leu repeat) of IL-10 that attach this flexible peptide segment to the helix bundle. Black boxes above the sequence correspond to α-helix A in unbound cIL-10 (2H24) and cIL-10 bound to sIL-10R1 (1Y6K). Residues that undergo conformational changes (at least 1Å Cα difference) between the unbound and IL-10R1 bound structures are shown by dashed lines. The location of IL-10R1 site 1a and 1b and IL-10R2 site 2a and 2b are shown. Residues important in IL-10R1 binding are boxed. Residues important for IL-10R2 binding are colored according to Fig. 4.

of Arg-24 and the carbonyl oxygen of IL-10R1 residue Arg-191 (Fig. 4). In addition to IL-10R1 binding, SPR studies and bioassays showed Arg-24 is important for sIL-10R2 binding and biological activity. Thus, Arg-24 appears to be the only residue in the cIL-10 ternary complex that is involved in interactions with both IL-10R1 and IL-10R2. As observed for Met-22, IL-10R1 binding changes the side-chain conformation of Arg-24. These changes presumably influence both IL-10R1 and IL-10R2 binding through the hydrogen bond with the LS receptor loop.

In contrast to Met-22 and Arg-24, Arg-32 is located at the other end of helix A in site 2a. As shown by the structural superpositions in Fig. 5, the side-chain position of cIL-10 Arg-32 is conserved in the IL-10R2 binding epitopes of cmvIL-10 and IL-22, which is consistent with its critical importance in cIL-10-IL-10R2 binding (Fig. 5) and STAT3 activation (supplemental Fig. S1). Despite general structural correspondence, the main-chain and side-chain positions of Arg-32 differ considerably among the three cytokines. In fact, the Ca atom positions of Arg-32 from cIL-10 and Arg-55 in IL-22 are separated by one turn of helix. Thus, structure-based primary sequence alignments of IL-10s and IL-22 do not identify the IL-10R2 binding residues (Fig. 6). Thus, the location of Arg-32 is only “loosely” conserved, which may reflect the inherent properties of the low affinity binding surface. In contrast, the main-chain and side-chains positions of 8 residues in the high affinity IL-10-IL-10R1 interfaces of cIL-10, ebvIL-10, and cmvIL-10 are highly conserved (33).

Previous studies have shown IL-22 exhibits a higher affinity for IL-10R2 ($K_d = 14$ μM) than cIL-10 ($K_d = 234$ μM). For IL-22, the energetic hotspot consists of Asn-54 and Arg-55 located in site 2a. As shown in Fig. 5B, Arg-55 in IL-22 forms an extensive hydrogen bond/salt bridge network with Tyr-51 and Glu-117, which might be responsible for the IL-22 higher affinity interaction with IL-10R2. In contrast to IL-22, the three energetically important IL-10R2 binding residues in cIL-10 (Met-22, Arg-24, and Arg-32) are spread out over site 2a (Arg-32) and site 2b (Met-22 and Arg-24). Interestingly, the most important residue in the cIL-10-IL-10R2 binding surface, Met-22, is located in site 2b rather than in site 2a, as observed for IL-22. Residues important in the cmvIL-10-IL-10R2 binding interface have not yet been identified.

However, it is interesting that the N-terminal region of cmvIL-10 is very well ordered and contains two additional turns of α-helix. This suggests structural differences in site 2b may contribute to the increased IL-10R2 binding affinity observed for cmvIL-10 (11).

Mechanism of IL-10 Ternary Complex Assembly—The combination of SPR, crystallographic, and bioactivity data demonstrate the importance of cIL-10 conformational changes in promoting the assembly of the cIL-10-IL-10R1-IL-10R2 signaling complex. Previous studies have shown cIL-10-IL-10R1 affinity is regulated by the AB loop, which adopts alternate structures in the unbound and IL-10R1 bound states (11, 21). Data from the current study now suggest productive engagement of the IL-10R2 chain is dependent on IL-10R1-induced conformational changes in the N terminus of helix A. A more detailed structural com-
parison reveals 24 of 34 (71%) cIL-10 residues, between Cys-12 and Leu-46, change their positions by at least 1 Å (Ca-Ca distance) upon IL-10R1 binding. All 24 residues map to the N terminus (residues Cys-12—Met-22) and AB loop (residues Val-33—Asn-44) segments of cIL-10.

For cIL-10 to accommodate these large conformational changes it must contain structural features that stabilize the ends of the flexible regions. For cIL-10, these regions are the Cys-12—Cys-108 disulfide bond at the N terminus and the Leu-Leu-Leu motif (residues 46—48) found at the C-terminal end of the AB loop (Fig. 6). The N-terminal and AB loop regions are separated by residues 23—32, which form the structurally invariant core of helix A. Consistent with our studies, the Cys-12—Cys-108 disulfide bond has previously been shown to be critical for the stability and biological activity of cIL-10 (34). Interestingly, these structural features are conserved in all IL-10R2 binding cytokines, suggesting manipulation of the conformations of the N-terminal region may be a common mechanism to regulate receptor binding and complex assembly for other class-2 cytokines.

Structural studies on glycosylated IL-22, which contains 6 molecules in the asymmetric unit, have identified considerable conformational variability in the IL-10R2 binding site on IL-22 supporting this hypothesis (35, 36). Furthermore, an anti-IL-22 antibody has been shown to increase the high affinity IL-22:IL-22R1 interaction, apparently by inducing conformational changes in the IL-10R2 binding site that are subsequently transmitted to the high affinity IL-22R1 binding site (37). These studies together with our current results emphasize the importance of conformational changes in promoting receptor assembly and biological activity of cIL-10, IL-22, and most likely, other members of the class-2 cytokine family. Further mechanistic studies on these molecules should aid in the design of novel methods to antagonize or enhance their broad biological activities.

The requirement of an IL-10R1-mediated conformational change in cIL-10 for productive IL-10R2 binding provides a mechanistic explanation for why cIL-10 ternary complex formation is sequential. For example, complex assembly depends on an initial interaction between cIL-10 and IL-10R1 followed by engagement of the IL-10R2 chain. The IL-10R1-induced conformational change in cIL-10 is thought to be at least partially responsible for the ~10-fold increase in IL-10R2 affinity in the presence of IL-10R1 (10). However, even the cIL-10 M22A mutant retains some IL-10R2 binding activity (Fig. 5), suggesting additional interactions between IL-10R1 and IL-10R2 chains may occur and contribute to the overall stability of the cIL-10:IL-10R1:IL-10R2 ternary complex. Such receptor-receptor interactions have been observed by crystallography in the growth hormone, IL-6, and IL-2 receptor systems and are predicted for cIL-10 (30, 31, 38—40).

Recently, the IL-10R1 and IL-10R2 chains were shown to associate with one another on the cell surface in the absence of cIL-10 (28). Our data suggests one function of the IL-10R2 chain in the preassembled IL-10R1:IL-10R2 complex is to monitor IL-10R1-induced conformational changes in cIL-10, which promotes assembly of the “active” ternary complex. The low affinity and fast binding kinetics of the IL-10R2 chain are predicted to make it exquisitely sensitive to the formation or dissociation of the cIL-10:IL-10R1 binary complex, which would facilitate the rapid activation or inactivation of cIL-10 transmembrane signaling.

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The IL-10R2 Binding Site on IL-10

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