Internal deletions of amino acids 29–42 of human interleukin-6 (IL-6) differentially affect bioactivity and folding

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Internal deletions of the human interleukin-6 (IL-6) cDNA have been generated in the region encoding residues 29 to 42. Mutant proteins were produced by in vitro transcription–translation or in *Escherichia coli* and tested for their biological activity using the hybridoma growth factor (HGF) assay or a transcriptional activation assay on human hepatoma cells. The folding of the mutants was also checked by immunoprecipitation with conformation-specific monoclonal antibodies. The results show that only residues 29 to 34 are crucial for IL-6 activity and that the first two amino acids are probably involved in the definition of the IL-6 active site.

Interleukin-6; Internal deletions; HGF assay; CRP/CAT assay; Monoclonal antibody

1. INTRODUCTION

Interleukin-6 (IL-6) is a cytokine produced and secreted by several cell types and active on a variety of target cells including B cells, T cells, cells of the hematopoietic lineage, hepatocytes and neurons (for reviews, see [1,2]). The mature form of IL-6 is a 184 amino acids-long polypeptide which interacts at low affinity with the IL-6 receptor (IL-6R), an 80 kDa transmembrane protein present on the surface of target cells [3]. Transduction of the IL-6 message within the cell is triggered by the association of the IL-6/IL-6R complex with GP130, another transmembrane protein, which is accompanied by the appearance of high affinity IL-6 binding sites [4]. IL-6 is therefore believed to possess two distinct active sites which interact with IL-6R and GP130 respectively.

IL-6 overproduction has been postulated as playing an important role in the pathogenesis of several neoplasic and autoimmune diseases [1,5]. It is therefore of practical importance to design IL-6 receptor antagonists which could be used as drugs in the therapy of diseases characterized by IL-6 overproduction. In the absence of a thorough understanding of the three-dimensional structure of IL-6, important information

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Abbreviations: IL-6, interleukin-6; IL-6 R, interleukin-6 receptor; HGF, hybridoma growth factor; CRP, C-reactive protein; CAT, chloroamphenicol acetyltransferase gene; IPTG, isopropylthiogalactopyranoside.

on the composition of the active site of this cytokine has been gained by site-directed mutagenesis of the protein and the use of neutralizing monoclonal antibodies (mAb's) [6–12]. These studies have led to the conclusion that two distinct portions of the protein play an important role in IL-6 structure and activity: (a) a region located towards the amino terminus and starting from the glutamine at position 28 [6]; (b) a carboxy-terminal domain from amino acid 154 to 184, which is believed to assume α-helical conformation [13].

In this paper we report the generation of a set of small internal deletions of human IL-6 spanning amino acids 29 to 42. Functional analysis of the mutant proteins and the recognition by conformation-specific monoclonal antibodies allow a precise definition of the amino terminal domain and the contribution of individual amino acids to IL-6 folding and bioactivity.

2. MATERIALS AND METHODS

2.1. *Chemicals*

L-[^{35}S]methionine (>37 TBq/mmol) was purchased from Amersham International. T7 RNA polymerase was obtained from BRL (Gibco BRL).

2.2. *Construction of human IL-6 internal deletions*

A *Bgl*II–*Hind*III fragment carrying the T7 promoter upstream to the human IL-6-coding region from the plasmid pT7.7/IL-6 [14] was subcloned in plasmid pEMBL 131 [15] to give plasmid pEMBL T7/IL-6.

Internal deletions of human IL-6 were generated by oligonucleotide-directed mutagenesis on a single stranded DNA template. Single stranded pEMBL T7/IL-6 was prepared as described [15].

Four oligonucleotides were synthesized to generate the following mutations:

Δ29–30	(Ile-Arg)	5'TCGAGGATGTATTGTTT-GTCAATTCG 3'
Δ31–34	(Tyr-Ile-Leu-Asp)	5' GCTGAGATGCCCCGA-ATTTGTTTG 3'
Δ35–38	(Gly-Ile-Ser-Ala)	5' GTCTCCTTTCTCAGG-TCGAGGATG 3'
Δ39–42	(Leu-Arg-Lys-Glu)	5' TGTTACATGTGGCTG-AGATGCC 3'

Mutagenesis was performed according to Zoller and Smith [16]. Individual mutations were checked by direct sequencing with the dideoxy-chain terminator method [17]. Individual mutants were also transferred in the expression vector pT7.7, making use of the same restriction sites.

