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Involvement of the Arg179 in the active site of human IL-6

Véronique FONTAINE[1], Rocco SAVINO[2], Rosaria ARCONE[3], Lucas De WIT[1], Just P. J. BRAKENHOFF[4], Jean CONTENT[1] and Gennaro CILIBERTO[2,3]

[1] Institut Pasteur du Brabant, Dept. of Virology, Bruxelles, Belgium
[2] Istituto di Ricerche di Biologia Moleculare, Pomezia, Roma, Italy
[3] CEINGE, Centro d'Ingegneria Genetica, Napoli, Italy
[4] Central laboratory of the Netherlands Red Cross, Dept. of Autoimmune Diseases, Amsterdam, The Netherlands

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Three internal-amino acid deletions of amino acids 171–179 of human interleukin 6 (IL-6) were introduced at the cDNA level. While all deletion proteins were biologically inactive, immunoprecipitations with a set of conformation-specific anti-(IL-6) monoclonal antibodies showed that only mutant Δ177–179 does not present major alterations in folding. This finding, together with the observation that Δ177–179 is not able to compete with IL-6 for binding to the soluble human IL-6 receptor, suggested that some or all of these three residues participate to the composition of the receptor-binding site of human IL-6. A large number of single-amino-acid-substitution mutants were generated in residues 177, 178 and 179. Their detailed analysis revealed that Arg179 is crucial for activity in mouse cells, because all amino acid substitutions in this position cause a dramatic drop of biological activity on murine hybridoma cells without affecting the overall protein folding. The only substitution which preserved some residual activity was the conservative Arg to Lys change. This demonstrates the absolute requirement for a positive charge in position 179 for the interaction of human IL-6 with its receptor.

Interleukin 6 (IL-6) is a pleiotropic cytokine mainly involved in the regulation of inflammatory and immunological processes. Amongst its major roles, it triggers the production of acute-phase proteins by hepatocytes, activates proliferation and cytotoxic differentiation of T cells, stimulates terminal differentiation of B lymphocytes and is a growth factor for murine hybridoma and plasmacytoma cell lines (reviewed in [1]). IL-6 exerts its action via a specific high-affinity receptor, present on various target cells and constituted by the IL-6-induced heterodimerization of two distinct transmembrane proteins [2–4].

In the absence of the IL-6 three-dimensional structure, mutagenesis studies have been initiated by several investigators in order to identify regions responsible for the various IL-6 functions, such as receptor binding and signal transduction. A first important region maps to residues 28–36 and is surrounded by residues which can be deleted without functional impairment [5, 6]. A second region, essential for IL-6 activity, is located at the C-terminus. Here progressive deletions of one, two or three amino acids or also single amino acid substitutions at positions 182 and 184 lead to substantial impairment of biological activity without affecting the overall folding [7–9]. The role of these two distinct blocks of amino acids residues (N-terminus and C-terminus) is further emphasized by epitope mapping studies with a set of neutralizing monoclonal antibodies, which also suggested that the N-terminus and C-terminus are located next to each other in the folded IL-6 molecule [7].

Computer-aided protein-sequence alignments have recently led to the hypothesis that several cytokines, including erythropoietin, IL-6, granulocyte-colony stimulatory factor and their related receptors not only could share a common folding but that amino acids involved in ligand/receptor interactions are distributed in regions of these molecules that constitute homologous structural and functional domains [10]. The prototypic molecules of this group are growth hormone (GH) and its receptor, for which X-ray crystallographic studies have provided detailed information about their three-dimensional structure [11]. In practice, GH is composed by a bundle of four anti parallel α-helices connected by three loops of different length [12]. Amino acids composing the receptor-interacting surface of GH are distributed part in helix A, part in the A-B loop and largely in helix D. The portion of the IL-6 molecule that, according to the aforementioned model, should form helix D of IL-6 are the last 30 C-terminal amino acids. If the model is correct, the prediction is that, in analogy to GH, more than just the two residues, 182 and 184, which have been studied in detail so far, establish crucial contact with the receptor.

With this in mind, we started a detailed analysis of the region upstream to the extreme C-terminal residues, generating mutant proteins both by internal deletions and point mutations and testing folding and biological activity. Next to the already identified Arg182 and Met185 [8, 9], the present study points at Arg179 as a crucial residue of the C-terminal

Correspondence to J. Content, Institut Pasteur du Brabant, Rue Engeland 642, B-1180 Bruxelles, Belgium
Fax: +32 2 373 31 74.

