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Saturation Mutagenesis of Human Interleukin-3*

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A deletion variant of human interleukin-3, hIL-$3_{15–125}$, was produced in the periplasmic space of *Escherichia coli* and had full activity in an AML193.1.3 cell proliferation assay. Libraries of random single-amino acid substitutions were constructed at each of 105 positions in the gene for hIL-$3_{15–125}$. Approximately eight single-site substitutions at each position were produced in osmotic shock fractions and screened for activity. 15 mutants were found with bioactivity of 5–26-fold greater than that of native hIL-3. The majority of amino acids in hIL-$3_{15–125}$ could be substituted without substantial loss of activity. Substitution of residues predicted to be in the hydrophobic core of the protein often resulted in reduced activity and/or low accumulation levels. Only five residues predicted to be on the surface of the protein were intolerant of substitution and hence are candidates for sites of interaction with the receptor. We therefore propose that the majority of residues in hIL-3 serve a structural role and permit the display of a few key residues in the correct configuration for recognition by the receptor.

Human interleukin-3 (hIL-3)[1] is a multilineage hematopoietic cytokine acting in the bone marrow to promote the growth of most lineages of blood cell precursors (1). Recently, exogenously administered hIL-3 has shown promise for the clinical relief of neutropenia and thrombocytopenia induced by cancer chemotherapy (2, 3). Sequence homology comparisons of hIL-3 with other proteins indicate that it is a member of the hematopoietic cytokine family (4–6) and that it adopts a four-α-helix bundle topology (7–10). The protein binds to a receptor comprising at least two nonidentical subunits (11, 12). Although the precise nature of interaction between hIL-3 and its receptor is not known, studies using site-specific mutants have shed some light on which portions of the protein are important for function (8, 13–17). In particular, mutagenesis of the adjacent helices A and D indicate that these regions are important for interaction with the receptor. This is similar to the findings for human interleukin-5 and human granulocyte-macrophage colony stimulating factor, whose receptors share a common β subunit with the hIL-3 receptor (11, 18, 19). Other members of the hematopoietic cytokine family also have important residues in helices A and D (19–23) and in helix C (20, 22, 24, 25).

In this paper we have undertaken an extensive mutagenesis of hIL-3 in order to discover mutants with enhanced proliferative activity and to define residues necessary for activity. Although alanine scanning mutagenesis has been successfully used to derive structure-activity information (20, 26–32), we chose to perform a more extensive mutagenesis, permitting the incorporation of any of the possible 19 substitutions (33).

MATERIALS AND METHODS

Production of hIL-3 and Variants in the Escherichia coli Cytoplasm—General techniques for manipulation of DNA are described elsewhere (34). The hIL-3 gene (35) was obtained from British Biotechnology (Cambridge, United Kingdom) and expressed in the cytoplasm of *E. coli* using pMON5847 (ATCC 68912), which contains the *recA* promoter and *g10*-L ribosome binding site (36). Human IL-3 and hIL-3 variants were expressed with an initiator methionine followed by an alanine at the N terminus of the genes. The initiator methionine was removed in *E. coli* by methionine aminopeptidase (data not shown). Production of hIL-3 in cultures of JM101 cells (Ref. 37; ATCC 33876) harboring hIL-3 expression plasmids and examination of total cell protein on polyacrylamide gels was performed as described previously (34). For measurements of the solubility of hIL-3 variants, cell pellets from induced cultures were lysed by sonication (38), and the proteins were fractionated by centrifugation.

Production of hIL-$3_{15–125}$ Variants in the Periplasm of E. coli—hIL-$3_{15–125}$ variants were produced in the periplasm of *E. coli* JM101 using a secretion vector (39). These plasmids contained the *E. coli araBAD* promoter (40) governing expression of fusions of the LamB signal peptide to hIL-3 variants. Culture growth was performed as described previously (36) except that the medium contained 0.2% glycerol in place of glucose. Arabinose was added to cultures to a final concentration of 0.05% to induce expression from p_{araBAD}, and cultures were grown at 30 °C for a further 3–4 h.

Osmotic Shock Release of Secreted hIL-$3_{15–125}$ from E. coli—The osmotic shock method used was similar to that of Neu and Heppel (41). A 1-ml aliquot of cells was harvested by centrifugation for 5 min at 5,400 × *g*. The pellet was resuspended gently by pipetting in 500 μl of a room temperature sucrose solution (20% sucrose (w/v), 30 mM Tris-HCl, pH 7.5, 1 mM EDTA). Following a 10-min incubation at room temperature, the cells were spun for 10 min at 8,400 × *g*. The sucrose fraction was carefully removed from the cell pellet and saved. The cells in the pellets were then resuspended gently by pipetting in 500 μl of ice-cold water. Following a 10-min incubation on ice, the cells were spun for 10 min at 14,200 × *g*. The supernatant water fraction was carefully removed and saved. Equal volumes of the sucrose and water fractions were pooled and used in the hIL-3 ELISA and AML cell proliferation assay. Some cultures and fractions were analyzed by immunoblots (34) using a rabbit polyclonal antibody raised against an hIL-$3_{20–41}$ peptide. The level of loading on the gel was normalized to the densities of the cultures, and protein corresponding to 4 Klett units was loaded in each lane.