2.3. Production of IL-6 mutant proteins

IL-6 mutant proteins were produced in two alternative systems.

(1) In vitro transcription and translation. Plasmids of the pEMBL series were Sa/1-linearized, transcribed with T7 RNA polymerase in the same conditions as described by Fontaine et al. [12] and subsequently translated in a rabbit reticulocyte lysate in the presence of [35S]methionine [12]. Translated proteins were analyzed on reducing 17% SDS/PAGE followed by fluorography and autoradiography. The products were normalized either by densitometric analysis of autoradiograms, or by cutting bands corresponding to individual proteins from the gel and measuring the radioactivity incorporated. These preparations were directly tested in the hybridoma growth factor (HGF) assay and in the hepatoma assay.

(2) Production in Escherichia coli. Plasmids of the pT7.7 series were introduced in strain BL 21 (DE3) Lys E and recombinant IL-6 mutant proteins were produced according to Arcone et al. [14]. This gave rise to large amounts of mutant IL-6 forms which were present in inclusion bodies. After renaturation in the presence of a glutathione redox system, proteins were quantified by a Bio-Rad protein assay, visualized on non-reducing 17% SDS/PAGE, stained with Coomassie brilliant blue R-250 and immunodetected following transfer to nitrocellulose filters (not shown). These IL-6 mutants were used for competition experiments.

2.4. IL-6 bioassays

IL-6 assays in human hepatoma Hep 3B cells were performed according to Ganter et al. [18]. Plasmid 3'Δ-121/-146 CRP-CAT [19] carrying a fusion between a segment of C-reactive protein (CRP) promoter linked to the SV 40 early promoter and to the bacterial chloramphenicol acetyltransferase gene (CAT) was transfected in the cells using the calcium phosphate precipitation technique [20]. After 15 h, precipitates were washed off and the cells were treated with equal amounts of in vitro synthesized wild-type or mutant IL-6. Induction was carried out for 24 h; cell extracts and CAT assays were performed as described [21] using 20 μg cell extracts incubated at 37°C for 15 min. After autoradiography, individual spots were cut, eluted and the percentage of acetylated versus total [14C]chloramphenicol was determined.

IL-6 hybridoma growth factor (HGF) assays were performed on the mouse B cell hybridoma 7TD1 as described previously [22]. Briefly, cells were washed and seeded at a density of 2×10^3 cells/microwell in a final volume of 0.2 ml. Cultures were incubated at 37°C in 5% CO_2 in the presence of serial dilutions of the deletion mutants produced in reticulocyte lysate. After 4–5 days, the number of living cells was evaluated by measuring hexosaminidase levels as described [23].

2.5. Conformational analysis of the IL-6-derived proteins by immunoprecipitation with anti-IL-6 mAb's

Immunoprecipitations were performed as described previously [9]. Briefly, goat anti-mouse antibodies coupled to Sepharose were incubated overnight with a fixed amount of the mAb's together with the deleted proteins. After washing, the Sepharose bound protein was dissolved in sample mix and subjected to SDS/PAGE under reducing conditions, followed by autoradiography. mAb 7 recognizes with a

higher affinity the unfolded wt hIL-6 than the native IL-6 and binds hexapeptides with amino acids Thr^{142}-Ala^{145} of hIL-6 in common [24]. The neutralizing hIL-6 conformation-specific mAb 8 binds at the carboxy terminus [9]. Immunoprecipitations with a rabbit anti-recombinant hIL-6 polyclonal antiserum in presence of Staphylococcus aureus Cowan I-protein A were used as a positive control [12].