Abbreviations. CAT, chloramphenicol acetyl transferase gene; CRP, C-reactive protein; IL-6, interleukin 6; HGF, hybridoma growth factor; h, human; r, recombinant; GH, growth hormone; PCR, polymerase chain reaction.

Enzymes. Chloramphenicol acetyl transferase (EC 2.3.1.28); T7 RNA polymerase (EC 2.7.7.6).

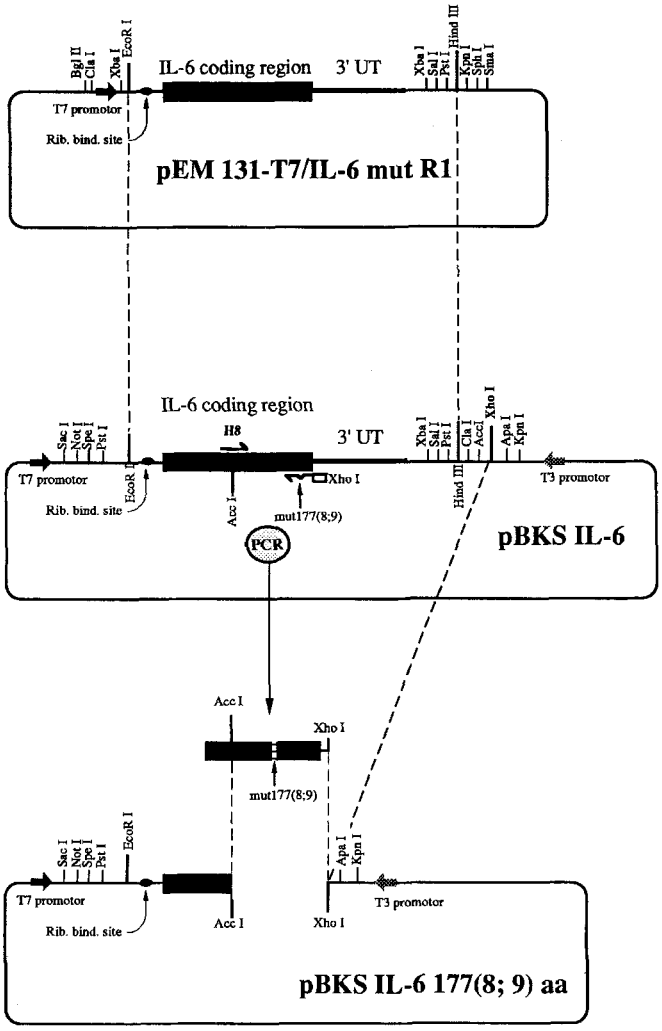

Fig. 1. Saturation mutagenesis within specific IL-6 codon sequence by PCR. In order to simplify future replacement of wild-type sequences by PCR amplified fragments, we constructed pBKS IL-6. This was performed by subcloning the IL-6-coding region of pEM 131-T7/IL-6 mut RI (where an *Eco*RI restriction site was introduced by mutagenesis) in plasmid Bluescript KS(−) (Vector Cloning Systems), making use of *Eco*RI and *Hind*III restriction sites. To generate IL-6 variants by PCR, we used mutated downstream (*Xho*I) and wild-type upstream (H8) primers, as described in Materials and Methods. The primers are shown in arrows. UT, untranslated region; Rib. bind. site, ribosome-binding site; mut., mutation.

region because, with the exception of the Arg to Lys substitution, every other single amino acids substitution in this position causes loss of biological activity without affecting IL-6 folding.

MATERIALS AND METHODS

Generation of deletion mutants

All the deletion mutants were derived from pEMBL T7/IL-6, which contains the T7 promoter upstream of the human IL-6-coding region [9]. Internal deletions of human IL-6 were performed by oligonucleotide-directed mutagenesis according to Zoller and Smith [13] on the single-stranded DNA template of pEMBL T7/IL-6. Single-stranded pEMBL T7/IL-6 was prepared as described [14].

