Interleukin-3 Binding to the Murine $\beta_{IL-3}$ and Human $\beta_c$ Receptors Involves Functional Epitopes Formed by Domains 1 and 4 of Different Protein Chains

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Interleukin-3 (IL-3) is a cytokine produced by activated T-cells and mast cells that is active on a broad range of hematopoietic cells and in the nervous system and appears to be important in several chronic inflammatory diseases. In this study, alanine substitutions were used to investigate the role of residues of the human $\beta$-common ($h\beta c$) receptor and the murine IL-3-specific ($h\beta_{IL-3}$) receptor in IL-3 binding. We show that the domain 1 residues, Tyr$^{15}$ and Phe$^{59}$, of the $h\beta c$ receptor are important for high affinity IL-3 binding and receptor activation as shown previously for the related cytokines, interleukin-5 and granulocyte-macrophage colony-stimulating factor, which also signal through this receptor subunit. From the x-ray structure of $h\beta c$, it is clear that the domain 1 residues cooperate with domain 4 residues to form a novel ligand-binding interface involving the two protein chains of the intertwined homodimer receptor. We demonstrate by ultracentrifugation that the $h\beta_{IL-3}$ receptor is also a homodimer. Its high sequence homology with $h\beta c$ suggests that their structures are homologous, and we identified an analogous binding interface in $h\beta_{IL-3}$ for direct IL-3 binding to the high affinity binding site in $h\beta c$. Tyr$^{151}$ (A-B loop), Phe$^{159}$, and Asn$^{167}$ (E-F loop) of domain 1; Ile$^{326}$ of the interdomain loop; and Tyr$^{448}$ (B-C' loop) and Tyr$^{401}$ (F-G' loop) of domain 4 were shown to have critical individual roles and Arg$^{156}$ and Tyr$^{317}$ major secondary roles in direct murine IL-3 binding to the $h\beta_{IL-3}$ receptor. Most surprisingly, none of the key residues for direct IL-3 binding were critical for high affinity binding in the presence of the murine IL-3 $\alpha$ receptor, indicating a fundamentally different mechanism of high affinity binding to that used by $h\beta c$.

Interleukin-3 (IL-3)$^1$ is a cytokine produced by activated T-cells and mast cells that has been shown to stimulate regrowth of pluripotent hematopoietic stem cells and to be a potent regulator of many hematopoietic cell lineages (1–3). Its role appears to be in stimulating inducible hematopoiesis in response to parasite infections (4), and it has also been implicated in the pathogenesis of several chronic inflammatory diseases, including asthma (1), and neurodegenerative disorders, such as multiple sclerosis (5). The effect of IL-3 on human cells is mediated by a receptor system composed of a ligand-specific $\alpha$ subunit and a $\beta$ subunit (denoted $\beta c$) that is also part of the receptor systems for the related cytokines, interleukin-5 (IL-5) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (6–9). Signaling through the $\beta c$ receptor requires the formation of a high affinity complex involving each cytokine and its respective $\alpha$ subunit (7–10). Whereas the $\alpha$ subunits bind their ligands with low affinity, $\beta c$ does not measurably bind any of the ligands alone. Upon receptor activation, the cytoplasmic portion of the $\beta c$ subunit, which lacks any intrinsic kinase activity (11), initiates a number of signaling pathways including the Janus kinase 2/signal transducers and activators of transcription, phosphatididylinositol 3-kinase, and Ras/mitogen-activated protein kinase pathways (reviewed in Ref. 12).

Mice also possess a $\beta$ subunit (m$\beta c$) but have an additional IL-3-specific $\beta$ receptor ($h\beta_{IL-3}$). $h\beta_{IL-3}$ differs from the m$\beta c$ subunit in its ability to bind murine IL-3 (mIL-3) directly (13), although the presence of the mIL-3 $\alpha$ subunit is absolutely required for signaling (14). The properties of mIL-3-responsive precursor cells from gene knock-out mice lacking expression of the $h\beta_{IL-3}$ subunit indicate that this subunit plays an important role in the response to mIL-3 stimulation (15).

The human $\beta c$ ($h\beta c$), m$\beta c$, and $h\beta_{IL-3}$ receptors are closely homologous in sequence and belong to the hematopoietin (or class I cytokine) receptor superfamily. Members of this family have extracellular domains containing the cytokine-receptor homology module composed of two fibronectin III domains that have a number of conserved sequence elements (16, 17). X-ray structures of ligand-receptor complexes of simpler members of the family, including growth hormone (18), erythropoietin (19) and interleukin-4 (20), have demonstrated that in each case the structural epitopes for ligand binding involve loops at approximately the orthogonal interfaces formed between the two fibronectin III domains of these receptors. In addition, mutagenesis studies of these receptors have shown that within the structural epitopes only a relatively small number of residues, which compose the functional epitope, are critical determinants of ligand binding (21–25). These residues are contributed by a combination of the A–B and E–F loops of the membrane-distal fibronectin III domain and the B’–C’ and F’–G’ loops of the membrane-proximal domain.

