The EMBO Journal vol.10 no.8 pp.2125-2131, 1991

Structure-activity relationship study of human interleukin-3: role of the C-terminal region for biological activity

Nathalie A.Lokker[1], Gerhard Zenke, Ulrike Strittmatter, Barbara Fagg and N.Rao Movva[2]

Preclinical Research, Sandoz Pharma Ltd, CH-4002 Basle, Switzerland
[1]Present address: Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA
[2]Corresponding author

Communicated by T.Staehelin

A structure-activity relationship study of human interleukin-3 (huIL-3) was performed by functional analysis of huIL-3 deletion and substitution variants combined with epitope mapping of huIL-3 specific neutralizing monoclonal antibodies (mAb). Analysis of the huIL-3 variants was accomplished by defining their capacity to compete with wild-type huIL-3 for binding to the huIL-3 receptor and to induce the proliferation of the huIL-3 dependent cell line M-O7. HuIL-3 variants with either 14 amino acids (aa) deleted from the N-terminus or eight aa from the C-terminus retained full biological activity *in vitro*. An huIL-3 variant, with 18 N-terminal aa deleted, exhibited a >7-fold reduced receptor binding capacity and proliferative activity. No biological activity could be detected with a variant where 22 C-terminal aa have been deleted. Neutralizing mAb recognizing presumed discontinuous epitopes failed to interact with the latter deletion variant indicating a possible location of their epitopes within the C-terminal region. Computer-aided structure prediction and sequence homology analysis of this region indicated the presence of an amphiphilic α-helix with highly conserved residues like Lys110 and Leu111. Substitution of Lys110 with either Glu or Ala resulted in variants with a 10-fold reduced activity in the receptor binding assay and the proliferation assay. Further variants, where Leu111 was substituted by Pro or Met, were totally inactive in these assays. Analysis of the binding of the two neutralizing mAb to these substitution variants showed that they did not bind to either of the Leu111 variants suggesting that Leu111 is part of an active site. Based on our results, a possible model for the structure of the huIL-3 molecule can be constructed with two active sites in close proximity.

Key words: epitope mapping/mutagenesis/neutralizing monoclonal antibodies/receptor binding site/structure prediction

Introduction

Interleukin-3 (IL-3), a glycoprotein isolated from T-cells, has been shown to act directly on hemopoietic progenitor cells to stimulate their proliferation and differentiation (for review see, Morris *et al.*, 1990). In addition to its growth promoting activities, IL-3 also affects the functional activity of mature blood cells (Rothenberg *et al.*, 1988; Kurimoto *et al.*, 1989). The cDNA of mouse IL-3 (mIL-3; Fung *et al.*, 1984) and human IL-3 (huIL-3; Yang *et al.*, 1986) have been cloned and the recombinant proteins produced. Interleukin-3 exerts its activities through a specific high affinity cell surface receptor (Urdal *et al.*, 1989; Valent *et al.*, 1989). A component of the mIL-3 receptor has recently been characterized (Itoh *et al.*, 1990), while the cloning of the huIL-3 receptor has not yet been achieved. The tertiary structures of huIL-3 and mIL-3 and their relationship to their multiple biological activities remain to be elucidated, though some observations have been made towards the structure-activity relationship (SAR) of mIL-3 (Clark-Lewis *et al.*, 1988; Clark-Lewis, 1989). A synthetic peptide of mIL-3 with the N-terminal residues 1-80 deleted, had some detectable biological activity while the C-terminal variants with >33 residues deleted were inactive. HuIL-3 and mIL-3 share an overall nucleotide homology of 45% and an aa homology of only 29% (for review see, Ihle, 1990). It is thus not surprising that IL-3 has often been found to be species specific. However, the broad spectra of activities of both molecules largely overlap and thus raises the interesting question as to whether their functional domains were conserved through evolution.

We have previously reported the initial results on the SAR analysis of huIL-3 and have identified residues important for receptor binding site on huIL-3 (Lokker *et al.*, 1991a,b). We mapped and genetically engineered the continuous epitopes of two neutralizing monoclonal antibodies (mAb) that encompassed residues Leu32 to Asp36 of huIL-3 and showed that residues Pro33 and Leu34 are essential for modulating the biological activity of huIL-3.

In the present study, we extended this SAR analysis, as several lines of evidence suggested the presence of additional receptor binding sites on huIL-3: (i) deletion of Pro33 and Leu34 severely reduced but did not completely abolish receptor binding; (ii) two neutralizing mAb, which were identified for possibly recognizing discontinuous epitopes, partly retained the ability to bind to all huIL-3 variants which were modified at Pro33 and Leu34 and; (iii) the continuous epitopes of two neutralizing mAb were shown in cross-competition experiments, to be linked to the other, unidentified discontinuous epitopes. These latter epitopes, which might be in spatial proximity to residues Pro33 and Leu34, are good candidates to form part of the huIL-3 receptor binding domain.

In the present study we examined the aa requirements at various regions, including N-terminal, internal and C-terminal parts of huIL-3 for either binding to the huIL-3 receptor or inducing the proliferation of the huIL-3 dependent human megakaryocytic leukemia cell line M-O7 (Avanzi *et al.*, 1988). By deletion and substitution analysis, a region was identified as being essential for the biological activity of huIL-3. The conformation of the rhuIL-3 analogs was probed by analyzing the binding of the variants to various

mAb. Based on these studies and our previous work a model of the tertiary structure of huIL-3 molecule is proposed.