3. RESULTS AND DISCUSSION

Brakenhoff et al. [6] have reported that removal of the first 27 residues of the amino terminus of human IL-6 does not affect a certain number of bioactivities of this cytokine. However, deletion of the following residues leads to partial (28–29) or total (30–34) loss of activity. This region also interacts with conformation-specific neutralizing monoclonal antibodies (mAb's) [9]; in particular, there is a subset of neutralizing mAb's which recognize a composite epitope formed by amino acids in the 29–34 region and residues located at the carboxy-terminal end of the molecule [9].

In order to gain insight into the extension of this amino terminal domain we decided to generate internal deletions of two to four residues, leaving the rest of the molecule intact. The cDNA coding for mature hIL-6, preceded by the T7 polymerase promoter, was cloned in pEMBL 131 plasmid [15], ssDNA was prepared and subjected to oligonucleotide directed mutagenesis [16]. We generated four mutants Δ29–30, Δ31–34, Δ35–38 and Δ39–42. The sequence of these mutants is schematically reported in Fig. 1.

The various IL-6 wild-type and mutated genes were in vitro transcribed with T7 RNA polymerase and the resulting mRNAs were in vitro translated with rabbit reticulocyte lysate in the presence of L-[35S]methionine. Normalization of the translated proteins was carried out as described in section 2. In vitro translated proteins were used in two independent bioassays: (a) the HGF assay performed on the mouse B cell hybridoma 7TD1 [22–23]; (b) transcriptional activation of the IL-6 inducible human C-reactive protein (CRP) promoter linked to the CAT gene and transfected in human Hep 3 B cells [18].

As can be seen in Table I, all mutants behave similarly in both systems: Deletion of amino acids 31–34 leads to complete loss of activity; deletion of amino acids 29–30 is accompanied by a 100- to 300-fold reduction of activity. Mutant Δ35–38 is only 10- to 20-fold less efficient than wild-type, whereas mutant Δ39–42 is only moderately impaired. These experiments, thus, define the boundaries of the amino-terminal domain between residues 29 and 38.

Biologically inactive IL-6 mutants can still bind to the IL-6 receptor if the structure of the active site has only been partially destroyed and the folding of the protein has been conserved. We have tested this idea by producing large amounts of the inactive mutants Δ29–30, Δ31–34 and Δ35–38. Mutated genes were subcloned in the pT7.7 expression vector and then introduced in E.

NH2	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	COOH
wt IL-6	Gln	Ile	Arg	Tyr	Ile	Leu	Asp	Gly	Ile	Ser	Ala	Leu	Arg	Lys	Glu	Thr	
Δ 29-30	Gln			Tyr	Ile	Leu	Asp	Gly	Ile	Ser	Ala	Leu	Arg	Lys	Glu	Thr	
Δ 31-34	Gln	Ile	Arg					Gly	Ile	Ser	Ala	Leu	Arg	Lys	Glu	Thr	
Δ 35-38	Gln	Ile	Arg	Tyr	Ile	Leu	Asp					Leu	Arg	Lys	Glu	Thr	
Δ 39-42	Gln	Ile	Arg	Tyr	Ile	Leu	Asp	Gly	Ile	Ser	Ala					Thr	

Fig. 1. Amino acid sequence of wild-type and mutant IL-6 in the region spanning residues 28 to 42. For each mutant blank spaces correspond to deleted amino acids.

coli strain BL 21 (DE3) Lys E. Mutant proteins were extracted from inclusion bodies and refolded as described in section 2. Competition experiments were performed in the Hep 3B bioassay using 2.5 ng/ml of wild-type *E. coli* produced IL-6 [14] and 1:1, 1:5 and 1:20 ratios of wild-type vs mutated proteins. The results shown in Fig. 2 clearly demonstrate that none of the inactive mutants is able to compete with IL-6. We obtained identical results when using a 10-fold excess of mutants to inhibit the HGF activity of the wild-type IL-6 (data not shown).

Recently, a set of mAb's has been developed which recognize different epitopes on the IL-6 molecule [9,24]. One of them, mAb 8, recognizes a group of carboxy-terminal residues better in the folded molecule. mAb 7 recognizes hexapeptides having IL-6 residues Thr^{142}-Ala^{145} in common. This epitope is located internally in the folded molecule because wild-type IL-6 is not very reactive, unless it is subjected to heat inactivation. We decided to use mAb 7 and mAb 8 as probes in immunoprecipitation experiments in order to obtain an idea of the molecule's conformational make-up. In Table II we report the absolute level of immunoprecipitation obtained with individual antibodies. We also include the results of immunoprecipitations with a rabbit polyclonal anti IL-6 antibody or without any antibody addition as positive and negative controls respectively.

