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Role of leucine residues in the C-terminal region of human interleukin-6 in the biological activity

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Site-directed mutagenesis of two sets of three periodic leucine residues which appear at every seventh position in the C-terminal region of human interleukin-6 (IL-6) was performed. Both receptor-binding and immunoglobulin (Ig)-induction activities of a triple mutant $Leu^{168,175,182}$→Val were only 1% compared with those of wild-type IL-6. However, the mutant $Leu^{152,159,166}$→Val had 13% receptor-binding and 2% Ig-induction activities of those of wild-type IL-6. In order to obtain more direct information on the receptor-binding region, we prepared two synthetic peptides. A significant binding activity was observed for the peptide Leu^{168}-Met^{185}, but not for the peptide Leu^{152}-Arg^{169}. These results indicate that leucine residues in the C-terminal region, especially Leu^{168}, Leu^{175}, and Leu^{182}, play an important role in the receptor-binding and Ig-induction activities.

Interleukin 6; Site-directed mutagenesis; Peptide fragment; Receptor-binding activity; B-Cell stimulatory activity; Human

1. INTRODUCTION

Interleukin-6 (IL-6) is important in modulating growth and differentiation of various cells [1]. Complementary DNA encoding human IL-6 (M_r = 21,000) has been isolated [2] and recombinant IL-6 has been expressed at a high level in *Escherichia coli* (*E. coli*) [3]. Only after the binding of IL-6 to its receptor [4] on the cell surface, can the receptor associate with a non-ligand signal transducer, gp130, and mediate its function [5]. Soluble human IL-6 receptor, which lacks the transmembrane and intracytoplasmic domains, has been expressed in Chinese hamster ovary cells [6].

Structure–function relationship of human IL-6 has been examined by producing IL-6 mutants and establishing a monoclonal antibody clone specific for IL-6. Brakenhoff et al. prepared various IL-6 mutants with deletions in the N-terminal region and concluded that the first 28 amino acid residues can be removed without significantly affecting the biological activity [7]. Arcone et al. tried internal deletions of IL-6, and concluded that only residues 29–34 are essential for the biological activity [8]. Ida et al. prepared a monoclonal antibody that strongly neutralizes the biological activity, and showed that the antibody recognizes the epitope comprising segment Thr^{150}-Phe^{174} [9]. Krüttgen et al. showed that removal of only three amino acids from the C-terminal region of IL-6 led to a complete loss of the biological activity [10]. However, it is still not clear which segments of IL-6 are actually involved in the receptor-binding.

In the previous study, on the basis of the chemical modification and ^{1}H NMR data, we have concluded that Trp^{158}, Met^{162}, His^{165}, and Met^{185} are in spatial proximity, comprising the receptor-binding region [11]. Furthermore, we have prepared several IL-6 mutants and suggested that hydrophobic side chains existing in Leu^{159}, Met^{162}, and Leu^{166} are significantly involved in the receptor-binding of IL-6 [12]. In the C-terminal region of human IL-6, there are two sets of three periodic Leu residues which appear at every seventh position, i.e. Leu^{152}, Leu^{159}, Leu^{166} and Leu^{168}, Leu^{175}, Leu^{185} (Fig. 1). In the present study, on the basis of our previous findings, we have tried two sets of triple mutations $Leu^{152,159,166}$→Val and $Leu^{168,175,182}$→Val and discussed the role of hydrophobic side chains of the Leu residues in the receptor-binding and immunoglobulin (Ig)-induction activities. We have also prepared two peptides, Leu^{152}-Arg^{169} and Leu^{168}-Met^{185}, corresponding to two sets of triple mutations ($Leu^{152,159,166}$→Val and $Leu^{168,175,182}$→Val), respectively, and measured their receptor-binding activities.

2. MATERIALS AND METHODS

2.1. *Oligonucleotide-directed mutagenesis*

The mutagenic oligonucleotides were synthesized by phospho-

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Abbreviations: IL-6, interleukin-6; *E. coli*, *Escherichia coli*; Ig, immunoglobulin; hGH, human growth hormone; ELISA, enzyme-linked immunosorbent assay.

ramidite chemistry on an Applied Biosystems Model 381A synthesizer and purified on oligonucleotide purification cartridges. The various mutagenic oligonucleotides for construction of the IL-6 mutants were used (Fig. 1). The 1,000-base pair *KpnI-SalI* fragment derived from pBSF2.38-1 [3], coding human IL-6 (185 amino acids), was subcloned into M13mp19, and the single-stranded form of this plasmid was used as a template for mutagenesis. The mutagenesis was performed by using an oligonucleotide-directed in vitro mutagenesis system (Amersham) [13]. The nucleotide sequence of mutant clones was determined by dideoxynucleotide chain termination procedure to ensure that there were no other point mutations [14]. For IL-6 protein expression, mutagenic DNA was recloned into *E. coli* expression vector derived from pBSF2.38-1.