Results

Receptor binding capacity and biological activity of the deletion variants

Deletion mutagenesis was performed at internal as well as at N- and C-terminal regions of the huIL-3 sequence in order to identify the relative importance of these regions for receptor binding and biological activity. Extracts from *E.coli* cells transformed with the different plasmid constructs encoding either wt rhuIL-3 or variants, were analyzed by Western blotting and ELISA in order to monitor the size and the amount of the proteins (data not shown). Some internal deletion variants (del-Asp44-Leu53, del-Arg54-Arg63 and del-Ala64-Ala73) did not express the protein to levels detectable either by Western blotting or ELISA and thus were not investigated further. These truncated forms of huIL-3 might have been extremely labile and proteolytically degraded in *E.coli* cells (Baker *et al.*, 1984). All remaining variants were tested for their ability to compete for binding to the huIL-3 receptor and to induce the proliferation of M-O7 cells (Table I). Their dissociation constant (K_d) and specific activity (SA) varied in a similar way, suggesting that high affinity binding of huIL-3 to its receptor is an essential step for the *in vitro* activity of huIL-3. The deletion variants, del-Ala1-Val14 and del-Thr126-Phe133 bound the huIL-3 receptor with high affinity (K_d = 0.3 and 1.2 nM) and had an SA of 4.2×10^6 and 2.7×10^6 U/mg, similar to wild-type rhuIL-3, indicating that residues Ala1-Val14 and Thr126-Phe133 are not required for the *in vitro* activity of rhuIL-3. Variant del-Ala1-Asn18 had a 7-fold higher K_d and a 10-fold reduced SA demonstrating that residues Asn15-Cys16-Ser17-Asn18 are required for efficient binding of huIL-3 to the receptor. This is probably due to the removal of Cys16, which abrogates the formation of the unique disulfide bridge and thus might destabilize the huIL-3 molecule. In mIL-3, the Cys residue at the comparable position has been identified as essential for mIL-3 activity (Clark-Lewis et al., 1988).

As can be seen in Table I the C-terminal (e.g. del-Thr112-Phe133) as well as the internal deletion variants (del-Ile74-Pro83, del-Trp104-Phe113, del-Tyr114-Ala123) had residual activities of <3% compared to wt rhuIL-3. In summary, we can deduce by analysis of the activities of the deletion variants that the aa located between Asn15 and Glu125 are necessary for huIL-3 activity. However, the N- and C-terminal aa identified as dispensable for the huIL-3 activity *in vitro* might nevertheless have a role *in vivo* (e.g. half-life of huIL-3). Similarly, studies with mIL-3 indicated that the 102 aa fragment Cys17-Val118 (corresponding to Cys16-Gln124 of huIL-3 when the sequences are aligned for maximum homology) represents the minimal sequence exhibiting high biological activity (Clark-Lewis, 1989).

Binding of the deletion variants to different mAb

The binding of eight different mAb to wild-type rhuIL-3 and variants were tested in order to probe their conformation. These selected mAb probably recognize different epitopes since they bind differently to denatured rhuIL-3 and Glu-C or Lys-C proteinase-digested rhuIL-3 (Lokker *et al.*, 1991a). This permitted the selection of mAb recognizing either continuous or discontinuous epitopes. Five of the selected mAb (F14−393, F15−371, F14−570, F14−746 and F16−21) are likely to recognize conformational epitopes as they failed to bind to huIL-3 peptides and might thus be sensitive to alterations in the tertiary structure of huIL-3. The results summarized in Table II indicate the B_{50} values (concentration of antibody required for half maximal binding) obtained with the binding of the different mAb to each variant. These data show that all mAb bound to the biologically active variants del-Ala1-Val14 and del-Thr126-Phe133 as to wild-type rhuIL-3. Similarly, all mAb also recognized variant del-Ala1-Asn18 suggesting that the conformation of huIL-3 is not grossly altered in the absence of the disulfide bridge. Moreover, all mAb recognizing continuous epitopes (columns A−D), bound to most of the variants suggesting a preservation of their epitopes. On the other hand, all mAb recognizing presumed discontinuous epitopes (columns E−I) showed a significant reduction in their binding capacities to the internal deletion variants del-Ile74-Pro83 and del-Trp104-Phe113, suggesting a substantially altered conformation of these variants. However, the binding pattern of these five mAb to the deletion variant del-T112-F133 was slightly different. The binding of four mAb (F15−371, F14−570, F14−746 and F16−21) was severely affected, whereas mAb F14−393 was able to interact with this variant. Interestingly, the nonbinding of the neutralizing mAb F14−570, F14−746 and F15−371 to del-Thr112-Phe133 suggests that at least a part of their epitopes are located within the C-terminal

Table I. Dissociation constants and specific activities of wild-type rhuIL-3 and variants

Variant	k_d (nM)	SA$\times10^{-6}$ (U/mg)
Wild-type rhuIL-3	0.4±0.2	4.1±1.2
Deletions: N-terminal		
del-Ala1-Val14	0.3±0.1	4.2±0.6
del-Ala1-Asn18	2.9±0.2	0.4±0.1
Deletions: Internal		
del-Ile74-Pro83	>13.0	<0.1
del-Trp104-Phe113	>12.0	<0.1
del-Tyr114-Ala123	>8.0	<0.1
Deletions: C-terminal		
del-Thr126-Phe133	1.2±0.7	2.7±1.8
del-Thr112-Phe133	>13.0	<0.1
Substitutions: C-terminal		
Ala110	4.0±0.4	0.4±0.1
Glu110	2.5±0.9	<0.1
Met111	>10.0	<0.2
Pro111	>8.0	<0.2

