Sequence randomization through functional phage display of single chain human interleukin (IL)-5 was used to investigate the limits of replaceability of the Glu\textsuperscript{110} residues that form a part of the receptor-binding epitope. Mutational analysis revealed unexpected affinity for IL-5 receptor α chain with variants containing E110W or E110Y. Escherichia coli-expressed Glu\textsuperscript{110} variants containing E110W in the otherwise sequence-intact N-terminal half, including a variant with an E110A replacement in the sequence-disabled C-terminal half, were shown by their CD spectra to be folded into secondary structures similar to that of single chain human IL-5 (scIL-5). Biosensor kinetics analysis revealed that (E110W/A5)scIL-5 and (E110W/A6)scIL-5 had receptor α chain binding affinities similar to that of (wt/A5)scIL-5. However, (E110W/A6)scIL-5 had a significantly reduced bioactivity in TF-1 cell proliferation compared with (E110W/A5)scIL-5 and (E110W/A6)scIL-5, and this activity reduction was disproportionately greater than the much smaller effect of Glu\textsuperscript{110} mutation on receptor binding affinity. The marked and disproportionate decrease in TF-1 proliferation observed with (E110W/A6)scIL-5 suggests a role for Glu\textsuperscript{110} in the biological activity mediated by the signal transducing receptor β chain subunit of the IL-5 receptor. This is also consistent with the lack of stimulation of JAK2 phosphorylation by the (E110W/A6)scIL-5 mutant in recombinant 293T cells, as compared with the concentration-dependent stimulation seen for scIL-5. The results reveal the dispensability of charge in the Glu\textsuperscript{110} locus of IL-5 for receptor α chain binding and, in contrast, its heretofore underappreciated importance for receptor activation.

The cytokine interleukin-5 (IL-5)\textsuperscript{1} plays a key role in controlling the maturation, proliferation, and activation of eosinophils, which have been implicated in the pathogenesis of asthma and other allergic inflammatory diseases associated with hypersensitivity reactions in the lung (1–3). IL-5 exerts its biological functions through binding to a heteromeric cell surface receptor composed of two types of subunit, α and β, each containing a glycosylated extracellular domain with repeating fibronectin-like sequences, a single transmembrane domain, and a cytoplasmic domain. The α chain is IL-5-specific, although structurally related to the α chains for GM-CSF and IL-3 receptors. The β chain is identical to the β chains of GM-CSF and IL-3 receptors (4, 5), hence the denotation β for common β. The extracellular domain of α subunit can bind to IL-5 in the absence of β chain (4, 6, 7). Human α chain alone binds IL-5 with a K\textsubscript{D} of 0.3–0.6 nM when expressed in COS7 cells (6). That β subunit also contributes to ligand binding (7, 8) is indicated by several findings, including the increased (2–4 fold) hIL-5 binding affinity when α and β chains are co-expressed (9). However, direct binding of IL-5 to β alone has never been quantitated. The β subunit is essential for signaling.

IL-5 is a member of a broad family of structurally related cytokines that contain a helical bundle core (10). The crystal structure of the IL-5 dimer shows it is composed of a pair of four-helix bundles (11, 12). Each bundle is similar to the growth hormone fold (13) found in many cytokines including IL-2 (14), IL-4 (15, 16), GM-CSF (17), macrophage CSF (18), and granulocyte CSF. In IL-5, helix D of one polypeptide chain combines with helices A, B, and C of a second, identical chain by domain swapping (19) to form the stable dimer.

Understanding of the IL-5 receptor activation process is still incomplete but is advancing. A growing body of evidence suggests that helices A (residues 11–31) and D (residues 93–118) of IL-5 both contribute important components to receptor binding. Evidence obtained in our own work by site-directed mutagenesis (20–22) as well as in other reports (23, 24) confirms the involvement of residues in the C-terminal D helix region of human IL-5 (in particular Glu\textsuperscript{110}) and nearby residues in the CD loop (in particular Glu\textsuperscript{13} and Arg\textsuperscript{21}) in receptor α chain interaction. Evidence for involvement of the IL-5 A helix in recruiting β has been obtained with a chimeric protein containing an N-terminal segment (residues 5–29) of mouse IL-5 and the remaining sequence from human GM-CSF (25). A role for Glu\textsuperscript{13} of the A helix in biological activity but not α chain interaction has been shown by mutagenesis of wild type IL-5 (23, 24) and of scIL-5 (21). The Glu\textsuperscript{13} of IL-5 is analogous to the functionally important Glu\textsuperscript{22} in IL-3 and Glu\textsuperscript{2} in GM-CSF. This is consistent with a role of Glu\textsuperscript{13} in β chain interaction. The CD loop and Glu\textsuperscript{110} compose an epitope around the four-helix bundle interface of the IL-5 cylinder, whereas the Glu\textsuperscript{13} stimulating factor; GM-CSF, granulocyte-macrophage CSF; PAGE, polyacrylamide gel electrophoresis.

