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# A Discontinuous Eight-Amino Acid Epitope in Human Interleukin-3 Binds the α-Chain of Its Receptor*

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**We have previously reported that, within the first helix of human interleukin (IL)-3, residues $Asp^{21}$ and $Glu^{22}$ are important for interaction with the α- and β-chains of the IL-3 receptor, respectively. In order to define more precisely the sites of interaction with the receptor, we have performed molecular modeling of the helical core of IL-3 and single amino acid substitution mutagenesis of residues predicted to lie on the surfaces of the A, C, and D helices. The resulting analogues were characterized for their abilities to stimulate proliferation of TF-l cells and for binding to the high affinity (α- and β-chain; IL-3Rα/Rβ) or low affinity (α-chain alone; IL-3Rα) IL-3 receptor. We found that in addition to $Asp^{21}$, residues $Ser^{17}$, $Asn^{18}$, and $Thr^{25}$ within the A helix and $Arg^{108}$, $Phe^{113}$, $Lys^{116}$, and $Glu^{119}$ within the D helix of IL-3 were important for biological activity. Analysis of their binding characteristics revealed that these analogues were deficient in binding to both the IL-3Rα/Rβ and the IL-3Rα forms of the receptor, consistent with a selective impairment of interaction with IL-3Rα. Molecular modeling suggests that these eight amino acid residues are adjacent in the tertiary structure, consistent with a discontinuous epitope interacting selectively with IL-3Rα. On the other hand, $Glu^{22}$ of IL-3 was found to interact preferentially with the β-chain with bulky and positively charged substitutions causing greater than 10,000-fold reduction in biological activity. These results show fundamental differences between IL-3 and granulocyte-macrophage colony-stimulating factor in the structural basis for recognition of their receptors that has implications for the construction of novel analogues and our understanding of receptor activation.**

Human interleukin (IL)[1]-3 is a glycoprotein of 133 amino acids produced principally by activated T-cells (1). IL-3 has a broad range of activities that includes regulation of proliferation of blood cell progenitors (1), adhesion of monocytes (2), and the respiratory burst of eosinophils (3). IL-3 has also been shown to support the growth of acute myeloid leukemia cells (4) and follicular B-cell lymphoma cells (5). More recently, IL-3 has been shown to activate endothelial cells (6), suggesting a direct involvement in leukocyte adhesion and transmigration to sites of inflammation. To understand the mechanism by which IL-3 carries out its pleiotropic functions it is essential to define the structural and functional basis of IL-3 interaction with its receptor.

IL-3 is a member of the hemopoietic cytokine family and is most closely related to granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 (7), two cytokines that both exhibit a bundle of four α-helices as their principal feature. Although the tertiary structure of IL-3 is not yet known, a preliminary report (8) suggests that it consists of a similar structure. Structure-function studies using site-directed mutagenesis have revealed that residues 21, 22, 27, 33, 43, 44, 48, 53, 58, 61, 64, 94, 101, 110, 111, 115, and 116 are important for activity (9–13), although several of these residues may be buried in the IL-3 structure. In addition, mouse-gibbon chimeras (14) and site-directed mutagenesis (15) of IL-3 identified two noncontiguous regions near the N and C termini that are required for biological activity.

The receptor for IL-3 has been shown to comprise an α-chain (IL-3Rα) that binds IL-3 specifically and with low affinity and a β-chain (Rβ) that does not detectably bind IL-3 by itself but which provides high affinity binding when coexpressed with the α-chain (16). The β-chain also acts as the affinity-converting subunit of the GM-CSF (17) and IL-5 (18) receptors. The co-expression of both receptor chains is required for biological signaling (19), an event that involves IL-3 inducing a covalent as well as noncovalent association of IL-3Rα and Rβ (20), phosphorylation of the β-chain (21), and interaction with intracellular signaling molecules (22, 23). IL-3 analogues selectively deficient in interaction with either the α- or the β-chain of the receptor have been shown to exhibit reduced biological potency (12). In order to be able to design more potent IL-3 agonists and antagonists, it is important to determine which parts of IL-3 are involved in binding each receptor chain.

We have previously shown that a double mutant, D101A/K116V, exhibited 10-fold enhanced biological activity with a commensurate increase in affinity for IL-3Rα (15). This suggested that the C-terminal region encompassing the fourth helix of IL-3 may play a role in binding to IL-3Rα. On the other hand, dissection of the IL-3 triple mutant (D21A/E22L/T25A) deficient in high affinity binding and function (12) revealed $Asp^{21}$ also to be important for binding to the receptor α-chain, thus implicating the first helix of IL-3 in binding to IL-3Rα (12). In contrast, charge reversal substitution of residue $Glu^{22}$ led to a molecule that was apparently deficient in association with the receptor β-chain but was still biologically active, albeit at high concentrations. An acidic residue within the first helix has been recognized as a common motif probably important for function in several cytokines (24). Indeed, charge reversal substitution of the analogous residue ($Glu^{21}$) in human GM-CSF

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[1] The abbreviations used are: IL, interleukin; IL-3Rα, IL-3 receptor α-chain; Rβ, β-chain; GM-CSF, granulocyte-macrophage colony-stimulating factor; CHO, Chinese hamster ovary.

not only causes selective loss of binding to Rβ (25) but leads to the generation of a GM-CSF-specific antagonist (26). The observation of residual activity in IL-3:E22R (12) suggests that IL-3 possesses additional determinants for interaction with the Rβ that may lie in helix A or, by analogy with growth hormone (27), in helix C. We wished to define those residues in IL-3 responsible for recognition of both the α- and the β-chains of the receptor and characterize their contribution to biological function.