Table 1. Conformational analysis of Δ171–173, Δ174–176 and Δ177–179 variants using anti-IL-6 mAb. The wild-type and IL-6 deleted proteins, produced in a reticulocyte lysate, were immunoprecipitated as described in Materials and Methods using anti-(IL-6) mAb either binding different epitopes of the native IL-6 (mAb 8, 12 and 16) or recognizing the unfolded wild-type IL-6 with a higher affinity than the native IL-6 (mAb 7) [19]. (+), immunoprecipitated; (−), non-immunoprecipitated; n.d., not determined. The ratios of the amount of proteins immunoprecipitated by mAb 7 and mAb 12 (mAb 7/mAb 12) or immunoprecipitated by mAb 7 in the absence of SDS and in the presence of 0.1% SDS (mAb 7/mAb 7 0.1% SDS) have been calculated on the basis of densitometric scanning of autoradiograms.

Protein	Immunoprecipitation with					
	mAb 7	mAb 8	mAb 12	mAb 16	mAb 7/ mAb 12	mAb 7/ mAb 7 +0.1% SDS
					%	
Wild-type IL-6	−	+	+	+	0.35	0.55
Δ171−173	+	+	+	−	2.55	0.94
Δ174−176	+	−	+	+	n.d.	n.d.
Δ177−179	−	−	+	+	0.31	0.47

Three oligonucleotides were synthesized to generate the following mutations: Δ171−173 (Lys-Glu-Phe), 5′-CTGGACTGCAGAAAGCTGCGCAG-3′; Δ174−176 (Leu-Gln-Ser), 5′-GCCCTCAGGCTGAACTCCTTAAAGC-3′; Δ177−179 (Ser-Leu-Arg), 5′-TTGCCGAAGAGCGGACTGCAGG-3′. To determine the end-point of the deletion in each clone, nucleotide-sequence analysis was performed using the dideoxy-chain-terminal method [15]. The *Bgl*II−*Hin*dIII fragment carrying the T7 promoter and the IL-6-coding region from Δ177−179 was also inserted into the *Escherichia coli* expression vector pT7.7 [6, 16] making use of the same restriction sites.

Generation of substitution mutants

A polymerase chain reaction (PCR) strategy was used to generate saturation mutations within a desired codon sequence as shown in Fig. 1. In all PCR, the upstream primer was a 31-residue oligonucleotide corresponding to positions 270−300 (sense strand), including the unique *Acc*I site of the IL-6 cDNA. The downstream mutagenic primers extending from position 513 to position 565 maximally, contained a new *Xho*I restriction site and ambiguous nucleotide sequences on the three bases of the codon to be mutated.

In each case, a 269-bp DNA fragment was amplified by the PCR according to the standard PCR amplification protocol [17]. Amplification was carried out for 35 cycles, each cycle consisting of incubations for 2 min at 94°C for denaturation, 2 min. at 50°C for annealing and 3 min. at 72°C for primer extension.

The amplified fragment was digested with *Acc*I and *Xho*I and purified from a 2% agarose gel. The double-digested PCR-generated fragment was ligated into the similarly cut recipient vector pBKS IL-6 (purified on a 1% agarose minigel) to replace the wild type sequence.

To construct pBKS IL-6, an *Eco*RI−*Hin*dIII fragment, carrying the human IL-6-coding region from the plasmid pEMBL T7/IL-6 [9], was subcloned into plasmid Bluescript

