Randomization of the Receptor α Chain Recruitment Epitope Reveals a Functional Interleukin-5 with Charge Depletion in the CD Loop

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We report the functional phage display of single chain human interleukin-5 (scIL-5) and its use for receptor-binding epitope randomization. Enzyme-linked immunosorbent assays and optical biosensor analyses verified expression of scIL-5 on the phage surface and binding of scIL-5 phage to interleukin-5 receptor α chain. Furthermore, an asymmetrically disabled but functional scIL-5 mutant, (wtASA)scIL-5, was displayed on phage. (wtASA)scIL-5-IL-5 was constructed from an N-terminal half containing the original five charged residues (EERRR) in the CD loop, including the Glu89 and Arg91 believed key in the α chain recognition site, combined with a C-terminal half containing a disabled CD loop sequence (AAAAAA) missing the key recognition residues. This asymmetric variant was used as a starting point to generate an scIL-5 library in which the intact 88–92 N-terminal CD loop was randomized. From this epilobe library, a receptor-binding variant of IL-5 was detected, (SLRGG/ASA)scIL-5, in which the only charged residue in the CD loop is an Arg at position 90. Characterization of this variant expressed as a soluble protein in E. coli shows that the IL-5 pharmacophore for receptor α chain binding can function with a single positive charge in the CD loop. Charge-depleted CD loop mimetics of IL-5 suggest the importance of charge distribution in functional IL-5 receptor recruitment.

Human interleukin-5 (hIL-5) is a T cell-derived cytokine that specifically stimulates differentiation, proliferation, and activation of eosinophils (1) and appears to play an important role in the pathogenesis of asthma. IL-5 is a homodimer that folds into a cylindrical molecule containing two four-helix bundles (2), each of which resembles the four-helix bundle seen in the monomeric cytokines IL-3, IL-4, granulocyte-macrophage colony-stimulating factor, and growth hormone.

The cell receptor for IL-5 is composed of two subunits, α and β. The α chain is cytokine-specific and by itself can bind IL-5 with high affinity (3). Extensive site-directed mutagenesis studies have shown that residues clustered near the helix bundle interface, in the CD loop, including Glu89 and Arg91, and at the carboxyl-terminal end of helix D, notably Glu110, engage in receptor α chain binding (4–6). Glu13, which is at each of the distal ends of the IL-5 cylinder away from the interface between the two four-helix bundles and is not involved directly in α chain interaction, is a key residue mediating productive interaction of the β chain of IL-5 receptor (4, 5). It is the β chain recognition site, (αβ), that leads to triggering of the intracellular signaling cascade.

Although site-directed mutagenesis studies have identified important residues for receptor binding, the one-residue-at-a-time substitution allowed by this technique limits a full mechanistic understanding of the specific structural and electrostatic features of the IL-5 surface required for receptor-ligand interaction. However, through the use of randomly generated side chain libraries, it should be possible to measure the effect of replacing individual side chains or sets of side chains in local regions on the IL-5 surface and hence determine 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 (7). Typically a foreign gene is fused in frame with phage coat protein pIII encoded by a phagemid vector, and the surface-displayed recombinant foreign protein 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 (8). Phage display of multichain proteins has also been achieved, for example for antibody Fab fragments (9) and vascular epidermal growth factor (10). Nonetheless, in our hands, phage display of the dimeric wild type IL-5 has proven difficult, perhaps reflecting confusing findings that have previously led to insoluble inclusion bodies in intracellular Escherichia coli expression (11).

We have sought to overcome limitations in phage display of IL-5 by using a single chain form of human IL-5 (scIL-5), in which two IL-5 molecules are tandemly linked by a Gly-Gly dipeptide linker (12). Single chain IL-5 and wtIL-5 have virtually identical biological activities and binding affinities for shIL-5Rα. Since scIL-5 requires the export and folding of only
one polypeptide chain, this form provides a new opportunity for *E. coli* expression and phage display. In this paper, we report the successful display of scIL-5 on filamentous phage and functional evaluation of scIL-5 fused to phage. In addition, an asymmetric mutant of scIL-5, denoted (wtA5)scIL-5, has been used as a starting point to prepare a scIL-5 library in which the N-terminal CD loop has been randomized in order to identify functional IL-5 variants of this region of the receptor α chain pharmacophore. Following affinity selection and initial characterization of *E. coli*-expressed and purified recombinant protein, we have identified a (SLRGG/A5)scIL-5 variant that retains significant affinity for IL-5 receptor α chain as well as bioactivity. The findings reflect the mutability of a key component of the IL-5 receptor pharmacophore and confirm the utility of phage display for examination of the IL-5 receptor recruitment mechanism.

**EXPERIMENTAL PROCEDURES**

**Construction of pMK-scIL-5 Expression Vector—**pCDN-IL-5 (6), which encodes human IL-5, was used as a template for the PCR to generate the IL-5 gene fragment. The primers used in this PCR reaction were as follows: primer A (forward primer), 5'-GCCATGCGATGGCCGAGCCACAGTCTGAATTG-3' (NeoI and BglII sites underlined); primer B (reverse primer), 5'-CTAGTCTAAGATCTTATCACATT-3' (BglII site underlined).

The resulting 350-bp PCR product was ligated with NeoI and XbaI and was used to replace the NeoI/SpeI fragment of pMK-IL-4-G3 to yield pMK-IL-5. Plasmid pMK-IL-4-G3 was derived by modification of pMK-GSH (13), in which the MuIII/Xbal fragment of pMK-GSH was deleted and an IL-4 coding sequence was ligated between the NeoI/SpeI sites. Phage preparations from pMK-IL-4-G3 plasmid-transformed cells specifically bind to the soluble IL-4Rα and anti-IL-4 antibodies by enzyme-linked immunosorbent assay (ELISA).^2^ The single chain IL-5 DNA was derived by BglII digestion of pCDN-IL-5-G3Hp (12). The 350-bp ScI-II fragment was ligated into the BglII site of pMK-IL-5, yielding pMK-scIL-5. pMK-scIL-5 encodes a scIL-5 protein containing two tandem IL-5 sequences linked by a Gly-Gly sequence. This scIL-5 sequence is the same as the COS-expressed scIL-5 (12) except that there are two extra amino acids (Met-Ala) at the C-terminal end of scIL-5, respectively. The vector pCR-IL-5A has NcoI sites on the 5'-end and 3'-end, respectively, of the IL-5 gene. The pCR-IL-5B vector contains the N-terminal half and C-terminal half of scIL-5, respectively. The vector pCR-IL-5A has NeoI and BglII sites on the 5'-end and 3'-end, respectively, of the IL-5 gene. The pCR-IL-5B vector has BglII and BamHI sites on the 5'-end and 3'-end, respectively, of the IL-5 gene. To generate an asymmetric CD loop mutation in scIL-5, the sequence EERRR^2^ in pCR-IL-5B was mutated to five Ala residues using a QuickChange site-directed mutagenesis kit (Stratagene). The combination of wild-type CD loop in the N-terminal half and an Ala block (A5) CD loop in the C-terminal half of scIL-5 yielded an asymmetric mutant phagemid, designated as pMK-wtA5.