To what extent the principles of ligand binding established...
for the two domain receptors apply to the hβc receptor and its close relatives has been unclear because the hβc receptor does not engage ligand in the absence of the α subunits and possesses an extracellular domain with two cytokine-receptor homology modules. Residues in the domain 4 B′–C and F′–G loops have been identified previously (28–30) by mutagenesis studies modeled on the growth hormone receptor as critical for formation of a high affinity ligand binding complex. Prior to the structure of the hβc receptor being elucidated, it was expected that domain 3 would partner with domain 4 in forming the ligand-binding interface, but no binding residues in domain 3 of the hβc receptor were identified. The hβc receptor structure gave new insight into the ligand-binding interface thus predicting the involvement of domain 1. The crystal structure of the complete extracellular domain of the hβc subunit showed that hβc exists as a preformed homodimer (Fig. 1), in which the G-strands of domains 1 and 3 are swapped between the two monomers (26). As a result of the intertwining of two monomers, domains 1 and 4 of symmetry-related chains form an approximately orthogonal interface that resembles the ligand-binding interfaces of two-domain hematopoietin receptors. The participation of domain 1 in ligand binding has been supported by recent mutagenesis studies that have shown that residues in the A–B and E–F loops of domain 1 of hβc are required for the formation of the high affinity human GM-CSF (hGM-CSF) complex and for receptor activation by hGM-CSF and human IL-5 (hIL-5) (31). The involvement of domain 1 also provides further evidence for the biological relevance of the hβc receptor structure.

The murine βIL-3 subunit has the interesting feature of binding m IL-3 directly with low affinity as well as forming a high affinity complex in the presence of the mIL-3α receptor (14). It has been shown that the carboxyl-terminal cytokine-receptor homology module of βIL-3 makes a unique contribution to the direct binding of mIL-3 and that the domain 4 B′–C loop has a critical role (32). The amino-terminal cytokine-receptor homology module is also essential, but the corresponding portion of mβc can effectively substitute for that of βIL-3 without compromising direct mIL-3 binding (32). However, a detailed analysis of the residues involved in direct and high affinity IL-3 binding has not been carried out previously nor has the structure of the βIL-3 subunit extracellular domain been determined.

In the present work, we show by using analytical ultracentrifugation and cross-linking that the expressed extracellular domains of the mβc and βIL-3 receptors, like hβc, are homodimers. This, together with the high sequence homology between the murine receptors and hβc, suggests that both mβc and βIL-3 are likely to have intertwined homodimer structures analogous to that determined for hβc (26). Consistent with this prediction, we demonstrate here that domains 1 and 4 cooperate to form the functional epitope for direct IL-3 binding to the murine βIL-3 receptor and similarly form the high affinity IL-3-binding site of the hβc receptor. Most surprising, however, we have shown that the residues in the “elbow” region of the murine βIL-3 receptor have little effect on the high affinity binding of m IL-3, indicating that this receptor forms a high affinity complex in a different way to the hβc receptor.

**IL-3-binding Epitopes of mβIL-3 and hβc**

**Expression Constructs**—For expression in COS-7 cells, the cDNAs encoding mIL-3 α and wild-type or mutant βIL-3 subunits were subcloned into the vector, pCEX-V-X3-Vhaa (31). Wild-type, Y15A, and F79A hβc cDNAs were prepared and subcloned into pCEx-V-X3-Vbaa as described previously (31). The cDNA encoding hIL-3α, in the expression vector PM1ESS, was kindly provided by A. Miyajima (Tokyo, Japan). For expression in CTLL-2 cells, cDNAs encoding hIL-3α or mIL-3α were subcloned into pEF-IRE-S-N, and the hβc or βIL-3 subunits were subcloned into pEF-IRE-P (33) (from S. Hobbs, Institute of Cancer Research, London, UK).

**Expression and Purification of hβc, mβc, and βIL-3 Extracellular Domains**—cDNAs encoding the extracellular domain of the hβc, mβc, and βIL-3 subunits were subcloned into the EcoRI site of pBacPK8 for baculovirus expression. Recombinant baculovirus was produced, and protein was expressed and purified essentially as described previously (34). Briefly, concentrated baculovirus supernatant, containing expressed protein, was applied to a Sephacryl S-200 (Amersham Biosciences) gel filtration column, and fractions containing protein were concentrated before being chromatographed on a MonoQ HR 5/5 column (Amersham Biosciences) at pH 6.5 using a fast protein liquid chromatography apparatus and eluted using a 0–350 mM NaCl gradient. Protein concentrations were determined using a Bradford assay (Bio-Rad), with bovine γ-globulin as the protein standard. The identities of these proteins were confirmed by amino-terminal sequencing, which was performed as described previously (34).

**Chemical Cross-linking of β Subunit Extracellular Domains**—Purified hβc, mβc, and βIL-3 extracellular domain proteins (1 μg), in 0.1 M sodium phosphate buffer (pH 7.5), were reacted with 2 mM BS3 (Pierce) at 25 °C for 1 h, before the reaction was quenched with glycine (50 mM). Nonreducing SDS-PAGE was carried out by using 8% Tris-glycine gels with silver staining, as described previously (34).