2.2. *Protein expression and purification*

All mutant and wild-type IL-6s were produced as described by Yasukawa et al. with some modifications [15]. The *E. coli* RB791 containing the plasmid coding IL-6s was cultured for 5 h by using LB medium (60 ml). The cells were harvested by centrifugation, and the cell pellet was suspended in 50 mM Tris-HCl (pH 8.0) and disrupted by sonication. After centrifugation, the inclusion body of human growth hormone (hGH)-IL-6 fusion protein in the pellet fraction was dissolved with 8 M urea in 50 mM Gly-NaOH buffer (pH 9.0). The protein solution was dialyzed against 1 M urea in 50 mM Tris-HCl (pH 8.0) in the presence of a glutathione redox system, and the resultant refolded fusion protein was cleaved by thrombin into hGH and IL-6. After the addition of 20 mM $CaCl_2$, the solution was centrifuged, and the resultant pellet was dissolved by 6 M guanidine-HCl in 50 mM Tris-HCl (pH 8.0). The IL-6 solution was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) in the presence of the glutathione redox system. Wild-type and mutant IL-6s were purified by ammonium sulfate precipitation, and apparent 21 kDa single band was observed by SDS-polyacrylamide gel electrophoresis. From 60 ml of culture medium, 200–500 μg of wild-type and mutant IL-6s was obtained. Protein concentrations of IL-6s was obtained. Protein concentrations of IL-6s were determined by the dye binding assay (Bio-Rad Laboratories) with bovine serum albumin as a standard.

2.3. *Radioisotope competitive assay for testing the binding activity of IL-6 mutants*

Anti-(IL-6 receptor) antibody (4 μg/ml) [16] was coated in the 96-well microplates in 100 μl of 0.1 M carbonate/hydrogen carbonate (pH 9.6) at 4°C overnight. The wells were blocked with 1% bovine serum albumin in 20 mM phosphate, 120 mM NaCl (pH 7.4) at room temperature for 2 h. Soluble IL-6 receptor (10 ng) [6] was added and incubated for 2 h. After washing, 100 μl of test samples containing ^{125}I-labeled IL-6 (15,000 cpm) was incubated at room temperature for 2 h. The wells were washed and radioactivity of each well was measured with a γ-counter (Aloka).

2.4. *B-cell stimulatory assay*

Four thousand SKW6-CL4 cells, EBV-transformed B-cell lines, were cultured in 200 μl of RPMI1640 medium containing 10% fetal calf serum, and wild-type and mutant IL-6s were added into the culture medium [17]. After three days, the concentration of IgM produced in the culture medium was determined by enzyme-linked immunosorbent assay (ELISA).

2.5. *Synthesis of peptide fragments*

Peptide fragments, Leu^{152}-Arg^{169} and Leu^{168}-Met^{185}, were synthesized using an Applied Biosystems Model 430A peptide synthesizer. Crude peptides were washed with diethylether, extracted with acetic acid, and lyophilized. Purification was carried out by reverse-phase high performance liquid chromatography. The amino acid composition of each peptide was checked. The whole amino acid sequence of each peptide was also confirmed by a Applied Biosystems Model 470A gas-phase sequencer.

3. RESULTS

The competitive binding activities of wild-type and mutant IL-6s to its soluble receptor were measured by using [^{125}I]IL-6. The radioactivity derived from bound [^{125}I]IL-6 decreased with increasing concentration of the competing IL-6 analogs and generated the competition binding curves as shown in Fig. 2. More pronounced effects on the binding activity were observed with mutant Leu^{168,175,182}→Val than with mutant Leu^{152,159,166}→Val. The amount of wild-type and mutant IL-6 proteins required for 50% displacement of [^{125}I]IL-6 (IC_{50}) was determined for each IL-6 analogs (Table I). The binding activities of mutants Leu^{152,159,166}→Val and Leu^{168,175,182}→Val were 13% and 1% compared with that of wild-type IL-6, respectively.

The biological activities of IL-6 analogs were estimated by the amount of IgM produced from IL-6-analog-treated SKW6-CL4 cells (Fig. 3). Both mutants Leu^{152,159,166}→Val and Leu^{168,175,182}→Val showed a pronounced decrease in the Ig-induction activity. The amount of IL-6 analogs required for the half-maximal Ig-induction activity (EC_{50}) was determined for each IL-6 analog (Table I). The Ig-induction activities of mutants Leu^{152,159,166}→Val and Leu^{168,175,182}→Val were 2% and 1% of that of wild-type IL-6, respectively.