We show here that the recognition site for IL-3Rα is contained in an epitope consisting of at least eight residues distributed between the A and D helices of IL-3. Additionally, we find no residue other than $Glu^{22}$ to be specifically involved in association with Rβ. Furthermore, molecular modeling suggests that the helical core of IL-3 is packed similarly to that of GM-CSF but that, unlike GM-CSF, helix A is able to interact with both IL-3Rα and Rβ, suggesting a different orientation of IL-3 with respect to the receptor chains.

## EXPERIMENTAL PROCEDURES

*Molecular Modeling of Human IL-3*—A model of the helical core of IL-3 was developed based on the crystal structure coordinates of GM-CSF (28), and an additional helix was introduced between the A and B helices based on the preliminary nmr data of Feng and co-workers (8). The coordinates of GM-CSF were kindly provided by Dr. P. A. Karplus (Cornell University). Although clearly related to GM-CSF, IL-3 exhibits only 13% amino acid identity (26% similarity) overall (7). Secondary structure predictions of IL-3 and GM-CSF were performed using the GOR II algorithm (29) and compared with the known structure of GM-CSF. Sequences in predicted helical regions were aligned using a hydrophobicity matrix scoring method and manually adjusted where necessary according to the heptad repeat method (30). In regions predicted to be involved in the helical core of IL-3, the sequences exhibited 22% identity and 59% similarity with those of GM-CSF, permitting reliable alignment of these sequences (Fig. 1). An Indigo$^2$ computer (Silicon Graphics) was used to run the molecular modeling programs Insight II, Homology, and Discover (Biosym Technologies Inc., San Diego, CA). Coordinates for regions of IL-3 thought to be conserved structurally, corresponding to the proposed helices, were assigned from the homologous backbone coordinates of GM-CSF and some side-chain coordinates. The C helix of IL-3 was predicted to differ from those of GM-CSF and IL-5, since GM-CSF contains a kink induced by $Pro^{76}$ and the C helix of IL-5 is relatively long and bridges the two domains of the IL-5 dimer. The location of helix C of IL-3 was constrained, however, by the presence of $Cys^{84}$, which forms a disulfide bond with $Cys^{16}$ (31). Helix D was modeled to extend for an extra turn at the C terminus beyond that of GM-CSF, since there was continuance of the heptad pattern and no helix-breaking proline residue. An inspection of the model revealed a well packed hydrophobic core with only moderate steric clashes between the hydrophobic side chains of adjacent helices. Manual and automated methods were used to select appropriate conformations for the hydrophobic side chains of residues proposed to be buried in the core of the IL-3 molecule. Residues 42–50, which have been reported to constitute an additional helix (denoted A′) (8) were manually placed in a helical conformation and docked against the face of the B and D helices, principally via the side chains of $Leu^{48}$ and $Met^{49}$ in order to be consistent with the reported nuclear Overhauser effect constraints (8). The model was evaluated for stereochemical parameters using Procheck (32). The sequences corresponding to the predicted four-helix bundle of IL-3 and their calculated exposure to the solvent, using the dssp program (33), are shown in Fig. 1.

*Oligonucleotide-directed Mutagenesis*—A synthetic human IL-3 cDNA (34) was subcloned by standard techniques into the *Hin*dIII/*Bam*HI sites of pAlter (Promega Corporation, Sydney, New South Wales, Australia). Oligonucleotide-directed mutagenesis was carried out as described in the pAlter protocol using mutagenic oligonucleotides synthesized on a DNA synthesizer (Applied Biosystems Inc., Melbourne, Victoria, Australia). Mutants were subcloned using the polymerase chain reaction into the *Hin*dIII-*Bgl*II sites of the *Escherichia coli* secretion vector pFLAG (International Biotechnologies Inc., Sydney, New South Wales, Australia). The full-length cDNA insert was sequenced in full to confirm the presence of the desired mutation and the absence of other mutations. All IL-3 analogues also carried a substitution of Tyr in place of Phe at residue 133. This substitution had no effect on either biological or binding activities (data not shown).