Dissociation constants (k_d) and specific activities (SA) of wild-type rhuIL-3, deletion and substitution variants (mean and standard deviation of three independent assays). For competitive binding, M-O7 cells were incubated with 150 pM [^{125}I-]rhuIL-3 in the presence of various dilutions of *E.coli* protein extracts. Proliferative activity was determined by incubating M-O7 cells with serial dilutions of the protein extracts. After 72 h incubation, the degree of proliferation was determined by absorbance using the MTT colorimetric method (Lokker *et al.*, 1991b). The k_d and SA of each variant were graphically defined from the inhibition and proliferation curves. Control experiments showed that the contaminating proteins from *E.coli* extracts had no effect either on binding of wild-type rhuIL-3 to the huIL-3 receptor or on the proliferative activity of rhuIL-3. If a > or < sign is used, this corresponds to the highest k_d or the lowest specific activity which could be defined.

region of huIL-3. This encouraged us to analyze structural predictions and perform substitution mutagenesis of the C-terminal region of huIL-3, to obtain further insights into its secondary structure and the residues essential for huIL-3 activity.

Computer-aided structure homology analysis of huIL-3 and mIL-3: rationale for substitution mutagenesis

The aa sequences of huIL-3 and mIL-3 were aligned and showed a region of maximal homology (human residues 103–120 and mouse 96–114; Figure 2A). Computer-aided secondary structure predictions (Chou and Fasman, 1978) were generated to obtain information concerning its putative structure. These predictions indicated an α-helix at the C-termini of both molecules including this region of high homology. This predicted α-helix was also seen when other algorithms were applied on the huIL-3 sequence (e.g. Garnier *et al.*, 1978; data not shown). This α-helical structure was also predicted for C-terminal sequences of rat and gibbon IL-3.

The C-terminal proximal huIL-3 sequence was projected on an α-helical wheel and interestingly, a small hydrophobic and a larger hydrophilic side could be seen (Figure 2B). To examine the relevance of this structure to the biological activity of huIL-3, we decided to perform specific alterations of the side-chains at either face of this α-helix.

Analysis of the Lys110 and Leu111 substitution variants

Two different residues Lys110 and Leu111 were chosen for substitution experiments because of their respective hydrophilic and hydrophobic side-chains. These residues are conserved between human, gibbon, mouse and rat IL-3 (Morris *et al.*, 1990) and thus, could be critical for IL-3 activity.

Specific substitutions of residues Lys110 and Leu111 were performed to investigate the contribution of the side-chains to both the biological activity and the formation of an α-helix. Residue Lys110 (basic polar side-chain) was replaced by Glu (acidic polar side-chain, Glu110) or by the smaller residue Ala (Ala110). Residue Leu111 (non-polar side-chain) was replaced by the smaller residue Met (Met111) or by Pro (Pro111), which is known to disrupt helices. Competitive binding and proliferation assays of the four C-terminal substitution variants clearly indicated that K_d and SA were affected (Table I). Variants Pro111 and Met111 showed <3% residual receptor binding capacity and proliferative activity compared with wt rhuIL-3. The analysis of the binding of mAb to these variants indicated that they were still recognized by all mAb, except by the two C-terminal sensitive mAb F14–570 and F14–746. This suggests that Leu111 is part of the epitopes of these neutralizing mAb and that the side-chain of Leu111 is of direct importance for huIL-3 receptor binding. Indeed, several indications favor this hypothesis: (i) replacement of Leu111 by the smaller hydrophobic residue Met results in loss of biological activity, though the putative α-helix is expected to be maintained, (ii) Leu111 is centered in a presumed hydrophobic site of the putative C-terminal α-helix as illustrated in Figure 2C, (iii) mIL-3 can induce the proliferation of human M-O7 cells when used in a 100-fold excess and this activity is specifically inhibited with the neutralizing anti-huIL-3 mAb F14–746

Table II. Binding of antibodies to wild-type and mutant rhuIL-3 proteins

	MAb								
	(A) F13–267	(B) F15–216	(C) F13–415	(D) F13–947	(E) F14–393	(F) F15–371	(G) F14–570	(H) F14–746	(I) F16–21
Neutralizing[a]	+	+	–	–	+	+	+	+	n.t.
IL-3 peptide recognition: Lys-C[b]	+	+	–	+	–	–	–	–	–
IL-3 peptide recognition: Glu-C[b]	+	+	+	–	–	–	–	–	–
Wild-type rhuIL-3	30	2	20	26	4	2	13	16	2
Deletions: N-terminal									
del-Ala1-Val14	95	3	79	55	2	2	53	120	5
del-Ala1-Asn18	14	0.5	10	12	2	0.5	6	10	1
Deletions: Internal									
del-Ile74-Pro83	178	1	8	9	>10000	>10000	>10000	>10000	>10000
del-Trp104-Phe113	28	0.5	480	2	>10000	>10000	>10000	>10000	>10000
Deletions: C-terminal									
del-Thr126-Phe133	15	0.5	21	11	1	1	6	13	2
del-Thr112-Phe133	33	0.5	0.5	2	2170	>10000	>10000	>10000	>10000
Substitutions: C-terminal									
Ala110	31	2	32	35	7	2	26	26	5
Glu110	58	2	9	33	3	3	32	58	3
Met111	132	2	1	18	800[c]	81[c]	>10000	>10000	3
Pro111	28	2	1	10	3750[c]	15[c]	>10000	>10000	1

The binding of mAb to wild-type and mutant rhuIL-3 proteins was determined with ELISA. The values represent the concentration of antibody required for half-maximal binding (B50) in ng/ml.

[a]Ab were tested for their ability to neutralize the activity of rhuIL-3 in the CML proliferation assay (Lokker *et al.*, 1991a).

