Spatial Orientation of the α and βc Receptor Chain Binding Sites on Monomeric Human Interleukin-5 Constructs*

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Interleukin-5 (IL-5), a disulfide-linked homodimer, can be induced to fold as a biological active monomer by extending the loop between its third and fourth helices (Dickason, R. R., and Huston, D. P. (1996) Nature 379, 652–655). We have designed eight monomeric IL-5 proteins to optimize biological activity and stability of the monomer. This was achieved by (i) inserting the joining loop at three different positions, (ii) by introducing an additional intramolecular disulfide bridge onto these backbones, and (iii) by creating circular permutations to fix the position of the carboxyl-terminal helix relative to the three other helices. The proteins dimerize with Kd values ranging from 20 to 200 μM and are therefore monomeric at the picomolar concentrations where they are biologically active. Introduction of a second disulfide confers increased stability, but this increased rigidity results in lower activity of the protein. Contrary to wild type IL-5, mutation of the βc contact residue on the first helix, Glu12, to Lys, into the circularly permuted constructs, did not abolish TF-1 proliferative and eosinophil activation activities. These results indicate that activation of the IL-5 receptor complex is not mediated solely by Glu12 on the first helix, and alternative mechanisms are discussed.

Interleukin-5 (IL-5)1 is the key cytokine involved in the differentiation and maturation of eosinophil precursors and the activation and survival of mature eosinophils (1–5). The association of eosinophilia with chronic inflammatory conditions such as asthma, rhinitis, and atopic dermatitis (6–8) indicates that blocking the action of IL-5 may provide therapeutic benefit in these allergic disorders. Indeed, neutralizing antibodies to IL-5 have been shown to reduce pulmonary eosinophilia, tissue damage, and bronchial hyperactivity in animal models of asthma (9–11). Experiments with mice in which the IL-5 gene has been deleted have further validated the central role of IL-5 in eosinophilia (12).

IL-5 was originally identified from a murine T cell culture supernatant (13) and was shown to be a disulfide-linked homodimer consisting of two glycosylated subunits (14). Glycosylation is not required for activity, as the human recombinant protein produced in Escherichia coli is fully active (15). The three-dimensional crystal structure of the E. coli-derived protein showed that the disulfide-linked dimer forms two domains, each containing four helices which pack with the cytokine fold (16). The cytokine fold is common to many other cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF) and growth hormone (17). However, all the other cytokines are monomeric. The dimeric topology of IL-5 is unique in that each four helix bundle consists of three helices from one subunit, whereas the fourth is provided by the other subunit.

IL-5 binds to a heterodimeric receptor complex composed of an α-chain that binds IL-5 uniquely and a common signaling βc-chain, which is also a component of the GM-CSF and IL-3 receptors (18, 19). Eosinophils bear 300–1000 such binding sites, to which IL-5 binds with Kd of 200–400 pM (20, 21). Surprisingly, given the homodimeric structure of IL-5, it can bind to the receptor α-chain to form a 1:1 complex of IL-5 dimer to receptor monomer (22, 23). Residues of both IL-5 and the IL-5 receptor α-chain required for receptor-ligand interaction have been mapped by extensive mutagenesis studies (24–27). The residues of IL-5 that bind the α-chain are found in the carboxyl-terminal region as follows: Glu109 and Trp110 on the fourth helix and Glu121 and Arg220 on the β-sheet preceding it, while activation of the βc-chain has been shown to be transmitted by a single glutamic residue on the first helix. The two other cytokines that share the βc-chain similarly have a Glu on the first helix as a βc-chain contact, Glu11 for GM-CSF (28) and Glu22 for IL-3 (29). Mutating this acidic residue in the IL-5 protein to glutamine which is polar (25) or the positively charged lysine (30) and to arginine in GM-CSF (31) results in protein antagonists that are able to bind the α-chain but are unable to activate the signaling βc-chain.

Asymmetric mutagenesis of single chain IL-5 molecules suggests that a single IL-5 domain suffices for biological activity but that optimal binding of the α-chain may require residues from both of the four helix bundles (32, 33). Extension of the loop linking the third and fourth helices of IL-5 in a manner analogous to GM-CSF allows the protein to fold as a monomer (34). However, this monomeric IL-5 was 15-fold less active than the wild type molecule. This may reflect either a lack of receptor interaction points normally contributed by the second four helix bundle or reduced stability of the monomeric protein.

