Leucine-58 in the putative 5th helical region of human interleukin (IL)-6 is important for activation of the IL-6 signal transducer, gp130

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Abstract A model of the tertiary structure of human IL-6, derived from the crystal-structure of granulocyte-colony stimulating factor, reveals a 5th helical region in the loop between the first and second α-helix. To investigate the importance of this region for biological activity of IL-6, residues Glu-52, Ser-53, Ser-54, Lys-55, Glu-56, Leu-58 and Glu-60 were individually replaced by alanine. IL-6·Leu-58Ala displayed a 5-fold reduced biological activity on the IL-6-responsive human cell lines XG-1 and A375. This reduction in bioactivity was shown to be due to a decreased capacity of the mutant protein to trigger IL-6 receptor-α-chain-dependent binding to the IL-6 signal transducer, gp130.

Key words: Interleukin-6; Structure–function analysis; gp130

1. Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine with a central role in host defense mechanisms [1]. The biological functions of IL-6 result from binding to a specific low-affinity IL-6 receptor (IL-6Rα) [2]. The IL-6/IL-6Rα complex induces disulphide-linked homodimerization and tyrosine phosphorylation of a signal transducing receptor component, gp130 [3,4]. Both IL-6 and IL-6Rα cannot independently bind to gp130 [5]. Gp130 is not specific for IL-6, but is also part of the receptor complexes of leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, IL-11 and probably also of the recently described novel cytokine cardiotrophin-1 [5,6]. In a variety of human diseases uncontrolled expression of IL-6 is observed and thought to play a role in the pathogenesis of these diseases (for review see [1]). Reagents which can block IL-6 activity such as IL-6 receptor antagonists may therefore have therapeutic value. For a rational design of such molecules detailed knowledge of the structure–function relationships of human (h) IL-6 is required.

IL-6 is a member of the long-chain family of cytokines [7,8] with a similar anti-parallel four α-helical bundle topology as growth hormone, granulocyte-colony stimulating factor (G-CSF) and leukemia inhibitory factor, the crystal structures of which have been determined [9–11]. Based on the structure of G-CSF, which is most homologous in primary structure to IL-6, a three-dimensional model of IL-6 has been built by using molecular modelling techniques [12]. In this model IL-6 contains a core of 4 α-helices (A,B,C,D) which are connected by long loops in an up-up-down-down topology. In addition to helices A-D, G-CSF contains a small helical region in the A-B loop consisting of a so-called '3_{10}-helix' of four residues and a six-residue α-helix (Fig. 1A) [10]. In G-CSF this region is relatively exposed and protrudes from the main body of the structure [10]. Interestingly, this helical region may be important for the biological activity of G-CSF because it overlaps with epitopes recognized by G-CSF specific neutralizing monoclonal antibodies (mAb) [13].

Based on the G-CSF structure, a fifth helical region in the IL-6 A-B loop was predicted to run from residues Glu-52 or Ser-53–Leu-65 (Fig. 1B) [12]. Previous experiments suggest that this helical region of IL-6 may be important for the interaction of the IL-6/IL-6Rα complex with gp130: in the three-dimensional model it is in close proximity to the start of helix D, which has been identified to be important for the IL-6Rα-dependent gp130 interaction (site I or β1-region) [14,15]. More importantly, human/mouse chimeric proteins of IL-6 in which residues Lys-42–Ala-57 (the 2A or β2-region, [12,15]), or Cys-51–Glu-56 ([16], Fig. 1B) of hIL-6 were replaced with the corresponding residues of mouse IL-6 showed a strongly reduced capacity to trigger the binding of the IL-6/IL-6Rα complex to gp130. To identify single residues in IL-6 important for the IL-6Rα-dependent binding to gp130, we have constructed alanine substitution mutants of residues Glu-52–Glu-60 of hIL-6 (indicated in Fig. 1A) and analyzed their bioactivity and receptor binding characteristics.

