Receptor and Antibody Interactions of Human Interleukin-3 Characterized by Mutational Analysis*

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Human interleukin-3 (hIL-3) is a regulator of proliferation and differentiation of multipotent hemopoietic progenitor cells. Mutants of hIL-3 have been constructed by oligonucleotide-directed mutagenesis and expressed in Escherichia coli and Bacillus licheniformis. Purified muteins were assayed for induction of DNA synthesis in IL-3-dependent human cells and for binding to the IL-3 receptor. Residues at the NH₂ and COOH termini together comprising one-quarter of the molecule could be removed without loss of biological function. Deletions of 6–15 residues within the central part of the molecule caused a large reduction (up to 5 logs) but no complete loss of activity. Substitution of evolutionary conserved residues resulted in a strong decrease of biological activity and demonstrated that the S-S bridge is an essential structural element in hIL-3. Interestingly, four muteins displayed a significantly higher potency of binding to the IL-3 receptor than in stimulating DNA synthesis. These results demonstrate that receptor binding may be (partly) disconnected from activation of DNA synthesis. Analysis of hIL-3 muteins demonstrated that the majority of monoclonal antibodies are directed against a small portion of the IL-3 molecule. The neutralizing potential of individual monoclonal antibodies could be increased by a combination of antibodies directed against nonoverlapping epitopes.

Interleukin-3 (IL-3) is a hemopoietic growth factor that regulates proliferation and differentiation of immature blood cell progenitors (Metcalfe, 1986; Clark and Kamen, 1987; Wagemaker et al., 1990a). Murine IL-3 was shown to stimulate proliferation of multipotent hemopoietic progenitors (Ihle and Weinstein, 1986). The human counterpart was identified and characterized by binding to specific cell surface receptors (Metcalf, 1986; Nicola and Metcalf, 1988). To assess which regions of the protein are involved in receptor binding we have constructed a series of deletion and substitution mutants. These mutants were expressed in Escherichia coli and Bacillus licheniformis, purified and tested for biological function and receptor binding. In addition, these muteins were used to define the binding sites of neutralizing monoclonal antibodies (mAbs) directed against hIL-3.

MATERIALS AND METHODS

Bacterial Expression Vectors—All modifications on human IL-3 were performed on the IL-3 cDNA insert, contained in the eukaryote expression plasmid pLB4 (Dorssers et al., 1987) after removal of the repeat sequences within the 5'-noncoding region by blunt end ligation of filled AvaI (nucleotide residue 545) and XhoI (nucleotide residue 857) sites. The shortened IL-3 cDNA was inserted into the polylinker of pUC8 in phase with the NH₂-terminal amino acids of the lacZ polypeptide. Since the 5'-noncoding sequences, as well as the sequences encoding the IL-3 leader polypeptide, were included in this construct (pUC/lacZ), a 176-amino acid fusion polypeptide of 19,866 daltons was produced. This fusion protein was produced efficiently in E. coli and used for raising antibodies.

To produce a polypeptide closely resembling the mature human IL-3, a construct was made lacking the 5'-nontranslated and leader IL-3 sequences. The IL-3 cDNA insert of pUC/lacZ was digested with HindII and HindIII, ligated to a synthetic oligonucleotide containing a high copy number (pUC/HindII/III). The resulting plasmid (pUC/lacZ) was used for transformation of E. coli strain DH5α. The expression of IL-3 was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The conditioned medium was harvested after 4 hours of incubation and used for the preparation of the fusion protein. The fusion protein was purified by affinity chromatography on a affinity matrix containing immobilized protein A, followed by gel filtration on a Superdex 75 column. The purity of the fusion protein was assessed by SDS-PAGE under reducing conditions and by Western blotting using a monoclonal antibody directed against the C-terminus of IL-3. The fusion protein was used for the preparation of polyclonal antibodies, which were used for the production of monoclonal antibodies. The monoclonal antibodies were prepared using standard procedures and were used for the analysis of the receptor interactions of IL-3.