We have shown that in single chain dimers of IL-5 only a single copy of the receptor α-chain contact residues, Arg220 and Glu121, is necessary to attain full biological activity. This observation led us to design a series of monomeric IL-5 constructs.

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1 The abbreviations used are: IL-5, interleukin-5; IL-3, interleukin-3; GM-CSF, granulocyte-macrophage colony stimulating factor; PCR, polymerase chain reaction.
that we have used to separate effects of protein stability from those that may be due to interaction of the second domain of IL-5 with its receptor. We also created monomers stabilized by an additional disulfide bond and two circularly permuted monomeric proteins designed to introduce tighter packing of helix D with the other three helices. When the β-chain blocking mutation, E12K, was introduced into the circularly permuted constructs, monomeric IL-5 proteins were found to retain agonist activity in vitro. This is in marked contrast to the results observed for the wild type protein containing this mutation and suggests that residues other than E12K play a role in β-chain activation and that the E12K mutant may act by introducing negative interactions with the β-chain rather than removing positive interactions.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Unless otherwise stated, all chemicals were purchased from Sigma. Enzymes were from New England Biolabs, and chromatographic material was from Pharmacia Biotech Inc.

**Construction, Expression, and Purification of IL-5 Mutants**—IL-5 constructs were made using a synthetic gene coding for the mature sequence of human IL-5 (25) which begins with sequence MTEIP . . . , where T is Thr27 of the human IL-5 precursor. Residue numbering begins with the amino-terminal methionine. The single chain dimer was constructed by the insertion of a Gly residue between two copies of the synthetic human IL-5 gene using overlap PCR. PCR products corresponding to the single chain length were subcloned into two copies of the synthetic human IL-5 gene using overlap PCR. PCR chain dimer was constructed by the insertion of a Gly residue between the first residues of the insert sequence and a cysteine introduced by an I112C mutation at the carboxyl-terminal end of the protein. Insertion mutants and circular permutations were made by mega-primer and overlap extension PCR, respectively. In all cases, the genes were inserted into NcoI/HindIII-digested pET23d (Novagen) and expressed in E. coli BL21 (DE3).

After the first TF-1 proliferation bioassay, the IL-5.3 backbone was identified as producing the most active protein and the mutants IL-5.3, IL-5.4, IL-5.5, C29, and IL-5.6, which all had the loop inserted after Lys84, were subsequently fermented in 5-liter fermentors for further characterization. The proteins were purified from inclusion bodies and renatured as described for the recombinant wild type protein (15, 56). Renaturation was carried out by rapid dilution of the purified protein in 6 M guanidine/HCl into 0.1 M Tris/HCl, pH 8.5. Disulfide Bond Determination—Disulfide bond formation was determined by the analysis of the amino acid composition of the proteins after alkylation of reduced and oxidized samples and derivatization of free Cys residues with diithiobispropionic acid (37). The proteins were hydrolyzed at 112 °C for 24 h, and the analysis was carried out using the Waters AccQ.Tag Chemistry Package.

**Analysis of Apparent Molecular Weight and Aggregation State**—The proteins were analyzed by gel permeation chromatography using a SMART system equipped with a Superdex 75 column equilibrated with 0.1 M Tris/HCl, pH 8.5, containing 15 M NaCl. The column was calibrated with standards of known molecular weights, as well as recombinant IL-5 and GM-CSF. 50 µl were applied at concentrations between 35 and 150 µg/ml.

Sedimentation equilibrium analytical ultracentrifugation of native IL-5, the various IL-5 mutants, and GM-CSF was performed using a Beckman XL-A (Palo Alto, CA) centrifuge with six-channel 12-mm charcoal-filled epon centerpieces. Runs were performed at 25,000, 30,000, 32,500, and 35,000 rpm at 4 °C with scans taken at 220 or 280 nm at 1-h intervals. Equilibrium was judged to be achieved by the absence of change between plots of several successive scans after approximately 20 h. 100 µl of each sample in 100 mM Tris/HCl, pH 8.0, was centrifuged against 120 µl of the equivalent buffer blank. Solvent density was determined empirically at 4 °C using a Mettler DA-110 density-specific gravity meter calibrated against water. The partial specific volume of each protein was calculated using the method of Cohn and Edsall (38). Temperature differentials were incorporated using the appropriate equation modified from values of each amino acid at 25 °C (39). Data sets were obtained as radial distance versus absorbance and later converted to concentration units using an empirically derived extinction coefficient. Raw data were analyzed by the Beckman/Microcal Origin non-linear regression software package using multiple iterations of the Marquardt-Levenberg algorithm (40) for parameter estimation or by global fitting routines kindly provided by the National Analytical Ultracentrifuge Facility at Storrs, CT.