*Expression of IL-3*—Wild type IL-3 and analogues were expressed in *E. coli* in order to enable production of highly purified material in a sufficient quantity for full titration in biological assay and low affinity binding experiments. Mutants of helix D and K28E were expressed in the pFLAG system and purified as described previously (12). Other analogues as well as wild type IL-3 were expressed using the pEC611 vector (35), and the resultant insoluble protein was recovered from *E. coli* by dissolution in 8 M urea containing 10 mM dithiothreitol and 25 mM Tris-Cl, pH 8.5. The proteins were purified by anion exchange chromatography on S-Sepharose (Pharmacia, North Ryde, New South Wales, Australia) in the presence of 8 M urea using 25 mM *N*-ethylmorpholine at a pH of 9.5 for wild type IL-3 and more acidic analogues and at pH 10.2 for analogues more basic than wild type. Protein was eluted by a series of washes with increasing concentrations of NaCl, with most analogues eluting at 75 mM NaCl. The peak fractions were identified by SDS-polyacrylamide gel electrophoresis and subjected to reversed phase high pressure liquid chromatography on either a 4.6 × 100-mm RP-300 column or a 10 × 100-mm C8 column (Aquapore, Applied Biosystems Inc., Melbourne, Victoria, Australia) using a gradient of acetonitrile in the presence of 0.1% trifluoroacetic acid. The peak fractions, as determined by SDS-polyacrylamide gel electrophoresis, were pooled and quantified. IL-3 produced by either method showed equivalent biological activity and receptor-binding properties (data not shown).

*Purification and Quantitation of IL-3*—Direct protein quantitation was performed by laser scanning densitometry using samples of purified protein resolved on Coomassie-stained 13% SDS-polyacrylamide gel electrophoresis (with an acrylamide:bisacrylamide ratio of 19:1) and compared with lysozyme standards using an LKB-Pharmacia Ultrascan XL system including GSXL software.

*Stability of IL-3 Analogues*—The stabilities of IL-3 expressed in the pFLAG system or intracellularly in *E. coli* were assessed by transverse urea gradient gel electrophoresis (36). Samples (20 μg) of purified IL-3 or analogue were electrophoresed toward the anode through 8% acrylamide gels (acrylamide:bisacrylamide ratio of 19:1) containing a linear 0–8 M urea gradient and the high pH buffer of Ref. 36. Stability was estimated as described (36).

*Stimulation of Hemopoietic Cell Proliferation*—This was measured by incorporation of [$^3$H]thymidine into the human erythroleukemic cell line TF-1 (37) as described previously (3).

*Radioreceptor Assays*—IL-3 expressed in the pFLAG system and affinity-purified was radioiodinated to a specific activity of approximately 500 Ci/mmol by the ICl method (38). Direct competitive binding experiments were performed using CHO cells transfected permanently with either the IL-3Rα alone (39) or IL-3Rα and Rβ. CHO cells expressing both IL-3Rα and Rβ were obtained by retransfecting cells (25) that expressed IL-3Rα with a pRUFpuro-Rβ retroviral expression plasmid that had been linearized with *Sca*I. Pools of cells were obtained after selection with puromycin (5 μg/ml) for 2 weeks and were demonstrated to express both chains of the receptor by flow cytometry. For low affinity binding measurements, 96-well multiwell dishes containing confluent monolayers of CHO cells transfected with IL-3Rα were incubated with 60 μl of a buffer (50 mM HEPES, 0.5% bovine serum albumin, pH 7.4) containing $^{125}$I-IL-3 (5 nM), IL-3 (5 nM), and various concentrations of IL-3 or analogues. After 2 h at 25 °C, the medium was aspirated, and the cells were briefly washed with phosphate-buffered saline. The cell-associated radioactivity was dissolved in 10 mM Tris, 150 mM NaCl containing 1% Nonidet P-40 and transferred to 3DT tubes, and radioactivity was determined using a Cobra γ-counter (Packard, CT). For high affinity binding measurements, 96-well multiwell dishes containing confluent monolayers of CHO cells transfected with IL-3Rα and Rβ were incubated with 60 μl of a HEPES/bovine serum albumin buffer containing $^{125}$I-IL-3 (0.3 nM) and various concentrations of IL-3 or analogues and processed as above. Although these cells expressed approximately 10-fold more IL-3Rα than Rβ, the extreme low affinity of IL-3Rα means that 95% of specifically bound $^{125}$I-IL-3 is associated with the high affinity receptor.

## RESULTS

*Model of the Helical Core of IL-3*—In order to facilitate targeting of mutations to exposed residues, we developed a model for the structure of IL-3. Although clearly related to GM-CSF and IL-5, IL-3 only exhibits significant sequence identity in regions corresponding to the helical cores of these cytokines (23% identity to GM-CSF and 17% to IL-5) with 50–60% similarity, permitting reliable alignment of the sequences only in