- Wild-type IL-6 is recognized at high efficiency by mAb 8 but not by mAb 7.

- The active mutant Δ39-42 behaves similarly, but here there is a moderate reaction with mAb 7, probably a sign of partial denaturatic f the protein. This result is in line with the slight decrease of activity that this mutant protein shows.
- Mutant Δ31-34 behaves inversely: its recognition by mAb 8 is considerably decreased and at the same time the mutant protein is precipitated efficiently by mAb 7. This is clear evidence that this protein is denatured, and explains its lack of activity.
- For mutant Δ35-38 recognition by mAb 8 still prevails over recognition by mAb 7, but in this case the protein is only partially denatured, which is in line with its partial loss of activity.
- The most interesting mutant is Δ29-30. Its immunoreactivity is similar to that of Δ35-38 and therefore the protein is only partially unfolded, but its bioactivity is 10- to 20-fold lower than that of Δ35-38.

If we compare the two mutants Δ29-30 and Δ35-38, we find that they show the same degree of structural alteration, but have different biological activities. This

Table I

Biological activity of the deletion mutants

Proteins	Relative HGF activity (%)	^{14}C-Chloramphenicol conversion (%)
wt hIL-6	100	100
Δ29-30	0.3	1-2
Δ31-34	<0.3	0
Δ35-38	6	5-10
Δ39-42	70	40-60

The results are given for a same amount of proteins, determined by densitometry analysis of SDS-PAGE autoradiograms.
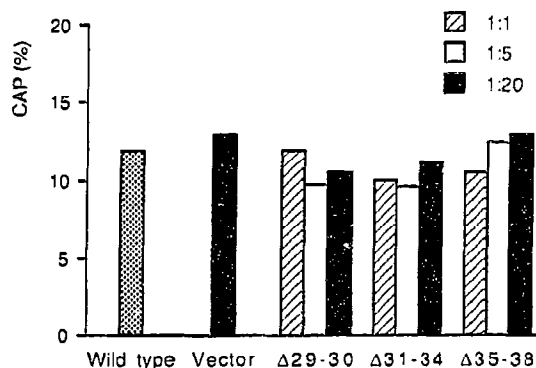
Fig. 2. Competition of IL-6 activity by mutant proteins. Hep3B CRP-CAT bioassays were performed with 2.5 ng/ml of wild-type *E. coli* produced IL-6 as described in section 2. Competition experiments were carried out either with a 20-fold excess of *E. coli* proteins extracted from cells carrying the expression vector without IL-6 insert (vector) or with a 1:1, 1:5 or 1:20 molar ratio of wild-type vs mutated proteins for each of the three mutants Δ29-30, Δ31-34 and Δ35-38. The results are represented as % of [^{14}C]chloroamphenicol conversion.

Table II

Conformational analysis of the deletion mutants

Proteins	Immunoprecipitation with			or without anti IL-6 Ab
	polyclonal Ab	mAb 7	mAb 8	
wt IL-6	+	−	+ + + +	−
Δ29–30	+	+	+ +	−
Δ31–34	+	+ +	+	−
Δ35–38	+	+	+ +	−
Δ39–42	+	+	+ + +	−

would suggest that the residues 29 and/or 30 participate more directly in the composition of the contact surface between IL-6 and the receptor or the GP 130 molecule.

Two main conclusions can be drawn from this analysis: (a) in the amino terminal region of IL-6 only a limited set of amino acid residues (29–38) is required for optimal biological activity; (b) in this region it is possible to distinguish between residues required to maintain IL-6 folding (31–38) and residues which also contribute to defining of the IL-6 active binding site (29 or 30 or both), probably in conjunction with residues at the carboxy-terminal end of the molecule. We will be able to arrive at a more rigorous definition of the importance of this region with the analysis of single amino acid substitutions.

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