**Analysis of Secondary Structure**—Circular dichroism (CD) spectral analysis was performed using an Aviv model 62DS CD spectropolarimeter. Proteins were scanned repetitively in 0.1-cm quartz cuvettes from 199 to 340 nm in 1-nm wavelength increments. Ellipticity was converted to molar ellipticity for comparisons.

**Thermal Stability Determination Using Circular Dichroism**—Thermal transitions were analyzed with the CD instrument described above by monitoring the proteins at 222 nm over a temperature range of 5–95 °C. Data were collected in 1 °C increments with a slope of 10 °C/min. The half-point of the thermal transition, Td, was determined by iterative fitting using the Boltzmann equation. Data were fitted to the following thermodynamic model (see Equations 1 and 2).

**RESULTS**

**Protein Design**—Increasing the length of the loop between helices C and D of IL-5 to resemble the loop of GM-CSF allows IL-5 helix D to fold back onto its parent monomer (Fig. 1). The amino acid sequences used for creating the monomeric IL-5s are depicted schematically in Fig. 2. In each case 8 amino acids, corresponding to the loop separating helices C and D in GM-CSF, were introduced into the analogous position in IL-5. The sequence (S/C)PPTEPTWS which corresponds to residues Ser160 to Ser178 of GM-CSF, just after Gln81 (IL-5.1 and 5.2), just following Lys84 (IL-5.3 and 5.4), or in place of Lys82-Lys84 (IL-5.5 and 5.6). In the even numbered constructs (IL-5.2, IL-5.4, and IL-5.6), two cysteine residues have been inserted to allow a potentially stabilizing disulfide bond to form between the first residues of the insert sequence and a cysteine introduced by an I112C mutation at the carboxyl-terminal end of the protein. Insertion mutants and circular permutations were made by megaprimer and overlap extension PCR, respectively. In all cases, the genes were inserted into NcoI/HindIII-digested pET23d (Novagen) and expressed in E. coli BL21 (DE3).

Circular dichroism (CD) spectral analysis was performed using an Aviv model 62DS CD spectropolarimeter. Proteins were scanned repetitively in 0.1-cm quartz cuvettes from 199 to 340 nm in 1-nm wavelength increments. Ellipticity was converted to molar ellipticity for comparisons.
dilution from the guanidine denaturant, a process which was not feasible for the wild type dimer. However, the yield using this procedure was lower than the longer protocol for the dimeric protein, which was therefore used for scale up purifications. The introduction of the charge reversal mutation at the \( \beta_c \)-chain binding residue, Glu\(^{12} \), to Lys, in the wild type dimeric IL-5 protein, caused an approximately 20-fold decrease in the expression level (lane 3, Fig. 3). However, when this mutation was introduced into the IL-5.cT29 and IL-5.cT63 circularly permutated constructs, the expression level was significantly higher than that observed for the E12K mutation in the wild type protein.

Correct folding of the purified proteins was established by CD spectroscopy. CD spectra (200–300 nm) indicated that the helical content of all the monomeric constructs was very similar to the dimeric protein (Fig. 4). Introduction of the charge reversal mutation, corresponding to E12K in the IL-5 sequence, into the circularly permutated constructs does not appear to perturb the overall secondary structure elements, as the spectrum for IL-5.cT63(E12K) overlaps well with the other spectra as shown in Fig. 4.

Disulfide bond formation was measured by analysis of amino acid composition. Fig. 2. Schematic representation of the monomeric constructs. The helices A, B, and C are shown as open boxes, and helix D as a shaded box. The \( \beta_c \) contact residue, Glu\(^{12} \), is shown in bold. The positions at which the loop has been introduced are indicated.

Fig. 3. SDS-polyacrylamide gel electrophoresis analysis of the expression of monomers compared with wild type IL-5 in *E. coli*. Lane 1, protein standards, molecular masses indicated; lane 2, wild type IL-5; lane 3, IL-5(E12K); lane 4, IL-5.3; lane 5, IL-5.cT29; lane 6, IL-5.cT29(E12K); lane 7, IL-5.cT63; lane 8, IL-5.cT63(E12K).