**Sedimentation Equilibrium Analysis of β Subunit Extracellular Domains**—Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge at 10,000 or 20,000 rpm by using 12-mm path length cells with carbon-filled double-sector centerpieces. The solution sector contained 100 μl of protein sample at a concentration of ~1 mg/ml dissolved in 20 mM bis-Tris propane, pH 6.5, with 110 μM of the solvent sector in the same buffer as reference. All experiments were performed at 25 °C for 20 h. Scans at sedimentation equilibrium of absorbance (A) versus radial distance (r) in centimeters from the axis of rotation were collected at 280 nm. Scans at 360 nm were collected and subtracted from the equilibrium scans to correct for anomalies arising from cell windows. The resulting A versus r data was analyzed by using software supplied with the instrument. Where the data indicated that the sample was homogeneous, the molecular weight was evaluated by employing the sedimentation equilibrium shown in Equation 1.

\[
M = \frac{2RT}{d \ln A} \frac{dA}{dr}
\]

where R is the gas constant; T is the absolute temperature; \( \omega \) is the angular rotation (radius), \( \theta \) is the partial specific volume of the protein (0.725 ml/g in this case), and \( \rho \) is the density of the buffer. The molecular weights of hβc and \( \beta \) were determined by running one sample of the protein in \( H_2O \) and one in \( D_2O \) in the sample experiment and solving the resulting two simultaneous equations. The best fit molecular weight for each sample was obtained using the program XLAEQ, and the homogeneity of the molecular weight was checked by evaluating molecular weights using the program XLMMW. The percentages of dimer and monomer in the samples were calculated using the program MULTMX1B.

**Cytokines and Radiolabeling—mIL-2 and mIL-3**—mIL-2 and mIL-3 were produced by using the baculovirus expression system (34). Recombinant hIL-3 was purchased from Sigma. Purified mIL-3 or hIL-3 was radiolabeled with \( \mathrm{Na}^{125} \mathrm{I} \) using the method of Fraker and Speck (35). Briefly, mIL-3 or hIL-3 was 30 μl of 0.1 M sodium phosphate buffer, pH 7.5, and 10 μl (1 mCi) of \( \mathrm{Na}^{125} \mathrm{I} \) in NaOH solution (Amersham Biosciences) were added to a microcentrifuge tube plated with 1 μl of IODO-GEN reagent (Pierce) and reacted on ice for 10 min. Radiolabeled cytokine was purified on a Sephadex G-25 column (Amersham Biosciences) and stored at 4 °C for use in cell experiments.

**Cells and DNA Transfections—COS7**—COS7 cells and CTLL-2 cells were maintained as described previously (31, 34). DNA constructs were introduced into cells by electroporation using a GenePulsor Xcell apparatus (Bio-Rad) (31, 34).
Equilibrium Binding Analysis for hIL-3 and mIL-3—Hot saturation binding experiments were performed on COS7 cells transfected with DNA constructs encoding the relevant receptors, as described previously (31). In order to assess the capacity of βIL-3 subunits to bind mIL-3 directly, cold competition assays were performed on COS7 cells transfected with cDNAs encoding wild-type or mutant βIL-3 subunits in the absence of the mIL-3α cDNA. 1 nM [3H]mIL-3 was added to 10⁶ cells in 200 μl of binding medium (RPMI 1640 supplemented with 0.5% v/v bovine serum albumin and 10 mM HEPES) with a serial dilution from a 200-fold excess of unlabeled mIL-3. Nonspecific binding was determined by performing the assay in the presence of 1 μM unlabeled mIL-3. After incubation for 2.5–3 h at 4 °C with intermittent agitation, the assay was terminated by centrifugation through 2:1 v/v dibutyl phthalate/dinonyl phthalate at 12,000 × g for 4 min. The tips of tubes and visible cell pellets were counted using a Packard 5750 Auto-gamma counter. The dissociation constants (Kd) were determined from specific binding data by using the programs EBDA (36) and LIGAND (37) as described (31).

Flow Cytometry—COS7 cells transfected with cDNAs encoding wild-type, K347A, I349A, Y21A/Y401A, or F85A/Y401A βIL-3 subunits were incubated with 10 μg/ml rat anti-Aic2 monoclonal antibody (MBL, Nagoya, Japan) for 30 min at 4 °C. Cells were washed and incubated with a phycoerythrin-conjugated goat anti-rat (μ) secondary antibody (BD Pharmingen) for 25 min at 4 °C and then washed before analysis of cell-surface expression was performed on a FACScan flow cytometer (BD Biosciences).

Proliferation Assays—[3H]Thymidine incorporation into the DNA of CTLL-2 cells expressing wild-type or mutant hIL-3 or mIL-3 receptors was used to measure the capacity of these receptors to deliver a proliferative signal in response to hIL-3 or mIL-3, respectively, as described (34).

Results

The βIL-3 and μβc Receptor Extracellular Domains Are Homodimers—Our studies of the hβc receptor extracellular domain expressed in the baculovirus system have demonstrated that it exists as a preformed homodimer both in solution and when crystallized (26). The full-length receptor has also been verified to exist as a dimer in the cell membrane (26). The novel intertwined dimer structure of the hβc receptor and the important ligand-binding loops of the elbow region formed by domains 1 and 4 of the two different protein chains are shown in Fig. 1. An alignment between the βIL-3 and μβc receptors and the hβc receptor is shown in Fig. 2. The figure demonstrates the high degree of sequence homology between the extracellular domains of the murine and human β receptors and the conservation of the cysteine residues known to form intramolecular disulfide bonds in hβc. It is therefore likely that they have homologous structures.

