Single Chain Human Interleukin 5 and Its Asymmetric Mutagenesis for Mapping Receptor Binding Sites*

(Received for publication, October 11, 1995)

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Wild type human (h) interleukin 5 (wt IL5) is composed of two identical peptide chains linked by disulfide bonds. A gene encoding a single chain form of hIL5 dimer was constructed by linking the two hIL5 chain coding regions with a Gly-Gly linker. Expression of this gene in COS cells yielded a single chain IL5 protein (sc IL5) having biological activity similar to that of wt IL5, as judged by stimulation of human cell proliferation. Single chain and wt IL5 also had similar binding affinity for soluble IL5 receptor α chain, the specificity subunit of the IL5 receptor, as measured kinetically with an optical biosensor. The design of functionally active sc IL5 allowed asymmetric mutagenesis of the symmetrical IL5 molecule. Such mutagenesis was exemplified by changes at residues Glu-13, Arg-91, Glu-110, and Trp-111. The receptor binding and bioactivity data obtained are consistent with a model in which residues from both IL5 monomers interact with the receptor α chain, while the interaction likely is asymmetric due to the intrinsic asymmetry of folded receptor. The results demonstrate a general route to the further mapping of receptor and other binding sites on the surface of human IL5.

Human interleukin 5 (hIL5) is a T cell-derived cytokine which plays an important role in the differentiation, proliferation, and activation of eosinophils (Sanderson et al., 1992; Bentley et al., 1992). Natural hIL5 is a disulfide-linked, homodimeric glycoprotein with 115 residues per chain. The high resolution crystal structures of both Escherichia coli-expressed (Milburn et al., 1993) and Drosophila-expressed hIL5 (Johanson et al., 1995) have revealed a core of two four-helix bundles. Each four-helix bundle resembles the four-helix bundle interface, was suggested to interact with the β chain of IL5R, since mutation at this position resulted in loss of biological activity but did not affect the binding affinity to the α chain (Tavernier et al., 1995; Graber et al., 1995).

While emerging data suggest models for the topography of receptor binding sites in hIL5, a more defined understanding is impeded by the homodimeric nature of the protein. Because of this, mutagenesis of wild type hIL5 inevitably has resulted in symmetrical changes in side chains on both sides of the 4-helix bundle interface. It is thus unclear whether one or both of the residues found to be important participate equally in receptor binding and, furthermore, whether the topology of binding is different for hIL5Rα versus hIL5Rβ. One way to overcome this limitation is asymmetric mutagenesis. Here, we report construction of an active single chain form of hIL5 dimer, denoted sc IL5, and its use to construct asymmetric mutations at residues Glu-110, Trp-111, Arg-91, and Glu-13.

MATERIALS AND METHODS

Construction of the Single Chain hIL5 COS Expression Plasmid

Construction of the COS expression vector pCDNIL5 containing the hIL5 coding sequence was described before (Morton et al., 1995). For construction of the sc IL5 gene, a DraII/BglII linker containing the amino-terminal 5 amino acids of hIL5 and 2 glycine residues was mixed with a BglII/Sad fragment (14 kilobases) from pCDNIL5, and the mixture was ligated into pCDNIL5 which had been digested with

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1The abbreviations used are: hIL5, human interleukin 5; GM-CSF, granulocyte/macrophage colony-stimulating factor; sc IL5 and wt IL5, single chain and wild type human interleukin 5, respectively; (sh)IL5Rα, (soluble) human interleukin 5 receptor α chain; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
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DraII and Sad. The product of the 3-piece ligation, called pCDN-IL5(sc), encodes sc IL5, a monomeric protein containing two tandem hIL5 sequences joined by a 2-amino acid linker, Gly-Gly. The sequence of this construction was verified directly by the dyeexoxy terminator kit from Applied Biosystems.

Mutagenesis

Site-directed mutagenesis was first carried out on pCDN-IL5 by “cassette mutagenesis” (Wells et al., 1985). To make a single site mutagenesis in sc IL5, the mutant pCDN-IL5 was digested with BglII and ligated with the 350-base pair BglII fragment of pCDN-IL5(sc). To make double mutations in single chain hIL5, PCR mutagenesis (Landt et al., 1990) was used to mutate the residue on the amino-terminal half site of sc IL5. The template for the PCR reaction is pCDN-IL5. The resulting PCR fragment was then ligated into the BglII site of mutant pCDNIL5(sc) in which the corresponding residue on the carboxyl-terminal half site had been previously mutated. Presence of the desired mutations and absence of PCR mistakes were verified by DNA sequencing.

