Dual asymmetric mutagenesis of single-chain interleukin 5 (scIL5) was used to obtain evidence that the normally homodimeric IL5 molecule, which contains two 4-helix bundle domains arranged symmetrically about a 2-fold axis, can recruit receptor α and βc subunits asymmetrically. Functionally active scIL5 was constructed using recombinant DNA methods by linking two IL5 monomers with a Gly-Gly linker. Mutants were constructed at residues Arg91, Glu110, and Trp111, previously shown to be involved in IL5 receptor α chain binding, and at residue Glu13, known to be involved in signal transduction presumably through interaction with the receptor βc chain. Mutants were examined for receptor α chain binding by an optical biosensor assay and for bioactivity using a cell proliferation assay. Substitution of the two binding site residues R91 and W111 in the same 4-helix bundle domain caused a 5-fold greater reduction in receptor binding affinity than when the two substitutions were distributed one in each domain. Substitution of E13 and R91 either in the same or in opposite domains gave comparable IL5Rc chain binding kinetics, essentially unchanged from those of scIL5. However, in contrast to the binding affinity pattern observed with R91A/W111A dual mutants, distributing the E13A/R91A mutations between the two 4-helix bundle domains caused a 5–6-fold greater loss of bioactivity than when the two changes were in the same domain, leaving the other domain unaltered. Taken with previous mutagenesis data, these results are consistent with a single shared-site model of IL5-IL5Rc chain recognition in which a single α chain can orientate in either of two modes, each one of which is stabilized preferentially by one of the two 4-helix bundles of IL5. Furthermore, the results suggest that a single βc molecule is activated for each IL5, through the Glu13 residue on the same helix bundle domain that dominates the IL5Rc interaction.

Human interleukin 5 (hIL5)1 is a disulfide-linked homodimeric protein that plays an important role in the differentiation and activation of eosinophils (1, 2). The high resolution crystal structures of both Escherichia coli-expressed (3) and deglycosylated, Drosophila-expressed hIL5 (4) show a core of two 4-helix bundles. Each 4-helix bundle resembles the 4-helix bundle first seen in bovine growth hormone (5) and more recently found for the functionally related but monomeric GM-CSF (6).

Human IL5 functions by interacting with a receptor system (hIL5R) composed of two different chains, denoted α and βc (7). The α chain has high affinity (≈Kd) and specificity for IL5 (8). In contrast, the βc (or common β) chain of hIL5R is not cytokine-specific but is instead shared with the receptors for IL3 and GM-CSF (7) and is required for signal transduction. Distinct sites have been observed for α and βc interaction on the surface of IL5. Hybrid constructs of mouse/human IL5 suggest that structural elements in the C-terminal 36-residue sequence of IL5 (which includes the helix D) interact directly with IL5Rc and confer species specificity (9). Extensive mutagenesis studies show that residues clustered in the CD loop and the amino-terminal end of helix D engage in receptor α chain binding (10–13). All these residues are around the dimer interface. In contrast, Glu13, which is at the distal ends of the IL5 dimer away from the helix bundle interface, has been suggested to interact with the βc chain of IL5Rc, since mutation at this position results in loss of biological activity but does not affect the binding affinity to α chain (10, 11). The effectiveness of Glu13-substituted IL5 mutants as antagonists of biological activity is consistent with a sequential receptor subunit binding scheme, whereby the cytokine initially binds the specific receptor α chain followed by interaction with βc to initiate signal transduction. That tight αβc complexes do not pre-form also is suggested from the observation of cytokine cross-competition using IL3 and GM-CSF to block IL5 activity (14).

The dual subunit composition of hIL5Rc, together with the observation that both dimeric IL5 and monomeric GM-CSF can trigger the same βc, led us to investigate the topological layout of receptor binding sites on IL5 that effect interaction and consequent bioactivity. A key observation has been that, despite the dimeric nature of IL5, the binding stoichiometry between soluble IL5 receptor α chain (shIL5Ra) and hIL5 is 1:1, that is, one receptor α chain bound per dimeric IL5 (4, 15). Since energetically important residues for α chain binding are distributed around the central interface between the two 4-helix bundles (12), the binding site for α chain could contain residues from both bundle surfaces, thus constituting a single central shared site. However, mutagenesis of wild-type IL5 (wtIL5) inevitably leads to symmetrical mutations of both bundles, thus restricting more high resolution topological mapping using the wild-type protein.