[b]MAb were analyzed for binding to huIL-3 peptides generated by digestion of rhuIL-3 with the proteinase Lys-C or Gly-C as previously described (Lokker *et al.*, 1991a).

[c]Plateau of the binding curve of the variant was low compared with wild-type rhuIL-3.

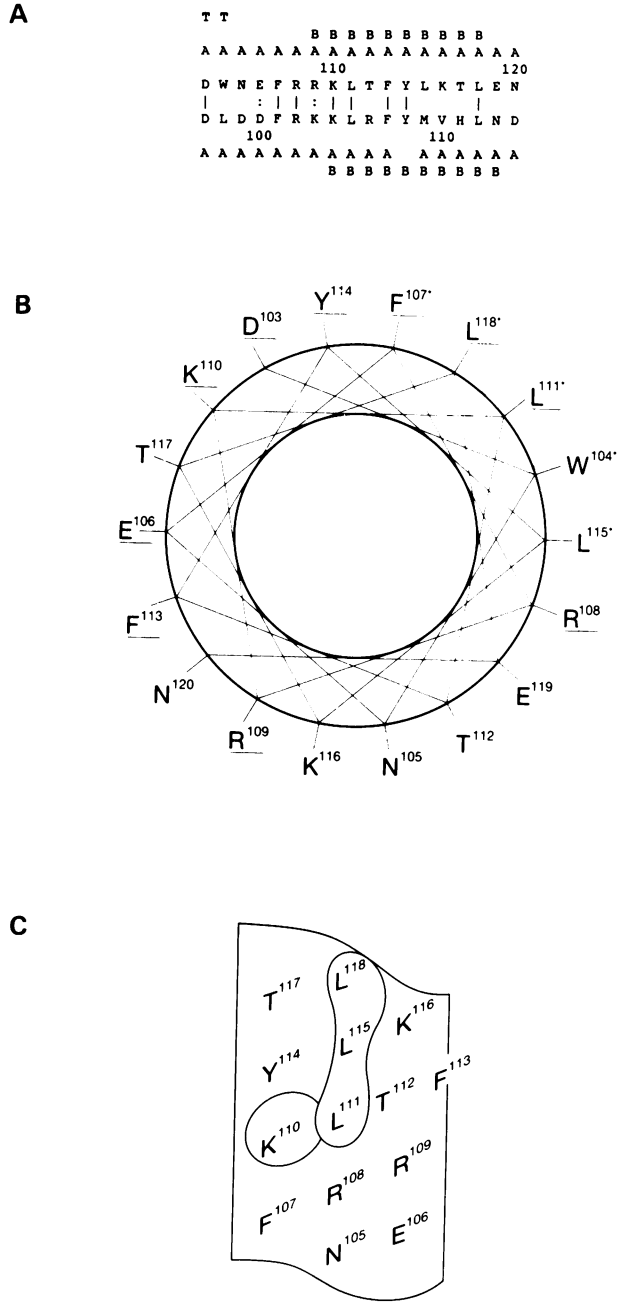

Fig. 1. A. Alignment of aa residues 103–120 of huIL-3 (upper sequence) and residues 107–114 of mIL-3 (lower sequence) proteins. Residues are indicated in their single letter code. Identical residues are marked by solid lines and similar residues by dotted lines. The secondary structure predictions (Chou and Fasman, 1978) are shown above the huIL-3 and below the mIL-3 sequence where A indicates an α-helix, B a β-sheet, and T a turn. **B.** Helical wheel projection of the putative C-terminal α-helix of huIL-3 from residues D^{103} to N^{120} (one turn every 3.6 residues, Rose *et al.*, 1985). Conserved residues are underlined, residues with an asterisk represent a hydrophobic face. **C.** A cylindrical plot of the putative α-helix residues N^{105} to L^{118} are represented commencing at the bottom of the cylinder. The residues identified as critical for the biological activity of huIL-3 are K^{110} and Leu^{111}.

(data not shown) which probably recognizes a region in mIL-3 homologous to huIL-3 (e.g. Leu105 in mIL-3).

Substitution analysis of Lys110 showed that Glu110 and Ala110 had an ~10-fold reduced residual receptor binding capacity and proliferation activity compared with wild-type

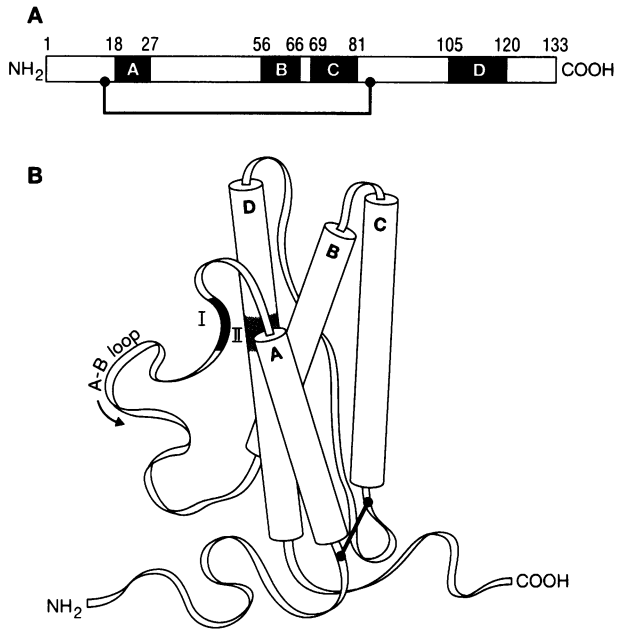

Fig. 2. Secondary and tertiary structure prediction for huIL-3. **A.** Location of secondary structural elements in the sequence of huIL-3. The four α-helices from the predicted structure are labeled A–D (filled boxes with residues limits). The unique disulfide bridge connection is marked by a black line. **B.** Drawing of the proposed conformation of huIL-3 emphasizing the helix bundle core and loop connectivity. In addition, the two distinct sites recognized by the neutralizing mAb (site I in loop A–B and site II in the C-terminal helix D) are indicated by shading. These antibody sites are believed to be in close proximity to each other in the three dimensional structure. The unique disulfide bond supplies further constraints on the huIL-3 folding.