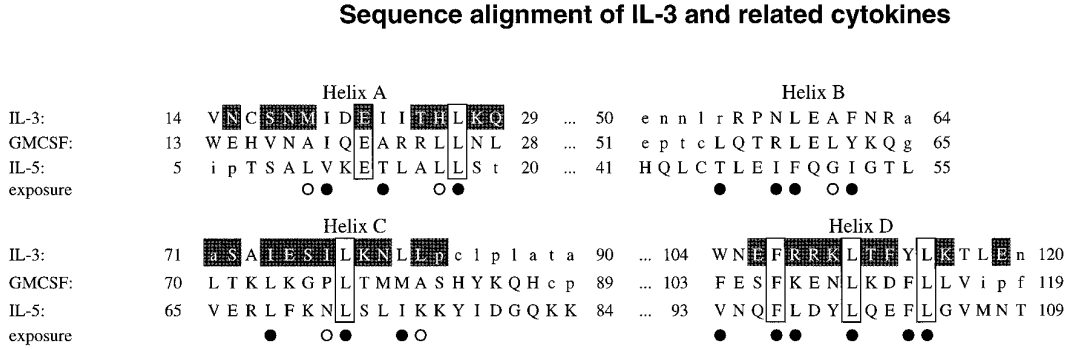


FIG. 1. **Sequence alignment of the proposed helices of IL-3.** The sequences were aligned as described under "Experimental Procedures." Residues observed (GM-CSF and IL-5) or proposed (IL-3) to be within helices are indicated in *capital letters* with conserved residues *boxed*. Residues of less than 5% (●) or between 5 and 20% (○) solvent exposure for GM-CSF and IL-5 are indicated. Residues in IL-3 selected for mutation are *shaded*.

these regions (Fig. 1). In particular, the high degree of conservation of hydrophobic residues in the putative helices A and D of IL-3 suggested that they pack against each other in a very similar manner to that of GM-CSF and IL-5. We predicted the core helical regions of IL-3 to consist of residues 14–29, 55–63, 72–82, and 104–119, in close agreement with the nmr data (8). The assignment of these helices was used as a framework for building a molecular model, which has allowed the likely surface exposure of amino acid residues to be assessed (Fig. 1). The residues of helices A, C, and D that were predicted to be exposed were then subjected to mutagenesis, and the resulting analogues were examined for biological activity and their receptor-binding properties.

*Helix A of IL-3*—We have demonstrated previously that, within the putative helix A of IL-3, residue $Asp^{21}$ is important for association with the receptor α-chain and residue $Glu^{22}$ is crucial for association with the receptor β-chain (12). We have now sought to define further the contacts between helix A of IL-3 and its receptor by the mutation of additional residues $Asn^{15}$, $Ser^{17}$, $Asn^{18}$, $Met^{19}$, $Thr^{25}$, $His^{26}$, $Lys^{28}$, and $Gln^{29}$ that are predicted to lie on the surface of the molecule. We chose to introduce positively charged residues in place of hydrophilic residues, Ala for Met and His, and perform charge reversal at position $Lys^{28}$. The analogues N15K, S17K, N18K, M19A, T25R, H26A, K28E, and Q29K were prepared from *E. coli* and tested for their abilities to stimulate the proliferation of TF-1 cells. The H26A, K28E, and Q29K analogues exhibited wild type levels of bioactivity, while the N15K and M19A analogues exhibited only 2–3-fold reduced biological potency (Fig. 2*A*). The S17K and T25R analogues were approximately 30-fold less potent than wild type IL-3 (Fig. 2*A*) and exhibited a similar reduction of potency of competition for both the high affinity IL-3 receptor (Fig. 2*B*) and for the α-chain of the receptor alone (Fig. 2*C*). This indicates that the residues $Ser^{17}$ and $Thr^{25}$ behave similarly to $Asp^{21}$ (12) and are required for the selective interaction with the α-chain of the receptor. The N18K analogue was 60-fold less potent biologically than wild type IL-3 (Fig. 2*A*), exhibited a 250-fold reduction of potency of competition for the high affinity IL-3 receptor (Fig. 2*B*) and a 5-fold reduction of potency of competition for the α-chain of the receptor alone (Fig. 2*C*), suggesting that residue $Asn^{18}$ may be involved in interactions with both chains of the receptor. These biological and receptor binding data are summarized in Table I. Since it was possible that the reduced binding of the S17K, N18K, and T25R analogues to the α-chain of the IL-3 receptor may result from a general structural perturbation, we examined their stabilities by transverse urea gradient electrophoresis. Each analogue was apparently fully folded in the absence of urea and exhibited a urea-induced transition from a fast migrating (folded) form to a more slowly migrating (unfolded) form. The estimated stability of these analogues was only slightly less than wild type IL-3 (Table I), consistent with normal folded structure. In addition, this systematic investigation of helix A shows that no residue in helix A other than $Glu^{22}$ reported previously (12) interacts exclusively with Rβ. Since the N18K analogue showed some apparent loss of interaction with Rβ, we combined this mutation with the E22R mutation in order to further abrogate interaction with Rβ. The resulting double mutant retained a small measure of biological activity (data not shown) and thus presumably a weak ability to interact productively with Rβ.

Since only residue $Glu^{22}$ has been found to be significant for association with Rβ and for bioactivity and its substitution by Arg leads to a dramatic loss of high affinity binding and biopotency (12), we chose to investigate a series of basic and/or bulky substituents at this position. Residues His, Arg, Lys, Phe, Trp, and Tyr were introduced in place of $Glu^{22}$, and the proteins were expressed and purified as described under "Experimental Procedures." These analogues were tested for their abilities to stimulate the proliferation of TF-1 cells as shown in Fig. 3. All analogues exhibited weak biological activity with the following range of potencies (expressed as percentage of wild type): His (0.2%) > Lys ≈ Tyr ≈ Arg (0.005%) > Phe ≈ Trp (<0.001%). The extremely low activity of the Phe and Trp analogues suggests that the introduction of bulky, hydrophobic groups is highly deleterious to interaction with Rβ.