Two-step Site-directed PCR Mutagenesis—Libraries of all 19 possible single-site variants were constructed at each position from residues 17–123 in the hIL-$3_{15–125}$ molecule, using two PCR mutagenesis steps. This two-step approach facilitated the rapid identification of single amino acid substitutions using differential DNA hybridization. Libraries at positions 94–105 were created from an intermediate plasmid that

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[1] The abbreviations used are: hIL-3, human interleukin-3; hIL-$3_{15–125}$, a deletion variant of hIL-3 consisting of residues 15–125; ELISA, enzyme-linked immunosorbent assay; HGH, human growth hormone; PCR, polymerase chain reaction; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

contained a 12-base replacement. This approach for the creation of a library at residue 105 is illustrated (see Fig. 2*a*). In the first mutagenesis step, one of the PCR primers replaced codons 102–105 in the hIL-3_{15-125} gene with 12 bases encoding two translation stop codons and six additional bases (5′-TAATAAGTCGAC-3′). The reverse PCR primer was complementary to the 5′-end of the hIL-3_{15-125} gene. Plasmid DNA containing the hIL-3_{15-125} gene in a secretion expression vector served as the template in the PCR reaction. PCR mutagenesis (42) was performed using reagent kits, and thermal cycler from Perkin-Elmer Cetus. Primer extension was carried out using 20 pmol of each oligonucleotide and 0.5 pmol of template plasmid DNA for 35 cycles (94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min). The PCR-generated DNA product was extracted with phenol/chloroform and precipitated with ethanol. The DNA was digested with *Nco*I and *Eco*RI and ligated into the corresponding restriction sites in the hIL-3_{15-125} secretion plasmid. The resulting "intermediate plasmid" served as the template in the second mutagenesis step. The 12-base segment described above was restored with a PCR primer encoding the parental DNA sequence at codons 102–104 and a 32-fold degeneracy at codon 105. The degenerate oligonucleotides were synthesized to have G, A, T, or C (in equal proportions) in the first and second positions and G or C in the third position of the codon. The reverse PCR primer was complementary to the 5′-end of the hIL-3_{15-125} gene. The PCR reaction mixture was purified as above, digested with *Nco*I and *Eco*RI, and ligated into the corresponding sites of the intermediate plasmid. Single colonies were selected at random and grown in liquid culture in a 96-well plate format. Plasmid DNA was prepared from the resulting cultures in a 96-well format and DNA dot-blot hybridization was used to distinguish mutants from parental plasmids using a probe specific for the parental plasmid. This process was repeated in a similar manner to create libraries of mutants at positions 94–104. A limited number of positive clones from the hybridization screen were sequenced to confirm the quality of the libraries.

Libraries at positions 17–93 and 106–123 were created using intermediate plasmids that contained an 18-base deletion. This approach for the creation of a library at a single position, residue 71, is illustrated (see Fig. 2*b*). Again, a two-step PCR mutagenesis approach was used, but in this case, the first mutagnesis step led to an intermediate plasmid with a portion of the coding region deleted. In the second step, the mutagenic PCR primer encoded the parental DNA sequence at codons 72–76 and a 32-fold degeneracy at residue 71. Similarly, the same intermediate plasmid was used for creation of libraries at residues 72–76. Other intermediate plasmids carrying 6-codon deletions throughout the remainder of the hIL-3_{15-125} (residues 17–22, 23–28, 29–34, 35–40, 41–46, 47–52, 53–58, 59–64, 65–70, 71–76, 77–82, 83–88, 88–93, 106–111, 112–117, and 118–123) were constructed and used as templates for the remaining libraries.

Mutants from each library were selected at random and identified by DNA sequencing. In addition, a small number of selected single-site mutants were constructed by substitution of a portion of the gene with appropriate synthetic oligonucleotides.

hIL-3 ELISA—Concentrations of hIL-3 variants in osmotic shock fractions were determined using a sandwich ELISA. Dynatech Immulon II microtiter plates were coated with affinity-purified polyclonal goat-anti-hIL-3 (see below) at 1 μg/ml in 100 mM $NaHCO_3$, pH 8.2. Plates were incubated overnight at room temperature in a humidified chamber. The plates were blocked with Dulbecco's phosphate-buffered saline (D-PBS) containing 3% bovine serum albumin and 0.05% Tween 20, pH 7.4, for 1 h at 37 °C in a humidified chamber. Plates were then washed 4 times with 150 mM NaCl containing 0.05% Tween 20. Unknowns were serially diluted in assay buffer (Dulbecco's phosphate-buffered saline containing 0.1% bovine serum albumin, 0.01% Tween 20, pH 7.4). A standard curve of purified recombinant hIL-3 diluted in assay buffer ranged from 0.125 to 5 ng/ml. Plates were incubated for 2.5 h at 37 °C in a humidified chamber. After four washes, goat anti-hIL-3 conjugated to horseradish peroxidase was added to each plate. Plates were incubated 1.5 h at 37 °C in a humidified chamber. Plates were washed 4 times and ABTS peroxidase substrate solution (Kirkegaard and Perry Labs, Gaithersburg, MD) was added. The plates were read at a test wavelength of 410 nm and a reference wavelength of 570 nm on a Dynatech microtiter plate reader (Chantilly, VA). Concentrations of hIL-3 in unknown samples were calculated from the standard curve using software supplied with the plate reader. To raise polyclonal antibodies to recombinant hIL-3, goats were initially immunized with 3 mg of purified recombinant hIL-3 in complete Freund's adjuvant. Each received two monthly boosts of 1 mg in incomplete Freund's adjuvant. Thereafter, each goat was given monthly boosts with 0.25 mg of hIL-3 in incomplete Freund's adjuvant. Blood was collected 10 days after each boost and allowed to coagulate at room temperature. Serum was separated from whole blood by centrifugation, sterile filtered, and stored at −70 °C. Antibody titers were verified prior to purification using Ouchterlony immunodiffusion. Goat polyclonal antibodies to hIL-3 were purified from serum using an affinity chromatography column of 10 mg hIL-3 coupled to 1 ml Affi-Gel 10 agarose beads (Bio-Rad). Purified antibody was conjugated to horseradish peroxidase for use in the ELISA assay.