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1 The abbreviations used are: IL, interleukin; hIL, human interleukin; wt, wild type; scIL, single chain human interleukin; shIL-5R, soluble human interleukin-5 receptor α chain; Fe, Fe chimeras; ELISA, enzyme-linked immunosorbent assay; cfu, colony-forming units; mAb, monoclonal antibody; PBS, phosphate-buffered saline; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage CSF; PAGE, polyacrylamide gel electrophoresis.

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residues are at the distal ends of the cylinder (Fig. 1). A view has arisen in which receptor α chain recruitment occurs around the four-helix bundle interface, whereas β, recruitment leading to signaling occurs at the distal ends (26).

Although site-directed mutagenesis studies have identified important residues for receptor recruitment and activation, the one-residue-at-a-time substitution allowed by this technique limits a full mechanistic understanding of what specific structural and electrostatic features of the IL-5 surface are required for receptor binding. Such understanding can be obtained more completely by examining the replaceability of individual side chains, or sets of side chains, by all other side chain types, in other words by examining libraries of sequences containing all possible side chains in local regions of the surface and determining which combinations allow productive binding. Such random epitope mutagenesis can be achieved with sequence libraries formed by phage display. This technique has been applied successfully to

in vitro antibody maturation and protein engineering (27). Typically a foreign gene is fused in frame with phage coat protein PRII encoded by a phagemid vector. The surface displayed recombinant foreign protein can be selected by affinity selection procedures (biopanning). Human growth hormone has been displayed on phage and higher receptor affinity variants selected through random mutagenesis and phage panning (28).

We have displayed scIL-5 on phage using a two-stage construction that now allows asymmetric randomization mutagenesis of the IL-5 molecule and therefrom a deeper understanding of the IL-5-receptor recognition process (29). An asymmetrically disabled but functional scIL-5 mutant was displayed on phage. This mutant was constructed of an N-terminal half containing the original five charged residues (88EERRR92) in the CD loop combined with a C-terminal half containing a disabling CD loop sequence (88AAAAA92) in which the charged side chains of both Glu were

identifying by site-directed mutagenesis as important for α chain recruitment) are replaced. This asymmetrically disabled variant was used as a starting point to generate an asymmetric scIL-5 library in which the 88–92-residue N-terminal CD loop was randomized to identify functional IL-5 variants. From this epitope library, a receptor-binding variant of IL-5 was selected in which the only charged residue in the CD loop is Arg. The results obtained argue that the key receptor recognition element in the CD loop is an Arg residue, that the position of this charged side chain but do not induce receptor activation. We consider two mechanistic possibilities by which Glu may function in receptor activation, including its direct involvement in β, contact or alternatively its more indirect impact on β, recruitment via the mode of a chain binding that it helps to effect.

**EXPERIMENTAL PROCEDURES**

**Construction of Glu Mutants by Site-directed Mutagenesis** —The phagemid vector, pMK-wt/A5-G3 (29), was used as a starting point to prepare different Glu mutants. Two working vectors, pCR-IL-5A and pCR-IL-5B encoding one copy of IL-5 sequence, have been used for asymmetric mutation as reported previously (29). To generate mutations in Glu position, the vector of Glu in pCR-IL-5A was mutated to 19 other amino acids using a QuickChange site-directed mutagenesis kit (Stratagene). The combination of mutated Glu in the N-terminal half and a wild type Glu in the C-terminal half of scIL-5 yielded an asymmetric mutant phagemid, designated pMK-E110/A5-G3. X represents all residues with the exception of glutamic acid. The resulting constructs were verified by DNA sequencing.