In summary, the results presented in this article demonstrate that hIL-3 is capable of stimulating hemopoiesis in all bone marrow lineages (Wagemaker et al., 1990; Gansser et al., 1990, 1990b). DNA mutagenesis (Botstein and Shortle, 1985) has been applied extensively as a tool to study the mechanism of action of proteases, enzymes, growth factors, receptors, transcription factors, oncogenes, and other cell components (Knowles, 1987; Stone et al., 1988; Russell and Fersht, 1987; Bass et al., 1988; Cohen et al., 1986a; Kuga et al., 1989; Yanofski and Zurawski, 1990; Izbizes et al., 1990). Analysis of fusion proteins, deletion, insertion, and substitution mutants has enabled the precise localization of functional domains in some cases. Amino acid residues involved in receptor binding of IL-2 (Cohen et al., 1986; Zurawski and Zurawski, 1988, 1989) and growth hormone (Cunningham and Wells, 1989; Cunningham et al., 1989) have been identified by using these procedures. Such information may result in the development of mutant proteins with either enhanced function (Russell and Fersht, 1987), reduced antigenicity, or even antagonistic activity (Marcucci and De Maeyer, 1986; Baird et al., 1988; Hannum et al., 1990; Eisenberg et al., 1990; Carter et al., 1990).
purifying the sequence encoding the NH2-terminal 14 amino acids of the IL-3, as inserted into pTZ19R (Pharmacia LKB Biotechnology Inc.) digested with Sall and HindIII. After verification of the sequence (Sanger et al., 1977), the complete IL-3 insert on the Sall-HindIII fragment was introduced into pUC8 (digested with Sall and HindIII) for protein production. To allow for direct sequencing after expression, the fl-ori and HindIII to substitute part of the polylinker and 5’terminal IL-3 sequences (nucleotide region 137-497) and the a-amylase terminator contained in plasmid pGB/IL-322 (van Leen et al., 1991). The resulting plasmid (pPH3) was digested with BamHI and HindIII to substitute the polylinker and 5’terminal IL-3 sequences for kinased synthetic oligonucleotides. Thus, the IL-3 gene was reconstructed with a heterologous leader sequence (14 amino acid residues, M, = 16,541). The DNA sequence of this plasmid (pPH4) was verified. The DNA and protein sequence of the heterologous leader are shown.

**MTMitNSRGSSVD**

ATGACCATGATAGATCCCCGGGATCGGAC

An alternative expression plasmid was constructed for transfer of interesting mutants into large scale production vectors for different hosts. For this purpose, the pPH1 plasmid was digested with HpaI and HindIII to remove the 3’terminal part of the IL-3 cDNA, made blunt, and ligated with a blunt fragment carrying the corresponding IL-3 sequences (nucleotide region 137-497) and the B. licheniformis a-amylase terminator contained in plasmid pGB/IL-322 (van Leen et al., 1991). The resulting plasmid (pPH3) was digested with BamHI and HindIII to substitute part of the polylinker and 5’terminal IL-3 sequences for kinased synthetic oligonucleotides. Thus, the IL-3 gene was reconstructed with a heterologous a-amylase leader peptide (14 amino acid residues, M, = 16,541). The DNA sequence of this plasmid (pPH4) was verified. The DNA and protein sequence of the heterologous leader are shown.

**MTMitNSRGSSAAAAA**

ATGACCATGATAGATCCCCGGGATCGGAC

*In Vitro Mutagenesis*—In vitro mutagenesis was performed using synthetic oligonucleotides (mutations were flanked by 12–15 homologous nucleotides) according to the procedure developed by Kunkel et al. (1987). Single-stranded template DNA was prepared by transformation of pPH1 or pPH4 DNA into E. coli strain C3236 (Bio-Rad) and superinfection with M13K07 (Pharmacia) helper phage. Phage DNA was prepared and fractionated on 16,384. The DNA and protein sequence of the heterologous leader are shown.