AML193.1.3 Cell Proliferation Assay for hIL-3—AML193 cells (43) were obtained from the ATCC. The cells were adapted for long term growth in hIL-3 by starving for growth factor (granulocyte-macrophage colony stimulating factor) for 24 h and replating in media containing hIL-3 (100 units/ml, Amgen, Thousand Oaks, CA). After 2 months, the cells could grow rapidly in hIL-3. These cells were designated AML193.1.3 and were maintained in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum, 50 μM 2-mercaptoethanol and 4 ng/ml hIL-3. AML193.1.3 proliferation in response to hIL-3 or hIL-3 variants was determined by [^{3}H]thymidine incorporation. Cells were seeded at 2.5×10^4/well in 96-well microtiter plates (Costar, Cambridge, MA). Human IL-3 samples were added in Iscove's modified Dulbecco's medium supplemented with 500 μg/ml bovine albumin, 100 μg/ml human transferrin, 50 μg/ml soy bean lipids (all from Boehringer Mannheim) and 50 μM 2-mercaptoethanol. After 72 h, 0.5 μCi of [^{3}H]thymidine was added to each well. After 16–24 h, the cells were harvested onto a glass fiber filter mat (Pharmacia Biotech Inc.) and counted in an LKB 1205 Betaplate scintillation counter (Bromma, Sweden). Osmotic shock fractions with hIL-3_{15-125} accumulation levels greater than 1 μg/ml were tested in the AML193.1.3 bioassay, and the concentration of hIL-3_{15-125} variants that gave 50% of maximal proliferation was determined. Each assay included secreted hIL-3_{15-125} as an internal standard and was performed 1–7 times. All activity values were expressed relative to this control. Purified protein variants were assayed at least 3 times, and EC_{50} values were calculated by fitting a four-parameter logistic model to the data.

Purification of hIL-3—Inclusion bodies from *E. coli* cell pellets were isolated by sonication in 10 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride followed by centrifugation (38). One gram of inclusion body pellet was extracted for 15–30 s with 5 ml of 6 M guanidine HCl, 50 mM CHES, pH 9.5, 20 mM dithiothreitol, using a Bio-Homogenizer. The solution was gently rocked for 2 h at 5 °C and dialyzed (1,000 molecular weight cut-off) overnight at 5 °C against 100 volumes of 4 M guanidine HCl, 50 mM CHES, pH 8.0. Dialysis was repeated against 2 M guanidine HCl, 50 mM CHES, pH 8.0. The protein solution was acidified by the addition of an equal volume of 40% acetonitrile (CH_3CN), 0.2% trifluoroacetic acid. After clarification by centrifugation (16,000 × *g* for 5 min), the supernatant was loaded onto a Vydac C-18 reversed phase column (10 × 250 mm), equilibrated in 20% CH_3CN, 0.1% trifluoroacetic acid. The column was eluted at 3 ml/min with a linear gradient (0.2% CH_3CN/minute) between 40 and 50% CH_3CN, 0.1% trifluoroacetic acid. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and the appropriate fractions were pooled and dried by lyophilization or in a Speed Vac concentrator. The dry powder was reconstituted with 10 mM ammonium bicarbonate, pH 7.5, and clarified by centrifugation (16,000 × *g* for 5 min). The amino acid composition and concentration of purified proteins were determined from samples that had been subjected to acid hydrolysis (6 M HCl, evacuated sealed tubes, 90 min, 150 °C). All analyses were performed after postcolumn derivatization of the hydrolysates using ninhydrin as modified from Ref. 44. A Beckman model 6300 Autoanalyzer was employed for the actual determinations. All proteins were recovered at a purity of >90%, as determined by densitometric scanning of Coomassie-stained SDS-polyacrylamide gels.

RESULTS

N- and C-terminal Deletions of hIL-3 Define a Core Required for Full Activity—Deletions of the N and C termini of hIL-3 were constructed in order to determine the minimal protein required for full activity. The variants were expressed in the cytoplasm of *E. coli*, refolded *in vitro*, purified, and tested in the AML193.1.3 cell proliferation bioassay. As shown in Table I, residues 15–118 were required for full activity, while C-terminal deletions extending beyond residue 118 retained less than 1% activity. Our data on the minimal size of a bioactive hIL-3 molecule differ somewhat from those of Dorssers *et al.* (14) who found that hIL-3_{15-115} retained approximately 10% bioactivity.

TABLE I
Deletion analysis of interleukin-3

Deletion variant	Solubility[a] in *E. coli*	Activity[b]
1–133	−	1.00 (reference)
1–125	++	3.2
1–114	ND[c]	0.001
15–114	ND	0.006
15–115	−	0.002
15–116	−	0.001
15–117	+	0.004
15–118	+	2.1
15–119	+	1.6
15–123	+	2.5
15–124	++	3.0
15–125	++	2.4

[a] Apparent solubility in *E. coli* was determined by SDS-polyacrylamide gel analysis of proteins fractionating either in the cell pellet or in the supernatant (see "Materials and Methods"), followed by Coomassie staining and visual inspection. The (−) symbol demotes no detectable hIL-3 variant in supernatant; (+) indicates detectable material in sonication supernatant; and (++) denotes approximately 1:1 partitioning between the pellet and supernatant.

[b] Proliferative activity of refolded, purified variant protein is expressed relative to that of hIL-$3_{1–133}$.