**Phage ELISA for Glu Mutant Binding to shIL-5Rα-Fc** —E. coli XLI Blue cells (Stratagene, La Jolla, CA) harboring pMK-E110/A5-G3 or pMK-scIL-5-G3 were grown overnight in 100 ml of super broth medium in the presence of M13 VCS helper phage (Stratagene). Phage pellets were resuspended in PBS buffer as described (29). Phage titers, defined as carbencillin-resistant-colony-forming units/ml (cfu/ml), were determined according to Zhong and Smith (30).

**PRO-BIND ELISA plates (Falcon) were coated with 100 μl (10 mg/ml) of shIL-5Rα-Fc (31). Protein-charged ELISA plates were incubated with 100 μl of phage samples in PBS buffer for 1 h at 37 °C, and the wells then were washed with PBS plus 0.5% Tween 20. Phage binding was detected with a 1:2500 dilution of horseradish peroxidase-conjugated goat anti-M13 IgG (Amersham Pharmacia Biotech) and color development with 3,3',5,5'-tetra-methylbenzidine dihydrochloride. This reagent was dissolved in 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.03% sodium perborate (Sigma). The reaction was measured by absorbance at 450 nm. Phage, which did not carry any insert, was used as background control.

Competitive phage ELISA on microtiter plates coated with shIL-5Rα-Fc (29) were performed to confirm the relative receptor binding affinities of different phage selectants. The experiments were carried out according to Jones et al. (32). A concentration of phage sample, predetermined to elicit 60% signal in titration assays, was incubated with different concentrations of soluble IL-5Rα, ranging from 0 to 100 nM. These phage mixtures were then added to the shIL-5Rα-Fc coated wells. Following incubation, microtiter plates were washed thoroughly, and bound phage were detected with horseradish peroxidase-conjugated anti-phage antibody (1:9000 dilution). ECvalues were determined by fit to a four-parameter equation to determine the concentration of competing shIL-5Rα that resulted in half-maximal phage binding.

**Expression and Purification of scIL-5 and Glu Mutants from E. coli** —The phagemid vector pHage 3, which is derived from M13, was used for protein expression (Maxim Biotech, Inc., San Francisco, CA). Transcription was under the control of the lac promoter. This vector contains an Amber stop codon before the insertion, was used as background control.

**Kinetic and equilibrium constants for the interaction between hIL-5Rα and Glu 110 mutants was analyzed by SDS-10% polyacrylamide gel electrophoresis (PAGE) according to Laemmli (33).**

**Kinetic Analysis of Receptor Binding by E. coli-expressed scIL-5** —Kinetic and equilibrium constants for the interaction between IL-5Rα and Glu 110 mutants was analyzed by SDS-10% polyacrylamide gel electrophoresis (PAGE) according to Laemmli (33).
and E. coli-expressed scIL-5 or Glu\(^{110}\) mutants were measured using a BIACORE X optical biosensor (Biacore Inc., Piscataway, NJ). The monoclonal antibody 4A6 was first immobilized onto the biosensor chip (31). The expressed scIL-5 or Glu\(^{110}\) variants from bacterial supernatants were anchored noncovalently to the antibody. The binding of various concentrations of soluble hIL-5Rα-Fc (12) to the antibody-anchored scIL-5 or mutant protein was then measured. Alternatively, soluble hIL5Rα-Fc was directly immobilized on the sensor chips, and scIL-5 variants were used as analytes. Conditions for immobilization and the sensor assay were the same as those described by Morton et al. (20). For the calculation of rate constants, the association and dissociation phases of sensograms obtained for a series of soluble hIL-5Rα-Fc or scIL-5 variant at different concentrations were fitted to a 1:1 Langmuir model, \(A + B \leftrightarrow AB\), to yield, respectively, an association rate constant \(k_a\) and a dissociation rate constant \(k_d\). Data analysis was conducted using the BIAcore evaluation software version 3.0. The equilibrium dissociation constant, \(K_D\), was determined from ratios of \(k_d/k_a\) (35).

**Cell Proliferation Assay for Biological Activity—**Biological activity was measured using a subclone of a highly responsive subclone of the human erythroleukemia cell line TF-1 (21) and an assay with 3,4-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (Thiazolyl blue assay) (36). Cells were cultured in RPMI 1640 medium supplemented with 1-glutamine, penicillin-streptomycin, and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). Plates with 96 flat-bottomed wells were seeded with 5000 cells/well and incubated for 48 h in a humidified incubator at 37 °C. Media were changed every 48 h. Concentrations of scIL-5 or mutant proteins were determined by Western blot analysis and by quantitative IL-5 ELISA using the monoclonal antibodies 4A6 and TRFK-5 (R & D Systems).