**Construction of pMK-scIL-5-G3 Phagemid Vector—**In pMK-scIL-5 (above), there is a stop codon between scIL-5 and gene III. In order to construct a vector encoding the scIL-5-III fusion protein, the scIL-5 gene fragment was amplified from pMK-scIL-5 by PCR using primer A (above) and a new primer D, 5'-CTAGTCTAAGATCTTATCACATT-3' (BglII site underlined).

The resulting PCR fragment was digested with NeoI and XbaI and ligated into the NeoI and SpeI site of pMK-IL-4-G3, yielding pMK-scIL-5-G3 (12). The sequence constructed by fusing scIL-5 to pIII is as follows: Met-Ala/NeoI-SacI-SacII-Thr^3^ (El2^2^). 

**Phage Preparation—**E. coli XL1-Blue cells (Stratagene, La Jolla, CA) harboring pMK-scIL-5-G3 were grown in 100 ml of super broth medium containing 50 μg/ml carbenicillin and 1% glucose at 37 °C until the A^600^ reached 1.0. The culture was then infected with 50 μl of M13 VCS helper phage (Stratagene, La Jolla, CA). After 1 h, kanamycin was added to a final concentration of 75 μg/ml, and the culture was shaken at 250 rpm at 37°C overnight. Phage were precipitated from cell-free medium by adding one-fourth volume of 3.5 M sodium chloride, 20% polyethylene glycol 8000 (Fisher Scientific, Fair Lawn, N J), mixed thoroughly, kept on ice for at least 30 min, and centrifuged at 17,000 × g for 15 min at 4°C. Phage pellets were resuspended in 2 ml of PBS buffer (pH 7.4). Phage titers, defined as carbencillin-resistant colony-forming units per ml (cfu/ml), were determined according to Zhong and Smith (14).

**ELISA for Phage Binding to mAb 4A6 and shIL-5Ra-Fc—**PrO-BIND ELISA plates (Falcon) were coated with 100 μl (10 μg/ml) of either the anti-IL-5 monoclonal antibody 4A6, which does not neutralize the IL-5 receptor α chain interaction, or shIL-5Ra-Fc (15). 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 (PBST). Phage binding was detected with a 1:2500 dilution of horseradish peroxidase-conjugated sheep anti-M13 IgG (Amersham Pharmacia Biotech) and color development with 3,3',5,5'-tetramethyl-benzidine dihydrochloride. This reagent was dissolved in 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.03% paraformaldehyde (Sigma). The reaction was detected by reading the absorbance at 450 nm. Null phage, which did not carry any insert, were used as background control.

Competitive phage ELISA on microtiter plates coated with shIL-5Ra-Fc (20 μg/ml) were performed to confirm the relative receptor binding affinities of different phage selectants. The experiments were carried out according to Jones et al (16). A concentration of phage sample, predetermined to elicit 60% signal in titration assays, was incubated with different concentrations of soluble IL-5Ra, ranging from 0 to 500 nM. These phage mixtures were then added to the shIL-5Ra-Fc-coated microwells. Following incubation, microtiter plates were washed thoroughly, and bound phage were detected with horseradish peroxidase-conjugated anti-phage antibody (1:900 dilution). EC^50~ values were determined by fit to a four-parameter equation to determine the concentration of competing shIL-5Ra that resulted in half-maximal phage binding.

**Optical Biosensor Analysis of Phage Binding—**A BIAdorer Microsensor biosensor was used to detect receptor binding of scIL-5-Phage. IL-5 receptor α chain, shIL-5Ra-Fc, was immobilized directly onto the biosensor CM5 chip using EDC/NHS chemistry (6). Single chain IL-5 phage at 2 × 10^13^ cfu/ml were injected onto the receptor-coated surface at 2 μl/min for 4 min. Phage samples were incubated with different concentrations of soluble IL-5Ra, ranging from 0 to 1.0 μl/min, and were then passed over the IL-5-Ra-Fc surface. After this association phase, the surface was washed with running buffer, PBS plus 0.01% Tween 20, at a rate of 100 μl/min for 1 min. The net amount bound was determined as the difference in signal between points before sample injection and after the 1.0-μl buffer wash. Regeneration of the sensor surface was achieved with 10 mM HCl (pH 2.0).

**Affinity Bend Selection of scIL-5 Phage—IL-5Ra-Fc was affinity—**captured on protein A-Sepharose (Amersham Pharmacia Biotech) at a density of 2 μg of IL-5Ra-Fc/g of drained resin. scIL-5 phage and IL-4 phage were mixed at a ratio of 1:100 (based on titer) to a total titer of 1.0 × 10^14^ cfu/ml. One hundred μl of the mixed phage were incubated with 20 μl of IL-5Ra-Fc resin with constant rotation for 1.5 h at room temperature to facilitate the binding interaction. The mixture was transferred off a MultiScreen filtration system (Millipore Corp., Bedford, MA) and rapidly washed four times with 400 μl of PBS containing 0.5% Tween 20. The resin was eluted with 100 μl of 0.1 M glycine-HCl (pH 2.0) and neutralized with 25 μl of 1 M Tris-base (pH 8.0). The eluted phage samples were amplified by infection of *E. coli* XL1-Blue cells, followed by superinfection with M13 VCS helper phage. An aliquot of phage sample was used to infect XL1-Blue cells, and the transfected cells were plated on LB agar containing 50 μg/ml ampicillin. Colonies were randomly picked for DNA mini-prep of phagemid vectors. The identity of the phage was determined by restriction-digestion analysis.