- Fig. 1. Molecular models of IL-5, GM-CSF, and monomeric IL-5. A, super-position of GM-CSF onto one monomeric domain of IL-5. GM-CSF is depicted in green, and the IL-5 subunits are shown in light and dark blue. The intermolecular disulfide bonds of IL-5 are shown in yellow, and the intramolecular disulfides of GM-CSF in red. B, model of the monomeric IL-5.4 construct, which is based on the IL-5.3 backbone with the eight amino acid loop (shown in green) inserted after Lys\(^{84} \). The disulfide linking Cys\(^{46} \) to Cys\(^{83} \) and the additional disulfide created by the introduction of a Cys as the first residue of the loop and the I112C mutation are shown in red.

\(^2 \) A. Proudfoot, unpublished results.
acid composition. All of the constructs had one disulfide bond. The three constructs, IL-5.2, IL-5.4 and IL-5.6, which had an additional pair of Cys residues introduced with the aim of forming a disulfide bridge analogous to the second disulfide bond in GM-CSF, were found to effectively contain this second disulfide. Nonreducing SDS-polyacrylamide gel electrophoresis demonstrated that there was no formation of inter-molecular disulfide bonds (results not shown).

The quaternary state of the IL-5 constructs was analyzed by size-exclusion chromatography and analytical ultracentrifugation. When subjected to gel filtration at concentrations around 1 mg/ml (approximately 70 μM), the proteins eluted at the volume observed for IL-5 indicating that they were associating as dimers (data not shown). However, at concentrations of 150 μg/ml or less (<10 μM), the proteins co-eluted with GM-CSF, indicating that a weak monomer-dimer self-association was occurring at concentrations well above the concentrations at which IL-5 exhibits biological activity. To further refine these observations, analytical centrifugation was employed to determine dissociation constants (K_d) for these interactions (Table I). The insertion of the loop after Gln 81 in IL-5.1 and IL-5.2 produced proteins that had the least tendency to dimerize, since they had K_d values of 400 μM, whereas the other monomers analyzed had dissociation constants between 20 and 60 μM. The circular permutations shared intermediate dissociation constants, IL-5.cT29 having a K_d of 200 μM and IL-5.cT63 a K_d of 67 μM. In each case the K_d for dimerization is far greater than the concentrations at which biological activity was measured.

Biological Activity—The single chain protein was almost equipotent to wild type IL-5 in the TF-1 proliferation assay, where it had an EC50 of 2.8 pM compared with 1.6 pM for the wild type. Introduction of a single R90A or E109A mutation at the dimer interface of wild type IL-5 with Glu did not prevent dimerization since both the single T42E and N43E mutations as well as the double T42E/N43E mutants all showed a K_d of 40 μM. This suggests that the dimerization process in these mutants may involve a difference in the quaternary packing compared with the wild type protein.

The stability of four monomeric constructs determined by thermal denaturation followed by CD showed that the self-folding monomers were significantly less stable that the parent IL-5 dimer and GM-CSF monomer. The T_1/2 values for IL-5.3, IL-5.cT29, and IL-5-cT63 were 58.8, 51.6, and 55.9 °C respectively, compared with 71.1 °C for IL-5 and 71.4 °C for GM-CSF. However, the introduction of the second disulfide inferred increased stability to the protein, as demonstrated by the T_1/2 of 64.9 °C obtained for IL-5.4.