[c] ND, not determined.

Interestingly, we routinely found that variants lacking residues 119–133 had a 2–3-fold increase in activity.

Human IL-3 produced in the cytoplasm of *E. coli* accumulated as insoluble inclusion bodies and was recovered in the cell pellet (see Table I). During the course of our deletion analysis of hIL-3, it was found that the deletion of the last eight amino acids (TTLSLAIF) resulted in approximately 50% of the hIL-3 variant fractionating in the cell supernatant rather than the cell pellet. The reason for this apparent change in solubility is unclear, but it might be due to the removal of a number of hydrophobic amino acids.

Secretion of hIL-$3_{15–125}$ in E. coli—Although refolding and purification of cytoplasmically produced hIL-3 variants was suitable for testing a small number of proteins, it was too cumbersome for an extensive mutagenesis study. We have previously demonstrated the utility of secretion in *E. coli* as a route for generating soluble, correctly folded recombinant bovine somatotropin (45), and therefore tested whether hIL-3 could expressed as a secreted protein when fused to the *E. coli* LamB signal peptide. The truncated protein, hIL-$3_{15–125}$, could be expressed using this vector (Fig. 1*a*). Efficient signal processing of the LamB signal peptide was observed, releasing hIL-$3_{15–125}$, which was soluble and could be extracted from the periplasmic space by osmotic shock. Accurate processing of the signal peptide was confirmed by N-terminal sequence analysis. As shown in Fig. 1, *b* and *c*, the secreted hIL-$3_{15–125}$ had equivalent activity to cytoplasmically-produced hIL-$3_{15–125}$, which had been refolded, purified, and accurately quantified. Hence, secretion provided a simple route for the production of correctly folded hIL-$3_{15–125}$ and provided a critical tool for the extensive mutagenesis of hIL-3.

Construction and Screening of Libraries of Single-site Mutants of hIL-$3_{15–125}$—An extensive site-directed mutagenesis of the essential portion of hIL-3 was undertaken. At the outset of this study, we wished to avoid any preconceived ideas about which residues would be important for biological activity and therefore constructed a series of 105 libraries of single-site mutants at most positions in the hIL-$3_{15–125}$ molecule (Fig. 2). Approximately eight mutants were selected at random from each library, and expression of the mutants in *E. coli* was induced by the addition of arabinose. After extracting the proteins by osmotic shock, the level of hIL-3 variant was measured by ELISA. Typically, secreted hIL-$3_{15–125}$ could be recovered from osmotic shock fractions of *E. coli* at a level of about 5–10