rhuIL-3. Analysis of binding of the various mAb to Glu110 and Ala110 (Table II) indicated that all mAb including F14–570 and F14–746 bound to these variants suggesting that no major alterations in the huIL-3 conformation had occurred. Residue Lys110 can not play such a pivotal role as Leu111 in the interaction of huIL-3 with the receptor because either variant, Glu110 or Ala110, retained a substantial receptor binding capacity. We suggest that the positively charged side-chain of Lys may have an indirect role as a consequence of its close spatial proximity to the three hydrophobic Leu residues, Leu111, Leu115 and leu118 (Figure 2C). This idea is consistent with the finding that residue Leu111 is essential for the biological activity of huIL-3. The functional importance of such an hydrophobic patch located in the proximity of a critical hydrophilic residue has also been reported for mIL-2, which like huIL-3, showed the importance of both the hydrophilic and the hydrophobic side-chains (Zurawski *et al.*, 1989). Moreover, many investigators have found that, residues critical for the function of polypeptide hormones such as granulocyte-macrophage colony-stimulating factor (GM-CSF), map to an α-helical structure as described here (Kaushansky, 1990).

Discussion

The observation that the binding of huIL-3 to KG-1 (Gesner *et al.*, 1988), M-O7 (Avanzi *et al.*, 1990), AML cells as well as monocytes (Budel *et al.*, 1990) and eosinophils (Lopez *et al*, 1989) can be specifically inhibited by human GM-CSF suggests the existence of shared structural elements