**MTMitNSRGSSAA**

ATGACCATGATAGATCCCCGGGATCGGAC

Recombinant IL-3 protein—E. coli JM109 cultures were exported into the culture medium (van Leen et al., 1991) and superinfection with the parental IL-3 construct. After sequence verification these clones were used for protein production. For expression in BL21, the fragment of the pPH4-derived mutant containing the a-amylase signal peptide, the mutant IL-3 cDNA, and the a-amylase terminator was isolated and transferred to the Bacillus expression vector pGB/IL-322 (van Leen et al., 1991).

Molecular biology procedures not detailed were performed according to the standard procedures (Maniatis et al., 1982; Berger and Kehoe, 1984; Sambrook et al., 1989). Molecular biology reagents were obtained from Gibco-BRL and Pharmacia.

Purification of Recombinant IL-3 Protein—E. coli JM109 cultures (100 ml) were inoculated with 0.5 ml of a fresh overnight culture of the (mutant) IL-3 clone and grown at 37°C until an OD of 0.4–0.6 at 550 nm. Plasmid-directed protein synthesis was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (Pharmacia). After culture for 3–6 h the cells were collected by centrifugation (10 min, 3,000 x g at 4°C) and stored frozen. Lysozyme was added (500 µg/ml) to the E. coli suspension in 10 mM of TE (10 mM Tris/HCl, pH 8, 1 mM EDTA). After incubation for 30 min at room temperature, MgCl2 and DNase were added to final concentrations of 10 mM and 20 µg/ml, respectively. After incubation at 37°C for 15 min, Tween 20 (0.2%), DTT (2 mM), and phenylmethylsulfonyl fluoride (0.1 mM) were added. The suspension was cooled on ice and sonified vigorously (two x 35 s). The homogenate was clarified by centrifugation (30 min at 15,000 x g) at 4°C, and the supernatant was discarded. The pellet was resuspended in 4 ml of 55% sucrose in buffer TED (50 mM Tris/ HCl, pH 8, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mM DTT) by sonication and layered onto a discontinuous sucrose gradient (2-m1 portions of 75 and 60% of sucrose in buffer TED). After centrifugation at 200,000 x g at 25°C for 2 h the inclusion bodies containing the IL-3 proteins were recovered from the 75% sucrose interface (essentially as described by Clark et al. 1987). At least 4-fold dilution the inclusion bodies were pelleted at 25,000 x g (30 min) and sonified in 5 ml of 8 M urea containing 50 mM Tris/HCl, pH 8.9, and 2 mM DTT and left overnight at 4°C. The clarified solution was next applied to a 3-ml DEAE-Sepharose Fast Flow column (Pharmacia), equilibrated with 8 M urea, 50 mM Tris/HCl, pH 8.9, and 1 mM DTT buffer. The IL-3 protein was bound to the column and step eluted with 75 mM NaCl in the same buffer. The eluted protein was dialyzed against several portions of 10 mM Tris/HCl, pH 8.0, and 1 mM DTT buffer and made isotonic by adding 10-fold concentrated RPMI cell culture medium (Sigma) containing 1% BSA. The filter-sterilized solution was used for biochemical and biological characterization.

Mutant proteins produced by *B. licheniformis* T9 were exported into the culture medium (van Leen et al., 1991) and do not require such drastic purification procedures. The clarified supernatant (1 liter) was adjusted to 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride

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2 S. C. Clarke, A. B. Ciarietta, and Y. C. Yang, International Patent Application, WO 86/00598.
fluoride, 1 M ammonium sulfate and passed over a 15-20-mL Fractogel TSK Butyl 650(C) column (Merck) equilibrated with 1 M ammonium sulfate, 10 mM Tris/HCl, pH 7.0, buffer. The bound IL-3 protein was eluted with 10 mM Tris/HCl buffer and subsequently passed over a 1.5-mL DEAE-Sepharose Fast Flow column equilibrated with 10 mM Tris/HCl, pH 8.0, buffer. The flow-through was collected and adjusted to 70% saturation with ammonium sulfate to concentrate the IL-3 protein. The precipitate was collected by centrifugation at 15,000 × g, dissolved, and dialyzed against 10 mM Tris/HCl, pH 8.0, 1 mM DTT buffer.