2. Materials and methods

2.1. Reagents

Recombinant wild type (wt) hIL-6 (Ala-1–Met-185) was purified from *E. coli* BL21 (DE3) (a kind gift from Dr. G. Pruyn, Nijmegen, The Netherlands) carrying the pET8c-hIL-6 cDNA expression vector as described [14].

2.2. Construction of expression plasmids and expression and purification of IL-6 mutant proteins

Site-directed mutagenesis on the hIL-6 cDNA was done by a two-step PCR mutagenesis technique as described before [15,17]. To facilitate subcloning of PCR fragments, we introduced a unique *Eco*RI site into the IL-6 coding region by introducing two silent substitutions in the codons of Glu-94 and Phe-95 (GAGTTT→GAATTC). We then used the unique *Eco*NI and *Eco*RI sites for subcloning of PCR fragments with alanine substitutions of Glu-52, Ser-53, Ser-54, Lys-55, Glu-56, Leu-58 and Glu-60, in the T7 promotor-vector pRSET6D (a kind gift from Dr. T. Stoyan, Aachen, Germany). If possible, restriction sites were concomitantly introduced, with the alanine substitutions, to

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Abbreviations: IL-6, interleukin-6; sIL-6Rα, soluble 80 kDa IL-6 receptor; wt, wild type.

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facilitate identification of positive clones. Primers were synthesized on an Applied Biosystems DNA synthesizer type 381A (Warrington, UK). Mutagenesis primers (antisense) used were Glu-52Ala; 5′-CTT TGC TGC TTG CAC ACA TGT T-3′, Ser-53Ala (*Hin*dIII); 5′-GTG CCT CTT TGG AAG CTT CAC ACA TGT TA-3′, Ser-54Ala (*Eco*47III); 5′-GTG CCT CTT TAG CGC TTT CAC AC-3′, Lys-55Ala (*Hae*II); 5′-CCA GTG CCT CAG CGC TGC TTT CAC-3′, Glu-56Ala (*Hae*III); 5′-CTG CCA GTG CGG CCT TGC TGC TTT C-3′, Leu-58Ala (*Pvu*II); 5′-GTT GTT TTC TGC AGC TGC CTC TTT GC-3′, and Glu-60Ala (*Hae*III); 5′-GTT CAG GTT GTT GGC CGC CAG TGC CTC-3′. The IL-6.Arg-31Ala plasmid (kindly provided by Dr. R. Kastelein, DNAX Research Institute, Palo Alto, CA, USA) was constructed by the method of Kunkel et al. as described [18]. The integrity of the constructs was verified by restriction enzyme digestion and nucleotide sequence analysis on dsDNA of the *Eco*NI-*Eco*RI fragments with the dideoxy chain termination method by using the Sequenase kit (Biochemical Corp., Cleveland, USA). For expression of the IL-6 mutant proteins, the vectors were transformed into *E. coli* BL21 (DE3) and the proteins were purified from inclusion bodies and quantitated as described [14]. IL-6 bioassays The capacity of IL-6 and IL-6 mutant proteins to induce the proliferation of B9 mouse hybridoma cells and XG-1 human myeloma cells ([19]; kindly provided by Dr. B. Klein, Institute for Molecular Genetics, Montpellier, France), was measured as described elsewhere [14,15]. IL-6 can inhibit the proliferation of the human melanoma cell line A375 [20]. A375 cells (kindly provided by Dr. C. Figdor, NKI, Amsterdam, The Netherlands) were maintained in Iscove's modified Dulbecco's medium supplemented with 5% (v/v) fetal calf serum, 5×10^{-5} M 2-mercapto-ethanol, 100 IU penicillin, 100 μg/ml streptomycin, 20 μg/ml human transferrin (Behringwerke, Marburg, Germany). To test the ability of IL-6 mutant proteins to inhibit proliferation of A375 cells, the cells were incubated in 96-well flat-bottomed microtiter plates (4×10^3 cells/well) in triplicate for 72 h in the presence of serial dilutions of the samples to be tested. After this culture period, the cells were pulse-labeled with 7.4 kBq of [^{3}H]thymidine (74 Gbq/mmol) for 5 h, detached with 10 mM EDTA and the radioactivity incorporated in the nuclei was measured as described [14].