**Generation of an Asymmetric CD Loop Library—**To design an N-terminal half CD loop library in which resides Gua^6^, Gua^8^, Arg^6^, Arg^8^, and Arg^8^ were randomly mutated, the library was constructed using the parental unique Clal and DraI restriction sites, which were 27 nucleotides upstream and 54 nucleotides downstream, respectively, of the CD loop DNA coding region in the IL-5 gene, were used to generate a randomly mutated cassette. This cassette was constructed from the following mutagenic oligonucleotides: primer CD101, 5'-ATACATGATGATTGCCCAGGAAAAAAAGTGTGGA/NND/N/ GTAAAACCAATTTGCTAGA/3'- (Clal site underlined); primer CD102,
Selection of CD Loop Library for IL-5Rα Binding—IL-5Rα-Fc was immobilized directly onto CNBr-activated Sepharose 6MB (Amersham Pharmacia Biotech) overnight at 4 °C, at a density of 2 µg of IL-5Rα-Fc/µl of drained resin. The coupling procedure was performed according to the manufacturer’s instructions. Sixty µl of CD loop library phage samples were incubated with 40 µl of IL-5Rα-Fc resin. One hundred µl of PBS containing 0.5% Tween-20 were added to the reaction mixture. Incubation was carried out with constant rotation for 1.5 h at room temperature to facilitate the binding interaction. The resin was washed, and the bound phage was eluted with 100 µl of 0.1 M glycine-HCl (pH 2.0) and neutralized with 25 µl of 1 M Tris-base (pH 9.0). An aliquot of eluted phage solution was used to titer the number of phage selected. The remaining phage eluted from IL-5Rα-Fc resin were propagated for use in the next affinity selection cycle. After four rounds of selection, 20 individual clones were selected and sequenced.

Expression of scIL-5 and Mutants from E. coli—pMK-scIL-5, and mutants were electroporated into TOPP3 cells (Stratagene, La Jolla, CA). The recombinant cells were grown in super broth medium (32 g of Trypton, 20 g of yeast extract, and 5 g of NaCl in 1 liter of medium) containing 50 µg/ml carbenicillin and 1% glucose at 37 °C, until A600 reached 0.6. The cells were then induced with 1 mM IPTG and grown overnight at 37 °C. Cells from 1 liter of the induced culture were pelleted by centrifugation (7000 g for 15 min, frozen), and thawed twice in 20 ml of 0.1 M HEPES buffer (pH 7.5). The lyate in HEPES buffer was incubated at 37 °C for 2 h. The lysate was centrifuged at 22,000 × g for 20 min, and the supernatant was frozen and saved for further purification. Purification of E. coli-expressed scIL-5 Protein and Variants—To purify E. coli-expressed scIL-5 or mutant, the lysate supernatant from 1 liter of the induced culture was dialedyzed against PBS buffer (pH 7.4). The supernatant was loaded onto an affinity column packed with mAb 2E3-Sepharose 4B matrix. The 2E3 mAb is a neutralizing antibody that blocks IL-5 interaction with the α chain of the IL-5R receptor. The column was washed with PBS buffer, and bound scIL-5 or mutant was eluted with 0.1 M glycine buffer (pH 2.5). Pooled fractions from the affinity column were neutralized with 2x Tris-HCl buffer (pH 8.0) and dialyzed against PBS buffer (pH 7.4). The final yield of scIL-5 and mutants was analyzed by SDS-10% polyacrylamide gel electrophoresis according to Laemmli (21).

TF-1.28 Cell Proliferation Assays—Biological activity was measured using a subclone of a highly responsive subclone of the human erythroleukemia cell line TF-1 (12) and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (22). Cells were cultured in RPMI 1640 medium supplemented with l-Glutamine, penicillin-streptomycin, and 10% heat-treated fetal calf serum (Life Techniques, 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 scIL-5 and mutant proteins. Then, 20 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue (Sigma) in 1× PBS was added per well, and after 5 h 100 µl of 10% SDS in 0.1 N HCl was used to solubilize the precipitated crystals. After overnight incubation at 37 °C, the absorbance at 570 nm was detected. Concentrations of E. coli-expressed scIL-5 or mutant proteins were determined by Western blot analysis and by quantitative IL-5 ELISA using the monoclonal antibodies 24G9 (20) and TRFK-5 (R & D Systems).

Kinetic Analysis of Receptor Binding by E. coli-expressed scIL-5—Kinetic and equilibrium constants for the interaction between hIL-5Rα and E. coli-expressed scIL-5 were measured using a BLAcore optical biosensor (Biacore AB, Uppsala, Sweden). To detect receptor binding of scIL-5 and variants, shIL-5Rα-Fc was immobilized directly onto the biosensor CMS chip using EDC/NHS chemistry (6). Conditions for immobilization and the sensor assay were the same as in Morton et al. (6). Protein samples from 0 to 100 nM were passed over the shIL-5Rα-Fc surface. Sensorgram data were represented visually by linearized plots as described before (23). For the calculation of rate constants, the linear portions of the association and dissociation phases of sensorgrams obtained for a series of scIL-5 or different variant concentrations were calculated by fitting these data to a two-component model, $A + B \rightarrow AB$, to yield an association rate constant $k_{on}$ and a dissociation rate constant $k_{off}$. $K_d$ values were determined from ratios of $k_{on}/k_{off}$ (23). The dissociation rate constant $k_{off}$ was calculated by fitting dissociation phase data to the equation,

$$\ln(R/R_0) = k_{off}(t - t_0)$$

(Eq. 1)

The association rate constant $k_{on}$ was calculated by fitting association phase data to the equation,

$$dR/dt = k_{on}[C][R_{max} - (k_{off}[C] + k_{diss})R]$$

(Eq. 2)

where $R_0$ is the response at the start of dissociation; $t$ is the independent variable, time in seconds; $t_0$ is the start time for the dissociation or
RESULTS

Functional Expression of scIL-5 in E. coli—We constructed an expression vector, pMK-scIL-5, in which scIL-5 was fused to a pelB leader sequence under the control of a lacZ promoter (Fig. 1A). Soluble scIL-5 was detected in the media after isopropyl-1-thio-β-D-galactopyranoside induction, as expected for recombinant proteins that are expressed and secreted into the periplasm. In contrast, no soluble form of the dimeric wtIL-5 could be detected in the supernatant using a similar construct (pMK-IL-5). The purified, E. coli-expressed scIL-5 protein was analyzed by SDS-polyacrylamide gel electrophoresis, revealing a single band approximately 30 kDa.