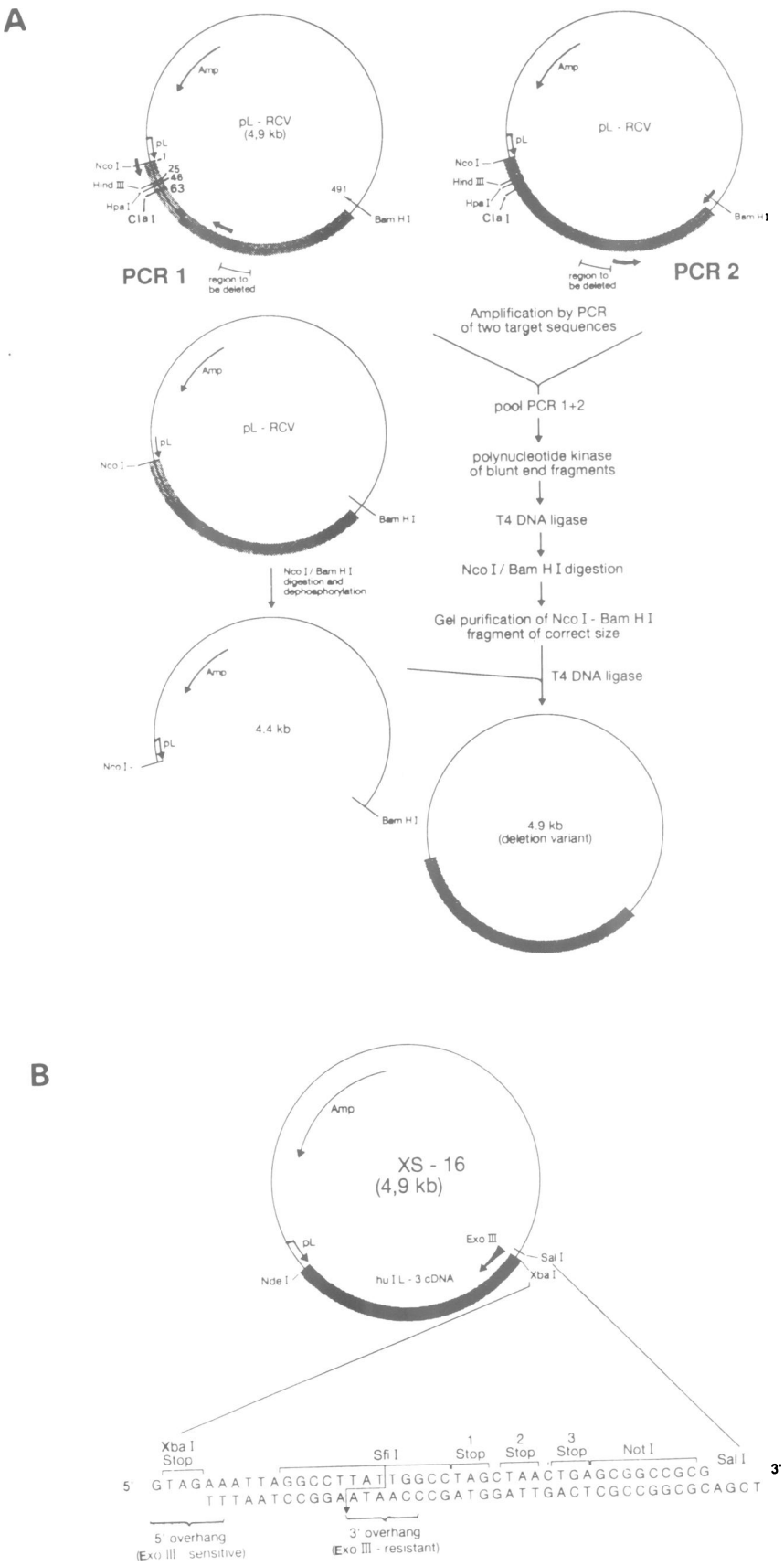

Fig. 3. A. Construction scheme for the generation of internal deletions of the huIL-3 gene by PCR using the pL-RCV plasmid. Thick arrows within the plasmid circles indicate the oligonucleotide primers for PCR. **B**. Construction scheme for the generation of C-terminal deletion mutants of huIL-3. Plasmid XS-16 was obtained by inserting a *XbaI–SalI* synthetic linker in the GI609-IL-3 plasmid. This linker contained two new restriction sites, *SfiI* creating a 3′ overhang after *SfiI* digestion (resistant to ExoIII) and NotI which has been included for control. An additional three stop codons were inserted in all three reading frames.

in both ligands and their respective receptors. The C-terminal half of several cytokines proved to be important for the biological activity including GM-CSF (Kaushansky *et al.*, 1989), G-CSF (Kuga *et al.*, 1989), IL-2 (Collins *et al.*, 1988), mIL-3 (Clark-Lewis, 1989) and IL-6 (Brakenhoff *et al.*, 1990). A relatedness in the C-terminal proximal regions of these cytokines has been found which may represent a conserved binding domain (Parry *et al.*, 1988). Moreover, the corresponding receptor components characterized so far indicate that they all belong to the class I cytokine receptor family (Bazan, 1990; Gearing *et al.*, 1989, Itoh *et al.*, 1990). Therefore, a similarity in the ligand structures is anticipated. Interestingly, mutagenesis of GM-CSF (Kaushansky *et al.*, 1989), G-CSF (Kuga *et al.*, 1989) and IL-6 (Kruttgen *et al.*, 1990) yielded similar results. Substitutions in the N-terminal half and C-terminal proximal regions of these molecules abolished their activity as well. The characteristic four α-helical bundle core which has been determined for molecules such as the growth hormone (Abdel-Meguid *et al.*, 1987) and in a model of GM-CSF (Kaushansky *et al.*, 1990) is in striking concordance with the predicted structure for huIL-3 when various algorithms were applied. As depicted in Figure 3A, the helices are expected to range approximately from 18–27, 56–66, 69–81 and 105–120.

Furthermore, some evidence exists that the huIL-3 receptor is composed of more than one subunit (Budel *et al.*, 1990; Itoh *et al.*, 1990; Tamura *et al.*, 1990). Consequently, several sites on the huIL-3 molecule could be involved in receptor binding. The molecular interaction of huIL-3 with its receptor perhaps resembles the model proposed for the multiple interactions of IL-2 (Collins *et al.*, 1988) or IL-6 (Brakenhoff *et al.*, 1990) to subunits of their receptors. We have found two active sites of huIL-3 involved in the receptor binding and/or the transmission of the proliferation signal. One of these sites could be located in a loop region including residues Pro33 and Leu34 since the variants created at this site severely affect the biological activity of huIL-3 (Lokker *et al.*, 1991b) whereas the second site could be located in the C-terminal α-helical region including residue Leu111. These sites are distant in terms of primary structure but they may be in close proximity in the tertiary structure of huIL-3. The observation that the epitopes of all neutralizing mAb (one series recognizing residues Leu32-Asp36 and one series binding to the C-terminal region) are spatially related (Lokker *et al.*, 1991a), is in agreement with this hypothesis. We have also found that in addition to their ability to inhibit the proliferation of cells of a patient with chronic myelogenous leukemia, these mAb neutralized the binding of huIL-3 to its receptor and the huIL-3 stimulated proliferation of M-O7 cells (unpublished data). Based on all these results including the predicted presence of four α-helices, a model of huIL-3 can be constructed, that is similar to that of growth hormone (Abdel-Meguid *et al.*, 1987; Figure 3B). In this model helix D lies spatially close to helix A but is somewhat tilted so that the C-terminal region approaches the loop region between helix A and B. The characteristically spaced helices A–D (and associated connections: a long A–B and C–D loop and a short B–C loop) is in striking concordance with the growth hormone structure. Our findings with the variants generated within residues 30–36 (loop A–B) and helix D as well as the presence of two spatially related active sites are in agreement with this model. Additionally, the deletion variant del-Ile74-Pro83 (helix C) had an altered conformation and was inactive. This region remains to be further characterized by analyzing either smaller deletion or substitution variants. Possible isolation of huIL-3 antagonists will elucidate the nature of participation of these sites as distinct domains both in receptor binding and biological activity.

Identification of the huIL-3 receptor in the near future, together with the continued description of the receptor binding domain(s) of huIL-3 will shed light on the ligand–receptor interactions at the molecular level.

Materials and methods

Bacterial strains and plasmids

The *E.coli* strain W3110c_I, which has the CI857 gene (coding for the temperature-sensitive repressor of the phage λ) integrated into the chromosome (Semon *et al.*, 1986), was used to propagate plasmids containing the thermo-inducible P_L promoter. Undermethylated DNA for digestion with *ClaI* was prepared by growing plasmids in GM33, a dam^-, dcm^- strain of *E.coli*.

Oligonucleotides and enzymes

Synthetic oligonucleotides used for *in vitro* mutagenesis and sequencing primers, were prepared using the Applied Biosystems 380A DNA synthesizer as described previously (Matteicci *et al.*, 1981). All restriction endonucleases and DNA modifying enzymes were purchased from Boehringer. Polymerase chain reactions (PCR) were performed using the GeneAmp kit (Perkin-Elmer Cetus). DNA sequencing was carried out using the sequenase kit (United States Biochemical Corporation). The double-stranded Nested Deletion Kit (Pharmacia) was used for deletion mutagenesis.