2.3. Receptor binding experiments

To investigate the binding of IL-6 mutant proteins to IL-6Rα, a solid phase assay was used in which the capacity of the IL-6 mutant proteins was measured to compete for binding of biotinylated IL-6 to immobilized soluble (s) IL-6Rα as described [15,21]. To measure gp130 interaction, the capacity of IL-6 and the IL-6 mutant proteins to induce binding of a sgp130-IgG1 fusion protein to immobilized sIL-6Rα was measured as described [21].

2.4. MAb binding experiments

To study the structural integrity of the IL-6 mutant proteins their reactivity with conformation specific mAb was measured in sandwich ELISA's with mAb CLB.IL-6/8, mAb CLB.IL-6/15 or mAb CLB.IL-6/16 as coating antibodies and affinity purified biotinylated sheep polyclonal anti-IL-6 as detecting antibody as described before [22].

3. Results

3.1. IL-6·Leu-58Ala has a reduced activity on IL-6 responsive human cells

A panel of IL-6 variants was constructed in which residues Glu-52, Ser-53, Ser-54, Lys-55, Glu-56, Leu-58 and Glu-60 were individually replaced by alanine. The biological activity of the mutant proteins was measured in three different bioassays for IL-6: induction of proliferation of mouse B9 hybridoma cells and of human XG-1 myeloma cells, and inhibition of proliferation of the human melanoma cell line A375. In general the observed differences in bioactivity between the mutant proteins were small. All mutant proteins had a similar activity as wtIL-6 in the mouse B9 assay with a maximum two-fold decrease observed for the Ser-53Ala protein (Table 1). In the bioassays with human cells only the Leu-58Ala mutant protein displayed a consistent 5-fold reduction in specific activity in both assays (Table 1). The activities of the other mutant proteins varied less than three-fold from that of wtIL-6, whereas they all were more active on XG-1 cells then on A375 cells. The experimental error inherent to these measurements is approximately a factor two (data not shown). Additional experiments would therefore be required to firmly establish these small differences. As suggested by the full activity of the Leu-58Ala mutant protein on B9 cells, the reduced activity on the human cells was probably not due to a perturbation of the overall tertiary structure of the Leu-58Ala protein. Also, the Leu-58Ala mutant protein bound with comparable affinity as wtIL-6 and the other mutant proteins to conformation specific mAbs 8, 15 and 16 (Table 1). These results suggested therefore that the reduced bioactivity of the Leu58Ala protein is due to a defect in receptor binding.

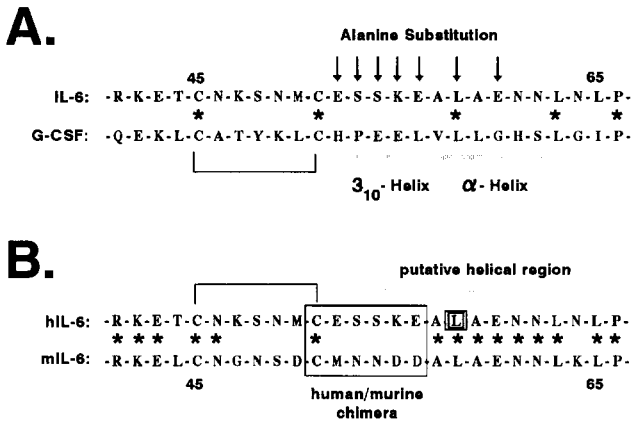


Fig. 1. (A) Part of the alignment of the hIL-6 and hG-CSF primary sequence. Identical residues are indicated by asterisks. The alignment was obtained from ref. [12]. The location of the '3_{10}'- and α-helix and the disulfide bond of G-CSF are indicated [10]. The amino acids of IL-6 that have been substituted to alanine in this study are indicated by an arrow. (B) Part of the alignment of the primary sequence of human and mouse (m) IL-6. Identical residues are indicated by asterisks. The alignment was obtained from [12]. The location of the putative helical region, the disulphide bond, and L58 are indicated. The amino acids which have recently been identified to be important for gp130 interaction, by studying mouse/human chimeric molecules of IL-6 are also indicated by the box [16].