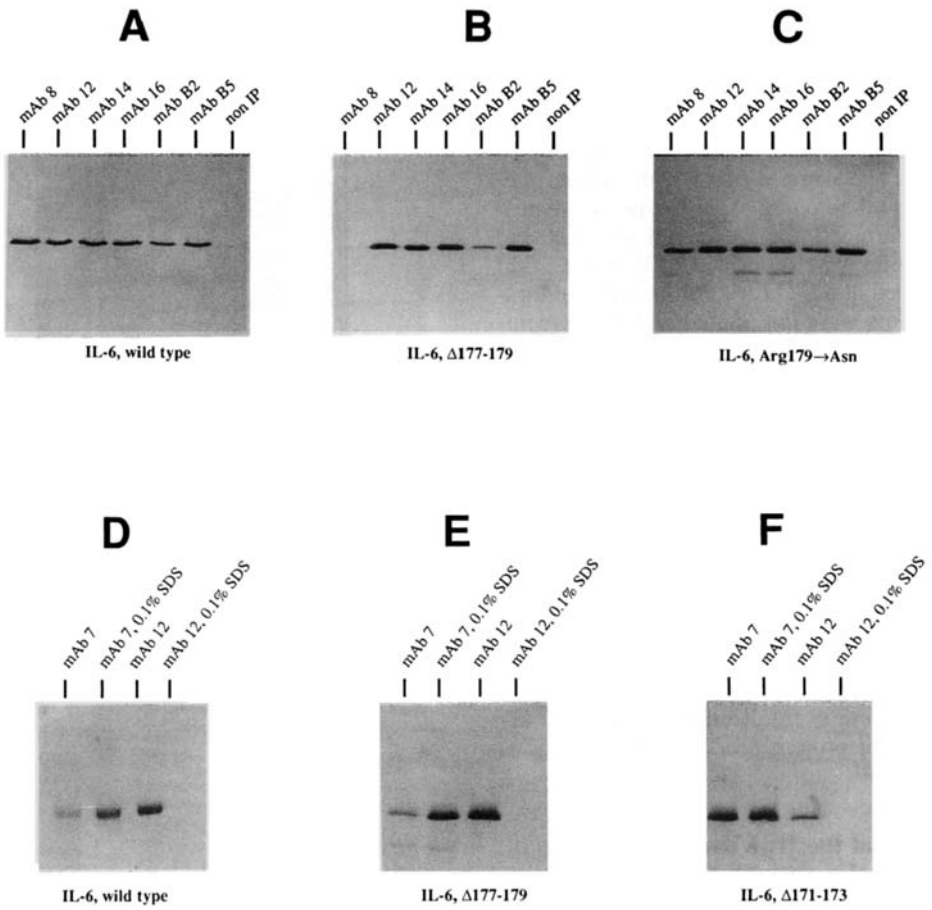

Fig. 2. Structural integrity analysis of deletion and substitution variants by immunoprecipitation with anti-(IL-6) mAb. Wild-type or mutated IL-6, produced in reticulocyte lysate, were immunoprecipitated in presence of 500 ng mAb/ml and goat anti-mouse antibodies coupled to Sepharose, as described in Materials and Methods. After an overnight incubation, the Sepharose-bound proteins were washed, dissolved in 20 µl sample mix and subjected to 0.1% SDS/17% PAGE, followed by autoradiography. (A–C) immunoprecipitation of wild-type IL-6, Δ177–179 deletion mutant and Arg179→Asn mutant, respectively, with conformation-specific mAb (mAb 8, 12, 14, 16, B2 and B5). These mAb species recognize different conformational epitopes: mAb 12 and B2 recognize among others N-terminal amino acids, mAb 8 binds close to mAb 14 at the C-terminal end of the IL-6 molecule and mAb 16 recognizes residues around Trp157 [7, 28]. (D–F) immunoprecipitation of wild-type IL-6, Δ177–179 deletion mutant and Δ171–173 deletion mutant, respectively. The immunoprecipitations have been realized in the absence or in the presence of 0.1% SDS, either with mAb 7 recognizing with a higher affinity the unfolded IL-6 protein or with the conformation-specific mAb12. Control lanes are non IP, immunoprecipitation without anti-(IL-6) mAb.

KS(−) (Vector Cloning Systems). All mutated plasmids were characterized by restriction mapping [18] and sequence analysis [15].

Production and quantification of IL-6 mutant proteins

IL-6 proteins were produced in two alternative systems.

(a) *In vitro* transcription and translation. Plasmids of the pEMBL or pBKS series, linearized by *Sal*I or *Xho*I restriction endonucleases, respectively, were transcribed with T7 RNA polymerase in the same conditions as previously described [19]. The corresponding mRNA species were subsequently translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine [19].

Translated proteins were analyzed by 0.1% SDS/17% PAGE, followed by fluorography and autoradiography using standard procedures. The amount of IL-6 mutant proteins in each preparation was then determined by densitometric scanning of the SDS/polyacrylamide gels autoradiograms and normalized to that of wild-type IL-6, allowing the specific activity of each sample to be estimated. Three separate protein preparations and protein concentration analyses were performed on each mutant.

(b) Production in *E. coli.* Plasmid pT7.7 Δ177–179 was introduced in strain BL21 (DE3)Lys E, the recombinant deleted protein was produced according to Arcone et al. [16]. This gave rise to large amounts of mutant proteins 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 0.1% SDS/17% polyacrylamide gel stained with Coomassie brilliant blue R-250.