Construction of plasmids for the expression of rhuIL-3 variants

Plasmid DNA isolation, polyacrylamide and agarose gel electrophoresis were performed as described (Maniatis *et al.*, 1982).

Three series of expression plasmids were used for mutagenesis of the huIL-3 gene, GI609-IL-3, previously described (Lokker *et al.*, 1991b), pL-RCV and XS-16 (Figure 1). All constructs coding for the mature huIL-3 of 133 aa, are numbered from alanine (following the initiating codon methionine) as aa number 1 while the nucleotide numbering starts at the initiation codon.

The pL-RCV plasmid (kindly provided by R.Schmitz-Guenin) as shown in Figure 1A contained a modified version of the huIL-3 coding region, under the regulation of lambda P_L promotor. The modifications contained the presence of several unique restriction sites at the nucleotide positions indicated and a single *Bam*HI site at the end of the structural gene. This plasmid was used to produce internal decapeptide deletions spanning the huIL-3 sequence. As illustrated in Figure 1A, each of these variants was constructed using the pL-RCV plasmid as template DNA in two independent PCRs. The first PCR (PCR 1) was performed using an upstream primer, which started at the 5′ end of the huIL-3 gene and included the unique *NcoI* site, and a downstream primer placed immediately 5′ of the sequence to be deleted. The second PCR (PCR 2) was performed with an upstream primer which started 3′ of the sequence to be deleted and a downstream primer located at the 3′ end of the gene, which included the unique *Bam*HI site. For each variant the same upstream primer of PCR1 and downstream primer of PCR2 were used and only the primers, which were located 5′ and 3′ of the sequences to be deleted, were newly designed. The PCRs were performed as described (Lokker *et al.*, 1991b). The resultant fragments were gel-purified, Klenow treated, kinased, ligated and digested with *NcoI* and *Bam*HI. Each *NcoI–Bam*HI fragment of the expected size was gel-purified and ligated into the similarly cut recipient vector, pL-RCV, to replace the wt sequence.

The N-terminal deletion variant with the first 14 aa deleted was obtained by digesting pL-RCV with *NcoI* and *HpaI* and treated with Klenow fragment. The linearized plasmid was gel-purified and reclosed. In order to delete 18 N-terminal aa, the sequence between *NcoI* and *ClaI* was replaced by a synthetic linker encoding a deletion of these residues.

The C-terminal deletion variants were produced using Exonuclease III (ExoIII) protocol as described (Henikoff, 1984). The expression plasmid XS-16 was constructed for this purpose by inserting between *XbaI* and *SalI* of GI609-IL-3 the synthetic linker which is shown in Figure 1B. This plasmid was digested with *SfiI* and *XbaI*, and the large *XbaI–SfiI* fragment was gel-purified. The linearized DNA-fragment with only one susceptible 3′

end, preceding the target sequence to be deleted, was treated with ExoIII which resulted in progressive removal of nucleotides from only one strand of the huIL-3 sequence. The remaining single-stranded region was removed using S1 Nuclease and ligated to recircularize the DNA fragment.

A sample of each of the ligation reactions was used for transformation of competent W3110c_I cells (Hanahan, 1983). All modified plasmids were characterized by restriction mapping and the nucleotide sequences of variants were verified (Sanger *et al.*, 1977). The two C-terminal deletion variants which are described in this paper contain two additional amino acids, Pro and Ser (Figure 1B).

Preparation and analysis of the huIL-3 analogs

Expression of the huIL-3 proteins, the ELISA and Western blotting as well as the competitive binding and M-O7 proliferation assays were performed as described (Lokker *et al.*, 1991b).

Variations in the production of huIL-3 analogs also seen by visual inspection of immunoblots were quantified by ELISA. This allowed us to correct for differences in the level of protein expression between variants and to define for each variant the dissociation constant (K_d) from the inhibition curves and the specific activity (SA) from the proliferation dose-response curves.

Computer-aided structure predictions of the wt rhuIL-3 and variants

Secondary structure analysis (Chou and Fasman, 1978) was generated using the Intelligenetics software (Intelligenetics, Inc., Mountain View, CA). Additional structure prediction of huIL-3 was performed using three different Sybil/Biopolymer programs (Tripos Associates, Inc., St Louis, MO).

Acknowledgements

The authors wish to thank B.Loher for synthesis of the oligonucleotides and Dr M.Schreier for critical review of the manuscript.

References

Abdel-Meguid,S.S., Shieh,H.S., Smith,W.W., Dayringer,H.E., Violand,B.N. and Bentle,L.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6434–6437

Avanzi,G.C., Lista,P., Giovinazzo,B., Miniero,R., Saglio,G., Benetton,G., Coda,R., Cattoretti,G. and Pegoraro,L. (1988) *Brit. J. Haematol.*, **69**, 359–366

Avanzi,G.C., Brizzi,M.F., Lista,P., Porcu,P., Rosso,A., Giovinazzo,B., Clark,S.C. and Pegoraro,L. (1990) In Clark,S.C., Golde,D.W., (eds) *Hematopoiesis.* UCLA symposia on Molecular and Cellular Biology, New series, Vol. 120, pp. 181–189

Baker,T.A., Grossman,A.D. and Gross,C.A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6779–6783

Bazan,J.F. (1990) *Immunology Today*, **11**, 350–354

Brakenhoff,J.P., Hart,M., de Groot,E.R., Di Padova,F. and Aarden,L. (1990) *J. Immunol.*, **141**, 1975–1881