We recently reported creation of a single-chain form of human IL5 (scIL5), in which two IL5 molecules were tandemly linked by a peptide linker (16). Importantly, scIL5 and wtIL5 had equivalent biological activity and binding affinity for shIL5Ra. The design of functionally active scIL5 allowed asym-
metric mutagenesis of the symmetrical IL5 molecule. Through asymmetric mutagenesis, we obtained preliminary data to suggest that the single receptor α chain binding site on hIL5, that is positioned around the interface of the two four-helix bundles of the cytokine, uses residues from the two bundles in an asymmetric fashion (16). More recently, Dickason and Huston (13) described a biologically active IL5 monomer (mono 5) in which the length of the CD loop of hIL5 was increased to that of monomeric GM-CSF. The results with mono 5 showed that a single 4-helix bundle domain of IL5 contains sufficient structure to induce biological function. However, the IL5 monomer had about a 1 order of magnitude lower level for EC_{50} (13) and lower affinity than wtIL5 (17). The lower affinity of “mono 5” leaves open the possibility that both domains of IL5 are involved in receptor interaction in wtIL5. In the present work, we sought to investigate further the topology of the IL5Rα binding site on IL5 by making dual asymmetric Ala substitutions at residues E110, R91, E110, and W111. Residues R91, E110, and W111 have been identified as important residues for receptor α chain binding by extensive mutagenesis studies (10–12, 16), while E13 is the only residue so far identified to be important for bioactivity but not receptor α chain binding. We report here the results of these mutation studies with scIL5 and the insights gained into the composition of the binding sites for hIL5α and -αβ.

MATERIALS AND METHODS

Gene Expression—Construction of the COS expression vector pCDN-IL5(sc) containing the hIL5 coding sequence was described before (12, 16). COS-1 cells were transfected with this vector using DEAE-dextran (18) and grown in serum-free medium. Cell-free supernatants were collected after 3 days and stored at 4°C. Levels of expression were measured by Western blot using polyclonal anti-hIL5 antibodies.

Mutagenesis—Site-directed mutagenesis was first carried out on pCDN-IL5 by a “cassette mutagenesis” method (19). To make a single site mutation in scIL5, the mutant pCDN-IL5 was digested with BglII and ligated with the 350-base pair BglII fragment of pCDN-IL5(sc). To make multiple mutations in the scIL5, single mutations were first made individually in pCDN-IL5 or pCDN-IL5(sc), and then combined by restriction digestion and ligation. Presence of desired mutations was verified by DNA sequencing. The mutants made in this study were: R91A(a); R91A(b); W111A(b); R91A(a)/W111A(b); R91A(b)/W111A(b); R91A(a)/E110A(b); R91A(b)/E110A(b); E13A(a)/R91A(b); and E13A(b)/R91A(b). The N-terminal half of scIL5 is defined as (a), the C-terminal half as (b). For example, R91A(a)/W111A(b) has R91 replaced by A on the N-terminal half of scIL5, and W111 replaced by A on the C-terminal monomer of scIL5. The rest of the mutants are named analogously.

Receptor Binding Analysis of Mutants in Crude Expression Supernatants—Kinetic and equilibrium constants for the interaction between hIL5α and different forms of hIL5 were measured using an SPR optical biosensor (BIAcore, Pharmacia Biosensor, Uppsala) in an assay similar to that described previously (12). The expressed hIL5 from COS supernatants was anchored noncovalently but tightly to the non-neutralizing antibody 24G9 (20), and the binding of various concentrations of shIL5Rα to the attached hIL5 was then measured. The mutants studied here were bound to the anchoring antibody as tightly as wild-type hIL5 and dissociated only very slowly from the antibody within the time of each run of the assay. This slow dissociation had no significant affect on the binding analysis (12). Conditions for the assay were the same as those reported previously (12), except that the flow rate was changed to 50 μl/min in the dissociation phase of the IL5-shIL5Rα complex. The linear portions of the association and dissociation phases of sensorgrams obtained for a series of shIL5Rα concentrations were analyzed to give k_{on} and k_{off}, K_{d} values were determined from ratios of k_{off}/k_{on}.