Protein samples were analyzed on 13.5% SDS-polyacrylamide gels (acylamide/bisacrylamide = 29:1). Proteins were visualized by either Coomassie Brilliant Blue G-250 staining or immunological methods. Proteins were quantified (±15%) using densitometric analysis (model 620, Bio-Rad) with the purified recombinant IL-3 preparation (0.3-1 µg) as a reference. For preparation of large amounts of 21-kDa fusion protein (pUC/Hmulti) for antibody production, the bacterial pellet was homogenized in TE buffer containing 0.2% Nonidet P-40 using sonication. After centrifugation the pellet was solubilized in 1 M urea, 1% SDS, 1% 2-mercaptoethanol and fractionated on preparative 12.5% SDS-polyacrylamide gels. After completion of the run, 1-cm side strips were cut from the gel and stained briefly. The 21-kDa fusion protein band was located in the gel using the stained strips, excised, and used for immunization.

Immunological Procedures—Monoclonal antibodies were raised by injecting BALB/c mice with the gel-purified IL-3 fusion protein. The excised gel band was minced in saline with a mortar and emulsified in a 1:1 ratio in incomplete Freund's adjuvant. Splenic lymphocytes were fused 3 days later with SP2/0 myeloma cells according to standard procedures (Galfre and Milstein, 1981). Hybridoma supernatants were screened with an enzyme-linked immunosorbent assay with a lysate of E. coli cells containing the fusion protein as a positive control and a lysate of E. coli/pUC28 as negative control. Hybridoma cultures specific for IL-3 were selected and stabilized. Monoclonal antibodies were mostly of IgG1, IgG2A, and IgG2B type. A limited number of hybridoma cell lines was injected into mice for large scale production of antibodies. The ascites fluids were purified using protein A affinity chromatography.

For immunological detection of IL-3 proteins the gel-fractionated proteins were transferred onto nitrocellulose (0.2 µm) using a semidry blotting system (Pharmacia) with a continuous buffer system (39 mM glycine, 48 mM Tris, 0.0575% SDS, and 20% methanol) at 1.2 mA/cm² for 90 min. The nitrocellulose filter was subsequently air dried, excised gel band was minced in saline with a mortar and emulsified in a 1:1 ratio in complete Freund's adjuvant containing 1 mg of Mycobacterium tuberculosis H37RA/ml. Booster injections were given at week 2 in incomplete Freund's adjuvant. Splenic lymphocytes were fused 3 days later with SP2/0 myeloma cells according to standard procedures (Galfre and Milstein, 1981). Hybridoma supernatants were screened with an enzyme-linked immunosorbent assay with a lysate of E. coli cells containing the fusion protein as a positive control and a lysate of E. coli/pUC28 as negative control. Hybridoma cultures specific for IL-3 were selected and stabilized. Monoclonal antibodies were mostly of IgG1, IgG2A, and IgG2B type. A limited number of hybridoma cell lines was injected into mice for large scale production of antibodies. The ascites fluids were purified using protein A affinity chromatography.

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**RESULTS**

**Protein Production and Purification**—The expression vectors pPH1 and pPH4 carrying the IL-3 cDNA fused to the NH₂-terminal amino acids of β-galactosidase were modified by the oligonucleotide-directed mutagenesis procedure described by Kunkel et al. (1987). A total of 36 mutant IL-3 proteins, consisting of 18 deletion mutants and 18 substitution mutants, has been generated and analyzed (see Table II). The expression of pPH4-derived IL-3 proteins in E. coli was generally slightly higher than the same mutant protein from the pPH1 expression vector. The employed purification procedures were not designed for complete purification of IL-3 proteins but to remove toxic bacterial components and allow for quantification. In general, minor high molecular weight contaminants were observed on SDS gels. Using densitometric scanning of stained SDS gels (Fig. 1), the amount of IL-3 protein was determined in preparations that were also used for bioassays and to which BSA was added. The total yield was generally 0.1-1 mg of semipurified IL-3 protein from 100-mL cultures. Exceptions were mutants 811 and 933 carrying deletions between residues 29 and 49, which gave no detectable fusion protein production in either pPH1- or pPH4-derived expression vectors. Northern blot analysis indicated a strong reduction of mutant-specific RNA in the bacteria without a significant difference in plasmid DNA content. These results suggest that transcription or mRNA stability is reduced in E. coli through the deletions introduced in the eukaryote IL-3 cDNA sequence for reasons as yet unknown.