The interaction between E. coli-expressed recombinant scIL-5 and IL-5Rα was measured using a BIAcore optical biosensor assay. Fig. 2 shows sensorgrams of the specific binding of shIL-5Rα-Fc to scIL-5. Analysis of data from the early association phase (from 0 to 50 s) and the first 100 s of the dissociation phase yielded \( K_D \) and \( k_{on} \). The dissociation phase of the sensorgram at 100 nM scIL-5 in A was replotted according to Equation 1 as Ln (response at time 0 of dissociation/response at time n) versus time. The straight line shows the best fit to the first 100 s of dissociation. The slope of the line gives the dissociation constant.

![Graph A](image1.png)

**FIG. 2.** The interaction of E. coli-expressed scIL-5 with shIL-5Rα-Fc. A, overlays of sensorgrams showing binding by various concentrations of scIL-5 (12.5, 25, 50, and 100 nM) to shIL-5Rα-Fc. The increase in response shows the binding of scIL-5. The decay represents the dissociation of bound scIL-5 upon washing with running buffer alone. B, calculation of on-rate constant for the interaction of scIL-5 with shIL-5Rα-Fc. The association phases of the sensorgrams in A were replotted as the slope of the curve at a given time versus relative response at the time. The straight lines show the best of the data to fit a straight line. The slopes of these lines give values for \( k_{on} \), at each concentration. C, plot of \( k_{off} \) versus concentration. The slope of the line to these points gives the association rate constant. D, determination of dissociation rate constant \( k_{off} \). The dissociation phase of the sensorgram at 100 nM scIL-5 in A was replotted according to Equation 1 as Ln (response at time 0 of dissociation/response at time n) versus time. The straight line shows the best fit to the first 100 s of dissociation. The slope of the line gives the dissociation constant.

The purified E. coli-expressed scIL-5 also was tested for its biological activity in a TF-1 cell proliferation assay (22), yielding an EC50 of 35.4 pM (see also Fig. 8 and Table II). Hence, the E. coli-expressed scIL-5 was soluble and highly active in both receptor binding and bioactivity assays.

Functional Phage Display of scIL-5—Phagemid vector pMK-scIL-5-G3, in which the scIL-5 DNA was fused in-frame with residue 198 of sequence of gene III codon, was used to transform E. coli XL-1 blue cells. To verify the display of the scIL-5 sequence on the phage surface, ELISAs were performed using anti-IL-5 monoclonal antibodies and shIL-5Rα-Fc. Null phage were used as a control. ELISAs showed that scIL-5 phage could be recognized specifically by mAb 4A6 (Fig. 3A). Phage-displayed scIL-5 bound detectably to mAb 4A6 at titers higher than 1.0 × 10^7 cfu/well, compared with much weaker binding of null phage, which could not be detected at titers up to 10^9 cfu/well. The significant net signal over the background signal with null phage suggested that an immunoreactive scIL-5 was being expressed on the phage surface.
To test the binding activity of scIL-5 phage to IL-5 receptor, shIL-5Rα-Fc was coated onto ELISA plates, and bound phage were detected by binding an anti-phage antibody-horseradish peroxidase chimera followed by a colorimetric substrate assay. Null phage were used for background comparison. Phage displaying scIL-5 were detected bound to shIL-5Rα-Fc plates at titers down to less than $1.0 \times 10^8$ cfu/well (Fig. 3B). By contrast, background binding by null phage was detectable only at titers in excess of $10^9$ cfu/well. These data show that scIL-5 displayed on the phage surface can bind selectively to IL-5 receptor α chain.

We confirmed the receptor binding activity of scIL-5 phage using an optical biosensor assay. The shIL-5Rα-Fc fusion protein was immobilized directly on the sensor chip. Single chain IL-5 phage at a titer of $6 \times 10^{12}$ cfu/ml was preincubated with sIL-5Rα (0–25 μM) and then passed over the IL-5Rα-Fc surface for 4 min. The abrupt change of resonance signal at the beginning and end of association phases is due to differences in refractive index between the running buffer and phage samples. Inset, table showing the net amount of signal bound, after buffer wash of the sensor surface, versus the amount of sIL-5Rα competitor.

**Fig. 3. Functional phage display of scIL-5.** A, ELISA of binding to anti-IL-5 mAb 4A6. ELISAs for scIL-5 phage and null phage binding to mAb 4A6. Phage pellets were resuspended in PBS buffer. Different titers of phage samples were added to mAb 4A6-coated microwells. Null phage was used as a control. The read-out is $A_{450}$, with the mean ± S.D. of triplicate wells shown. ☐, scIL-5 phage; ●, null phage. B, ELISA of binding to shIL-5Rα-Fc. ELISAs for scIL-5 phage and null phage binding to shIL-5Rα-Fc. Phage pellets were resuspended in PBS buffer. Different titers of phage samples were added to shIL-5Rα-Fc-coated microwells. Null phage was used as a control. The read-out is $A_{450}$, with the mean ± S.D. of triplicate wells shown. ☐, scIL-5 phage; ●, null phage.

C, optical biosensor analysis of binding to immobilized shIL-5Rα-Fc. ~14,000 response units of shIL-5Rα-Fc was immobilized directly on the sensor chip. Single chain IL-5 phage at a titer of $6 \times 10^{12}$ cfu/ml was preincubated with sIL-5Rα (0–25 μM) and then passed over the IL-5Rα-Fc surface for 4 min. The abrupt change of resonance signal at the beginning and end of association phases is due to differences in refractive index between the running buffer and phage samples. Inset, table showing the net amount of signal bound, after buffer wash of the sensor surface, versus the amount of sIL-5Rα competitor.
1 min (100 μl). The net amount of phage bound to the immobilized sIL-5Ra-Fc, after the 100-μl buffer wash, decreased proportionately as the competitor concentration increased during preincubation (Fig. 3C). These results, taken with the ELISA data, argue that phage-displayed scIL-5 was functionally active and interacted specifically with IL-5 receptor α chain.