*Helix C of IL-3*—The presence of residual biological activity of the E22R analogue (12) and other position 22 analogues, as well as the failure to identify additional residues specifically involved in Rβ binding within helix A of IL-3, suggests that additional determinants of IL-3 binding to Rβ may be present elsewhere in the molecule. Helix C of growth hormone has been implicated in its binding to the second molecule of the growth hormone receptor, and several residues of IL-3 ($Glu^{75}$ and $Lys^{79}$) have been reported to be required for activity (40), suggesting that mutations within this region of IL-3 may lead to decreased interaction with Rβ. Molecular modeling suggested that helix C extends from residue 72 to 82 and that the hydrophobic residues $Ile^{74}$, $Ile^{77}$, $Leu^{78}$, and $Leu^{81}$ may be buried and important for the structure of this helix (Fig. 1). We hypothesized that the introduction of charged residues or prolines at these positions would destabilize the IL-3 molecule. Conversely, we predict that the surface of helix C of IL-3 includes residues $Ser^{72}$, $Glu^{75}$, $Ser^{76}$, $Lys^{79}$, $Asn^{80}$, and $Leu^{82}$ and tested whether this may form an additional site for recognition of Rβ. We produced the IL-3 analogues of residues predicted to be buried (A71E/K/P, I74R/P, and I77A) and of residues predicted to be exposed (S72K, E75K/A/Q, S76K, K79A/E, N80A, L82A/

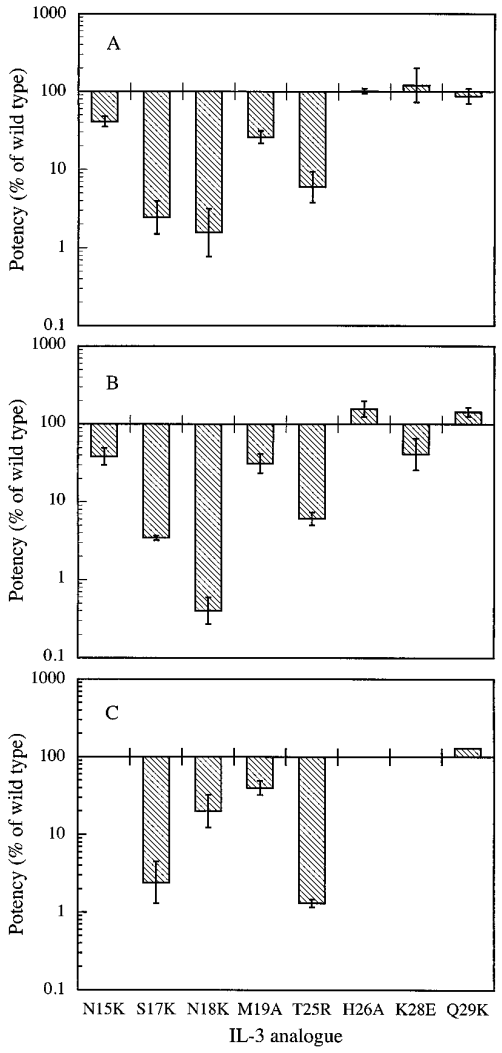


FIG. 2. **Characterization of helix A analogues.** The indicated analogues were tested for their abilities to stimulate the proliferation of TF1 cells as described (*A*), compete for binding of $^{125}$I-IL-3 to high affinity (IL-3Rα/Rβ) receptors (*B*), or compete for binding of $^{125}$I-IL-3 to low affinity (IL-3Rα) receptors (*C*) as described under "Experimental Procedures." Data are expressed as potency relative to IL-3 (100%) with values calculated from the $ED_{50}$ values of IL-3 and analogues where potency = ($ED_{50}$ (wild type)/$ED_{50}$ (analogue)) × 100%. For each assay, *error bars* representing the S.E. for results from separate experiments are shown.

TABLE I
*Relative activities of helix A analogues*

The biological activity, receptor binding, and stability of analogues substituted in helix A are expressed relative to wild type IL-3 (100%). For the bioassay and receptor binding data, the values are calculated from the $ED_{50}$ values of IL-3 and analogues, where potency = ($ED_{50}$ (wild type)/$ED_{50}$ (analogue)) × 100%. For stability data, the values are calculated from the estimated $\Delta G_0$ values of IL-3 and analogues, where relative stability = ($\Delta G$ (analogue)/$\Delta G$ (wild type)) × 100%. Assays were performed as described under "Experimental Procedures," and each value is the mean of the estimates from experiments performed at least twice.