**TABLE I**

| Epitope mapping of IL-3 monoclonal antibodies |
|-----------------------------------------------|
| Mutant | 510 | 933 | 811 | 934 | 812 | 935 | 813 |
| (μ amino acid residue) | 20-30 | 29-37 | 31-49 | 47-54 | 54-61 | 60-70 | 68-80 |
| mAb | | | | | | | |
| A1, 2, 12, 15 | ++ | ++ | ++ | + | + | + | + |
| A18, 32, 35 | ++ | + | + | + | + | + | |
| A4, 27 | + | + | + | + | + | + | + |
| 3, A5, 14 | + | + | + | + | + | + | + |
| A8, 6, 10, 26 | ++ | ++ | ++ | + | + | + | + |
| 13 | + | + | + | + | + | + |

*Mutants 820, 821, 822, 932, 936, 977, 938 and 939 are represented in this column.
Introduction of these mutant DNA sequences into a different host (B. licheniformis), using an expression vector carrying the α-amylase promoter, also resulted in poor production levels.

Characterization of Monoclonal Antibodies—Monoclonal antibodies of 17 hybridomas directed against hIL-3 were characterized by Western blot analysis of all deletion mutants. The majority of the monoclonal antibodies raised against SDS gel-purified fusion protein is directed against epitopes located between residues 30 and 55 of the mature IL-3 polypeptide (Table I). Since single deleted domains gave loss of binding of most mAbs these epitopes are likely to reflect linear amino acid chains. Further characterization by competition enzyme-linked immunosorbent assay of monoclonal antibodies from ascites fluids showed clear cross-competition between A1 and A18, between A5 and A8, and between A8 and A18, indicating that the binding epitopes of A1 and A18 overlap but are distinct from the overlapping epitopes of A5 and A8. Cross-competition was not observed with mAb A4. These data are in agreement with the immunoblotting experiments (Table I). Furthermore, all monoclonal antibodies were found to react with hIL-3 preparations from mammalian, bacterial, and yeast cultures, irrespective of glycosylation state (data not shown).

Analysis of key residues in the binding epitopes was performed using various substitution mutants and recombinant rhesus monkey IL-3 protein, which shows 19.5% amino acid sequence divergence with hIL-3 (Burger et al. 1990a, 1990b; see also Fig. 5). The Western blot analysis (Fig. 2, right panel) revealed that ascerts mAbs A1 and A18 recognize a similar domain. Binding is restricted to residues 31 to 37 and is slightly reduced upon substitution of 36D → R (1480, lane 5) and strongly affected by substitution of 34LL → SP in RhIL-3 (lane 9). Binding of mAb A8 is strongly influenced by substitution of residues 43ED → KR (1481, lane 4) and moderately by 46D → R (1482, lane 3), indicating that its epitope is located between residues 37 and 46. Substitutions in RhIL-3 at positions 42G → E and 46D → T apparently do
not interfere with A8 binding (lane 9). Analysis of deletion mutants showed that mAb A5 binding was located between amino acid residues 37 and 55. Substitutions of residues 46D → R (1482, lane 3), 50E → K (1483, lane 2), and 54RR → ED (1484, lane 1) showed clear reduction of binding whereas other NH2-terminal substitutions (such as 43ED → KR, lane 4) did not. This suggests that binding is confined to residues 44-55 and is sensitive to substitutions at positions 46 (D → R), 49 (M → V), and 51 (N → K) in RlIL-3 (lane 9). mAb A4 seems to bind to all available IL-3 mutants and variants (Fig. 2, right panel).