Budel,L.M., Elbaz,O., Hoogerbrugge,H., Delwel,R., Mahmoud,L.A., Lowenberg,B. and Touw,I.P. (1990) *Blood*, **75**, 1439–1445

Chou,P.Y. and Fasman,G.D. (1978) *Advances Enzymol.*, **47**, 45–148

Clark-Lewis,I., Hood,L.E. and Kent,S.B.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7897–7901

Clark-Lewis,I. (1989) In Rivier,J.E. and Marshall,G.R. (eds), *Peptides—Chemistry, Structure and Biology.* Escom Science Publishers, NY, pp. 749–750

Collins,L., Tsien,W.-H., Seals,C., Hakimi,J., Weber,D., Bailon,P., Hoskings,J., Greene,W.C., Toome,V. and Ju,G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7709–7713

Fung,M.C., Hapel,A.J., Ymer,S., Cohen,D.R., Johnson,R.M., Campbell,H.D. and Young,I.G. (1984) *Nature*, **307**, 233–237

Garnier,J., Osguthurpe,D.J. and Robson,B. (1978) *J. Mol. Biol.*, **198**, 425–4438

Gearing,D.P., King,J.A., Gough,N.M. and Nicola,N.A. (1989) *EMBO J.*, **8**, 3667–3676

Gesner,T.G., Mufson,R.A., Norton,C.R., Turner,K.J., Yang,Y.C. and Clark,S.A. (1988) *J. Cell. Physiol.*, **136**, 493–499

Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557–580

Henikoff,S. (1984) *Gene*, **28**, 351–359

Ihle,J.N. (1990) In Sporn,M.B., Roberts,A.B. (eds), *Peptide Growth Factors and their Receptors I. Handbook of Experimental Pharmacology.* Vol. 95, Springer-Verlag, NY, pp. 541–575

Itoh,N., Yonehara,S., Scheurs,J., Gorman,D.M., Maruyama,K., Ishii,A., Yanahara,I., Arai,K.I. and Miyajima,A. (1990) *Science*, **247**, 324–327

Kaushansky,K., Shoemaker,S.G., Alfaro,S. and Brown,C.B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1213–1217

Kaushansky,K., Brown,C.B. and O'Hara,P.J. (1990) *Int. J. Cell Cloning*, **8**, 26–34

Kruttgen,A., Rose-John,S., Moller,C., Wroblowski,B., Wollmer,A., Mullberg,J., Hirano,T., Kichimoto,T., Heinrich,P.C. (1990) *FEBS Lett.*, **262**, 323–326

Kuga,T., Yoshinori,K., Yamasaki,M., Sekine,S., Miyaji,H., Nishi,T., Sato,M., Yokoo,Y., Asano,M, Okabe,M., Morimoto,M. and Itoh,S. (1989) *Biochem. Biophys. Res. Comm.*, **159**, 103–111

Kurimoto,Y., de Weck,A.L. and Dahinden,D.A. (1989) *J. Exp. Med.*, **170**, 467–479

Lokker,N.A., Strittmatter,U., Steiner,C., Fagg,B., Graff,P., Kocher,H.P. and Zenke,G. (1991a) *J. Immunol.*, **146**, 893–898

Lokker,N.A., Movva,N.R., Strittmatter,U., Fagg,B. and Zenke,G. (1991b) *J. Biol. Chem.*, in press

Lopez,A.F., Englinton,J.M., Gillis,S., Park,L.S., To, Clark,S.C. and Vadas,M.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7022–7026

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Matteicci,M.D. and Caruthers,M.H., (1981) *J. Am. Soc.*, **103**, 3185–3191

Morris,C.F., Young,I.G. and Hapel,A.J. (1990) In Dexter,J.M., Garland,J.M., Testa,N.G. (eds), *Colony Stimulating Factor: Molecular and Cellular Biology.* Immunology series, Vol. 49, Marcel Dekker, NY, pp. 177–214

Parry,D.A.D., Minasian,E. and Leach,S.J. (1988) *J. Mol. Recognition*, **1**, 107–110

Rose,G.D., Geselowitz,G., Lesser,G.J., Lee,R.H. and Zehfus,M.H. (1985) *Science*, **224**, 834–838

Rothenberg,M.E.,Owen,W.F., Silberstein,D.S., Woods,J., Soberman,R.J., Austen,K.F. and Stevens,R.L. (1988) *J. Clin. Invest.*, **81**, 1986–1992

Sanger,F., Nicklen,S. and Coulsen,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467

Semon,D., Movva,N.R., Smith,T.F., Alama,M.E. and Davies,J. (1986) *Plasmid*, **17**, 46–53

Tadmura,S., Sugawara,M., Tanaka,H., Tezuka,E., Nihira,S., Miyamoto,C., Suda,T. and Ohta,Y. (1990) *Blood*, **76**, 501–507

Urdal,D.L, Price,H.M. Sassenfeld,H.M., Cosman D., Gillis,S. and Park,L.S. (1989) *Ann. N.Y. Acad. Sci.*, **554**, 167–176

Valent,P., Besemer,J., Muhm,M., Majolic,O., Lechner,K. and Bettelheim,P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5542–5546

Yang,Y.G., Ciarletta,A.B., Temple,P.A., Chung,M.P., Kovaciv,S., Gianotti,J.S., Leary,A.C., Kriz,R., Donahue,R.E. and Wong,G.G. (1986) *Cell*, **47**, 3–10

Zurawski,S.M. and Zurawski,G. (1989) *EMBO J.*, **8**, 2583–2590

Received on February 5, 1991; revised on April 22, 1991