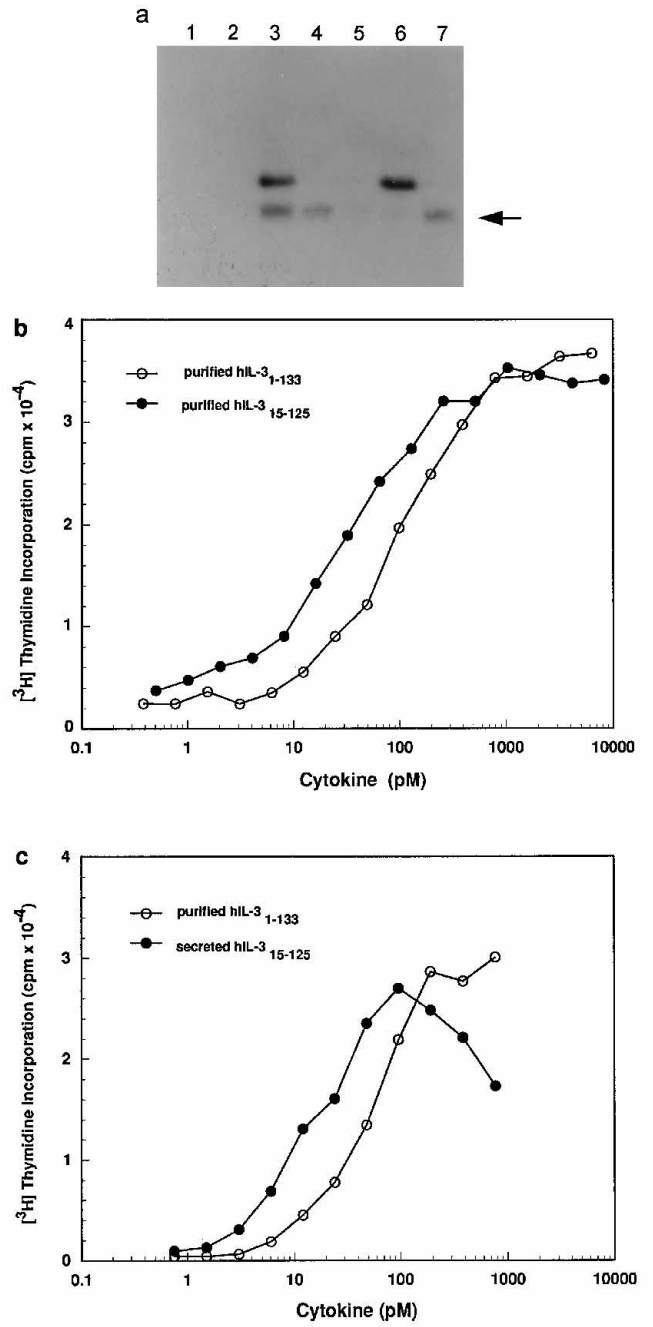

FIG. 1. **Secretion of bioactive hIL-$3_{15–125}$.** *a*, immunoblot analysis of secretion of hIL-$3_{15–125}$ from *E. coli*. Cultures were induced and proteins fractionated as described under "Materials and Methods." Loading in *lanes 1–6* was normalized to culture density prior to sample preparation (4 Klett units loaded). *Lane 1*, negative control, JM101 cells 3 h post-induction; *lane 2*, whole cell pellet of JM101 expressing secreted hIL-$3_{15–125}$, prior to induction; *lane 3*, whole cell pellet of JM101 expressing secreted hIL-$3_{15–125}$, 3 h post-induction; *lane 4*, osmotic shock water fraction of secreted hIL-$3_{15–125}$, 3 h post-induction; *lane 5*, sucrose fraction; *lane 6*, remaining cell pellet after osmotic shock; *lane 7*, 50 ng purified hIL-$3_{15–125}$ standard, denoted by the arrow. *b*, AML193.1.3 proliferation assay of purified hIL-$3_{15–125}$ and purified hIL-$3_{1–133}$. Purified proteins were quantified by amino acid composition. *c*, AML proliferation assay of crude, secreted hIL-$3_{15–125}$. Proteins were released from *E. coli* by osmotic shock and assayed for bioactivity. hIL-3 immunoreactive material was quantified by ELISA.

μg/ml. As illustrated in Fig. 1*c*, the secreted hIL-$3_{15–125}$ showed a biphasic dose-response relationship, with reduced proliferative activity at concentrations above approximately 100 pM. This effect was due to the presence of inhibitory material in the osmotic shock fraction, which was manifested at low sample

TABLE II

Structure-activity relationship of point-mutants of hIL-$3_{(15\text{-}125)}$. Unique clonal variants were assayed for AML proliferation activity and quantified by ELISA. All activities are expressed relative to that of native hIL-$3_{(15\text{-}125)}$ produced in the secretion vector. Amino acids are color coded according to the nature of the side chain: nonpolar (A, F, I, L, P, M, W), polar, uncharged (Y, C, G, H, S, T, N, Q); charged (R, K, E, D). COMMENTS: §1: All mutants [illegible]. [illegible] had [illegible] slightly below the [illegible] level required for accurate [illegible] because of [illegible] at these positions. *2: Mutants with these substitutions [illegible] had a [illegible]. *3: [illegible] also [illegible]. *4: [illegible] also [illegible]. *5: [illegible] also [illegible]. #6: [illegible] also [illegible]. *7: [illegible] had activity of [illegible]. *8: [illegible] had borderline activity of [illegible]. *9: [illegible] had borderline activity of [illegible].

Position	Native amino acid	Full activity (≥20% parental)	Moderate activity (5–19% parental)	Low activity (≤5% parental)	Low expression level	Comments
15	N					
16	C					
17	S	N Q G D R	K			
18	N	L I G H	F R			
19	M	I P	A G R		C	
20	I	A F		E R	C D	
21	D	P	A T S H	V F G Q N K		
22	E			A P G S C H	L Y W R D	
23	I	L S*	V G*	F§ W§ Y* M§*	F§ W§ K* R§	§1 *2
24	I	L V F		G Q		
25	T	A F S Q H R				
26	H	F A R	G	T	W	
27	L			A T	G D R	
28	K	C G R	V P		L W	
29	Q	L V H R			P	
30	P	G T S H			L K D	
31	P	L I A G D N R				*2
32	L	A G N Q E R		Y		*2
33	P	A Q H	L	T		
34	L	I V F A M T G S Q E R K				
35	L	V A P N Q		G		
36	D	L			V	
37	F	P S	I		W	
38	N	A				
39	N					
40	L				W R	
41	N	M H	L P C			
42	G	L I V A M Y S C N D H*				
43	E		N	L F A Y T S C Q D	G R	
44	D	N		L M W P S H K	A R T Q	
45	Q	L I V A M T S H E K	W	F	P	
46	D	I V F A Y G T S C H Q N E K				
47	I	V	H R	P G A		
48	L			I F S N H R	V C T K E	
49	M	V N D	A	F G H R		*4
50	E	A T S N D	P	I Q	V H K	
51	N	M P T S H R				
52	N	Q I	S	L H R		
53	L	N		P G T S E K	A	
54	R	A K	S	I L V G T H D	N	
55	R	L V Q T	S			
56	P	L Y A Q T S C N G H E R K				
57	N					
58	L			V S G D P	C	
59	E	L Y H	R		P	
60	A	T S N		Y	P	
61	F		S	P§ E R K	P§ R	§1
62	N	I V			H R D	
63	R	V F W H K	W	P		*6
64	A		T*	P N S Y		*8
65	V	L I S	C	P H		
66	K	M V G N T	V* E			*5 #3
67	S	I V F G N H	P	A		
68	L	I P H	K W	T N		
69	Q	A P G T E R			L W	
70	N				Y L W	
71	A	M P I Q E R	N	L	W	
72	S	A M N Q E R	H			
73	A	L P§ G T S Q E R				#5
74	I		M*	C	A P T R	*7
75	E	G S Q	D K		L W P	
76	S	V A P G N D E				
77	I	L S	R	T		
78	L			E	F A G S R	
79	K	I M S T N D R				
80	N	L Y Q H R	T		W	
81	L		V	A W G	Q N K	
82	L	A I F H M W Y V T G N H D R E	K			
83	P	A N W T			R	
84	C			C P	Y H R	
85	L	V		N S	N§ Q	§1
86	P				A C N K	
87	L	R			W S	
88	A	W	V R		R	
89	T	S N C	V K H	L	C	
90	A	M I E		P Q	F S	
91	A	L F S T S	S		H	
92	P	S			L T H E K	
93	T	L A P G Q D			N	
94	R			L I V P§ Q S§ H	F§ N§ K E	§1
95	H	L I V F N E G S T H K R	Y	G	F W	
96	P			I Q P§ T K	F§	§1
97	I	V	A	N§ R§	N§ K D	§1
98	H	L I F Y A M P T S Q N C R E K			S	
99	I	L V F		G P S C H D R		
100	K	L I P S G H R		Y		
101	D	V I L M F Y N T H K			G E	
102	G	S E	K	L P§ Y	P§	§1
103	D			S	L P Q R E	
104	W	L F	M V G	P T C	A C K	
105	N	L I A W F Y S G D H K	P			
106	E	A Q	S		I P T A	
107	F					
108	R	A Q S	T K	L I	F H D	
109	R	L T S E	G		F	
110	K		A*	M§ T H	L I M§ Q H F E	§1 *8
111	L				F M N D	
112	T	V Q H	S E	Y		
113	F		S*	H	F L Q N S C K D	*6
114	Y	M		L	C S H R	
115	L	A		M F H R	Y W T H	
116	K	A F I V M W Y T S H	C E R N			
117	T	S N		W K	I P	
118	L				A P Y N C C E	
119	E	L P Y T S	R	K		
120	N	L V P Q H		A		
121	A	I P G S N C K				
122	Q	I P M W F Y S C H K				
123	A	L M P Y G H E				
124	Q					
125	Q					