Experiments to test the efficacy of the purified ascites mAbs to block biological function of hIL-3 in vitro were performed on AML193 cells (Fig. 3). Monoclonal antibodies A1, A8, and A5 gave 50% inhibition of IL-3-stimulated (1.3 ng/ml, i.e. ± 20 units/ml) DNA synthesis at final dilutions of 1.8-4 × 10−4, whereas A4 showed little inhibition (±3 × 10−5). Mixtures of A1 + A5 and A1 + A8 were effective in 2.5 × 10−4 dilution whereas combination of all three mAbs showed 50% inhibition at 7 × 10−5 dilution (Fig. 3). Inhibition of AML193 DNA synthesis by the highest antibody concentration used (1%) could be completely reversed by the addition of excess IL-3, indicating the absence of nonspecific inhibitory substances. These results show that combinations of two or three monoclonal antibodies directed against different epitopes provide more potent inhibition (in comparison with the best single antibody: 4-14-fold, respectively) of in vitro biological function of human IL-3.

**Biological Activity of IL-3 Mutants**—IL-3 fusion proteins derived from both pH1 and pH4 expression plasmids containing the complete sequence for the mature human IL-3 protein were virtually identical in biological activity and not significantly different from the B. licheniformis “wild-type” reference hIL-3 (van Leen et al., 1991) preparation (Table II). Apparently, the heterologous NH2-terminal peptides of 12-15 amino acid residues exert no negative effect on biological activity. However, molecules with longer NH2-terminal peptides showed reduced biological activity. In-frame fusion of the complete IL-3 cDNA to lacZ of pUC8 (pUC/hmulti) resulted in a 175-amino acid product (including the wild-type leader peptide) displaying 100-fold reduced biological activity. Fusion of the mature IL-3 protein sequence to the COOH terminus of a full-length β-galactosidase protein resulted in an inactive polypeptide (not shown).

**IL-3 deletion mutants** were mostly significantly reduced in biological activity (Table II and Fig. 4A). Deletions of 6-15 amino acids between residues 47 and 106 resulted in more than 10,000-fold reduction in specific activity. However, in all cases residual biological activity was detected, which was
between binding affinity and the ability to stimulate DNA

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absent in control preparations from E. coli. These results indicate the sensitivity of the bioassay and the lack of large amounts of toxic components in the protein preparations used at high concentration. Biological activity was retained for deletion mutants 932 (1-14) and 939 (120-130) and the corresponding double mutant 932/9 (Fig. 4A). To demonstrate that the heterologous NH2-terminal peptide did not substitute for the natural amino acids, minimal mutants X (1-14/120-133) and Z (1-14/116-133) were expressed in B. licheniformis. These muteins exhibited close to full biological activity (Table II). In contrast, single substitutions of both cysteine residues (mutants 904, 16C + R) resulted in 3,000-fold reduction of activity (Fig. 4A). A similar effect was seen with a double cysteine mutant (904E, 16C+A), indicating that the S-S bridge plays an important stabilizing role in the IL-3 molecule. Substitution of (individual) amino acids by computer-assisted predictions (Chou and Fasman, 1978; Rose, 1978) available on the DNASIS (Pharmacia) software. Helix ends were chosen according to the amino acid preferences given by Richardson and Richardson (1988).