Bioassays

IL-6 C-reactive protein-chloramphenocol acetyl transferase (CRP-CAT) inducing assays were performed on human

hepatoma Hep3B cells according to Ganter et al. [20]. Plasmid 3′Δ-121/-146 CRP-CAT [21] carrying a fusion between a segment of the C-reactive protein promoter linked to the Simian virus 40 early promoter and to the bacterial chloramphenicol acetyltransferase gene was transfected into the cells using the calcium-phosphate precipitation technique [22]. After 15 h, transfected cells were washed with culture medium and treated with equal amounts of wild-type or mutated IL-6. Induction was continued for 24 h; cell extracts and CAT assays were performed as described [23] using 20 µl cell extracts and incubated at 37°C for 15 min. After autoradiography, individual spots were cut, eluted and the percentage of acetylated versus total [^{14}C]chloramphenicol was determined.

IL-6 hybridoma growth factor (HGF) assays were performed on the mouse B cell hybridoma 7TD1 as described previously [24]. 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 wild-type and mutated IL-6 produced in the reticulocyte lysate. After 5–6 days, the number of living cells were evaluated by measuring hexosaminidase levels as described [25]. Since high concentrations of the reticulocyte lysate interfere with the IL-6 assay, equal and subtoxic volumes of the translation mixtures were used. The measured IL-6 activities were normalized by taking into account the amount of mutant protein produced in the various preparations.

Conformational analysis of the IL-6 derived proteins by immunoprecipitation with anti-(IL-6) mAb

Immunoprecipitations were performed as described previously [6, 7, 19]. Briefly, goat anti-mouse IgG, coupled to Sepharose, were incubated overnight with a fixed amount of the mAbs together with the IL-6-derived proteins. After washing, the Sepharose bound protein was dissolved in sample mix and submitted to SDS/PAGE under reducing conditions, followed by autoradiography. In all experiments, input of materials non-immunoprecipitated or immunoprecipitated with a rabbit anti-rhuman IL-6 polyclonal antiserum in presence of *Staphylococcus aureus* Cowan-I–protein-A were used as a positive control [19].

RESULTS

Construction, expression and biological analysis of IL-6 deletion variants

In order to extend the analysis of the C-terminal domain, we have generated three internal amino acids deletions of residues 171–179 (Δ171–173, Δ174–176 and Δ177–179). This was carried out by oligonucleotide-directed mutagenesis of the IL-6 cDNA subcloned in pEMBL 131, as described in Materials and Methods. Wild type and mutated constructs were transcribed *in vitro* and the corresponding mRNA species were then translated in a rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine. The concentrations of the synthesized IL-6 variants were determined as described in Materials and Methods, allowing us to compare the biological activities of the mutated proteins to that of IL-6. This was carried out using two IL-6 bioassays; the HGF assay performed on the mouse B cell hybridoma, 7TD1, and a transcriptional activation assay performed on the human Hep3B hepatoma cells.