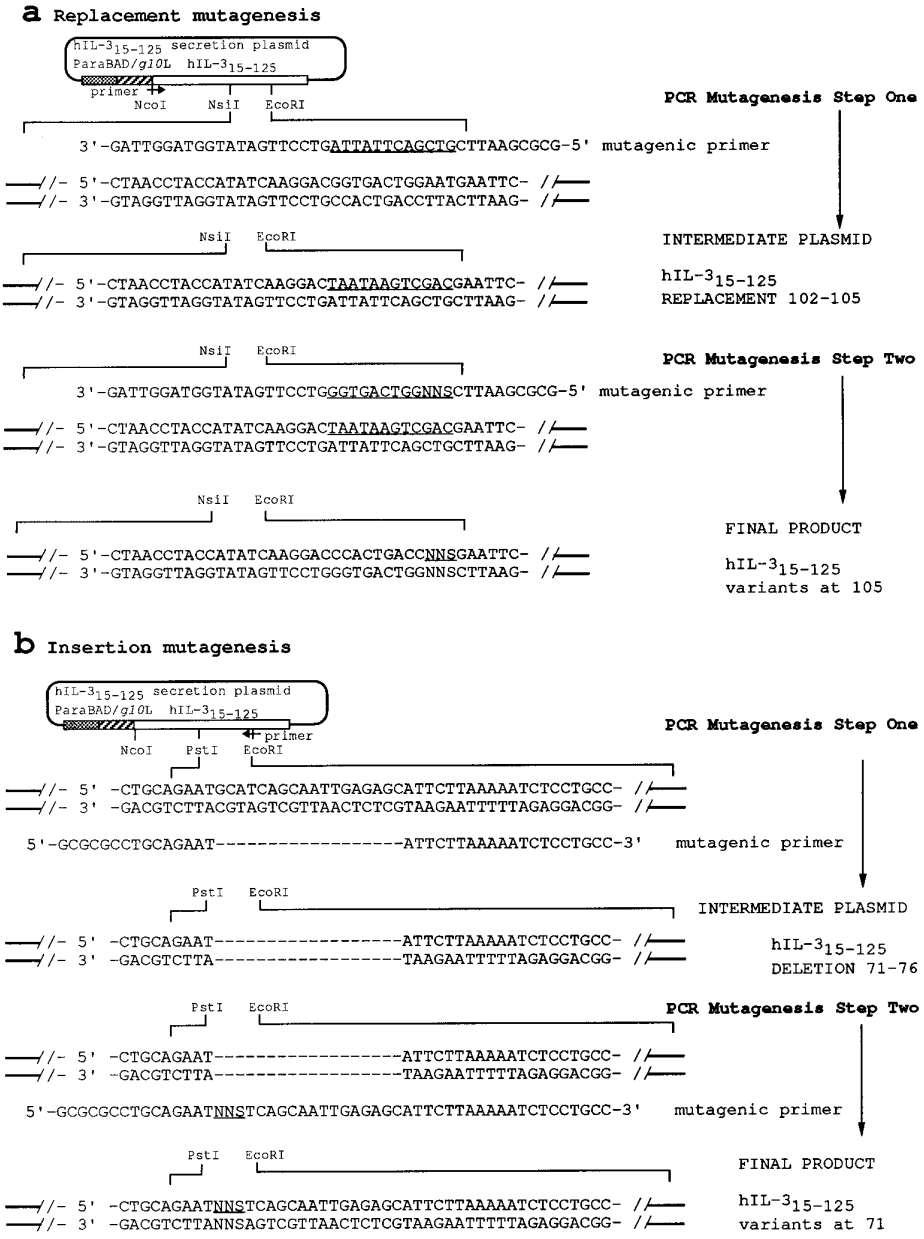

FIG. 2. **Construction of libraries of single-site mutants using two-step PCR mutagenesis.** Using the replacement mutagenesis approach (*a*), stop codons were first inserted in the hIL-3_{15-125} coding region, and the coding region was then restored in a second step, using a degenerate PCR primer. For insertion mutagenesis (*b*), a segment of hIL-3_{15-125} was first deleted, and then restored using a degenerate PCR primer.

dilutions (data not shown). Consequently, the bioassay screen was not used to measure potency when the concentration of hIL-3 variant was <1 μg/ml. Approximately 20% of the mutants were recovered at this level. The results of the 770 mutants characterized in this study are presented in Table II. Substitutions were divided into three activity classes: full ($\geq$20% specific activity of native hIL-3_{15-125}), moderate (5–19% specific activity) and low ($<$5%).