3.2. Leu-58 is involved in IL6Rα-dependent binding to gp130

We next studied the receptor binding characteristics of the IL-6 mutant proteins in binding assays with the soluble extracellular domains of both IL-6Rα and gp130. All mutant proteins bound with comparable affinity to sIL-6Rα as wtIL-6, with an observed maximum difference of a factor two (Glu-52Ala; Table 1). However, whereas the other mutant proteins had similar sgp130 binding characteristics as wtIL-6, the Leu-58Ala protein showed a reduction in the sIL6Rα-dependent binding to sgp130 (Table 1). To further substantiate this observation and to rule out the possibility that this reduction in sgp130 binding was in fact due to an indirect effect via sIL6Rα, the receptor binding characteristics of the Leu-58Ala protein were compared with those of an IL-6 mutant protein, IL-6·Arg-31Ala, with a reduced affinity for IL6Rα (J.P.J. Brakenhoff and R. Kastelein, unpublished results). Both sIL6Rα- and sgp130-binding were measured. Again the Leu-58Ala protein showed a similar affinity for IL6Rα as wtIL-6,

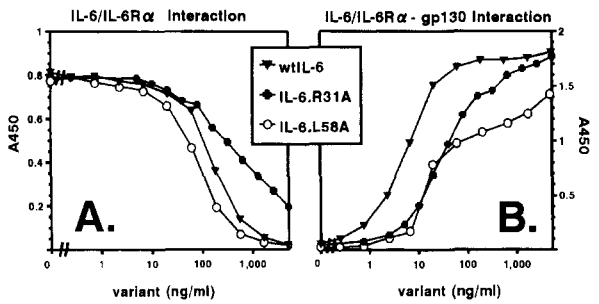


Fig. 2. Interaction of IL-6·Arg-31Ala and IL-6·Leu-58Ala with sIL-6Rα and sgp130. (A) Competitive inhibition of the binding of biotinylated IL-6 to sIL-6Rα by increasing concentrations of wtIL-6, Arg-31Ala and Leu-58Ala. (B) sgp130-IgG1 fusion protein was incubated with increasing concentrations of IL-6 (or IL-6-variants) and immobilized sIL-6Rα. Both assays were done on the same day with the same dilution series of each protein. The average values of duplicate measurements are shown of one experiment out of two.

whereas that of the Arg31Ala protein was ~6-fold reduced (Fig. 2A). In contrast, whereas the dose–response curve of the Arg-31Ala protein reached a similar maximum response as that of wtIL-6, indicating a similar capacity to bind to sgp130 as wtIL-6, again the maximum response of the Leu-58Ala protein was reduced. Interestingly, the dose response curves of IL-6·Leu-58Ala and IL-6·Arg-31Ala were shifted to the right, compared to that of wtIL-6. For IL-6·Arg-31Ala the extent of the shift corresponds to the reduced affinity for sIL6Rα. For IL-6·Leu-58Ala this shift must be due to the reduced affinity for sgp130.

4. Discussion

In previous studies three regions of hIL-6 have been shown to be important for the IL6Rα-dependent interaction with gp130, which we recently designated β1–β3 [21]. The β1 region corresponds to IL-6 residues Gln-153–His-165 [14,15], the β2 region to residues Lys-42–Ala-57 [12] and the β3 region to residues Tyr-32 and Gly-36 [23], Ser-119 and Val-122 ([24]; by using our numbering). It is unclear what the exact roles of the β-regions are in IL-6-receptor activation. The active IL-6 receptor complex is thought to consist of two molecules of each IL-6, IL-6Rα and gp130 [25,26]. Our current working hypothesis is that mutations in the β-regions may either affect dimerization of IL-6, which may be necessary for gp130 dimerization to occur, or a direct association with gp130. (See [21] for a more extensive discussion on this topic.)