Biological Activity Assays—Biological activity was measured using a subclone of the human erythroblast cell line TP-1 (subclone TP1-28), which is highly responsive to recombinant hIL5. The assay was described previously (16). Data were fitted to a four-parameter logistic curve (Grafit 3.0), and EC_{50} values were calculated. Results are given as the mean of three to six determinations. Coefficients of variation were <25%. Concentrations of wtIL5, scIL5, and mutants were determined by a combination of Western blot analysis (12) and quantitative IL5 enzyme-linked immunosorbent assay using the monoclonal antibodies 24G9 and TRFK-5 (R & D Systems, Minneapolis).

RESULTS

Expression of IL5 Mutants—COS cell supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with anti-hIL5 antiserum (12). As shown in Fig. 1, all mutants were secreted into the supernatant and had the same molecular weight (~34 kDa) as scIL5. COS-expressed wtIL5 homodimer also had the same molecular weight as scIL5 (16). In addition, all mutants were recognized by a panel of anti-IL5 monoclonal antibodies, demonstrating their structural integrity (data not shown).

Asymmetric Mutagenesis of scIL5 Residues Affecting IL5Rα Binding—We previously reported single-site Ala mutants of residues R91, E110, and W111 showing that, when only one residue of a given type is substituted in scIL5, affinity for Rα binding is far more than half that when both residues are mutated simultaneously (16). In the present study, we used these three key binding residues to construct different mutation combinations distributed over one or both domains of scIL5. The locations of these residues in the tertiary structure of IL5 are shown in Fig. 2. The binding kinetics of the interactions between shIL5Rα and mutants were measured by a sandwich biosensor assay (Fig. 3, Table I).

Mutants R91A(a), R91A(b), and W111A(b), which have only one residue substituted in the dimer, showed a small decrease (2–3-fold) in receptor affinity (Table I). In contrast, substitution of R91A or W111A in both domains gave ~40-fold reduction in receptor affinity. The much greater affinity decrease seen with a double mutant versus a single site mutant (e.g. R91(a,b) versus R91(a)) suggests a nonadditive role for these residues in stabilizing the IL5-receptor complex.

When substitutions R91A and W111A were combined, either in one domain R91A(b)/W111A(b) or in two different domains R91A(a)/W111A(b), markedly different receptor binding properties were obtained (Fig. 3 and Table I). R91A(b)/W111A(b), despite having one of its two 4-helix bundle domains unmodified, showed a weaker affinity (37 nM) than R91A(a)/W111A(b) (6.9 nM). This strongly suggests that the receptor-binding site is constituted of residues from both domains rather than from a half molecule.
Dual asymmetric mutants were also made for the combination of residues R91 and E110. In contrast to the results obtained with mutants R91A(a)/W111A(b) and R91A(b)/E110A(b), the $K_d$ values of R91A(a)/E110A(b) and R91A(b)/E110A(b) were similar (Table I). However, R91A(a)/E110A(b) and R91A(b)/E110A(b) showed 3- and 2-fold further reduction in $K_d$, respectively, versus the $K_d$ of their corresponding single mutants R91A(a) and R91A(b) (Table I). In other words, replacing residue E110 had an additional effect whether it was in the same domain in which the R91A mutation resides or whether instead it is on the other domain from that containing R91A. This suggests that both E110 residues at the dimer interface contribute to the binding of receptor α chain and is also consistent with the model that the receptor-binding site is constituted of residues from both domains. If the receptor only interacts with one domain of IL5, R91A(b)/E110A(b) should have a higher affinity than R91A(a)/E110A(b) since the former has an undisrupted domain while the latter does not.