Affinity-based Selection of scIL-5 Phage—The ability of scIL-5 phage to be differentially selected by its receptor α chain affinity was tested by a bead filtration selection assay. In this assay, scIL-5 phage were mixed with IL-4 phage at a titer ratio of 1:100, and the mixture was panned with IL-5Ra-Fc-conjugated Sepharose beads in suspension. Phage bound to the beads were examined for gene content of scIL-5 versus IL-4 by preparing DNA and carrying out a restriction digest analysis. A unique BamHI site, which does not exist in pMK-IL-4G3, was introduced in vector pMK-scIL-5-G3 (Fig. 1A) to allow differentiation of phage clones. Based on DNA sequence, digestion of pMK-scIL-5-G3 with BamHI and NheI yields a 700-bp DNA fragment, whereas the same treatment of pMK-IL-4G3 linearizes the vector without producing subfragments. As shown in Fig. 4A, before panning, all of the 10 randomly picked colonies contained pMK-IL-4G3, since only the linearized vector was seen on the gel. Fig. 4B shows the restriction analysis of 10 phage clones that were randomly picked after three rounds of receptor α chain panning; 9 of the 10 clones produced the ∼700-bp DNA band, indicating that 90% of the phagemid vectors were pMK-scIL-5-G3 after panning. The results confirm that scIL-5 phage can be enriched by panning on IL-5Ra beads.

Construction and Phage Display of an Asymmetrically Disabled scIL-5 Variant—In anticipation of using the scIL-5 phage display system for epitope randomization, an asymmetric scIL-5 mutant, designated (wt/A5)scIL-5, was designed. This (wt/A5)scIL-5 mutant was constructed (Fig. 5) from an N-terminal half containing the original five charged residues (88EEERRR92) in the CD loop, combined with a C-terminal half containing a disabling CD loop sequence (88AAAAA92). This variant, displayed on phage analogously to scIL-5, had binding properties comparable with those of scIL-5 phage, as shown by ELISA data in Fig. 6, A and B, for mAb 4A6 and IL-5 receptor α chain, respectively. Since the functionally critical CD loop in the (wt/A5)scIL-5 molecule was present only in the N-terminal half of the dimeric scIL-5, it can be surmised that the receptor binding activity of this molecule was due to this half molecule. This assumption is consistent with the retention of significant function observed previously for both monomeric forms of IL-5 (15) and asymmetrically mutated IL-5 dimers (12, 24). Hence,
(wt/A5)scIL-5 is a key intermediate to prepare scIL-5 libraries by randomization of the N-terminal CD loop randomization as a means to identify functional variations possible in the CD loop of IL-5.

**Design and Panning of a Phage-displayed Library of scIL-5 Variants with Randomization of the Receptor-binding Epitope in the CD Loop**—Affinity selection of a phage-displayed library of scIL-5 mutants with randomized sequences in the wild-type CD loop allowed us to search for hIL-5 receptor binding activity. An asymmetric CD loop library in the N-terminal half of (wt/A5)scIL-5 was constructed, in which the sequence of the targeted five residues (88EE-RRR92) was randomly mutated (Fig. 5). After four rounds of panning by selection with affinity beads containing immobilized scIL-5Rα-Fc, 20 clones were randomly selected and sequenced. As shown in Table I, 35% of the clones sequenced contained wild type CD loop sequence. Strikingly, several selectants were found that retained only a single charged residue, an Arg, in the CD loop, and this residue was found at either position 89 or 90. Within this latter group, there was an apparent preference (15%) for the sequence of 88SLRGG92 (Table I).

Phage selectants were analyzed for receptor binding activities using competitive phage ELISA (16) on plates coated with scIL-5Rα-Fc. Representative data are shown in Fig. 7. The (SLRGG/A5)scIL-5 phage selectant, which showed preferential receptor affinity head binding versus all sequences except for the wild type (Table I), was able to bind shIL-5Rα-Fc plates with a potency close to that of (wt/A5)scIL-5 phage. As shown in Fig. 7, (wt/A5)scIL-5 and (SLRGG/A5)scIL-5 both were competed significantly by soluble IL-5Rα. The shIL-5Rα binding affinities of phage-displayed scIL-5, (wt/A5)scIL-5, and (SLRGG/A5)scIL-5 were determined by this method, giving EC50 values of 42, 30, and 105 nM, respectively. The second Arg selectant, (LPRCG/A5)scIL-5, also showed significant receptor binding, as shown initially by the phage titration ELISA results and also by the follow-up competition ELISA data (Fig. 7). However, for this latter selectant, there is an apparently greater contribution of nonspecific to overall signal, resulting in incomplete competition with soluble receptor. The other phage selectants (Table I) generated largely nonspecific signals that could not be competed by soluble IL-5Rα. This is shown for the cases of (DGIWG/A5)scIL-5 and (SLRGA/A5)scIL-5 in Fig. 7. These selectants may be interpreted to have marginal if any receptor binding activity. Overall, despite the potential caveat of steric effects due to attachment of the scIL-5 variants to GIIH protein on phage (10), the ELISA data show that (SLRGG/A5)scIL-5 phage has a receptor binding efficacy similar to those of scIL-5- and (wt/A5)scIL-5 phage.

**Binding and Proliferation Properties of scIL-5 Variant Proteins**—Given its frequency of panning selection and relatively strong receptor binding activity in ELISA, (SLRGG/A5)scIL-5 was expressed in E. coli. The parent asymmetric mutant (wt/A5)scIL-5 was also expressed. Following purification using a 2E3 affinity column (Fig. 1B), the variants were tested for TF-1 cell proliferation and receptor binding activities.

ScIL-5, (wt/A5)scIL-5, and (SLRGG/A5)scIL-5 all elicited similar absolute activity maxima (absorbance at 570 nm) but different EC50 values. The increased proliferation of the TF-1 cells stimulated by the scIL-5 proteins are shown in Fig. 8.