| Analogue | Biological activity[a] | High affinity binding[b] | Low affinity binding[c] | Relative stability[d] |
|---|---|---|---|---|
| | | % | | |
| N15K | 41 | 38 | ND[e] | ND |
| S17K | 2.4 | 3 | 8.9 | 80 |
| N18K | 1.6 | 0.40 | 11 | 70 |
| M19A | 26 | 31 | 19 | ND |
| T25R | 5.9 | 6.1 | 1.31 | 80 |
| H26A | 102 | 157 | ND[e] | ND |
| K28E | 121 | 41 | ND[e] | ND |
| Q29K | 88 | ND | ND[e] | ND |

[a] Biological activity was measured using the TF-1 proliferation assay.
[b] High affinity receptor binding was measured by competition for binding of $^{125}$I-IL-3 to high affinity (IL-3Rα/Rβ) receptors.
[c] Low affinity receptor binding was measured by competition for binding of $^{125}$I-IL-3 to low affinity (IL-3Rα) receptors.
[d] Stability was measured by transverse urea gradient electrophoresis.
[e] ND, not determined.

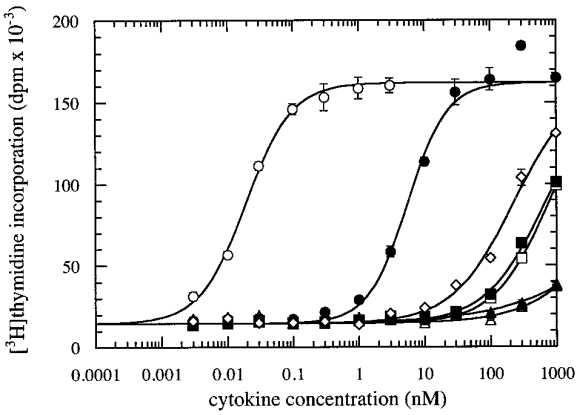


FIG. 3. **Characterization of analogues substituted at position 22.** A representative experiment is shown. IL-3 (○) and analogues E22H (●), E22R (□), E22K (■), E22F (△), E22W (▲), and E22Y (◇) were tested for their abilities to stimulate the proliferation of TF1 cells as described under "Experimental Procedures." *Standard error bars* are indicated where they are sufficiently large with respect to the *symbols*.

E/R, and P83L) and measured their abilities to stimulate the proliferation of TF-1 cells. These analogues demonstrated activities similar to wild type (Table II) with the exception the S72K, A71P, I77A, and I74R analogues, which were 8-, 9-, 15-, and 120-fold less potent than wild type IL-3, respectively. We then measured the stabilities of these four analogues as well as those of other analogues substituted at positions 71 and 74 and the L82E analogue by transverse urea gradient gel electrophoresis (Table II). The stabilities estimated differed widely, with the A71P/E, I74P, and I74R substitutions causing a 50–70% decrease in stability relative to wild type, consistent with major disruption of the normal IL-3 fold, whereas the A71R, S72K, and L82E analogues were of similar stability to wild type. The destabilizing effects of proline substitutions are consistent with residue 71 being in a type II β-turn and residue 74 lying within the C-helix of IL-3 as suggested previously (40). Residue Ile$^{74}$, but not Leu$^{82}$, was also sensitive to the introduction of a charged residue, consistent with predictions of Ile$^{74}$ being buried in the IL-3 structure and Leu$^{82}$ being exposed. The reduced biological activity of the S72K was found not to be due to selective loss of binding to Rβ, since this analogue was approximately 50-fold less potent than wild type IL-3 in both high and low affinity binding experiments (data not shown). It was possible that weak interactions of helix C residues with Rβ was masked by the strong interaction mediated by Glu$^{22}$. Analysis of the surface mutations of helix C in combination with the deleterious E22R mutation demonstrated no greater reduction in biological activity beyond that seen with the E22R mutation alone (data not shown). Thus, in IL-3, the region from residue 71 to 83 that encompasses helix C does not seem to play a direct role in biological function.

*Helix D of IL-3*—The D helix is proposed to extend from Trp$^{104}$ to Glu$^{119}$. In order to minimize disruption of the structure of IL-3, we chose to mutate only those residues predicted to lie on the surface of the D helix. Mutations were chosen to alter the chemical character of the residues; in particular, charged residues were replaced by amino acids of opposite

TABLE II
*Relative activities of helix C analogues*

The biological activity and stability of analogues substituted in helix C are expressed relative to wild type IL-3 (100%) as described in Table I. Each value is the mean of the estimates from experiments performed at least twice.

| Analogue | Biological activity | Relative stability |
|---|---|---|
| | % | |
| L68R | 28 | ND[a] |
| A71E | 260 | 50 |
| A71P | 11 | 40 |
| A71R | 169 | 80 |
| S72K | 13 | 120 |
| I74P | 25 | 50 |
| I74R | 0.79 | 30 |
| E75Q | 180 | ND[a] |
| E75K | 155 | ND[a] |
| S76K | 295 | ND[a] |
| I77A | 6.7 | ND[a] |
| K79A | 204 | ND[a] |
| K79Q | 150 | ND[a] |
| N80A | 63 | ND[a] |
| L82E | 63 | 110 |
| P83L | 150 | 110 |

[a] ND, not determined.