Effect of Asymmetric Mutagenesis on the Biological Activity of IL5—The TF1 cell proliferation assay was used to measure the biological activity of IL5 mutants. It was reported previously that residue E13 is not involved in IL5Rα binding activity, but is required for biological activity (10, 11). However, there was only a ~2–3-fold decrease in biological activity when one E13 residue was mutated to Ala in scIL5 (16). To test whether binding of IL5Rα caused activation of βγ through the E13 on the same domain which forms the major part of the receptor α chain binding site, we made combinations of asymmetric mutations of R91 and E13. As shown in Table I, E13A(a)/R91A(b) and E13A(b)/R91A(b) had the same receptor α chain binding kinetics, with $K_d$ values being almost the same as that for wtIL5. However, the biological activities of these two mutants differed significantly (Fig. 4 and Table I). Mutant E13A(a)/R91A(b), where changes were made in only one 4-helix bundle, showed no reduction in bioactivity. Mutant E13A(b)/R91A(b), which had substitutions made in different bundles, showed a 6-fold decrease in activity.

DISCUSSION

We began this study with a preliminary hypothesis that the receptor α chain binding site in IL5 is contributed dominantly by helices A and D and, reflecting the 1:1 receptor:IL5 binding stoichiometry, that the α receptor binding site involves both 4-helix bundle components of the IL5 homodimer (16). We were also aware of recent work showing that monomeric IL5 is at least partially functionally active (13, 17) and that hence only a single 4-helix bundle domain is required for bioactivity. Hence, we were interested in delineating more fully the extent to which the symmetrical IL5 may bind receptor subunits asymmetrically to produce a biological effect. We made several dual-residue asymmetric mutants of scIL5. In one case, we made dual mutants wherein both residues substituted were receptor α chain binding residues, such as R91 and W111. In a
The mutants contain denoted substitutions on both chains of homodimeric IL5 (16).

In A, in the rate constants $k_{on}$ and $k_{off}$ are from a BIAcore optical biosensor assay of shIL5Rα binding; the equilibrium dissociation constant $K_d$ is calculated from the ratio of $k_{off}/k_{on}$. Data were obtained from two individual experiments. TF-1 activity is given as the concentration required for half-maximal stimulation of TF-1 cell proliferation. Asymmetric mutants contain denoted substitutions in either the amino-terminal IL5 domain or the carboxyl-terminal IL5 domain (b). In B, related wtIL5 mutant binding data obtained previously with the IAsys biosensor.

| IL5 form       | $k_{on}$ $(M/s \times 10^3)$ | $k_{off}$ $(s^{-1} \times 10^{-3})$ | $K_d$ (nM) | Bioactivity of TF-1 |
|----------------|-------------------------------|--------------------------------------|------------|---------------------|
| A.             |                               |                                      |            |                     |
| wt IL5         | 7.9 ± 0.5                     | 2.0 ± 0.1                            | 2.5 ± 0.1  | 2                   |
| scIL5          | 7.9 ± 0.5                     | 1.8 ± 0.1                            | 2.3 ± 0.4  | 3                   |
| R91A(a)        | 3.9 ± 0.2                     | 2.7 ± 0.1                            | 6.9 ± 0.5  | 5                   |
| R91A(b)        | 5.1 ± 0.07                    | 2.3 ± 0.1                            | 4.5 ± 0.4  | 6                   |
| W111A(a)       | 5.4 ± 0.3                     | 3.2 ± 0.2                            | 5.9 ± 0.5  | 4                   |
| R91A(a)/W111A(a) | 4.3 ± 0.5                | 2.9 ± 0.0                            | 6.7 ± 0.7  | 9                   |
| R91A(b)/W111A(b) | 2.6 ± 0.2                  | 9.7 ± 0.3                            | 37 ± 0.2   | 10                  |
| R91A(a)/E110A(b) | 3.9 ± 0.0                 | 5.2 ± 0.3                            | 13 ± 1     | 5                   |
| R91A(b)/E110A(b) | 3.0 ± 0.3                  | 3.5 ± 0.2                            | 12 ± 2     | 5                   |
| E13A(a)/R91A(b) | 8.2 ± 0.3                  | 2.2 ± 0.1                            | 2.7 ± 0.2  | 3                   |
| E13A(b)/R91A(b) | 7.4 ± 1.6                  | 2.3 ± 0.2                            | 3.1 ± 0.9  | 17                  |

| B.             |                               |                                      |            |                     |
| wtIL5          | 7.2                           | 3.0                                  | 4.2        | 2                   |
| E110A          | 3.8                           | 18.5                                 | 49         | 15                  |
| W111A          | 2.9                           | 45.9                                 | 158        | 48                  |
| R91A           | 13.6                          | 220                                  | 161        | 181                 |
| E13A           | 9.9                           | 4.1                                  | 4.1        | 75                  |

Note: The two substitutions were made in different 4-helix bundle domains.