**Circular Dichroism—**Measurements were made on an Aviv 62A DS Circular Dichroism Spectrometer in a 0.1-cm water-jacketed cuvette. Solution conditions were 10 \(\mu\)M protein (pH 7.4), 20 mM sodium phosphate, and 150 mM NaCl at 25 °C. Spectra shown are the averages of three scan runs at 24 nm/min with a 5-s response time. Thermal stabilities of scIL-5 constructs were evaluated by monitoring the ellipticity at 222 nm. Temperature was increased in each case at a rate of 30 °C/h.

**JAK2 Phosphorylation Assays—**293T cells were a gift from Martin Carroll (Department of Hematology/Oncology, University of Pennsylvania). Cells were maintained in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum. Cloning of the cDNA for IL-5Rα, \(\beta\), and \(\delta\)HAK2 DNA samples by using CaPO\(_4\) method. After 48 h, cells were harvested and analyzed by immunoprecipitation and flow cytometry for receptor expression.

**RESULTS**

**Phage Display of Glu\(^{110}\) scIL-5 Variants**—The two glutamic acid 110 residues in the IL-5 dimer form a negatively charged patch on the surface of the cytokine (Fig. 1) and have been proposed from previous Ala replacement mutagenesis studies to be a key part of the pharmacophore for receptor \(\alpha\) chain binding that also has been proposed to include Glu\(^{89}\) and Arg\(^{91}\). Recent sequence randomization study of the CD loop region of IL-5 (29) has led to a significant reevaluation of how Glu\(^{89}\) and Arg\(^{91}\) participate in \(\alpha\) chain binding. The current study was undertaken to more deeply understand the role of Glu\(^{110}\) through a similar randomization approach.

To perform a rapid and efficient randomization of Glu\(^{110}\) in the N-terminal domain of (wt\(\alpha\))-scIL-5, an asymmetric construct reported previously (29), we combined site-directed mutagenesis and phage display technology to screen the IL-5 receptor \(\alpha\) chain binding ability. Phage particles displaying Glu\(^{110}\) scIL-5 variants were detected by mAb 4A6 immobilized on microtiter plates (29). These assays were used to verify that the Glu\(^{110}\) variants were displayed as proteins on the phage particles and accessible for receptor binding. Fig. 2A shows the phage ELISA results of this initial phage screening for receptor \(\alpha\) chain binding. To measure relative binding affinities of Glu\(^{110}\) phage variants, phage titrations were used, as suggested before (38), to identify phage concentrations that produced a similar signal for all 20 Glu\(^{110}\) variants. Representative curves are shown in Fig. 2B. Clearly E110D, E110W, and E110Y stand out as having relatively higher binding affinity. Hydrophobic residue replacements show a poor or nonexistent
binding affinity versus the other mutated forms. Competitive phage ELISA was carried out to further evaluate the relative receptor binding affinities for selected Glu\textsuperscript{110} variants. The shIL-5R binding affinities of phage displayed (E110W/A5)scIL-5 and (E110Y/A5)scIL-5 were determined by this assay, giving EC\textsubscript{50} values of 21.2 ± 0.4 and 19.6 ± 0.3 nM, respectively (Fig. 3).

Expression of Glu\textsuperscript{110} Mutants in E. coli—To confirm the binding affinities inferred from phage ELISA, we expressed and purified Glu\textsuperscript{110} mutants for bioactivity and kinetic analysis. The phage vector pH3-(E110X/A5)scIL-5, in which scIL-5 and Glu\textsuperscript{110} mutants were fused to a Gene III leader sequence under the control of a lac promoter, was engineered for protein expression. Soluble scIL-5 protein was detected in the supernatant after isopropyl-\(\beta\)-D-galactopyranoside induction, as expected for recombinant proteins that are expressed and secreted into the periplasm. The construction of (E110W/A5)scIL-5 is shown in Fig. 4A. The purified, E. coli-expressed scIL-5, (wt/A5)scIL-5 and Glu\textsuperscript{110} mutant proteins were analyzed by SDS-PAGE, revealing a single band of approximately 30 kDa (Fig. 4B). Unfortunately, (E110Y/A5)scIL-5 mutant was not sufficiently stable during the expression and purification process to allow for further characterization. Because of the unexpected receptor \(\alpha\) chain binding activity detected with (E110W/A5)scIL-5, we also constructed and produced (E110W/A6)scIL-5, as shown in Fig. 4A. In this construct, the Glu\textsuperscript{110} in the domain with CD loop mutated is replaced by Ala, resulting in a protein with no negative charges in the Glu110 locus (Figs. 1 and 4A). This additional mutant was expressed and purified similarly as the A5 mutant (Fig. 4B).