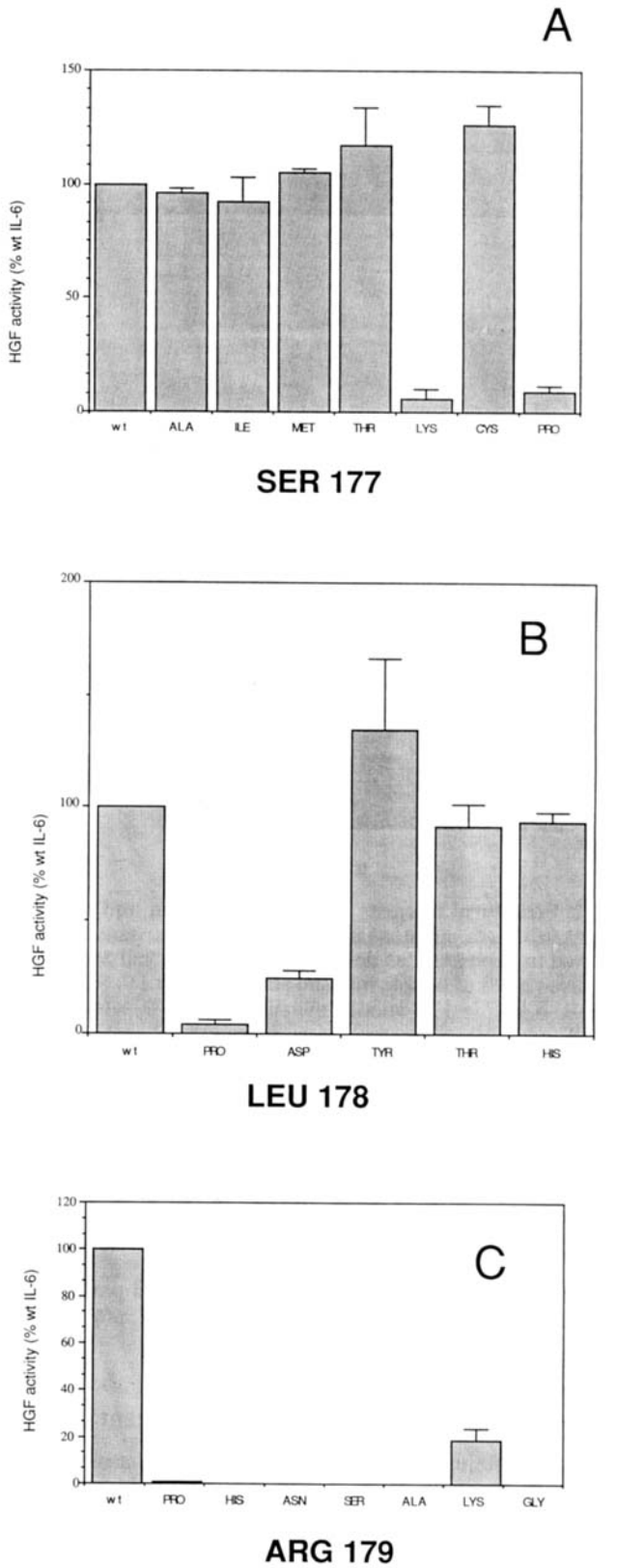

Fig. 3. Comparison of the HGF activity of the substitution variants to that of the wild-type IL-6. HGF bioassays were performed as described in Materials and Methods and the measured HGF activities were normalized by taking into account the amount of mutant or wild-type (wt) proteins produced in the various reticulocyte lysate preparations. The activity of wild-type IL-6 was always scored as 100% active. (A) Ser177 variants; (B) Leu178 variants; (C) Arg179 variants.

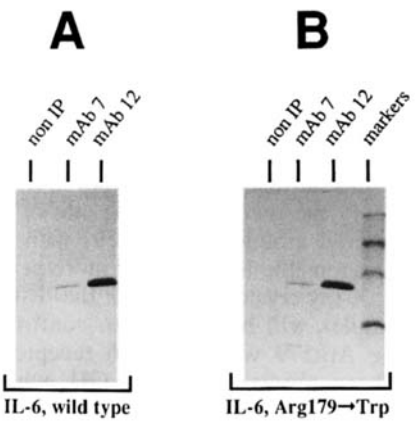

Fig. 4. Structural integrity analysis of the Arg179→Trp substitution variant by immunoprecipitation with anti-(IL-6) mAb. Wild-type or mutated IL-6, produced in reticulocyte lysate, were immunoprecipitated in presence of 500 ng mAb/ml and goat anti-mouse antibodies coupled to Sepharose, as described in Materials and Methods and Fig. 2. mAb 7 and the conformation-specific mAb 12 are described in Fig. 2. Control lanes are non IP, immunoprecipitation without anti-(IL-6) mAb.

Using subtoxic volumes of the translation mixtures, we could not detect any IL-6 biological activities for the IL-6 deletion variants. However, the sensitivity of the HGF assay was 200 U/ml or 0.5% of the wild-type IL-6 activity. Only a weak HGF specific activity, 3.5×10^5 U/mg, which corresponds to less than 0.1% of the wild-type activity, was measured for an *E. coli* preparation of the Δ177−179 protein (not shown).