The above results lead to a model of asymmetric receptor recruitment by IL5 that is shown in Fig. 5. Here, the α receptor binds to the single shared α chain site, which straddles the 4-helix bundle interface, but it can bind to this site in two alternative modes, favoring contact with either one or the other 4-helix bundle. Overlapping contact or at least physical occlusion would explain why the stoichiometry of α chain binding does not exceed 1:1. Both α chain binding modes are identical with respect to stabilizing contacts on the IL5 surface, since the two 4-helix bundle domains of IL5 are arranged symmetrically about the dyad axis. For asymmetric mutants such as R91A(b) and R91A(b)/W111A(b), the two binding modes are not equivalent, with one binding mode expected to have a much lower affinity. However, no obvious heterogeneity was detected in either the association or dissociation phases of the interactions between these asymmetric mutants and the receptor α chain, likely because the weaker binding mode was unavailable after the α chain occupied the higher-affinity site.

The model in Fig. 5 also shows only one receptor βε binding at a time, though there appear to be two sites, one around each of the two Glu13 residues, which could be crucial for βε recruitment. It is proposed that βε is only recruited to bind IL5 once α is bound and that the βε site favored is the “cis” site, namely the site involving the Glu13 on the 4-helix bundle domain which predominates in contacting α-chain. This would explain why there is less bioactivity with E13A(b)/R91A(b) than with E13A(a)/R91A(b), since the former has both of its bundle surfaces disabled with respect to α and βε interaction.

A key observation leading to the present study and to the proposed model in Fig. 5 is that monomeric IL5, which is biologically active, has a somewhat lower α chain binding affinity (17). Independently, we also designed and cloned a monomeric form of IL5, in our case by inserting the peptide segment GSGSGSGG between residues 85 and 86 in the wtIL5 sequence. With this form expressed in COS cells, we measured binding to receptor α chain directly by biosensor analysis using

$^a$ The two substitutions were made in different 4-helix bundle domains.

$^b$ The two substitutions were made in the same 4-helix bundle domain.
our model that a single receptor contributed by one 4-helix bundle of IL5. This is consistent with Kcal/mol, respectively. Thus the binding energy was mainly immediately bind to the other proximal site during its dissociation.

Both a and b bundle domains; B and the IL5R interacts with residues from only one 4-helix bundle domain, and the IL5R subunits of IL5 receptor are recruited asymmetrically.

It has been suggested that b subunit dimerization might be involved in signaling (21–23). Due to the 2-fold symmetry of IL5, IL5 itself can possibly link two b chains upon activation. However, our asymmetric mutagenesis data suggest that direct b dimerization is unlikely. First, single-chain mutant E13A(b) showed only a slight decrease in biological activity (16); second, there was a significant difference in biological activities of mutants E13A(a)/R91A(b) and E13A(b)/R91A(b). The fact that E13A(a)/R91A(b) showed close to wild-type activity indicates that the binding of IL5Rα causes the binding of one b chain to the same 4-helix domain where IL5Rα binds. Again, this is consistent with the finding that mono 5 is biologically active (13).

Interestingly, the marked reduction (16-fold) in the affinity for IL5Rα observed in the asymmetric mutant R91A(b)/ W111A(b) did not result in a parallel reduction in biological activity (Table I). Such a strong discontinuity between affinity and biological activity of IL5 mutants has been seen before (11). One interpretation is that the magnitude of cell proliferation, a response to b recruitment that no doubt involves several cell surface and intracellular steps (21–23), is nonlinearly related to (and hence seemingly permissive of) some variations in the binding of IL5Rα. A future effort to measure binding of the various scIL5 mutants to αβ complex versus α alone could yield a useful insight into the mechanism underlying the discontinuity between α chain affinity and bioactivity.

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