Protein Stabilities of Glu\textsuperscript{110} Mutants—We directly compared the secondary structures and stabilities of E. coli expressed scIL-5 mutants by their CD spectra. As shown in Fig. 5A, the similarity of CD for scIL-5, (wt/A5)scIL-5, (E110W/A5)scIL-5 and (E110W/A6)scIL-5 indicates that the Glu\textsuperscript{110} variants likely are folded into secondary structures similar to that of scIL-5. The shapes of the CD spectra are consistent with large \(\alpha\)-helical contents, consistent with the known structure of IL-5 as a four-helix bundle protein (11). Thermal stabilities of Glu\textsuperscript{110} scIL-5 mutants were evaluated by monitoring the effects of increasing temperature on molar ellipticity at 222 nm (Fig. 5B). The melting profiles show that the E110W mutants have a melting temperature similar to that of (wt/A5)scIL-5, although the progressive decrease in negative ellipticity for the E110W mutants, especially (E110Y/A6)scIL-5, suggest some instability in these proteins at lower temperatures (31). Importantly, though, all of the mutants were predominantly folded into helix-rich conformations at the temperatures used for subsequent receptor binding and bioactivity assays, namely 25 and 37 \(^{\circ}\)C, respectively.

Receptor Binding Activities of Glu\textsuperscript{110} Mutants—Kinetics of receptor binding were determined using a sandwich biosensor assay that measured binding of shIL5R to antibody-anchored scIL-5 or Glu\textsuperscript{110} mutants. Representative sensograms
FIG. 4. Glu\textsuperscript{110} scIL-5 variants and their expression in E. coli. A, construction of scIL-5 and Glu\textsuperscript{110} scIL-5 asymmetric variants. The gene of the single chain IL-5 was constructed by linking two hIL-5 genes in tandem separated by a spacer that encoded the dipeptide Gly-Gly (21). The N-terminal half of the molecule is defined as IL-5A, and the C-terminal half is defined as IL-5B. An asymmetric scIL-5 mutant, denoted (wt/A5)scIL-5, was designed composed of an N-terminal half containing the original five charged residues (88EERRR\textsuperscript{6}) in the CD loop combined with a C-terminal half containing a functionally disabling CD loop sequence (88AAAAA\textsuperscript{6}). This variant was used as a parent construction to prepare Glu\textsuperscript{110} scIL-5 variants with the Glu\textsuperscript{110} position randomized in the IL-5A domain. The last sequence, (E110W/A5)scIL-5, was constructed as a consequence of results of the IL-5A domain Glu\textsuperscript{110} randomization survey. It contains an E110A mutation in the IL-5B domain. B, expression of single chain IL-5 and Glu\textsuperscript{110} variants in E. coli visualized by Coomassie Blue-stained 10% SDS-PAGE. Lane 1, wild type scIL-5 protein; lane 2, (wt/A5)scIL-5 protein; lane 3, (E110W/A5)scIL-5 protein; lane 4, (E110W/A6)scIL-5 protein; lane 5, protein standards (Bio-Rad). Positions of molecular mass markers are indicated at right. Each lane contained 2–4 \( \mu \)g of protein.

![Fig. 4](image)

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for (E110W/A5)scIL-5 and (E110W/A6)scIL-5 are shown in Fig. 6 (A and B). For all cases, sensorgrams were obtained at a series of shIL5R-Fc concentrations, ranging from 25 to 100 nM. The calculated kinetic values are summarized in Table I. There was no significant difference in receptor binding affinity among (wt/A5)scIL-5, (E110W/A5)scIL-5, and (E110W/A6)-scIL-5; the \( K_d \) values determined were 8.4 ± 0.8, 4.1 ± 0.4, and 5.0 ± 0.2 nM, respectively. The IL-5R-Fc affinities for these asymmetric mutants, with a C-terminal half containing a disabled CD loop sequence (88AAAAA\textsuperscript{6}) missing the key recognition residues (Fig. 4A), were all lower by close to an order of magnitude than that for scIL-5 protein, with the major difference being the dissociation rate (Table I). This reduced binding is reminiscent of that found before for monomeric versus wild type IL-5 and may reflect the fact that all of the asymmetric mutants have only a single functional four-helix bundle rather than the two of wild type or scIL-5.