**Table I**

| Clone | Residue position | Frequency |
|-------|------------------|-----------|
|       | 88 89 90 91 92   |           |
| Wild type | E E R R R |           |
| CDla  | L  P  R  C  G   |  1        |
| CDlb  | D  G  I  W  G   |  1        |
| CDlc  | S  C  A  D  V   |  1        |
| CDld  | E  T  R  R  R   |  1        |
| CDle  | A  S  L  V  W   |  1        |
| CDlf  | H  L  G  C  C   |  1        |
| CDlg  | K  E  R  R  R   |  1        |
| CDlh  | E  E  R  R  R   |  7        |
| CDli  | C  L  F  S  S   |  1        |
| CDlj  | P  G  A  T  S   |  1        |
| CDlk  | S  L  R  G  G   |  3        |
| CDll  | S  R  L  G  A   |  1        |
The kinetics of binding of scIL-5 variants to shIL-5Rα-Fc were examined with the receptor components immobilized directly on sensor chips of an optical biosensor. Sensorgrams and replots of the data for (wt/A5)scIL-5 and (SLRGG/A5)scIL-5 according to Equations 1 and 2 are similar to those shown in Fig. 2. The calculated kinetic values are summarized in Table II. The affinity of (SLRGG/A5)scIL-5 binding to IL-5Rα-Fc was only slightly reduced from that of wild type sequence scIL-5 protein, with the major difference being the on rate (Table II). Interestingly, the (SLRGG/A5)scIL-5 protein (K_a = 15.6 nM) showed higher affinity to the IL-5Rα-Fc than the (wt/A5)scIL-5 construct (K_a = 53.3 nM), consistent with the higher bioactivity of the former. Overall, the data suggest that the wild type sequence of CD loop, EERRR, can be replaced by a novel sequence, SLRGG, which retains only one positive charge residue, Arg^{90}, versus the wild type sequence.

**DISCUSSION**

In the present study, we have shown that interleukin-5 can be functionally expressed on filamentous phage through a re-engineered form of scIL-5. Single chain IL-5 was solubly expressed in *E. coli* and was fully active in receptor binding and bioassays. Expression of scIL-5 in the form in *E. coli* justified using it to incorporate the IL-5 sequence into the phage coat protein III. Phage-displayed scIL-5 interacted specifically with both anti-IL-5 mAbs and IL-5 receptor α chain. Furthermore, we constructed a functional, asymmetrically mutated (wt/A5)scIL-5 variant on phage and showed that this variant background could be used to identify novel receptor binding sequences through CD loop epitope randomization. Receiver α chain selection of a CD loop library revealed that the normally occurring EERRR epitope for receptor α chain binding could be replaced by a substantially simplified sequence, SLRGG. Our results successfully demonstrate the feasibility of using affinity-based selections to screen phage libraries displaying scIL-5 variants and permit the identification of those variants with sequence variations in key receptor recognition epitopes that nonetheless still retain receptor binding affinity.

An important prerequisite for the generation of hIL-5 on phage is soluble expression of IL-5 in *E. coli*. Following translation in bacteria, recombinant protein to be packaged in phage is transported under the direction of pelB leader peptide to the periplasmic space (25). If expressed protein is not soluble, but instead forms inclusion bodies, it will not be secreted into the periplasm and hence will not be incorporated into phage. In our work, expression of both scIL-5 and wtIL-5 genes was attempted in *E. coli* using the pMK-G3H vector. Recombinant scIL-5 protein was produced in a soluble form and secreted into the periplasm, while the wtIL-5 produced in this system was insoluble. We thus focused phage display efforts on scIL-5.

We demonstrated the display of scIL-5 as a functional protein on filamentous phage M13 by a set of binding assays. Direct binding of scIL-5 phage to anti-IL-5 monoclonal antibodies was detected by ELISA. One antibody, 4A6, has been found not to bind to a panel of peptide fragments of the IL-5 sequence, hence arguing that the antigenic epitope is conformational. Hence, the specific interaction of scIL-5 phage with mAb 4A6 suggests that the scIL-5 on phage folds into a conformation similar to that formed in solution. The specific binding of scIL-5 phage to shIL-5Ra was detected by both ELISA and direct BIAcore assay (Fig. 3). In addition, indirect bead filtration selection (Fig. 4, A and B) clearly demonstrated that scIL-5 phage had receptor binding activity. Similar results were obtained with a batch affinity chromatographic capture phaging

**Fig. 7. Competitive phage ELISA evaluation of relative receptor binding affinities of loop library selectants.** Phage selectants after four rounds of panning were analyzed for binding to shIL-5R α. Different titers of phage samples that result in equivalent signal were incubated with different concentrations of sIL-5R α protein, ranging from 0 to 500 nM, and then the incubated mixtures were added after four rounds of panning were analyzed for binding to shIL-5R α. The data shown are the mean of two independent experiments, each carried out in triplicate.

**Fig. 8. Bioactivity of *E. coli*-expressed scIL-5 and 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 incubation, proliferation was evaluated as described under “Experimental Procedures.” The percentage of activity of proliferation which is the average of triplicate wells is shown. ○, scIL-5; ●, (wt/A5)scIL-5; □, (SLRGG/A5)scIL-5. Strikingly, (SLRGG/A5)scIL-5 gave an increase in proliferation (Table II), which was close to 40% of unmodified scIL-5 and measurably greater than that of the library parent (wt/A5)scIL-5.

3 R. Cook, personal communication.
method. While the overall data obtained for scIL-5 phage binding argue for specificity in both antibody and receptor α chain binding, the fine details of the biosensor data (Fig. 3C) are distinctly different than those obtained with soluble IL-5 as exemplified in Fig. 2 and previously (23). The most striking differences are the gradual increase in signal during association (after an abrupt bulk phase refractive index increase) and the very slow decrease in signal during dissociation upon washing (after an abrupt bulk phase refractive index decrease). Two factors that can occur uniquely with phage-displayed versus soluble scIL-5 could explain both phenomena. First, scIL-5 may be displayed in multiple copies on phage, leading to multivalent attachment of scIL-5 phage to sensor-immobilized antibody and receptor. Although such multivalency has been argued as unlikely in phagemid-based display due to the overall abundance of helper phage (26), this cannot be ruled out. Second, there may be a slowing of both association and dissociation of phage-displayed scIL-5 by mass transport into and out of the solid phase of the sensor surface. This could occur because phage, with a complex molecular surface, may bind nonspecifically even if weakly at multiple sites, with resulting nonspecific entrapment, by the carboxyextran matrix on which the receptor α chain is immobilized. If correct, this will limit the ultimate usefulness of the optical biosensor to measure binding kinetic constants for phage-bound molecules.