To map the 'functional epitopes' [27] of IL-6 that are involved in the IL6Rα-dependent interaction with gp130 in more detail, we have now performed alanine scanning mutagenesis [28] of residues close to or part of the β2 region. Only alanine substitution of residue Leu58, but not of residues Glu-52, Ser-53, Ser-54, Lys-55, Glu-56 or Glu-60 of IL-6 significantly reduced the binding of the IL-6/sIL-6Rα complex to sgp130 (Table 1, Fig. 2B). In the tertiary structure model of IL-6, Leu-58 is a solvent exposed residue in the central portion of the putative 5th helical region and could therefore very well be involved in a direct hydrophobic interaction with gp130 (Fig. 3). After all, in the human growth hormone-growth hormone binding protein complex, hydrophobic interactions were found to contribute most to the binding free energy of the hormone-receptor interaction [29].

The finding in this study that alanine substitutions of Glu-52–Glu-56 of IL-6 did not affect gp130 binding, contrasted the observations by Ehlers et al. who showed the β2 region and in particular residues Glu-52–Glu-56 thereof, to be important for gp130 interaction [12,16]. By studying human/mouse chimeric proteins of IL-6, they showed that simultaneous replacement of residues Glu-52–Glu-56 of hIL-6 with the corresponding mouse residues resulted in an ~30-fold reduction in bioactivity and IL-6Rα-dependent gp130 binding [16]. Assuming that we indeed deal with a gp130 contact site, the identification of such a region by this approach was in fact an unexpected observation because both mouse IL-6 in complex with the mouse α-chain and human IL-6 in complex with either the human or mouse α-chain, are capable of triggering signal transduction via human gp130 [30]. This would imply that homologous residues

Table 1
Characteristics of IL-6 mutant proteins with alanine substitutions of residues Glu-52–Glu-60[a]

	Bioassay[b]			Receptor binding[c]		mAb binding[c]		
	B9	XG-1	A375	IL-6Rα	gp130	8	15	16
wtIL-6	100	100	100	100	100	100	100	100
E52A	97	167	42	44	105	44	58	56
S53A	54	194	30	78	110	40	54	59
S54A	122	173	70	59	115	33	41	57
K55A	125	145	43	40	115	58	49	57
E56A	152	196	82	75	120	76	88	82
L58A	**93**	**20**	**18**	**94**[d]	**16**[e]	**31**	**60**	**75**
E60A	108	75	30	93	110	47	51	61

[a] Mutant phenotype is indicated in single letter code. Responses of the mutant proteins in the various assays are expressed as a percentage of that of wtIL-6 (set at 100%) and are derived from EC_{50} values (the effective concentration giving a half-maximal response).
[b] Biological activity of IL-6 mutant proteins in the B9, XG-1 and A375 assays was measured as described in section 2. Values represent averages of 2 experiments. The average EC_{50} in the B9 assay was 1 pg/ml, in the XG-1 assay 20 pg/ml and in the A375 assay 0.2 ng/ml, respectively.
[c] Binding of IL-6 mutant proteins to sIL-6Rα, sgp130 and the IL-6 specific mAbs 8, 15 and 16 was measured as described in section 2. Values are the average of 2 experiments, except for gp130 binding assay, where they represent the averages from duplicate measurements of one experiment. The average EC_{50} of wtIL-6 in the IL-6Rα binding assay was 100 ng/ml, in the gp130 binding assay 10 ng/ml, and in the mAb 8, 15 and 16 ELISA's 2, 2, and 0.5 ng/ml, respectively.
[d] Average of four assays.
[e] EC_{50} could not be determined precisely, as no maximal response was obtained. (See also Fig. 2.)