To investigate this further, we carried out cross-linking and analytical ultracentrifugation studies of the purified extracellular domains of the βIL-3 and hβc subunits expressed in the baculovirus system. The μβc subunit was also included for comparison. The amino-terminal sequences of the mature forms of βIL-3 and hβc had not been determined previously and were shown to be HEVETEETV and HGVTEAETV, respectively (as shown in Fig. 2). The amino-terminal sequence of hβc has been determined previously (34). Chemical cross-linking of all three β subunit extracellular domains using the water-soluble reagent, BS3, gave analogous cross-linked products (Fig. 3) supporting homologous structures.

Analytical ultracentrifugation studies were also carried out (Table I). Sedimentation equilibrium experiments were performed at pH 7.4 on the purified extracellular domains of βIL-3, μβc, and hβc. The average molecular weights determined for both μβc and βIL-3 were 83,000, compared with the average molecular weight of 91,300 obtained for hβc from HL60 eosinophils and 87,500 for hβc from TF1 cells. Because the predicted molecular weights (excluding glycosylation) of the βIL-3, μβc, and hβc (HL60) extracellular domains are 48,181, 48,331, and 47,739, respectively, the ultracentrifugation data indicate that the proteins exist as dimers at pH 7.4. There was some evidence of lower molecular weight protein in the samples. Although there was no evidence that this material was the monomer form of the subunits, fitting the data by using the program MULTIMX1B showed that the monomer, if present, would constitute less than 10% of the concentration of the dimer.

Ultracentrifugation experiments were also performed on the hβc extracellular domain at pH 3 (Table I). The average molecular weight obtained was 42,400 with some evidence of higher molecular weight material. These data indicate that the hβc extracellular domain dissociates to the monomer at pH 3. The calculated fractional ratio for the monomer was 3.9, suggesting that denaturation had occurred at this pH in addition to dissociation of the dimer. The corresponding value for the dimer was 1.32.

Thus, the cross-linking and analytical ultracentrifugation studies indicate that the βIL-3 and μβc receptors are homodimers like hβc. Based on the structure determined for hβc, together with the alignment in Fig. 2, we were able to make detailed predictions of the likely secondary structure of βIL-3 and the residues in potential ligand-binding loops.

Residues of the βIL-3 Receptor Required for Direct IL-3 Binding—The functional epitope for ligand binding and activation of the hβc receptor by hGM-CSF and hIL-5 includes Tyr89 and Phe79 of the A–B and E–F loops of domain 1, respectively (31),...
and the domain 4 residues Tyr347, His349, Ile350 (B/H11032-C/H11032 loop) (28, 29), and Tyr403 (F/H9252-G/H9252 loop) (30). By analogy with human c, we prepared alanine substitution mutants of residues in the relevant loops of the IL-3 subunit, and we examined their capacity to bind mIL-3 directly. Alanine substitution mutagenesis has been used extensively in the determination of ligand-binding sites in hematopoietin receptors, because alanine is found commonly in solvent-exposed and buried positions and in all types of secondary structure, and the methyl side chain does not cause steric or electronic interference to the protein chain. COS7 cells were transfected with expression vectors encoding wild-type or mutant IL-3 subunits and the resulting transiently transfected cells used in cold competition binding assays as described under “Materials and Methods.” Wild-type IL-3 was found to bind mIL-3 with a dissociation constant of 18.4 nM (Table II and Fig. 4). This is consistent with the $K_d$ values determined previously (13) by using a similar cold competition binding assay.

Involvement of Loops in Domain 1 of the IL-3 Subunit in Low Affinity mIL-3 Binding—The predicted A–B loop consists of residues Tyr21, Thr22, Asn23, and Arg24. Each of these residues was individually mutated to alanine, and the ability of these mutants to bind directly mIL-3 was assessed. The Y21A mutant IL-3 receptor exhibited no detectable binding, whereas the other three mutants were capable of mIL-3 binding with $K_d$ values within 2-fold of the $K_d$ for wild-type IL-3 (Table II). These results indicate that Tyr21 is a critical binding determinant and the only significant residue in the A–B loop involved in mIL-3 low affinity binding.

The predicted E–F loop consists of the residues Tyr82, Thr83, Arg84, Phe85, Ser86, and Asn87. Mutant IL-3 subunits were prepared containing individual alanine substitutions of these residues, and their ability to bind mIL-3 with low affinity was...
IL-3-binding Epitopes of mβIL-3 and hβc

| βIL-3 | Location of mutation | Kd ± S.E. | No. experiments |
|-------|----------------------|-----------|----------------|
| Wild type |                            | 18.4 ± 1.0 | 4              |
| Tyr21 → Ala | A-B loop | 20.6 ± 1.8 | 2              |
| Thr348 → Ala | A-B loop | 33.4 ± 1.1 | 2              |
| Tyr348 → Ala | F-G loop | 71.9 ± 6.5 | 2              |
| Thr348 → Ala | E-F loop | 20.2 ± 1.0 | 2              |
| Arg401 → Ala | E-F loop | 245.7 ± 73.1 | 2 |
| Phe386 → Ala | E-F loop | 74.2 ± 4.0 | 2              |
| Arg401 → Ala | E-F loop | 4.2 ± 0.1  | 2              |

To determine whether the residues critical for direct mIL-3 binding were in the same domain as the critical Tyr residues for low affinity mIL-3 binding, alanine substitution mutants of residues of the F′–G′ loop in domain 4 of βIL-3 were prepared and examined for mIL-3 binding. Both the I398A and S399A mutant βIL-3 subunits showed an 8-fold reduction in mIL-3 binding (32). The Y401A mutant βIL-3 subunit did not detectably bind mIL-3, implicating Tyr401 as a key residue for low affinity mIL-3 binding.