| TABLE I |
| Biological properties and physico-chemical characterization of the monomeric IL-5 constructs |
| Construct | Bioassays | Receptor binding | Aggregation, ultracentrifugation K_d (μM) | Stability, T_1/2 (°C) |
| Wild type | TF-1 proliferation | eosinophil adhesion | α-Chain (SPA) | αβ complex (TF-1 cells) | Dimer | 71.1 |
| GM-CSF | 17 ± 9 | 44 ± 10 | 11 ± 4 | 5.3 ± 1.8 | 84 ± 28 | 25 ± 8 | 78 ± 32 | Partial | 15 ± 2.1 | 796 ± 105 | 1295 ± 194 | 67 | 51.6 |
| IL-5.1 | 33 ± 13.8 | 7.4 ± 1.3 | 33 ± 13.8 | 3.4 ± 1.3 | 71 ± 16 | 135 ± 16 | 200 | 55.9 |
| IL-5.2 | 33 ± 13.8 | 3.4 ± 1.3 | 910 ± 220 | 1170 ± 280 | 532 ± 95 | 1032 ± 166 | 1413 ± 157 | 40 |
| IL-5.3 | 33 ± 13.8 | 3.4 ± 1.3 | 71 ± 16 | 135 ± 16 | 200 | 55.9 |
| IL-5.4 | 33 ± 13.8 | 3.4 ± 1.3 | 910 ± 220 | 1170 ± 280 | 532 ± 95 | 1032 ± 166 | 1413 ± 157 | 40 |
| IL-5.5 | 33 ± 13.8 | 3.4 ± 1.3 | 71 ± 16 | 135 ± 16 | 200 | 55.9 |
| IL-5.6 | 33 ± 13.8 | 3.4 ± 1.3 | 910 ± 220 | 1170 ± 280 | 532 ± 95 | 1032 ± 166 | 1413 ± 157 | 40 |
| IL-5.5Q43E | 34 ± 2 | 18.6 | 29.6 | 40 |
| IL-5.5L43E | 34 ± 2 | 18.6 | 29.6 | 40 |
| Q42EL43E | 34 ± 2 | 18.6 | 29.6 | 40 |
positions corresponding to the second subunit gave EC\textsubscript{50} values of 3.2 and 2.7 pM, respectively. When these mutations were made in the wild type protein, where by definition both copies of the amino acid residue were mutated, there was significant loss of potency with EC\textsubscript{50} values for the induction of TF-1 proliferation of 60 pM for R90A and 200 pM for E109 (Fig. 5).

The insertion of the 8 amino acid loop that enabled IL-5 to fold as a monomer resulted in proteins that elicit full biological activity in TF-1 proliferation, with a single exception, IL-5.cT29, where the protein was a partial agonist in the range of concentrations tested. The results are summarized in Table I. The most favorable position for insertion of the loop was after Lys\textsubscript{84}. IL-5.3 showed the highest activity in the TF-1 proliferation assay, with an 11-fold decrease compared with the wild type. This backbone was therefore chosen for the design of the circular permutations. The effects of inserting the loop after Gln\textsubscript{42} or by replacing Lys\textsubscript{82}-Lys\textsubscript{84} are small with respect to biological activity, as IL-5.1 and IL-5.5 had 17- and 44-fold increases in EC\textsubscript{50} values in the TF-1 proliferation assay, respectively. Although the creation of the second disulfide bond was favorable in terms of stability, these more rigid conformations were not advantageous with respect to bioactivity; IL-5.2, IL-5.4, and IL-5.6 had EC\textsubscript{50} values 3–8-fold higher than their parent constructs. The circular permutations similarly were active in this assay, where IL-5.cT63 had an EC\textsubscript{50} 33-fold higher than the wild type, but IL-5.cT29 was consistently only a partial agonist. Mutation of the polar residues Gln\textsubscript{42} and Thr\textsubscript{43} to Glu into the IL-5.5 backbone had little effect in this assay.

Eosinophil activation was used as a second in vitro bioassay. The monomers were more active in their capacity to induce eosinophil adhesion when compared with wild type IL-5 than in the TF-1 proliferation assay. IL-5.3 had an EC\textsubscript{50} of 5.3 nM and is only 4-fold less active than wild type IL-5, which has an EC\textsubscript{50} of 1.3 pM, and again the second disulfide introduced in IL-5.4 results in an approximately 4-fold drop in activity, with an EC\textsubscript{50} of 20 pM. Both circular permutation constructs exhibited full agonist activity in the induction of eosinophil adhesion, and in fact IL-5.cT63, with an EC\textsubscript{50} of 3.4 pM, was almost as potent as the wild type protein.

Receptor binding assays showed that the reduction in affinity of the monomeric proteins for either the recombinant α-chain or the αβ complex was significantly larger than the reduction in potency in biological assays. The IC\textsubscript{50} values for competition of \textsuperscript{125}I-IL-5 were between 100- and 1000-fold higher than the wild type IL-5. IL-5.3 had an IC\textsubscript{50} 140-fold larger than the wild type in the SPA assay for α-chain binding, which competes for \textsuperscript{125}I-IL-5 with an IC\textsubscript{50} of 1.2 nM and a 210-fold increase for competition for the αβ complex, where wild type IL-5 competes for \textsuperscript{125}I-IL-5 with an IC\textsubscript{50} of 0.16 nM. The introduction of the second disulfide into this backbone in IL-5.4 caused an additional 10-fold reduction in both binding assays. Of the two circular mutations, IL-5.cT63 showed a 10-fold greater affinity to both the α-chain and to the αβ complex than IL-5.cT29 compared with wild type IL-5. IL-5.cT63 had a 70-fold lower affinity for the α-chain and 135-fold lower for the αβ complex, whereas IL-5.cT29 showed decreases in affinity of 800 and 1300, respectively.