Phage display of scIL-5 should permit epitope randomization studies, allowing a deeper mechanistic investigation of receptor binding surfaces in IL-5 and perhaps leading to mutants with enhanced receptor binding. Sequence randomization using combinatorial cassette mutagenesis has been used previously to probe the information content of protein sequence of phage λ repressor (27), and the combinatorial phage library approach has been used for human growth hormone, permitting identification of a recombinant human growth hormone derivative with 400-fold increased receptor binding affinity (18). The same has been achieved with IL-6 (28) and ciliary neurotrophic factor (29), and other phage-cytokine systems such as IL-2 (30) offer similar opportunities. In the light of the promise of epitope randomization to enhance mechanistic understanding and identify novel IL-5 agonists and/or antagonists, IL-5 continues to present challenges due to its essentially homodimeric nature. Randomization of a homodimer on phage can lead to complex combinations of sequences with alterations that would be difficult to control on individual halves of the protein and also difficult to reproduce on both polypeptide chains.

To circumvent this limitation, we reconstructed the scIL-5 molecule in two halves to enable the facile formation of IL-5 variants with asymmetric modifications in either half of the sequence. This approach was used to construct (wt/A5)scIL-5, a form in which the previously identified receptor binding epitope in the 88FERRR92 CD loop was mutated to 88AAAAA92. (wt/A5)scIL-5 was expressed in E. coli as an active molecule and was also expressed displayed on the surface of phage in a form able to bind the IL-5 receptor α chain. The retention of substantial function by (wt/A5)scIL-5 is consistent with previous observations of activity in asymmetrically mutated forms of scIL-5 (12, 24) as well as in several monomeric forms of the protein (15, 31, 32).

As a first investigation of receptor binding sites in IL-5 by epitope randomization, we constructed a CD loop library using phage-displayed (wt/A5)scIL-5, by randomizing the wild type loop in the amino-terminal half of the molecule. Receptor α chain affinity beading panning of the library and random examination of affinity selectants revealed the preferential selection of a variant, (SLRGG/A5)scIL-5, with a highly altered CD loop sequence. The retention of functional viability suggested by preferential receptor affinity bead selection of the (SLRGG/A5)scIL-5 variant on phage was confirmed by its substantial receptor α chain binding activity revealed when bound to phage (Fig. 7) and by the functional properties of the soluble, E. coli-expressed form of this protein (Figs. 8 and Table II).

Strikingly, the functional (SLRGG/A5)scIL-5 lacks four of the five charged residues of the wild type sequence, including both Glu93 and Arg91 that have been identified previously to be important for receptor α chain binding, and instead contains a single Arg residue at position 90. Among the previous site-directed mutagenesis studies in the CD loop region, Tavernier et al. (4) found that an R90A IL-5 mutant was about 50% as active as wild type, while ES9A and RS91A mutants were only 1–10% active. Graber et al. (5) obtained similar findings on the importance of Arg91, although it must be noted that their numbering scheme was different that of others and they referred to this residue as Arg90 instead. More recently, Li et al. (24) have confirmed the importance of Arg91 using both symmetric and asymmetric R91A mutations. The overall agreement on the impact of single site mutagenesis of Glu93 and Arg91, and the conclusion from these studies of the importance of these CD loop residues in receptor recognition, has been reviewed recently (33, 34). In the face of these single-site mutation studies, including those from our own laboratory, the identification of the functional CD loop mutant (SLRGG/A5)scIL-5 by phage epitope randomization points to the need to rethink how the charged sequence elements in the receptor binding epitope of the CD loop are used in receptor recognition.

The functionality of the (SLRGG/A5) and to some extent (LPRCG/A5)scIL-5 variants versus the close to null receptor binding activities of many other X5/A5 mutants observed in this work (see for example Fig. 7) suggests that the SLRGG sequence itself and not the A5 in the second domain provides the epitope for receptor α chain recognition. One possibility is that the receptor binding epitope of the CD loop in the wild type sequence normally provides an electrostatic balance of one net positive charge R90°FERRR92 that is mimicked by the mutant sequence with Arg90. The importance of charge distribution in protein binding is recognized generally (35). Its role in IL-5 receptor recognition merits further investigation through an

**TABLE II**

| Variant                  | IL-5Ro-Fc binding | Proliferation assays (EC<sub>50</sub>) | Relative activity | 
|--------------------------|-------------------|--------------------------------------|------------------|
|                          | \(k_{in}\)         | \(k_{off}\)                          | \(K_d\)          | \(EC_{50}\) (wt/A5) / \(EC_{50}\) (mutant) |
| scIL-5                   | 7.0 \(\times 10^7\) | 7.0 \(\times 10^{-7}\)              | 10.0             | 35.4 \pm 3.4                             |
| (wt/A5)scIL-5            | 0.9 \(\times 10^{-6}\) | 4.8 \(\times 10^{-7}\)              | 53.3             | 288.0 \pm 38.2                           |
| (SLRGG/A5)scIL-5        | 5.0 \(\times 10^{-6}\) | 7.8 \(\times 10^{-7}\)              | 15.6             | 93.8 \pm 12.7                           |

\(k_{in}\) and \(k_{off}\) are from a BLAcore optical biosensor assay of shIL-5Ro-Fc binding; the equilibrium dissociation constant \(K_d\) is calculated from the ratio of \(k_{off}/k_{in}\). Data were obtained from a series of protein concentrations. TF-1 cell proliferation activities are given as the mean ± S.D. of three determinations. The rate constants \(k_{on}\) and \(k_{off}\) are from a BLAcore optical biosensor assay of shIL-5Ro-Fc binding; the equilibrium dissociation constant \(K_d\) is calculated from the ratio of \(k_{off}/k_{on}\). Data were obtained from a series of protein concentrations. TF-1 cell proliferation activities are given as the mean ± S.D. of three determinations. The correlation coefficient \(r\) is 0.9999.

S. Chen, S.-J. Wu, and I. Chaiken, unpublished results.
expanded binding and biophysical analysis of phage library selectants.

Such simplified, functional variants as (SLRGG/A5)scIL-5 may prove to be of substantial value as starting materials to investigate the mechanistic roles of other receptor recognition regions in IL-5 by additional epitope randomization. Two obvious examples are the N-terminal region of the D helix around residue Glu\(^{110}\) and the A helix around Glu\(^{13}\). These regions have been shown previously to play important roles, respectively, in IL-5 receptor \(\alpha\) chain and \(\beta\) chain recognition (see Ref. 34 and references therein). A charge-simplified mimetic background such as (SLRGG/A5)scIL-5 could allow more straightforward mechanistic interpretations of library variants by offering fewer possible varying parameters, in much the same way as a smaller mimetic with less sequence variables. Further definition of the key structural elements that lead to receptor recognition and signal triggering may lead not only to improved mechanistic understanding but also to novel types of rationally designed receptor antagonists. For example, the wtIL-5 mutant E13N has been found to be an IL-5 receptor antagonist, since it binds IL-5R\(\alpha\) and yet elicits no biological activity (4). Secondary libraries based on a combination of SLRGG and randomization around Glu\(^{13}\) potentially could lead to altered combinations of cytokine receptor specificity, affinity, and signal triggering.