Receptor Binding Analysis of Mutants in Crude Expression Supernatants—Kinetic and equilibrium constants for the interaction between hIL5Rα and different forms of hIL5 were measured using an IAsys optical biosensor (Fisons) in an assay similar to that described previously (Morton et al., 1995). Briefly, the non-neutralizing monoclonal antibody 24G9 (Ames et al., 1995) was first immobilized onto the sensor surface. The expressed hIL5 from COS supernatants was anchored noncovalently but tightly to the non-neutralizing antibody. The binding of various concentrations of shIL5Rα to the attached hIL5 was then measured. All mutants studied here were found to bind to the anchoring antibody as tightly as wt hIL5 and dissociated only very slowly from the antibody within the time of each run of the assay. The very slow dissociation had no significant affect on the binding analysis (Morton et al., 1996).

Biological Activity Assays: TF 1 Cell Proliferation—Biological activity was measured using a subclone of the human erythroleukemia cell line TF-1 (subclone TF-1.28), which is highly responsive to recombinant hIL5. For assay, cells were cultured in RPMI 1640 medium supplemented with l-glutamine, penicillin-streptomycin, and 10% heat-treated fetal calf serum (Life Technologies, Inc.). Plates with 96 round-bottomed wells were seeded with 5000 cells/well and incubated for 48 h in triplicate in the presence of serially diluted wt hIL5 or sc hIL5. Cultures were pulsed with 0.5 μCi of [3H]thymidine (Amersham) for the final 4 h and processed for scintillation counting. Data were fitted to a 4-parameter logistic curve (Grafit 3.0), and E50 values were calculated. Results are given as the mean of at least 3 determinations. Coefficients of variation were between 5 and 25%. Concentrations of wt hIL5, sc hIL5, and mutants were determined by Western blot analysis (Johnson et al., 1995) and by quantitative IL5 enzyme-linked immunosorbent assay using the monoclonal antibodies 24G9 and TRFK-5 (R & D Systems).

RESULTS

Design and Expression of Single Chain IL5—The crystal structure of hIL5 has shown that the carboxyl terminus of one hIL5 monomer is very close to the amino terminus of the second monomer (Milburn et al., 1993). Also, both the amino and carboxyl termini of hIL5 are exposed to the solvent and are very flexible (Milburn et al., 1993; Johnson et al., 1995). Therefore, we joined the two hIL5 monomers together covalently with a peptide linker. Since Gly residues are flexible and often found in protein β-turns (Chou and Fasman, 1978), we engineered a single chain hIL5 (sc IL5) by connecting two hIL5 monomers with a Gly-Gly linker (Fig. 1A).

sc IL5 and wt IL5 were expressed in COS cells, and the supernatants were analyzed by SDS-PAGE followed by immunoblotting with a polyclonal anti-hIL5 antisemur (Morton et al., 1995). As shown in Fig. 1B, sc IL5 had the same molecular mass (34 kDa) as that of the wt IL5 under nonreducing conditions. The levels of expression of wt IL5 and sc IL5 were similar. Under reducing conditions, sc IL5 still run as a dimer (34 kDa) while wt IL5 was reduced to two monomers (17 kDa) (Fig. 1B). The fact that hIL5 and sc IL5 had the same molecular masses under nondenaturing conditions indicated that both forms were glycosylated similarly and that no intermolecular disulfide bonds had formed in sc IL5.

Receptor Binding Activities of Single Chain IL5—Binding of shIL5Rα to antibody-anchored wt IL5 or sc IL5 was measured using a sandwich biosensor assay (Fig. 2). The linear portions of the association and dissociation phases of sensorgrams for a series of shIL5Rα concentrations were analyzed to give koff and koff as described previously (Morton et al., 1995). As summarized in Table I, the koff rates of sc IL5 were very close to those of wt IL5. The koff values of sc IL5 and wt IL5 were also similar (4.2 nm and 4.4 nm, respectively). This indicates that the single chain form of hIL5 retains full binding activity to the hIL5 receptor α chain.

The binding of COS-expressed sc IL5 and wt IL5 to the full length IL5Rα was also compared by competition for binding of 125I-IL5 to Drosophila cell membranes containing expressed IL5Rα (Johnson et al., 1995). Single chain IL5 was equally as effective as wt IL5 in inhibiting the binding of 125I-IL5 to the cell membranes (data not shown), consistent with the biosensor data, suggesting that sc IL5 and wt IL5 can bind to the same sites) of full-length IL5Rα with similar affinity.

The ability of sc IL5 to induce signal transduction was measured by cell proliferation. Single chain IL5 showed activity comparable to that of wt IL5 in the TF-1 cell assay (Table I). Overall, there were no major differences in either receptor binding or bioactivity of single chain and wild type IL5.