The introduction of the β\textsubscript{1} contact residue Glu\textsubscript{12} to Lys\textsubscript{84} mutation in the circular permutations did not abrogate agonist activity in either TF-1 proliferation or the induction of eosinophil adhesion, contrary to the results obtained by this mutation in the wild type protein (Figs. 6 and 7). IL-5.cT63(E12K) was a partial agonist in both assays, whereas IL-5.cT29(E12K) showed partial agonist activity in the TF-1 proliferation assay but was a full agonist with an EC\textsubscript{50} of 910 nM in the induction of eosinophil adhesion. The introduction of this mutation into IL-5.cT29 had very little effect on its binding to the receptor, whereas IL-5.cT63(E12K) showed a 15-fold decrease in affinity for the α-chain and a 10-fold increase in IC\textsubscript{50} for the competition of \textsuperscript{125}I-IL-5 from the αβ complex on TF-1 cells compared with their parent constructs.
The residues of IL-5 contributing to binding the specific \( \alpha \)-chain of the IL-5 heterodimeric complex have been identified by extensive alanine scanning mutagenesis studies (24, 25) and used to define the spatial location of essential groups, or pharmacophore, of human IL-5. These residues are located in the carboxyl-terminal region, a region previously shown to be responsible for the specificity between human and murine species (43). Glu^{88} and Arg^{90} are located on the \( \beta \)-sheet linking the third and fourth helices, and Glu^{109} and Trp^{110} are located toward the distal end of the fourth helix. In the three-dimensional structure solved for the \( E. coli \) protein (16), Trp^{110} is buried. Mutation of Trp^{110} to Ala is therefore thought to affect the orientation of Glu^{109}. However, the approximate distances between the other functional side chains can be determined. Glu^{88} and Arg^{90} are separated by 8 Å, and both are 26 Å from Glu^{109} on the same subunit but also from the Glu^{109} residue located on the other subunit. Because the original mutagenesis studies were carried out on the wild type protein, it is impossible to differentiate which Glu^{109} is involved in the pharmacophore.

Several lines of evidence have indicated that both four helix bundle domains may be not essential for bio-activity. First, the \( \alpha \)-chain binds to the IL-5 dimer with a 1:1 stoichiometry as demonstrated using the recombinant receptor in an \textit{in vitro} binding assay (22). Second, the construction of fully active single chain IL-5 proteins has allowed single copy mutations of the residues involved in \( \alpha \)-chain binding (32, 33, and this work). We have shown here that at least for TF-1 proliferation, despite the close proximity in the three-dimensional structure of the Glu residues located at the distal ends of the fourth helices, receptor activation requires only one of the Glu^{109} residues. Similarly, only one of the Arg^{90} residues is required. Since we were unable to produce IL-5 in \( E. coli \) bearing the E88A mutation, we did not investigate the effect of producing it in single copy. Several combinations of alanine mutations of both the \( \alpha \)-and \( \beta \)-chain binding sites have been made (32, 33), and these mutants were analyzed for their effects on binding to the \( \alpha \)-chain and their capacity to induce TF-1 proliferation. Although mutation of the \( \alpha \)-chain binding site residues in single copy lowers affinity for binding to the receptor, a factor attributed to increases in the dissociation constant, \( k_{d0} \), the effects on TF-1 proliferation are small suggesting that a single domain is sufficient for biological function.

IL-5 has been induced to fold with a monomeric topology by extension of the loop linking the third and fourth helices in a manner analogous to GM-CSF (34) as depicted in the model shown in Fig. 1. However, this protein was shown to have a 15-fold lower activity as measured by TF-1 proliferation compared with wild type dimeric IL-5, and little is known about the stability and oligomerization of the monomeric protein. We were interested to see if a fully active monomer could be formed by improving the packing of the fourth helix in the IL-5 monomer. The fourth helix carries one of the essential \( \alpha \)-chain binding sites, Glu^{109}, but has also been shown important in maintaining the integrity of the four helix bundle structure. Successive removal of the first two helical turns in the dimeric protein causes significant losses in activity, which are correlated to extensive changes in the structure, rather than the loss of the residue Glu^{109} (44). Monomeric IL-5 was made by inserting a sequence encoding the loop between GM-CSF helices C and D, Ser^{105} to Ser^{112}, into the analogous location of IL-5 in a region corresponding to the junction of exons 3 and 4 in IL-5. To obtain maximal packing of helix D, we engineered a disulfide bond designed to maintain a close packing of the helix D in the four helix bundle, as is found in GM-CSF. Finally, we made two circular permutations of the monomeric IL-5. This results in covalent attachment of helix D to the beginning of helix A.