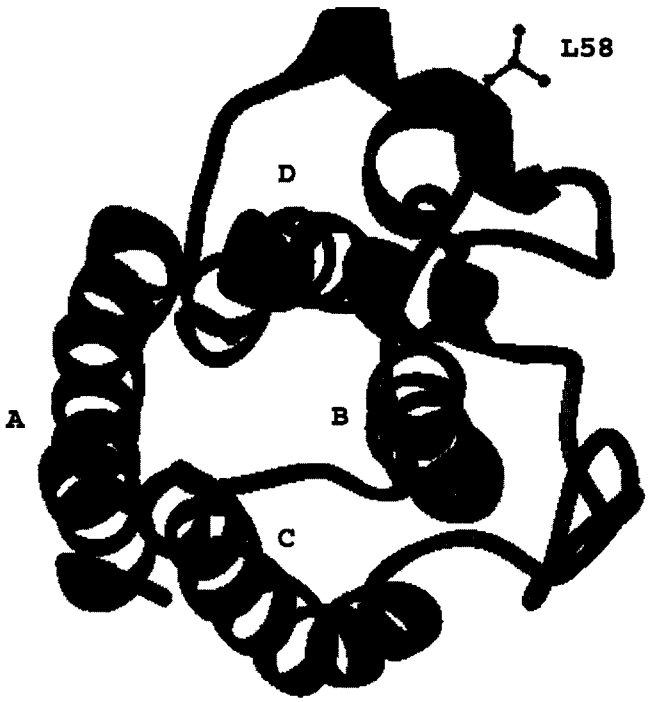


Fig. 3. Three-dimensional model of IL-6. Top view of a ribbon representation of the hIL-6 model. The four long-chain helices are numbered A–D. Leu-58 in the putative fifth helical region of IL-6 is highlighted.

between mouse and human IL-6 are involved. Introduction of non-homologous mouse residues in a human background would therefore be expected not to affect human gp130 activation. Viewed in this way, the observation that single substitution of the non-conserved residues Glu-52–Glu-56 (Fig. 1B) with alanine residues resulted only in minor effects in the various assays, is not unexpected. Also, the involvement of Leu-58, which is conserved between human and mouse IL-6 (Fig. 1B), in the IL-6Rα-dependent gp130 interaction, is in line with this. How then to explain the large effect of simultaneous Glu-52–Glu-56 substitution? One possibility could be that the charge reversal due to the Lys-55Asp substitution generates a repulsive force (see Fig. 1B). Otherwise, perturbation of the local tertiary structure of IL-6 due to the substitutions may affect the gp130 interaction capability of other residues.

Another puzzling observation is that the Leu-58Ala substitution does not seem to affect IL-6 activity on mouse B9 cells (Table 1). More in general, substitutions in human IL-6 that disrupt human gp130 binding, and bioactivity on human cells, have no or limited effects on mouse B9 cells [12,14,15]. These results suggest that residues in human IL-6 that interact with human gp130, may differ from the residues that interact with mouse gp130. More experiments are required to resolve these issues.

In conclusion, single alanine substitution of amino acid residues Glu-52–Glu-60 of hIL-6 revealed that Leu-58, a solvent exposed residue in the tertiary structure model of hIL-6, is involved in IL-6Rα-dependent binding to gp130. This might indicate that the putative fifth helix in IL-6 is involved in an interaction with gp130. Because of the small effect of Leu-58 substitution, it seems likely that besides Leu-58, other residues are involved. Further mutagenesis of this region is therefore required to identify additional residues involved in this interaction. Identification of single residues of IL-6 that are important for receptor interaction is a prerequisite for the rational design of small inhibitors of the molecule. Leu-58 is located just besides the IL-6 β2-region [12]. It will be interesting to combine the Leu-58Ala mutation with β1-substitutions [14,15] and to test wether this protein is inactive on myeloma cells and antagonizes IL-6 activity on these cells.

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