Asymmetric Mutagenesis of Residues Affecting IL5Rα Binding and IL5R Activation—The design of active sc IL5 made feasible asymmetric IL5 mutagenesis. We chose several residue positions for such mutations in this study to exemplify the approach. The mutations chosen were based on the crystal structure of IL5 and previous results showing a role in receptor binding or signal transduction (Devos et al., 1993; Johnson et al., 1995; Graber et al., 1995; Tavernier et al., 1995; Morton et al., 1995). These residues included Glu-110, Trp-111, and Arg-91 for IL5Rα binding and Glu-13 for signal transduction but not receptor α chain binding. When Ala residues were substituted in these positions symmetrically in the wt IL5 system, receptor binding and signal transduction activities were observed as shown in Table I. The results with the sym-
metrical mutants were consistent with previously reported data (Morton et al., 1995). Similar overall properties were observed (Table I) for symmetrical mutants made with sc IL5 for G110A and W111A, scIL5(G110A(a,b)) and scIL5(W111A(a,b)), respectively, although the extents of decrease in receptor binding and bioactivities were less for the sc IL5 mutants. The effects of asymmetric Ala mutagenesis in sc IL5 for Glu-110, Trp-111, Arg-91, and Glu-13 are shown in Table I. In all cases, with the exception of Glu-13, single site mutations in the b domain of sc IL5 led to small but finite decreases (−50%) in shIL5Rα binding activity compared with wt IL5. However, for each mutation, the reduction in binding affinity for the asymmetric construct was 4–19-fold less than that obtained with the corresponding double mutant (for example, sc IL5 (Glu-110(b)) versus scIL5 (Glu-110(a,b))). Consistent with the binding affinity data, asymmetric mutagenesis of Glu-110, Trp-111, or Arg-91 all resulted in an increased EC50 value compared with that of sc IL5, but these values were 4–30-fold less than those obtained with the corresponding double mutants.

Mutagenesis of Glu-13 in sc IL5 did not affect IL5Rα binding activity, as expected from earlier-reported results (Tavernier et al., 1995; Graber et al., 1995), but did cause a marked decrease in biological activity. The effect on activity of the single site E13A mutant in sc IL5 was nearly 10-fold less than that with the corresponding double mutant in wt IL5.

Finally, we also formed two asymmetric double mutants in the scIL5 system, namely (E13A(a), G110A(b)) and (E13A(b), G110A (b)). In the former, the mutations were in one 4-helix bundle, while in the latter, the two mutations were in different bundles. In both cases, the effects on IL5Rα binding were similar to that seen in the asymmetric E110A-alone mutants, while the decrease in bioactivity was far greater when each bundle contained one mutation than when one bundle had both while the other had none.

**DISCUSSION**

In this study, we constructed a tethered dimer of hIL5 with a two-Gly linker. This single chain hIL5 has properties very similar to native hIL5 dimer in both receptor α chain binding and biological activity. Single chain hIL5 offers an opportunity
to study the effect of changes in one of the two monomers, and hence one of the two 4-helix bundles, on properties of hIL5.

Since hIL5 has a 2-fold palindromic symmetry and binds to shIL5Rα with a 1:1 stoichiometry, we were interested in investigating whether the IL5Rα binding site is formed by one or both monomers and how the topology of binding sites for receptor α and β chains could lead to signal transduction. Previous mutagenesis studies have mapped the IL5Rα binding site to residues near the central symmetry axis of the dimer, with residues Glu-110, Trp-111, and Arg-91 being the most important (Tavernier et al., 1995; Graber et al., 1995; Morton et al., 1995). Accordingly, we made asymmetric mutations at these positions on sc IL5. Our data are consistent with a model in which residues from both monomers form a central shared patch to interact with the receptor molecule. It remains for further mutagenesis and, importantly, structural analysis of key mutants, to deduce a more refined and certain understanding of binding site topography for IL5Rα and ultimately the way this topography leads to signal transduction through IL5Rβ.

In conclusion, we have designed an IL5 system for asymmetric mutagenesis and have exemplified the approach of asymmetric mutagenesis with sc IL5. The results obtained suggest a model for IL5-IL5Rα receptor recognition in which residues from both 4-helix bundle domains contribute to the binding of a single molecule of IL5Rα, possibly by formation of a central patch as suggested previously (Morton et al., 1995). However, the receptor may use this symmetrical patch asymmetrically to stabilize the IL5-IL5Rα complex due to the asymmetry of the receptor molecule. It remains for further mutagenesis and, importantly, structural analysis of key mutants, to deduce a more refined and certain understanding of binding site topography for IL5Rα and ultimately the way this topography leads to signal transduction through IL5Rβ.

Acknowledgments—We thank Ann Breen and Robert Hertzberg for performing the radioligand assay of sc IL5 with the full-length IL5Rα. We are grateful to Ganesh Sathe, Jyce Mao, and Stephanie Van Horne for their assistance with oligonucleotide synthesis and DNA sequencing.

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Single Chain Human Interleukin 5 and Its Asymmetric Mutagenesis for Mapping Receptor Binding Sites
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J. Biol. Chem. 1996, 271:1817-1820.
doi: 10.1074/jbc.271.4.1817

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