charge, and the large hydrophobic residue, Phe[113], was replaced by a small, aliphatic alanine residue. The mutations made were E106R, R108E, R109E, K110E, T112R, F113A, K116E, and E119R. The proteins were expressed in *E. coli* using the pFLAG system, purified by affinity chromatography, and tested for their abilities to stimulate the proliferation of TF-1 cells. As shown in Fig. 4*a*, a range of potencies was observed with analogues R108E, K110E, F113A, K116E, and E119R, showing activities reduced 5–15-fold compared with wild type. These analogues were then examined for their abilities to compete for binding to the high affinity (IL-3Rα/Rβ) or low affinity (IL-3Rα) IL-3 receptors (except K110E, which expressed poorly). As shown in Fig. 4*b*, each of these analogues was less able than wild type to compete for binding to high affinity receptors, and the reduced potencies of these analogues were paralleled in binding to the IL-3Rα alone (Fig. 4*c*). The reduction in biological activity of these analogues apparently results from decreased association with the receptor, in particular the α-chain. These biological and receptor binding data are summarized in Table III. The stabilities of the least active analogues, R108E, F113A, K116E, and E119R, were tested by transverse urea gradient gel electrophoresis and found to be equivalent to wild type except for the R108E analogue, which showed a 40% reduction in stability (Table III). Thus, the reduced binding of these IL-3 analogues to the IL-3Rα probably results from the loss of receptor contacts rather than from general structural perturbation.

## DISCUSSION

We used a prediction of the four-helix bundle core of the IL-3 molecule to help target our mutagenesis to surface residues that may be available for receptor contact. Previous studies have implicated residues near both the N and C termini of IL-3 as being required for binding to the α-chain of the IL-3 receptor. Using the combination of binding experiments on both the high affinity (IL-Rα/Rβ) and low affinity (IL-Rα) receptors, we identified seven residues, Ser[17], Asn[18], and Thr[25] within helix A and Arg[108], Phe[113], Lys[116], and Glu[119] within helix D of IL-3 that are important for binding to IL-3Rα. An additional residue in helix A, Asp[21], has been shown previously to be important for binding to IL-3Rα (12). Although no single residue was found to be critical for binding to IL-3Rα, together these eight residues can fully account for the observed affinity of IL-3 for

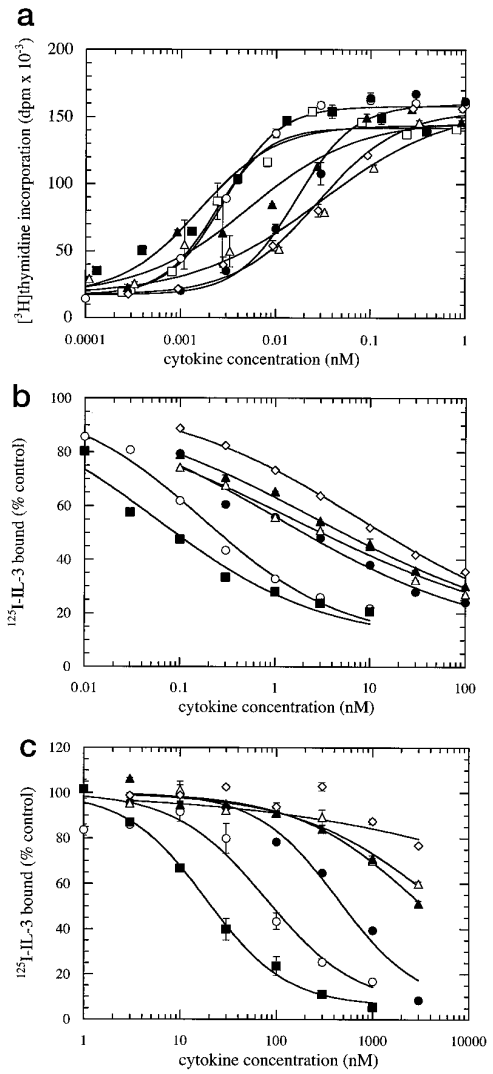


FIG. 4. **Characterization of helix D analogues.** Representative experiments for the indicated analogues are shown. IL-3 (○) and analogues R108E (●), R109E (□), T112R (■), F113A (△), K116E (▲), and E119R (◇) were tested for their abilities to stimulate the proliferation of TF1 cells (*a*), compete for binding of $^{125}$I-IL-3 to high affinity (IL-3Rα/β) receptors (*b*), or compete for binding of $^{125}$I-IL-3 to low affinity (IL-3Rα) receptors (*c*) as described under "Experimental Procedures." For the binding assays, data are expressed as a percentage of binding seen in the absence of competitor. Nonspecific binding has not been subtracted. *Standard error bars* are indicated where they are sufficiently large with respect to the *symbols*.