In contrast, mIL-3 binding by the Y348F and Y401F mutant βIL-3 subunits showed a reduction in affinity for mIL-3 binding of 2- and 6-fold, respectively (Table II). The result with K347A suggests that the F′–C′ loop makes any loss of binding ambiguous. The K344A and I345A βIL-3 subunits bound mIL-3 with near wild-type affinity. However, the K347A and I349A βIL-3 subunits showed a reduction in affinity for mIL-3 binding of 2- and 6-fold, respectively (Table II). The result with K347A is in agreement with the properties of a similar K347S mutant βIL-3 subunit studied previously (32). Complete abolition of mIL-3 binding was found with the Y348A mutant βIL-3 subunit, indicating a major role for Tyr401 in mIL-3 binding.

In order to determine whether the phenolic hydroxyl groups of the three critical Tyr residues (Tyr21, Tyr348, and Tyr401) were involved in binding, phenylalanine mutants were examined (Table II). Two different effects were found. Y21F βIL-3 subunits showed impaired binding of mIL-3 (a 6-fold reduction), indicating that the hydroxyl group of Tyr21 plays an important role in mIL-3 binding. In contrast, mIL-3 binding by the Y348F and Y401F βIL-3 subunits was not severely affected. Y348F βIL-3 subunits exhibited only a small reduction in affinity for mIL-3 (less than 2-fold), whereas the Y401F βIL-3 subunit bound mIL-3 with near wild-type affinity.

High Affinity mIL-3 Binding Properties of Mutant βIL-3 Subunits—To determine whether the residues critical for direct mIL-3 binding were in high affinity binding, COS7 cells were transiently co-transfected with expression vectors encoding the mIL-3 α receptor and wild-type or mutant βIL-3 Subunit in Low Affinity mIL-3 Binding—Alanine-scanning mutagenesis was performed on the interdomain loop predicted to connect domains 1 and 4. This loop is made up of the residues Tyr317, Tyr318, His319, and Ile320. Mutant βIL-3 subunits were prepared in which each residue was substituted with alanine, and the effect of these mutations on mIL-3 binding was assessed (Table II). The H319A βIL-3 receptor was capable of wild-type low affinity mIL-3 binding, whereas the Y318A mutant exhibited ~3-fold reduction in mIL-3 binding. Low affinity mIL-3 binding by the Y317A βIL-3 receptor was more severely impaired (~20-fold lower affinity than wild-type βIL-3), whereas the I320A βIL-3 receptor did not detectably bind mIL-3. These results implicate Ile320 as a key mIL-3 binding determinant and suggest that Tyr317 plays an important additional role in mIL-3 binding.

The residues of the predicted domain 4 B′–C′ loop were individually mutated to alanine, and the effect of the mutations on low affinity mIL-3 binding was assessed. Although previous work has implicated this loop in binding, to date no systematic analysis of the binding determinants in this loop has been performed. The loop comprises the residues Lys344, Ile345, Pro346, Lys347, Tyr348, and Ile349. The size and amino acid sequence of this loop differ from the B′–C′ loops of the murine and human βc subunits (Fig. 2) suggesting potential involvement in low affinity mIL-3 binding. Pro346 was not mutated to alanine in this study, as this substitution is likely to result in major structural perturbation of the B′–C′ loop making any loss of binding ambiguous. The K344A and I345A βIL-3 subunits bound mIL-3 with near wild-type affinity. However, the K347A and I349A βIL-3 subunits showed a reduction in affinity for mIL-3 binding of 2- and 6-fold, respectively (Table II). The result with K347A is in agreement with the properties of a similar K347S mutant βIL-3 subunit studied previously (32). Complete abolition of mIL-3 binding was found with the Y348A mutant βIL-3 subunit, indicating a major role for Tyr401 in mIL-3 binding.