In an alternative methodology, the kinetics of binding of scIL-5 variants to shIL5R-Fc was examined with the receptor immobilized directly on the sensor chips of an optical biosensor. The ability of Glu\textsuperscript{110} scIL-5 variants to induce signal was measured by TF-1 cell proliferation. There was no significant difference in TF-1 proliferation activity between (E110W/A5)scIL-5 and (wt/A5)scIL-5 (Fig. 7). Surprisingly, though, the data show an abruptly reduced signaling activity in the mutant protein having no Glu\textsuperscript{110}. In the cases of (wt/A5)scIL-5, (E110W/A5)scIL-5, and (E110W/A6)scIL-5, the

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The affinities obtained with this alternative configuration do suggest a modestly gradual weakening of affinity upon losing the charges of the Glu\textsuperscript{110} residues. However, the differences in affinity are small among the Glu\textsuperscript{110} mutants and (wt/A5)scIL-5. Spectra shown are the average of three scans run at 24 nm/min with a 5-s response time. O, scIL-5; \( \bullet \), (wt/A5)scIL-5; \( \triangle \), (E110W/A5)scIL-5; \( \blacklozenge \), (E110W/A6)scIL-5. B, thermal stabilities of scIL-5 constructs monitored by ellipticity at 222 nm. Temperature was increased in each case at a rate of 30 °C/h. Solid line, scIL-5; dashed line, (wt/A5)scIL-5; dotted line, (E110W/A6)scIL-5.

![Fig. 5](image)

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Table I. The affinities obtained with this alternative configuration do suggest a modestly gradual weakening of affinity upon losing the charges of the Glu\textsuperscript{110} residues. However, the differences in affinity are small among the Glu\textsuperscript{110} mutants and (wt/A5)scIL-5. Overall, the biosensor results confirm that the receptor α chain binding affinities of (wt/A5), (E110W/A5), and (E110W/A6) forms of scIL-5 are quite similar. The higher affinities seen with the assay of IL-5Rα-Fc binding to 4A6-anchored IL-5 than with IL-5 binding to directly immobilized IL-5Rα-Fc are likely due to the bivalency of the analyte (IL-5Rα-Fc) in the former configuration versus the monovalency of the analyte (IL-5) in the latter.

Signal Transduction—The ability of Glu\textsuperscript{110} scIL-5 variants to induce signal was measured by TF-1 cell proliferation. There was no significant difference in TF-1 proliferation activity between (E110W/A5)scIL-5 and (wt/A5)scIL-5 (Fig. 7). Surprisingly, though, the data show an abruptly reduced signaling activity in the mutant protein having no Glu\textsuperscript{110}. In the cases of (wt/A5)scIL-5, (E110W/A5)scIL-5, and (E110W/A6)scIL-5, the
Functional Role of Glu\textsuperscript{110} in IL-5 Receptor Activation

Concentrations of (E110W/A5)scIL-5 (analyte) to IL-5R\textsubscript{a}-Fc that was covalently immobilized on the sensor surface. The increase in response represents the binding of scIL-5; the decay represents the dissociation of bound scIL-5 upon washing with running buffer alone. Association rates and dissociation rates were evaluated separately using a 1:1 Langmuir binding model in BIAcore evaluation software version 3.0. The best fit curves are shown as lines. For dissociation, the initial 25 s of data were fitted using the 1:1 model. D, sensorgrams and data fits for binding by various concentrations of (E110W/A6)scIL-5 to sensor-immobilized IL-5R\textsubscript{a}-Fc. Experimental protocol is as described for C.