Conformational analysis of the IL-6 deletion variants

In order to determine whether the reduced activities of the deletion variants were produced by a modification in a specific region of IL-6 directly involved in binding to its receptor or by a change in the IL-6 tertiary structure, the conformational integrity of our variants was assessed. The use of mAb as sensitive probes for structural alterations in a mutant protein has already been extensively described [26, 27]. Therefore, we used the same approach in immunoprecipitation experiments to probe the structural integrity of our variants. Amongst a panel of anti-(IL-6) mAb that were recently developed [7, 28, 29], we selected one mAb (mAb 7) that shows preference towards denaturated hIL-6 and six conformation-specific mAbs (mAb 8, 12, 14, 16, B2 and B5) to perform our immunoprecipitation assays. The six selected conformation-specific mAb recognize different conformational epitopes; mAbs 12 and B2 recognize among others N-terminal amino acids (around Ile25), mAb 8 binds close to - mAb 14 at the C-terminal end of the IL-6 molecule and mAb 16 recognizes residues around Trp157 [7, 28]. mAb B5 and 14 are conformation-specific mAb but they do not neutralize human IL-6 bioactivity [7, unpublished results]. On the contrary, mAb 7 recognizes the unfolded wild-type IL-6 with a higher affinity than the native IL-6 and binds hexapeptides having amino acids Thr142 to Ala145 of human IL-6 in common [29]. Moreover, the binding of mAb 7 to human IL-6 in solution can be greatly enhanced by addition of 0.1−0.2% SDS [29]. As shown in Table 1 and Fig. 2 A, B, D, E, F, we observed that variants Δ171−173 and Δ174−176 have lost a part of the IL-6 tertiary structure. Indeed, though immunoprecipitated by some neutralizing mAb species, these two proteins are also immunoprecipitated efficiently by mAb 7.

On the contrary, the Δ177−179 protein, which is recognized and immunoprecipitated by some conformation-specific mAb species, has not undergone major structural changes because it is not significantly immunoprecipitated by mAb 7. Moreover, the enhanced reactivity of mAb 7 with the Δ177−179 protein in the presence of 0.1% SDS confirmed that Arg179 might not be part of the mAb 7 epitope. This is in agreement with pepscan analysis of the mAb 7 epitope [29]. Furthermore, in view of the selective absence of reactivity of mAb 8 toward Δ177−179, we can conclude that the epitope recognized by this mAb does not include only the last four amino acids defined by previous studies [7], but also more upstream residues.

Since Δ177−179 has conserved the conformation of wild-type IL-6 but is at least 1300-fold less active, we favor the idea that one or more amino acids of the 177−179 triplet could be functionally involved either in receptor interaction or, more selectively, in IL-6 signal transduction.

In order to distinguish between these two possibilities, Δ177−179 was expressed in *E. coli* [6], produced in large amounts and purified to homogeneity. The *E. coli* preparation failed to inhibit the HGF activity or CRP-CAT expression displayed by wild-type IL-6, even using a 100-fold molar excess (not shown). Moreover, a soluble IL-6 receptor competition assay [30] showed that this deletion variant, even at a 1000-fold molar excess, is unable to compete with wild-type ^{125}I-labelled IL-6 for binding to the gp80 soluble subunit (data not shown).

Construction and biological activity analysis of substitution variants

In order to identify the key residue(s) in the 177−179 amino acid region, multiple amino acids substitutions were generated at each position using a PCR strategy depicted in Fig. 1. Clones bearing mutations were selected on the basis of *Xho*I restriction mapping and further characterized by sequence analysis. These mutated constructs, as well as the wild-type IL-6 cDNA were transcribed *in vitro* and the corresponding mRNA species were translated in rabbit reticulocytes lysates, as previously described. The HGF activity of these preparations were tested and compared to that of IL-6, taking into account the translated protein concentration in each preparation. In each assay, the HGF activity of the wild-type IL-6 preparation was scored as 100% active.

As shown in Fig. 3A and B, most of the substitutions of Ser177 and Leu178 did not significantly alter the biological activity on mouse cells, suggesting, therefore, that these residues are not directly involved in the interaction with the murine IL-6 receptor. An important change was exhibited by proline substitutions (10% and 1% residual activity for positions 177 and 178, respectively). This may be interpreted as consequence of the disruption of the putative α helix caused by this amino acid. Indeed, the presence of an α helix at the IL-6 C-terminus has been predicted by molecular modeling [10] and further supported by some biological evidences [9].

A lower HGF activity (10−30%) was also observed when Ser177 or Leu178 were replaced by charged residues; Lys at position 177 or Asp at position 178 (Fig. 3 A and B).