In Fig. 5, Alignment of primate and rodent IL-3 amino acid sequences. IL-3 amino acid sequences of human (Hu, Dorssers et al., 1987), gibbon (G; Yang et al., 1986), rhesus monkey (Rh, Burger et al., 1990a, 1990b), mouse (Mu, Fung et al., 1984), and rat (Ra, Cohen et al., 1986b) were aligned using the Clustal algorithm (Higgins and Sharp, 1988). Alignment was based on conservation of splice sites in these IL-3 species. Gaps in the aligned protein sequence are represented by blanks, and identical residues are indicated by dashes. The numbering refers to hIL-3 protein sequence. Cylinders at the top represent proposed helices for human IL-3 as predicted by the algorithms of Garnier et al. (1978), Chou and Fasman (1978), and Rose (1978) available on the DNASIS (Pharmacia) software. Helix ends were also shown to be required for biological function in mature IL-3 (Clark-Lewis et al., 1988). The hydrophobic residues at the COOH-terminal boundary mentioned above are also completely conserved in primate and rodent IL-3 species (Fig. 5) and could thus be essential for function. Binding of monoclonal antibodies to the essential domain of hIL-3 neutralized the biological function by interference with receptor binding and signal transduction. Deletion of amino acid residues may result in removal of residues involved in receptor binding or affect protein folding and similarly reduce the affinity of the mutein for the IL-3 receptor. Amino acid deletions within the essential domain of hIL-3 either had a moderate effect on activity (between residues 20 and 47) or resulted in at least 10,000-fold reduction of biological activity (Table II). The finding that all deletion mutants had retained some specific biological activity was unexpected. Contamination of mutant proteins with wild-type IL-3 derived from the parental pH vector is unlikely. Plasmid DNA of characterized mutants has been recloned in E. coli prior to protein production. This implies that the residual biological activity of the mutant proteins must be caused by specific interaction with the receptor and subsequent activation of intracellular signalling pathways.

Analysis of the substitution mutants shows that alteration of highly conserved residues (Fig. 5) in both primate (human, gibbon, and rhesus monkey) and rodent (mouse and rat) IL-3 species gives more than 3 orders of magnitude reduction of biological activity (1481, 43ED + KR; 1484, 54RR + ED; 1491, 106E + K; 1492, 106RR + EDE; 1493, 113FY + AT). Substitution of residues that have not been conserved during evolution shows moderate reduction (2-3 logs) of activity. Since a functional species barrier exists between primate and rodent IL-3 species (Cohen et al., 1986b; Dorssers et al., 1987; Burger et al., 1990a) it is likely that conserved residues are important in protein architecture rather than in specific interaction with the receptor. Circular dichroism measurements of purified recombinant hIL-3 have indicated a high proportion (≥80%) of α-helix content.3 Combining computer-assisted predictions (Chou and Fasman, 1978; Rose, 1978), personal communication.
1978; Garnier et al., 1978) and observed amino acid preferences at the ends of helices (Richardson and Richardson, 1988), we propose α-helices between residues 18 and 29 (I), 42 and 54 (II), 57 and 82 (III), 85 and 91 (IV), and 105 and 121 (V) of hIL-3 (Fig. 5). Introduction of helix-breaking residues (Pro-Gly) at residues 63/64 (1486) strongly affects biological activity. This may be explained by disruption of the long helix III, which is predicted for all species by the mentioned algorithms. Helix stability may be increased by properly charged residues at its ends (Presta and Rose, 1988; Richardson and Richardson, 1988). Substitution of the conserved residues 43/44 (1481), 54/55 (1484), and 106 (1491) at the termini of the postulated helices II and V by residues with opposing charge resulted in loss of activity and may be explained by this model. Charge reversals of conserved charged residues at positions 108–110 (1492) also strongly affected activity. These residues are not located at the end of a helix and are not supposed to contribute notably to helix stability but could be involved in long range interactions. Substitution of the completely conserved hydrophobic residues 113/114 (1487) severely reduced biological function and may be explained by disruption of hydrophobic interactions within the molecule and/or with the receptor. Two-dimensional nuclear Overhauser effect spectroscopy-1H NMR measurements on recombinant hIL-3 and RhIL-3 showed virtually identical nuclear Overhauser effects from the aromatic side chains of Phe313, 4, indicating that the aromatic protons have highly conserved long range interactions (±0.5 nm) in both IL-3 species.