Most Residues in hIL-31_{5-125} Were Tolerant of Substitution—A representation of the structure-function relationship for hIL-3_{15-125} is shown in Fig. 3. As a rule of thumb, residues were considered tolerant of substitution if at least three substitutions were tested and found to have $\geq$5% bioactivity of the parental hIL-3_{15-125}. 71% of residues tested fell into this category. Residues considered to be intolerant of substitution had no more than one mutant with $\geq$5%, but at least two mutants with $<$5% activity. 16% of residues tested fell into this category. The assignment of 14 residues in hIL-3_{15-125} is uncertain, either because mutants were not recovered or because the accumulation level of the variants was low. It is likely that residues 36–40 are not necessary for biological activity, be-

TABLE III
Single-point mutations in hIL-3_{15-125} with increased activity

Purified hIL-3_{15-125} variants were tested in triplicate in the cell proliferation assay, and potency was calculated from a four-parameter logistics model. Activity was expressed relative to that of hIL-3_{1-133}.

Position	Native residue	Mutant residue	Relative activity
32	L	R	8
34	L	S	6
34	L	M	6
42	G	S	10
42	G	D	9
45	Q	M	6
45	Q	V	8
46	D	S	10
50	E	D	7
76	S	A	5
82	L	W	5
98	H	I	6
101	D	M	6
116	K	V	8
116	K	W	26
122	Q	F	6

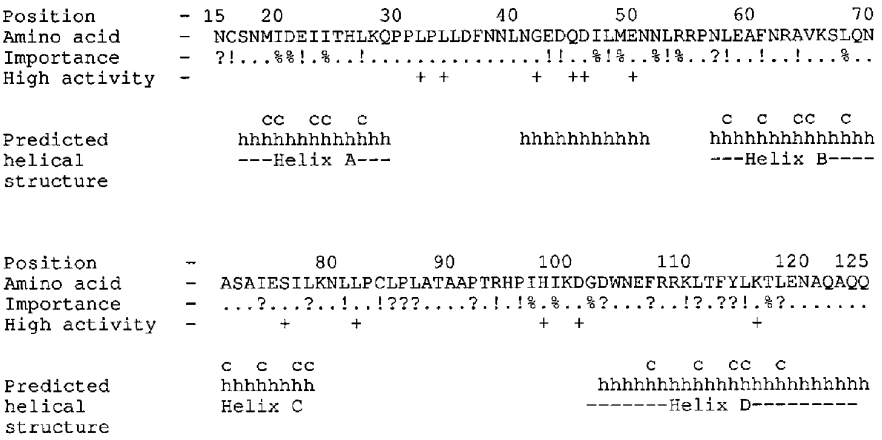

FIG. 3. **Relationship between predicted secondary structure of hIL-$3_{15–125}$ and bioactivity of single-site mutants.** Predicted helical regions (7, 47, 48) are indicated. !, intolerant of substitution; %, partially tolerant of substitution; ., not necessary for activity; ?, insufficient data; +, site of mutant(s) with increased activity; *c*, residue predicted to be on the hydrophobic side of an α-helix; *h*, predicted α-helical region. The predicted helical regions are similar to those recently identified from the NMR structure of a highly-substituted hIL-3 variant (60).

cause a hybrid protein containing the corresponding murine amino acids had substantial activity.[2] Residue Asn-70 is not likely to be important for receptor binding since it can be *N*-glycosylated in active hIL-3 produced in mammalian cells.

Mutants with Increased Activity—Approximately 1–2% of the single-site mutant proteins appeared to have elevated activity in the screening assay relative to that of hIL-$3_{15–125}$ (data not shown). In order to validate these results with purified proteins, 16 mutants were selected (see Table III and Fig. 3). The mutant hIL-$3_{15–125}$ genes were transferred to a cytoplasmic vector, permitting the proteins to be produced at high levels in inclusion bodies. The variants were then refolded *in vitro*, purified, and quantified by amino acid analysis. This approach eliminated the possible artifacts that might have arisen out of the screening approach. All 16 purified proteins showed at least 5-fold increased activity. Remarkably, K116W was 26-fold more active than native hIL-3.

DISCUSSION

We have measured the effect of 770 amino acid substitutions on the cell proliferative activity of hIL-$3_{15–125}$. Most surprising was that single-amino acid substitutions at 12 positions in hIL-$3_{15–125}$ resulted in at least 5-fold increased cell proliferative activity (see Table III and Fig. 3). It seems unlikely that these 12 residues make essential hIL-3 receptor contacts, since all of the positions were highly tolerant of substitution or, in the case of Gln-122, could be deleted without loss of cell proliferative activity (*cf.* Table I). Furthermore, two different substitutions with increased activity were found at positions 34, 42, 45, and 116. It is interesting to note that two critical residues, Glu-43 and Asp-44, are flanked by residues where substitutions with increased activity were observed (positions 42, 45, and 46). Perhaps mutations with increased activity function by providing an improved context for critical residues. Increased potency could result by bringing essential contact residues into more favorable alignment with the receptor by reducing steric hindrance through moving unfavorable residues away from the receptor or by having an effect on the flexibility of the ligand. For most of the variant proteins tested, increased bioactivity correlated with increased affinity for the hIL-3 receptor.[3]