**DISCUSSION**

In this study, we have used epitope randomization to identify the limits of sequence replaceability of Glu\textsuperscript{110} for IL-5 function. Randomization on phage displayed scIL-5 showed that replacements by E110Y and E110W, residues without negatively charged side chains, were only fractionally less effective than E110D in inducing IL-5Ra binding, despite the previously held view (20, 21) that Glu\textsuperscript{110} charge formed a key component for receptor \( \alpha \) chain recognition. When E110W mutations were expressed in soluble proteins, more quantitative measurement of binding properties than achievable with phage displayed proteins confirmed the effectiveness of E110W mutants in IL-5Ra binding. However, Trp replacement abruptly reduced bioactivity, except when Glu\textsuperscript{110} was present in the neighboring four-helix bundle domain. These results argue that the negative charge at Glu\textsuperscript{110} or the hydrogen bonding network provided by the carboxylate of Glu\textsuperscript{110} is dispensible for \( \alpha \) chain binding but is important for receptor activation. The data further argue that Glu\textsuperscript{110} can function in receptor activation from either four-helix domain, that is either within-domain or cross-domain. To what extent flexibility in the residue 110 side chain is important in enabling the interactions between IL-5 and receptor subunits that lead to receptor activation cannot be determined by the current data.

The acceptability of E110W for IL-5Ra binding, for example in (E110W/A6)scIL-5, contrasts with the significant loss of such binding activity with dual E110A replacement seen previously in wild type IL-5 (20, 21). This could suggest that the Trp side chain contributes stabilizing contacts which are lost upon Ala replacement. Two possibilities are \( \pi \)-stacking and hydrogen bonding. The former as a sole explanation seems unlikely as judged by the lower replaceability of Glu\textsuperscript{110} for IL-5 function. When E110W mutations were given the lower apparent efficacy of Ser and Thr in replacing Glu\textsuperscript{110} (Fig. 2). The unique acceptability of W and Y suggests that perhaps it is a combination of hydrogen bonding and \( \pi \)-stacking that may enable effective \( \alpha \) chain binding by the mutants. Obviously, \( \pi \)-stacking is not available with the native sequence Glu\textsuperscript{110}, whereas hydrogen bonding is. In this work, bioactivity was measured in two ways, mainly by proliferation of TF-1 cells and also by phosphorylation of JAK2 in recombinant 293T cells for one of the key mutants. For the mutant case compared, (E110W/A6)scIL-5, suppression of bioactivity was noted by concentrations of (E110W/A5)scIL-5 (analyte) to IL-5R\textsubscript{a}-Fc that was covalently immobilized on the sensor surface. The increase in response represents the binding of scIL-5; the decay represents the dissociation of bound scIL-5 upon washing with running buffer alone. Association rates and dissociation rates were evaluated separately using a 1:1 Langmuir binding model in BIAcore evaluation software version 3.0. The best fit curves are shown as lines. For dissociation, the initial 25 s of data were fitted using the 1:1 model. D, sensorgrams and data fits for binding by various concentrations of (E110W/A6)scIL-5 to sensor-immobilized IL-5R\textsubscript{a}-Fc. Experimental protocol is as described for C.
The rate constants $k_{\text{on}}$ and $k_{\text{off}}$ are from BLAcore optical biosensor assays (Fig. 6); the equilibrium dissociation constant $K_{d}$ is calculated from the ratio of $k_{\text{off}}/k_{\text{on}}$. TF-1 cell proliferation activities are given as the concentrations required for half-maximal stimulation. The binding parameters and EC$_{50}$ values given are the means ± S.D. of three determinations.

| Variant          | mAb 4A6-anchored scIL-5 proteins bind to IL-5Rα-Fc | scIL-5 proteins bind directly to immobilized IL-5Rα-Fc | Proliferation assays |
|------------------|-------------------------------------------------|--------------------------------------------------|--------------------|
|                  | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_{d}$ | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_{d}$ | EC$_{50}$ |
| scIL-5           | 1.5 ± 0.1  | 1.0 ± 0.2  | 0.7 ± 0.1 | 6.3 ± 0.1  | 10.3 ± 0.9 | 16.3 ± 1.4 | 36.3 ± 0.9 |
| (wt/A5)scIL-5    | 1.0 ± 0.1  | 8.4 ± 0.3  | 8.4 ± 0.8 | 0.4 ± 0.1  | 6.3 ± 0.8  | 157.5 ± 44.2 | 140.3 ± 17.1 |
| (E110W/A5)scIL-5 | 1.1 ± 0.1  | 4.5 ± 0.2  | 4.1 ± 0.4 | 0.4 ± 0.05 | 12.2 ± 2.2 | 305.0 ± 66.9 | 154.5 ± 10.6 |
| (E1110W/A6)scIL-5| 1.0 ± 0.04 | 5.0 ± 0.1  | 5.0 ± 0.2 | 0.2 ± 0.03 | 9.5 ± 2.1  | 475.0 ± 126.9 | 2835.3 ± 97.8 |