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REFERENCES

1. Sanderson, C. J. (1992) Adv. Pharmacol. 23, 163–177
2. Milburn, M. V., Hassell, A. M., Lambert, M. H., Jordan, S. R., Proudfoot, A. E. I., Graber, P., and Wells, T. N. C. (1993) Nature 363, 172–176
3. Murata, Y., Takaki, S., Migita, M., Kikuchi, Y., Tominaga, A., and Takatsu, K. (1992) J. Exp. Med. 175, 341–351
4. Tavernier, J., Tuyens, A. V., Plaetinck, R. D., Heyden, J. V., Cuisinier, Y., and Oefner, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5194–5198
5. Graber, P., Proudfoot, A. E., Talabot, F., Bernard, A., McKinnon, M., Banks, M., Fattah, D., Peitsch, M. C., and Wells, T. N. (1995) J. Biol. Chem. 270, 15762–15769
6. Morton, T., Li, J., Cook, R., and Chaiken, I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10879–10883
7. Clackson, T., and Wells, J. A. (1994) Trends Biotechnol. 12, 173–184
8. Lowman, H. B., Cunningham, B. C., and Wells, J. A. (1991) J. Biol. Chem. 266, 10982–10988
9. Baca, M., Presta, L. G., O’Connor, S. J., and Wells, J. A. (1997) J. Biol. Chem. 272, 10678–10684
10. Muller, Y. A., Li, B., Christinger, H. W., Wells, J. A., Cunningham, B. C., and de Vos, A. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7192–7197
11. Proudfoot, A. E. I., Fattah, D., Kawashima, E. H., Bernard, A., and Wingfield, P. T. (1990) Biochem. J. 270, 357–361
12. Li, J., Cook, R., Dede, K., and Chaiken, I. (1995) J. Biol. Chem. 270, 1817–1820
13. Tsui, P., Tornetta, M. A., Ames, R. S., Banksosky, B. C., Greigo, S., Silverman, C., Porter, T., Moore, G., and Sweet, R. W. (1996) J. Immunol. 157, 772–780
14. Zhong, G., and Smith, G. P. (1994) BioTechniques 16, 838–839
15. Li, J., Cook, R., Hensley, P., Doyle, M., McNulty, D., and Chaiken, I. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6694–6699
16. Jones, J. T., Ballinger, M. D., Picascia, F. I., Leigren, J. A., Fitzpatrick, V. D., Fairbrother, W. J., Wells, J. A., and Sliwowski, M. X. (1998) J. Biol. Chem. 273, 11667–11674
17. Kay, B. K., Adey, N. B., He, Y.-S., Manfredi, J. P., Mataragnon, A. H., and Fowlkes, D. M. (1993) Gene (Amst.) 128, 59–65
18. Lowman, H. B., and Wells, J. A. (1993) J. Mol. Biol. 234, 564–578
19. Toniatti, C., Cabibbo, A., Sorensen, E., Salvati, A. L., Cerretani, M., Serafini, S., Lahm, A., Cortese, R., and Ciliberto, G. (1996) EMBO J. 15, 2726–2737
20. Ames, R. S., Tornetta, M. A., McMillan, L. J., Kaiser, K. F., Holmes, S. D., Appelbaum, E., Cusimano, D. M., Theisen, T. W., Gross, M. S., Jones, C. S., Silverman, C., Porter, T. G., Cook, R. M., Bennet, D. and Chaiken, I. M. (1995) J. Immunol. 154, 6355–6364
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Monfardini, C., Ramamurthy, M., Rosenbaum, H., Fang, Q., Godillot, P. A., Canziani, G., Chaiken, I. M., and Williams, W. V. (1998) J. Biol. Chem. 273, 7657–7667
23. Morton, T., Bennett, D. B., Appelbaum, E. R., Cusimano, D. M., Johanson, K. O., Matico, R. E., Young, P. R., Doyle, M., and Chaiken, I. M. (1994) J. Mol. Recognit. 7, 47–55
24. Li, J., Cook, R., and Chaiken, I. (1996) J. Biol. Chem. 271, 31729–31734
25. Burton, D. R., and Barbas, C. F., III (1993) ImmunoMethods 3, 155–163
26. Bass, S., Greene, B., and Wells, J. A. (1990) Proteins 8, 209–314
27. Reidaarala, J., and Sauer, R. T. (1984) Science 221, 53–57
28. Cabibbo, A., Sorensen, E., Toniatti, C., Altamura, S., Savino, R., Panessa, G. and Ciliberto, G. (1995) Gene (Amst.) 167, 41–45
29. Saggio, I., Ghincu, I., Paiana, G., and Laufer, R. (1995) EMBO J. 14, 3045–3054
30. Bucki, P. J., Wu, Z., and Ciardelli, T. L. (1997) Arch. Biochem. Biophys. 339, 79–84
31. Dickason, R. R., and Huston, D. P. (1996) Nature 379, 652–655
32. Edgerton, M. D., Graber, P., Willard, D., Consler, T., McKinnon, M., Uings, I., Aroel, C. Y., Borlat, F., Fish, R., Peitsch, M., Wells, T. N. C., and Proudfoot, A. E. I. (1995) J. Biol. Chem. 272, 20611–20618
33. Chaiken, I. M., and Proudfoot, A. (1999) in IL 5: From Molecule to Drug Target (Sanderson, C. J., ed) Marcel Dekker Inc., New York, pp. 172–180
34. Bagley, C. J., Woodcock, J. M., Stomski, F. C., and Lopes, A. F. (1999) in IL 5: From Molecule to Drug Target for Asthma (Sanderson, C. J., ed) Marcel Dekker Inc., New York, pp. 189–203
35. Chong, L. T., Dempster, S. E., Hendish, Z. S., Lee, L.-P., and Tidor, B. (1998) Protein Sci. 7, 206–210