The majority of the monoclonal antibodies reacted with continuous epitopes within two small regions of the hIL-3 protein. The largest group of mAbs is capable of identifying the 933 (29–37) mutant but failed to react with the 811 (31–49) mutant (Table I). The epitopes recognized by these mAbs must reside between residues 37 and 50, which is a highly hydrophilic region and could be predicted to be an antigenic determinant (Hopp and Woods, 1981). Actually, mAb A5 appears to interact with the hydrophilic face (residues 46, 50, and 54) of helix II since alterations in these residues strongly affected binding. The other group of antibodies is characterized by the absence of reaction with mutant 933, which lacks 9 amino acids. This hydrophobic proline-rich region between helices I and II (Richardson and Richardson, 1988) and are not supposed to contribute notably to helix stability but could be involved in long range interactions. Substitution of the completely conserved hydrophobic residues 113/114 (1487) severely reduced biological function and may be explained by disruption of hydrophobic interactions within the molecule and/or with the receptor. Two-dimensional nuclear Overhauser effect spectroscopy-1H NMR measurements on recombinant hIL-3 and RhIL-3 showed virtually identical nuclear Overhauser effects from the aromatic side chains of Phe313, 4, indicating that the aromatic protons have highly conserved long range interactions (±0.5 nm) in both IL-3 species.

Deletion mutants do not cover both domains simultaneously. This hypothesis is supported further by the apparent discrepancy in receptor binding and induction of DNA synthesis of some mutants, indicating that the different receptor-binding domains on the growth factor do not induce signal transduction with identical efficiencies. Discontinuous polypeptide domains essential for biological function have also been suggested for other growth factors such as growth hormone (Cunningham and Wells, 1989; Cunningham et al., 1989), GM-CSF (Shanafel and Kastelein, 1989; Kaushansky et al., 1989) and tumor growth factor-α (Defeo-Jones et al., 1988). The principle of multiple domain interaction between growth factor and receptor has been demonstrated in detail for IL-2 (Collins et al., 1988; Robb et al., 1988; Zurawski and Zurawski, 1988, 1989). The high affinity receptor of IL-2 consists of two polypeptides that interact with different domains of the growth factor (Collins et al., 1988; Zurawski and Zurawski, 1989). The IL-3 receptor also seems to be a multicomponent complex. A low affinity murine IL-3-binding protein with structural homology to other growth factor receptors (such as IL-2Rα and erythropoietin receptor) has been molecularly cloned but lacks biological function (Itoh et al., 1990; Bazan, 1990). Apparently this molecule associates with a second polypeptide to constitute a high affinity receptor capable of transducing the signal (Hibi et al., 1990). A further complication is presented by receptor molecules on specific lineages of human hemopoietic cells (not murine) capable of binding both IL-3 and GM-CSF (Park et al., 1989; Lopez et al., 1989; Budel et al., 1990). Thus it may be speculated that the low affinity IL-3-binding protein can form a complex with at least two different polypeptides resulting in distinct high affinity receptors. Since the AML cells used in the binding experiments expressed both types of IL-3 receptors, differential binding of mutant proteins to either IL-3 receptors or common receptors may have been masked. However, preliminary experiments revealed no discrepancy in binding by various mutants (e.g., 810, 812, 822, and 904) to AML cells with specific IL-3 receptors and IL-3/GM-CSF common receptors. The mutational analysis presented here has shown that approximately 24% of the amino acid residues at the extremes of the mature hIL-3 are not essential to biological function. Similar results have been obtained for murine IL-3 (Clark-Lewis et al., 1986) and other growth factors such as IL-2 (Zurawski and Zurawski, 1988; Shanafel and Kastelein, 1989; Yanofsky and Zurawski, 1990), indicating that the terminal residues are often less important in protein folding and function. Comparison of primate IL-3 sequences revealed that the COOH-terminal 9 amino acids are absent in rhesus monkey IL-3, without affecting its stimulatory effect on human cells (Burger et al., 1990a). In contrast, hIL-3 displays 100-fold reduced activity on rhesus monkey hemopoietic cells in vitro and in vivo (Burger et al., 1990a; Wagemaker et al., 1990b). These results indicate that the protein sequence divergence in RhIL-3 does not significantly affect interaction with the various types of IL-3 receptors on human cells but also suggest that the RhIL-3 receptor(s) may have diverged notably during evolution. Finally, the observed higher binding activity of some mutants in comparison with their capacity to induce DNA synthesis suggests that receptor binding and induction of cell proliferation may be disconnected and that antagonists may be devised.

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4 D. Schipper, Gist-brocades, unpublished results.

5 L. Budel, unpublished results.