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TABLE III
Dissociation constants for mIL-3 binding to COS7 cells expressing mIL-3 α alone and with wild-type or mutant βIL-3 subunits determined using the hot saturation binding assay

| βIL-3 Location of mutation | No. sites detected | Kd | High affinity +/- S.E. | Low affinity +/- S.E. | No. experiments |
|---------------------------|-------------------|----|------------------------|------------------------|----------------|
| None                      | 1                 | 44.9 ± 18.2 | 1                      |
| Wild-type                 | 2 p < 0.001       | 208.5 ± 11.2 | 45                     | 2                      |
| Tyr21 → Ala               | A–B loop          | 298.1 ± 15.6 | 45                     | 2                      |
| Thr22 → Ala               | A–B loop          | 334.6 ± 15.6 | 45                     | 1                      |
| Asn37 → Ala               | A–B loop          | 338.4 ± 34.1 | 45                     | 1                      |
| Arg88 → Ala               | A–B loop          | 339.3 ± 20.8 | 45                     | 1                      |
| Tyr251 → Ala              | E–F loop          | 158.2 ± 14.7 | 45                     | 1                      |
| Thr252 → Ala              | E–F loop          | 308.9 ± 15.9 | 45                     | 1                      |
| Arg253 → Ala              | E–F loop          | 310.2 ± 31.0 | 45                     | 1                      |
| Phe254 → Ala              | E–F loop          | 353.0 ± 13.1 | 45                     | 3                      |
| Ser389 → Ala              | E–F loop          | 369.5 ± 29.6 | 45                     | 1                      |
| Asn23 → Ala               | E–F loop          | 322.7 ± 35.5 | 45                     | 1                      |
| Tyr+17 → Ala              | Interdomain       | 281.8 ± 33.8 | 45                     | 1                      |
| Tyr+18 → Ala              | Interdomain       | 211.1 ± 16.9 | 45                     | 1                      |
| His+27 → Ala              | Interdomain       | 264.8 ± 26.5 | 45                     | 1                      |
| Ile325 → Ala              | Interdomain       | 306.4 ± 24.2 | 45                     | 1                      |
| Lys+34 → Ala              | B–C’ loop         | 300.2 ± 27.0 | 45                     | 1                      |
| Ile345 → Ala              | B–C’ loop         | 155.3 ± 13.7 | 45                     | 1                      |
| Lys+37 → Ala              | B–C’ loop         | 491.2 ± 36.9 | 45                     | 2                      |
| Tyr+46 → Ala              | B–C’ loop         | 195.6 ± 12.6 | 45                     | 2                      |
| Ile349 → Ala              | B–C’ loop         | 517.6 ± 30.6 | 45                     | 2                      |
| Ile399 → Ala              | F–G’ loop         | 2 p = 0.008  | 45                     | 1                      |
| Ser399 → Ala              | F–G’ loop         | 259.5 ± 22.4 | 45                     | 1                      |
| Asp300 → Ala              | F–G’ loop         | 374.5 ± 23.1 | 45                     | 2                      |
| Tyr301 → Ala              | F–G’ loop         | 285.6 ± 12.9 | 45                     | 2                      |
| Asp302 → Ala              | F–G’ loop         | 331.6 ± 29.2 | 45                     | 1                      |
| Tyr+3 → Ala/Tyr+61 → Ala  | A–B loop/F–G’ loop| 354.2 ± 28.4 | 45                     | 2                      |
| Phe+65 → Ala/Tyr+61 → Ala | E–F loop/F–G’ loop| 576.6 ± 20.7 | 45                     | 2                      |

* All βIL-3 mutants exhibited high affinity binding. When a large number of high affinity binding sites were present, data were not recorded for all βIL-3 mutants. Likewise, studies of the human IL-4 α receptor have demonstrated that mIL-3 binding to COS7 cells, and their ability to bind mIL-3 with high affinity was assessed (Table III). Both of these double mutants were still capable of high affinity mIL-3 binding. The Y21A/F85A and Y401A mutations exhibited high affinity binding. When a large number of high affinity binding sites were present, data were not recorded for all βIL-3 mutants. Likewise, studies of the human IL-4 α receptor have demonstrated that mIL-3 binding to COS7 cells, and their ability to bind mIL-3 with high affinity binding.
**IL-3-binding Epitopes of mβIL-3 and hβc**

**Fig. 5. Flow cytometry of COS7 cell transfected with empty vector (A) or wild-type (B), K347A (C), I349A (D), F85A/Y401A (E), or Y21A/Y401A (F) βIL-3 cDNAs.** Cells stained by the secondary antibody alone were used as a control (broken line).

The finding that all of the βIL-3 alanine substitution mutants examined in the present work were able to bind mIL-3 with high affinity was surprising, as alanine substitutions of the analogous residues in the hβc receptor abolished high affinity ligand binding. In order to further probe this finding, we examined the ability of the wild-type Y21A mutant βIL-3 subunit to be activated and to deliver a downstream signal in response to mIL-3 by using cell proliferation as an absolute measure. This mutant βIL-3 was chosen for this study because of its obligatory involvement in direct mIL-3 binding and non-involvement in high affinity mIL-3 binding. Wild-type or mutant mIL-3 receptors were stably transfected into the mIL-2-dependent lymphoid cell line, CTLL-2, to examine its capacity to grow in response to mIL-3 in two steps. Initially a cDNA encoding the mIL-3 α subunit was stably transfected into CTLL-2 cells via the pEF-IRE-S-N vector, which encodes G418 antibiotic resistance. The wild-type or Y21A mIL-3α subunits were subsequently transfected into G418-resistant cells by using the vector, pEF-IRE-S-P, which encodes puromycin resistance. Cells resistant to these antibiotics, which had been maintained in mIL-2, were then used in proliferation assays to determine their responsiveness to mIL-3 (Fig. 6A). As evident from the curve in Fig. 6A, the mIL-3-stimulated growth responses of the CTLL-2 cell lines co-expressing mIL-3 α and wild-type βIL-3 or Y21A βIL-3 are comparable, whereas the parent cell line, CTLL-2 mIL-3α, did not detectably respond to mIL-3. This verifies that the high affinity binding observed for Y21A βIL-3 results in normal receptor activation.