It is obvious from the previously published report and this work that the principal factor required to achieve activation of the IL-5 receptor complex is the packing of the four helices into the “cytokine fold” so that the \( \alpha \)- and \( \beta \)-chain binding sites are correctly oriented to interact with their respective receptor subunits. Subtle effects can be seen on the positioning of the loop into the primary IL-5 sequence. Insertion of the loop after Lys^{84} resulted in the most active protein, since it was 11-fold less active than the wild type in TF-1 proliferation and 5-fold less in eosinophil adhesion. Using this backbone to create the circular permutations, one, IL-5.cT63, was only 3-fold less active than wild type IL-5 in the eosinophil adhesion assay. In general, higher activities for all the constructs were observed in the eosinophil adhesion assay than in the TF-1 proliferation. It could be reasoned that in view of their lower stability compared with the native dimer, the short incubation time of 30 min in the adhesion assay is more favorable than the 3-day assay of TF-1 proliferation, during which time protein destabilization and degradation could easily occur.

The receptor binding assays may also reflect the lower stability of the monomeric proteins, presumably due to a suboptimal packing. Although their overall conformation resembles that of IL-5 as demonstrated by circular dichroism, the monomers are over 100 times less efficient at competing for \( ^{125}I \)-IL-5 (with a single exception, IL-5.cT63 which has a 70-fold increase in IC_{50}) on both the \( \alpha \)-chain and the \( \alpha \beta \) complex. Equilibrium competition of the iodinated ligand from the receptor may be considered to be more demanding on structure,
in view of the fact that activity is triggered by picomolar concentrations, whereas competition occurs at nanomolar concentrations.

By introducing the charge reversal E12K into the IL-5 sequence, we have produced a potent antagonist of both TF-1 proliferation and eosinophil adhesion (30), but we were hampered in our attempts to continue our studies in animal models of allergic disorders by the difficulty of producing the protein in E. coli. Attempts to express this mutant at a high level in a baculovirus expression system were similarly unsuccessful. We were therefore interested in using the monomeric scaffolds, in particular the circular permutations where the Glu residue in question was no longer proximal to the amino terminus of the sequence, as a means of obtaining large amounts of the antagonist. Although the permuted proteins possess the characteristics necessary to confer IL-5 activity, introduction of this mutation surprisingly did not abolish activity. We had previously made the observation that the E12K mutation in the wild type protein, while creating a potent antagonist against IL-5-induced TF-1 proliferation and eosinophil adhesion, retained the ability to induce eosinophil survival, albeit with a 50,000-fold reduction in potency (30). This suggested that in the eosinophil there may be separate signaling pathways involved in adhesion and survival. Moreover, there may be other residues that are involved in activating and triggering the βγ-chain or, alternatively, that the α-chain itself may signal in the induction of eosinophil survival. However, in the two constructs described in this work bearing this charge reversal mutation activity is achieved in the two functional assays, TF-1 proliferation and induction of eosinophil adhesion, for which the wild type E12K mutant was devoid of activity.

We believe that these results support the hypothesis that the Glu residue on the first helix is not the only point of contact required for βγ activation. This argument is strengthened by the fact that mutations of the Glu residues identified as being βγ contact points for GM-CSF and IL-3, also on the first helix, have been found to retain their capacity as agonists. In the case of IL-3, the charge reversal mutation E21R retains agonist activity to induce TF-1 proliferation, with a 20,000 reduction in potency (45). The mutation E22A in murine GM-CSF likewise is devoid of agonist activity and produces a 200,000 reduction in activity to induce TF-1 proliferation and eosinophil adhesion, for which the wild type E12K mutant was devoid of activity.

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Spatial Orientation of the $\alpha$ and $\beta_c$ Receptor Chain Binding Sites on Monomeric Human Interleukin-5 Constructs

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