Fig. 7. Bioactivity of E. coli-expressed scIL-5 and Glu$^{110}$ variants. The human IL-5-dependent cell line TF 1.28 was used to test the activity of scIL-5 proteins to induce proliferation. 5000 cells/well were incubated with various dilutions of scIL-5 and variants. After 48 h of incubation, proliferation was evaluated as described under “Experimental Procedures.” The percentages of proliferation activity are the averages of results of triplicate wells. C, scIL-5; ○, (wt/A5)scIL-5; △, (E1110W/A5)scIL-5; ▲, (E1110W/A6)scIL-5.

Both assays. Intriguingly, though, the activity observed with the nonmutated scIL-5 was distinctly different in dose response dependence. For cell proliferation, the EC$_{50}$ was in the pM range, in keeping with the previously found discrepancy from the nM affinity of IL-5 for human receptor $\alpha$ and even $\alpha + \beta_c$ (9). This discrepancy has been interpreted to suggest that cell proliferation response can occur at fractional receptor occupancy, likely because of amplification of binding through the signaling cascade. In contrast, that the JAK2 phosphorylation response was in the nM range suggests that this early step in the signaling cascade is triggered with a more 1:1 linkage to receptor occupancy. This suggests that the amplification of signal leading to pM proliferation EC$_{50}$ values occurs beyond the JAK2 phosphorylation step.

The overall results of this work argue that the negative charge at position 110 plays a role in receptor activation. Two possible mechanisms may be envisioned: (i) Glu$^{110}$ makes a direct contact with $\beta_c$ in recruiting the latter into an activation complex with IL-5 and IL-5R$\alpha$ and (ii) Glu$^{110}$ participates in the orientation of R$\alpha$ into an alignment that is productive for recruitment and activation of $\beta_c$ through interactions other than at Glu$^{110}$. It is not possible to distinguish between these two possibilities by the current data. However, the latter seems more likely, because the former would require close overlap of R$\alpha$ and $\beta_c$ binding residues at the four-helix bundle interface. One might ask why, if mechanistic choice II were operative, mutations with no Glu at 110 were not observed to have lower Re binding affinity (Table I). One reason could be the possibility raised above that Trp might compensate for loss of negative charge interaction through a combination of $\pi$-stacking and hydrogen bonding. That the negative charge at Glu$^{110}$ can play a role in $\alpha$ binding affinity in the absence of such compensating interactions is consistent with the lower binding efficacy effected by simple Ala replacements, in E110A wtIL-5 (20). Intriguingly, the possibility that Glu$^{110}$ plays a role in receptor activation was hinted by the nonlinearly reduced bioactivity versus receptor $\alpha$ chain affinity of this E110A wtIL-5 mutation (20).

The results of this work have significant implications for designing IL-5 antagonists of potential use in asthma and other eosinophilia-associated disease (1–3). The heretofore held view that the receptor $\alpha$ chain-binding epitope was distributed over residues Glu$^{50}$, Arg$^{91}$, and Glu$^{110}$ (26) has suggested that the receptor $\alpha$ chain-binding epitope is large and hence that antag-
ozing this interaction with a small molecule would have a low likelihood of success. However, the current data suggest that the Glu\textsuperscript{89}-Arg\textsuperscript{91}-Glu\textsuperscript{110} epitope is divisible into two parts and that each may provide an opportunity for local contact disruption. In this view, interfering with the local CD loop contacts with receptor α chain might be sufficient for effective antagonism of receptor α chain binding. Alternatively, interfering with the Glu\textsuperscript{110} local epitope might interfere sufficiently with β, recruitment so that even though a chain binding might persist bioactivity would not result. The combined mutations of Glu\textsuperscript{110} and Glu\textsuperscript{13} the latter of which has previously been shown to lead to IL-5 antagonism, may produce a more effective IL-5 antagonist than achievable by mutation of either Glu position alone.

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