However, substitution of Arg179 has more drastic effects on bioactivity; replacement with either a Pro, His, Asn, Ser,

Ala or Gly residue resulted in variants with at least a 100-fold reduced activity in the HGF assay. Only the conservative replacement (Arg179→Lys) did not reduce the activity of the corresponding variant more than fivefold (Fig. 3C). Furthermore, using a random mutagenesis approach in a separate experiment, we have also observed that one of our mutant proteins, with Arg179 replaced by Trp, retains 1% of the wild type IL-6 biological activity, on the 7TD1 cells. The expression of this variant in *E. coli* allowed us to assess its affinity for the IL-6 receptor on UAC cells. This substitution variant recognized the IL-6 receptor with a weaker affinity, compared to wild-type IL-6 or to another IL-6 variant (substituted on the Arg168) which had retained 5% of its activity (Fontaine et al., unpublished results).

Consequently, these results suggest that the positive charge at position 179 is of critical importance for the biological activity of human IL-6 and for the interaction with its receptor.

Structural analysis of Arg179 substitution variants

To further assess the specific role of Arg179, the structural integrity of three substitution variants (in which Arg179 was replaced either by Ala, Asn or His) was analyzed by immunoprecipitation with mAb 7, 8, 12, 14, 16, B2 and B5 as previously described for the original set of deletion variants.

As shown in Fig. 2A and C for the Arg179→Asn substitution, and in Fig. 4 for an Arg179→Trp substitution, conformation-specific mAb (mAb 8, 12, 14, 16, B2 and B5) recognized these mutants, whereas mAb 7 did not. Moreover, in separated experiments, we observed that the ratio of the amount of mutant proteins immunoprecipitated by mAb 7 and immunoprecipitated by neutralizing mAb (mAb 8, 12 and 16) is the same as for the wild-type IL-6, corresponding to 0.43, 0.29 and 0.43, respectively. This immunoprecipitation pattern allows us to conclude that the inactive Arg179 variants retain, most probably, a tertiary structure similar to wild-type IL-6. Since the decrease in activity is most probably not due to an altered folding, all these results point to the involvement of Arg179, and, more precisely, of a positive charge at this position, in the human IL-6 receptor interaction.

DISCUSSION AND CONCLUSION

Using a deletion mutagenesis approach we have been able to emphasize the functional significance of the 177–179 amino acid region of human IL-6. The more N-terminal stretch of amino acids (171–176) apparently contributes to human IL-6 folding, but given the fact that our deletion mutants in this area are always showing major structural alterations as revealed by their reaction with mAb 7, we cannot exclude the possibility that other determinants for receptor binding are located here which can only be revealed by more subtile mutagenesis experiments.

Our substitution analysis of the restricted 177–179 region allowed us to identify Arg179 and, more precisely, its positive charge, as being of crucial importance for biological activity in the HGF assay. On a statistical basis, it is significant that in the murine system five out of seven amino acids 177 mutants are active, three out five amino acids 178 are also active but only one out of seven amino acid 179 mutants has a residual 20% activity. Since we cannot detect any major conformational change between IL-6 and a subset of our 179 substitution mutants, at least with the set of available monoclonals, our conclusion is that the positive charge located here must fit into the IL-6 receptor pocket. In this respect, it is interesting to observe that, in general, mutations of Arg179 cause an effect at least 10-fold more dramatic than that caused by substitutions of Arg182 and Met184. This implies that probably the receptor-binding site of human IL-6 is spatially centered around the Arg179, with Arg182 and Met184 being responsible for looser or more peripheral contacts. However, X-ray crystallography of the IL-6-soluble IL-6–receptor complex will be required to confirme direct interaction of the Arg179 with the IL-6 receptor. A similar hierarchy has been observed also for GH, where different exposed residues in the C-terminal region (helix D) contribute differentially to receptor binding [11].

The unexpected weak activity of the Lys177 and Asp178 variants could be explained by the formation of salt bridge (Asp178) or repulsive ionic interactions (Lys177) between these charged residues and the crucial Arg179, which could interfere with the normal function of this important residue.

Finally, in agreement with what has been previously observed by Lutticken et al. [9], we also find that introduction of prolines in this part of the molecule (positions 177 and 178) has always a dramatic effect. This strengthens the idea that an α-helical fold of the C-terminus is required for full biological activity of human IL-6.

Taken together, our results further stress the importance of the C-terminal region of human IL-6, but also reveal that important determinants in receptor binding are probably located over an extended region of the molecule whose complete identification will require further mutagenesis.

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