TABLE III
*Relative activities of helix D analogues*

The biological activity, receptor binding, and stability of analogues substituted in helix D are expressed relative to wild type IL-3 (100%) as described in Table I. Each value is the mean of the estimates from experiments performed at least twice.

| Analogue | Biological activity | High affinity binding | Low affinity binding | Relative stability |
|---|---|---|---|---|
| | | % | | |
| E106R | 228 | 29 | ND[a] | ND |
| R108E | 18 | 9.5 | 18 | 60 |
| R109E | 156 | 203 | ND[a] | ND |
| K110E | 14 | 40 | ND[a] | ND |
| T112R | 47 | 196 | 417 | ND |
| F113A | 7.3 | 14 | 1.7 | 100 |
| K116E | 18 | 12 | 2.4 | 100 |
| E119R | 5.6 | 5.5 | 0.56 | 120 |

[a] ND, not determined.

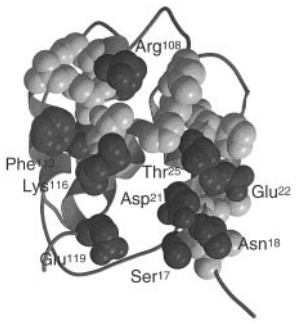


FIG. 5. **The proposed face on IL-3 responsible for binding to IL-3Rα.** The model of IL-3 is represented in diagram form, using Molscript (42) and Raster3d (43), viewed toward the face of the D and A helices. Residue α-carbon atoms and side chains are represented by Corey-Pauling-Koltun spheres shaded according to significance; residues for which a >5-fold effect was seen on mutagenesis are *dark*, while other mutated residues are *light*.

IL-3Rα, suggesting that the major part of the recognition site for IL-3Rα has been defined. Structurally, the juxtaposition of the A and D helices directs these eight residues to form a nearly continuous surface that is available for binding to IL-3Rα (Fig. 5). The apparent insensitivity to mutation of residue $Thr^{112}$, which is predicted to lie in the middle of this patch, may be the result of our choice of an Arg residue as replacement, which may deputize for the nearby side chain of $Arg^{108}$ in interacting with IL-3Rα. Olins *et al.* (13) report that the replacement of $Thr^{112}$ by Glu or Tyr results in greater than 5-fold loss of biological activity. In addition to the identification of the probable surface residues $Lys^{110}$ and $Phe^{113}$ observed by other workers (9, 13), we found residues $Lys^{116}$ and $Glu^{119}$ to be important for biological function. Our results extend these observations by demonstrating that the surface of helix D is specifically involved in binding to IL-3Rα.

The normal interaction of IL-3 with Rβ requires the integrity of the conserved residue $Glu^{22}$ but is not completely abrogated by charge reversal at this position (12). Growth hormone is known to employ helices A and C for binding to molecule 2 of its receptor. By analogy, we sought to locate additional IL-3 residues that interact with Rβ by mutation of residues in the A and C helices that were predicted to lie close to $Glu^{22}$ in the tertiary structure of IL-3. Within helix A of IL-3, we found $Ser^{17}$, $Asn^{18}$ and $Thr^{25}$ to be additional determinants for interaction with IL-3Rα rather than with Rβ, although $Asn^{18}$ apparently interacts with both chains of the receptor. Substitution of residues predicted to lie on the surface of helix C revealed that they were not important for biological function, and when combined with the E22R substitution, there was no reduction in biological activity beyond that with the E22R substitution alone. Our observations with the E75K and K79E mutations differ from those of Dorssers and co-workers (40), who observed greater that 100-fold reduction in activity with their E75R and K79E analogues produced from inclusion bodies in *E. coli*. Our IL-3 E75K and K79E analogues were produced with N-terminal Flag™ peptides and purified using affinity chromatography and quantified by Coomassie staining of SDS-polyacrylamide gels, which may have helped to overcome any potential problems caused by the poor solubility of some IL-3 analogues produced in *E. coli*.

$Glu^{22}$ is the only residue identified as required for association with Rβ uniquely, as judged by the selective loss of high affinity binding (Table I). However, neither charge reversal nor introduction of bulky residues was able to completely abrogate biological activity. This contrasts with GM-CSF, in which replacement of the homologous $Glu^{21}$ by Lys or Arg generated complete antagonists and the Phe and His substitutions led to weak partial agonism (26). Several observations suggest that Rβ may interact more intimately with IL-3 than with GM-CSF. First, the degree of affinity conversion effected by Rβ on the IL-3Rα is much greater than for the GM-CSF receptor α-chain (16, 41). Second, three individual mutations in the putative B–C loop of the membrane-proximal extracellular domain (domain 4) of Rβ are able to eliminate the high affinity binding of GM-CSF, but they exhibit only modest effects on the binding of IL-3 (41). In addition to the pivotal role played by residue $Glu^{22}$ of IL-3, the recognition of Rβ by the IL-3·IL-3Rα complex may result from the combination of several weak Rβ/IL-3 interactions, not detectable by our mutagenic strategy. Alternatively, Rβ may directly recognize IL-3Rα itself, since the two receptor chains must be brought into very close proximity by their both binding to helix A of IL-3. Either of these possibilities suggests a fundamental difference between the structure of the IL-3 receptor complex and the closely related GM-CSF receptor complex that may be resolved ultimately by analyzing the interaction of ligands and receptors in solution or by x-ray crystallography.

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