Only 16 residues of hIL-3 were identified that were critical for activity (see Fig. 3). Five of these critical amino acids, Glu-22, Glu-43, Asp-44, Arg-94, and Lys-110 are charged residues that are candidates for contact with the receptor. Our interpretation of the role of these amino acids is consistent with previous reports (8, 14, 15, 17) and supports the model that electrostatic interactions have been proposed to be important for ligand-receptor interactions of the hematopoietic cytokines (4, 49). Most of the other residues interpreted to be critical for activity of hIL-3 are nonpolar (Leu-27, Leu-48, Leu-53, Leu-58, Phe-61, Ala-64, Leu-81, and Leu-115). "Helical wheel" (7, 50) projections predict that all but residue Leu-81 are on the hydrophobic faces of amphipathic α-helices. It is likely that these nonpolar residues are in the interior of the protein and do not interact directly with the receptor. Substitution of these residues could result in disruption of the structure of hIL-3 and hence render the protein inactive (21, 51, 52). Other nonpolar residues, Ile-20, Ile-23, Ile-24, Ile-47, Met-49, Leu-68, Ile-97, and Ile-99 were found to be partially tolerant of substitution. At some positions, 23, 24, 97, and 99, only other nonpolar residues were identified in active hIL-$3_{15–125}$ variants. Interpretation of the effect of substitutions at residues Phe-107, Leu-111, Tyr-114, and Leu-118 is problematic since few mutants were expressed at sufficient levels for assay. However, all four residues are located on the predicted hydrophobic face of helix D and therefore seem unlikely to be involved directly in interaction with the receptor. It should be noted that atoms in the protein backbone can also interact with the receptor (24, 53).

We have not determined the nature of the expression defect seen for approximately 20% of the mutants. However, it is tempting to speculate that several of these single-site mutants might cause a disruption in protein structure that could result either in aggregation, increased susceptibility to host proteases and/or faulty recognition by the host secretion machinery (54). Examination of Table II and Fig. 3 suggests that substitution of residues predicted to lie in the hydrophobic core of the protein can often lead to a reduction in accumulation. This would be consistent with a disruption in protein folding, as has been observed for other proteins (21, 51, 52, 55). 12 hIL-$3_{15–125}$ mutants with levels of recovery slightly below 1 μg/ml (indicated by § in Table II) were assayed for proliferative activity. In all cases, the mutants had low activity, supporting the notion that the phenotype of low expression can provide useful biological information. We have not undertaken physical studies (such as CD spectroscopy) of poorly expressed mutants to determine whether substitutions do indeed disrupt protein folding or thermal stability. Tryptophan, lysine, and cysteine substitutions were strongly overrepresented in the low expression category (χ^2 analysis not shown). In the case of tryptophan, it is possible that the presence of the bulky side chain could result in a disruption of protein structure. The reason for overrepresentation of lysine is unknown. The overrepresentation of cysteine in this class of mutants might be a consequence of aberrant disulfide bond formation and/or protein dimerization (21). Cys-

[2] P. O. Olins, S. C. Bauer, S. Braford-Goldberg, K. Sterbenz, J. O. Polazzi, M. H. Caparon, B. K. Klein, A. M. Easton, K. Paik, J. A. Klover, B. R. Thiele, and J. P. McKearn, unpublished results.

[3] J. Thomas, unpublished results.

teine substitution did not always disrupt the protein, since we observed several examples of cysteine substitutions retaining considerable activity (at positions 41, 42, 46, 56, 104, and 122).

Our choice of mutagenesis was more extensive than two other commonly used approaches, alanine scanning (20, 26–32) and charge reversal (14, 19, 22, 56, 57). In general, the 46 alanine substitutions recovered in our random mutagenesis support the value of alanine scanning mutagenesis. Seven alanine substitutions gave low accumulation levels, so bioactivity could not be determined. At 36 positions, the conclusions from alanine substitutions we recovered agree with the interpretation from the substitutions of other amino acids at those positions. However, at residues Ser-67, Leu-115, and Asn-120, alanine substitution did not have the same effect as substitution of other amino acids. Hence, alanine scanning would have led to false interpretation about the importance of these residues. Moreover, it should be stressed that alanine scanning would not have identified most of the mutants with enhanced proliferative activity. An alternative mutagenic approach is charge reversal (14, 19, 22, 56, 57), in which charged residues are substituted with those with the opposite charge to test the effect on activity. The assumption is that electrostatic interactions may be an important element in ligand-receptor interaction (4, 49, 58). In our study, results from three charge reversal substitutions D21K, R54D, and E119K would have led to a conclusion that was in disagreement with that from our larger data base. Consequently, applying a charge reversal scanning mutagenesis strategy to hIL-3 would have led to a limited and potentially misleading interpretation.

Our mutagenesis work led to the identification of 770 amino acid substitution mutations in hIL-3_{15-125}. The catalogue of tolerated amino acid substitutions generated in this study permits the construction of highly-mutated hIL-3 variants (59). The solution NMR structure for one highly substituted variant of hIL-3 (SC-65369) has recently been solved (60). This structure should provide an excellent tool for refining the interpretation of the mutant data base presented in this paper. The random mutagenesis approach we have employed is an effective way to discover variants of a protein hormone with increased activity, particularly in cases where little structural information is available. The relatively large number of mutations of hIL-3_{15-125} with elevated cell proliferative activity was unexpected, considering that the potency of hIL-3 is already high (EC_{50} = 10–100 pM). Perhaps the optimal *in vivo* properties of hIL-3 do not include the maximal affinity for its receptor. We anticipate that mutants of other protein hormones may also have considerably increased activity. If so, this may be important for the development of clinically superior molecules.

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