**Residues of hβc Forming the Functional Epitope for High Affinity hIL-3 Binding**—Previous studies (31) in this laboratory demonstrated that alanine mutations of the domain 1 residues, Tyr15 and Phe79, of the hβc receptor abolished hGM-CSF binding and severely impaired receptor activation by hGM-CSF and hIL-5. In view of the surprising results with high affinity binding of the βIL-3 receptor, the role of these residues in high affinity hIL-3 binding and receptor activation by hIL-3 was examined.

COS7 cells were co-transfected with expression vectors encoding wild-type, Y15A, or F79A hβc and wild-type hIL-3 α subunits. Saturation binding assays were performed on these transiently transfected cells by using increasing amounts of [125I]-radiolabeled hIL-3, and the dissociation constants were determined (Table IV). COS7 cells expressing the wild-type hβc and hIL-3α receptors exhibited two binding sites. In agreement with earlier studies, it was found that accurate estimation of the low affinity site is not possible because of its very low Kd values (100 nM) resulting in a high associated error (28). Accordingly, the low affinity site Kd was fixed at 100 nM during analysis in LIGAND in order to obtain a more accurate estimate of the high affinity site (Kd, 107 pM). The dissociation constants we obtained are consistent with those reported in previous studies (28, 30) on the hIL-3 receptor. The hβc subunit used in these studies was derived from HL-60 eosinophils and contained a six-amino acid insertion in the C–D loop of domain 3 (34). We have shown that this does not affect wild-type high affinity binding of GM-CSF (31), and here we demonstrated that there was no effect on normal hIL-3 high affinity binding.

Alanine substitution of Tyr15 (A–B loop) and Phe79 (E–F loop) in domain 1 of hβc abolished high affinity binding (Table IV), implicating both of these residues in the formation of the high affinity hIL-3 complex. Because the formation of the high affinity hIL-3 complex is believed to be necessary for receptor activation and downstream signaling, we reconstituted the wild-type and mutant hIL-3 receptors in the mIL-2-dependent lymphoid cell line, CTLL-2, to correlate the loss of high affinity binding with an unequivocal signaling response, proliferation. Initially, CTLL-2 cells were transfected with a cDNA encoding the hIL-3 α subunit, and subsequently the wild-type or mutant βIL-3 subunit was introduced via vectors encoding antibiotic resistance (as described for the mIL-3 receptors above). Cells resistant to these antibiotics, which had been maintained in mIL-2, were then used in proliferation assays to determine their responsiveness to hIL-3 (Fig. 6B).

The amount of hIL-3 resulting in half-maximal stimulation of the cell line co-expressing wild-type hβc and hIL-3 α was set at 1 unit, and the relative amounts of hIL-3 required for 50% stimulation of cell lines expressing mutant receptors was estimated (Table IV and Fig. 6B). Y15A and F79A mutant hβc receptors were at least 64- and 5-fold, respectively, less responsive to hIL-3 confirming that the abolition of high affinity binding was reflected in reduced efficiency of signaling. As is evident in Fig. 6B, the proliferation curves of CTLL-2 cells containing the hIL-3 α and Y15A and F79A hβc subunits did not reach a plateau in this hIL-3 titration because of limitations on the volume of hIL-3 that could be applied. Conse-
quently, the reductions in hIL-3 responsiveness observed for cell lines expressing the mutant hβc receptors are likely to be underestimated. The parent cell line, CTLL-2 hIL-3, did not respond to hIL-3 even at doses in excess of 200 units (Fig. 6B).

The above results together with previous mutagenesis studies on domain 4 (30, 38) suggest that the functional epitope of the hβc subunit for high affinity hIL-3 binding consists of Tyr15 and Phe79 in domain 1 and Tyr403 in domain 4, with secondary contributions from Tyr347, His349, and Ile350. The reductions in high affinity binding and receptor activation by hIL-3 observed for the Y15A and F79A mutant hβc subunits are not a consequence of impaired receptor translation, folding, or cell-surface expression, as previously these mutants were shown to be expressed at wild-type levels in COS7 cells (31).

### DISCUSSION

A conserved ligand-binding interface has been identified for several members of the hematopoietin receptor superfamily including the growth hormone (18, 21, 22), erythropoietin (19, 23, 24), and IL-4 α receptors (20, 25). These ligand-binding interfaces are present at an elbow of ~90° that is formed between the two fibronectin III domains constituting the extracellular domains of these receptors. Residues are contributed from a combination of the A–B and E–F loops of the membrane-distal domain and the B–C and F–G loops of the membrane-proximal domain to form a cluster of residues critical for ligand binding. The crystal structure of the hβc receptor showed that a similar elbow, representing a potential ligand-binding interface, is formed between domains 1 and 4 of the two different monomers that compose the hβc homodimer (26). This elbow region resembled the ligand-binding interface conserved in the simpler two-domain hematopoietin receptors, despite domains 1 and 4 being non-contiguous. Indeed, mutagenesis studies suggest that residues in domains 1 and 4 of the hβc subunit form the functional epitope for human GM-CSF and IL-5 binding in a manner analogous to the simpler hematopoietin receptors (31, 28–30). In the present work we show that this hβc functional epitope is similarly involved in high affinity hIL-3 binding and receptor activation. The residues, Tyr15 and Phe79, of domain 1 in hβc were shown to be critical for high affinity hIL-3 binding and also required for optimal receptor activation. As discussed in detail previously (31), it is possible that the effect of the F79A mutation of hβc could be due to an indirect effect on Tyr15. A detailed understanding of the mechanism of binding awaits the determination of the structure of IL-3-binding Epitopes of mβIL-3 and hβc

#### TABLE IV

Human IL-3 binding and proliferation studies of wild-type or mutant βc subunits co-expressed with the hIL-3 α subunit

| βc     | Sites detected | High affinity $K_d \pm$ S.E. | No. experiments | Units of hIL-3 for 50% growth $^a$ |
|--------|----------------|-----------------------------|----------------|-----------------------------------|
| Wild-type | 2 $p < 0.001$ | 106.8 ± 47.3 $^b$ | 2              | 1                                 |
| Y15A   | 1              | $\sim$                      | 2              | $>63.6$                           |
| F79A   | 1              | $\sim$                      | 2              | $>4.5$                            |

$^a$ Proliferation assays were performed on CTLL-2 cells co-expressing the hIL-3 α and indicated βc subunits. Data for these proliferation assays are shown in Fig. 6B.

$^b$ A low affinity site of 100 nM was fitted in LIGAND (37) to enable a more accurate estimation of the high affinity binding site.

$^c$ No high affinity binding sites were detected.
the high affinity hIL-3 complex, but the functional epitope based on this and previous mutagenesis studies (30, 38) consists of Tyr211, Phe79, and Tyr403 with secondary interactions from residues Tyr47 to Ile350 in the B’–C’ loop.

The residues in the murine beta3L receptor critical for mIL-3 binding were also determined. This receptor provides the opportunity to compare residues involved in both low and high affinity binding. The murine beta3L and mβc receptors are highly homologous in sequence suggesting that they may have homologous structures. As part of the present work, cross-linking and ultracentrifugation studies provided evidence that the expressed extracellular domains of the beta3L and mβc receptors, like that of the hβc receptor, exist as homodimers in the absence of ligand. Taken together these findings suggest the beta3L and mβc receptors may be intertwined homodimers like hβc, predicting an involvement of domains 1 and 4 in forming the ligand-binding epitope.

Mutagenesis of the beta3L receptor revealed that Ala substitutions of Tyr211 (A–B loop), Phe85, and Asn87 (E–F loop) of domain 1; Ile329 of the interdomain loop; and Tyr348 (B’–C’ loop) and Tyr401 (F’–G’ loop) of domain 4 resulted in no detectable direct low affinity mIL-3 binding. We estimate that the dynamic range of the assay used extends to slightly beyond ~370 nM, or a little over 20 times the Kd value of wild-type beta3L for direct binding. The alanine substitutions of several other beta3L residues also impaired low affinity mIL-3 binding. Arg44 and Tyr317 were shown to have major secondary roles. Asp300 and Asp302, which neighbor the critical residue Tyr401, had detectable effects. The critical beta3L residues, Tyr211, Phe85, Tyr348, and Tyr401, align with 4 of the 6 residues of hβc shown to be involved in high affinity ligand binding (Fig. 2). Thus direct binding involves the cooperation of domains 1 and 4 in forming an analogous ligand-binding interface in beta3L to that shown for hβc (Fig. 1).

To understand further the role of Tyr211, Tyr348, and Tyr401 of beta3L in direct mIL-3 binding, phenylalanine mutants of these residues were prepared. Y211F beta3L was able to bind mIL-3 with 6-fold lower affinity than wild-type beta3L, indicating that Phe was able to restore some mIL-3 binding but that the phenolic hydroxyl group plays an important role. In contrast, Y348F beta3L was able to bind mIL-3 with an affinity within 2-fold of the wild-type receptor, whereas Y401F beta3L exhibited near wild-type affinity indicating that the phenolic hydroxyl is not critical in either case. In comparison, with hGM-CSF high affinity binding to hβc, phenylalanine substitution mutants of the critical tyrosines Tyr15 and Tyr347 were fully active (29, 31), whereas high affinity hGM-CSF binding was abolished by the Y401F mutation.

An unexpected finding in the present work is the major differences in the interactions between beta3L and beta3L in low (direct) and high affinity mIL-3 binding. None of the residues that are individually critical for direct IL-3 binding are obligatory for high affinity binding. The simplest prediction would have been that the residues critical for high affinity binding would be those involved in low affinity binding or that the latter residues might be involved in an obligatory intermediate step in the formation of the high affinity complex with the mIL-3α subunit. It would therefore appear that the mechanism of high affinity IL-3 binding in the murine beta3L receptor is fundamentally different from that of its close relative the hβc receptor. The possibility of an additional signaling role for the