Since the receptor-binding activity of mutant Leu^{168,175,182}→Val was 13-fold less than that of mutant Leu^{152,159,166}→Val, we have focused our attention on the former Leu residues, Leu^{168}, Leu^{175}, and Leu^{182} (Table I). The receptor-binding activities of mutants Leu^{168}→Val, Leu^{175}→Val, and Leu^{182}→Val, were 20%, 8%, and 17% of that of wild-type IL-6. This result shows

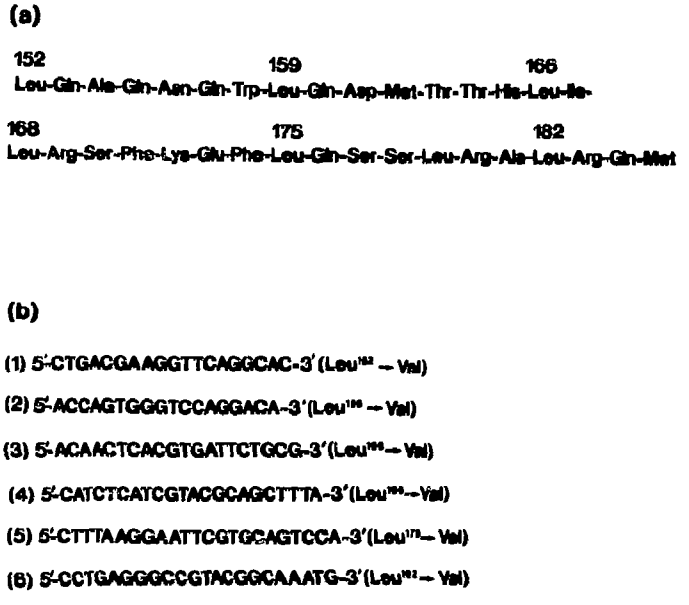

Fig. 1. Amino acid sequence in the C-terminal region of human IL-6 (a) and mutagenic oligonucleotides designed for site-directed mutagenesis (b).

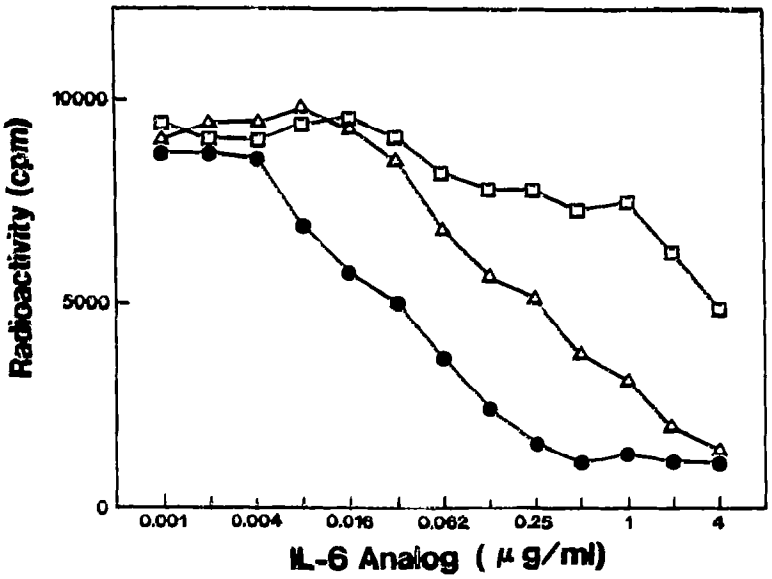

Fig. 2. The competitive binding activity to the soluble IL-6 receptor of wild-type (●), mutant Leu^{152,159,166}→Val (△), and mutant Leu^{168,175,182}→Val (□). The immunosorbent assay was performed as described under Materials and Methods. Radioactivities of [^{125}I]IL-6 binding to soluble IL-6 receptor were measured by a γ-counter. Data represent means of triplicate assays.

that Leu^{175} plays the most important role in the receptor-binding of IL-6. More significant effects on the receptor-binding activity were observed for double substitutions of Leu^{168}, Leu^{175}, and Leu^{182} than the single substitutions. Mutants Leu^{168,175}→Val and Leu^{175,182}→Val showed 3% and 6% receptor-binding activities of that of wild-type IL-6. The decrease in the Ig-induction activity induced by single or double substitutions of Leu^{168}, Leu^{175}, or Leu^{185} was consistent with that of the receptor-binding activity.

Table I

Amino acid substitutions and the receptor-binding and Ig-induction activities

Substitutions	Relative activity	
	Receptor-binding activity (IC_{50})[a]	Ig-induction activity (EC_{50})[b]
Wild-type	100	100
Leu^{152,159,166}→Val	13	2
Leu^{168,175,182}→Val	1	1
Leu^{168}→Val	20	33
Leu^{175}→Val	8	13
Leu^{182}→Val	17	19
Leu^{168,175}→Val	3	2
Leu^{175,182}→Val	6	7

[a]The amount of IL-6 analogs required for 50% displacement of [^{125}I]IL-6 was determined.

[b]The amount of IL-6 analogs required for the half-maximal Ig-induction activity was determined.

In order to show that the C-terminal region is directly involved in the receptor-binding, two peptides, Leu^{152}-Arg^{169} and Leu^{168}-Met^{185}, were synthesized. A significant activity of the receptor-binding was observed for the peptide Leu^{168}-Met^{185} (Fig. 4). On the basis of the IC_{50} value, the activity of the peptide Leu^{168}-Met^{185} was estimated as 10^4-fold less than that of wild-type IL-6.

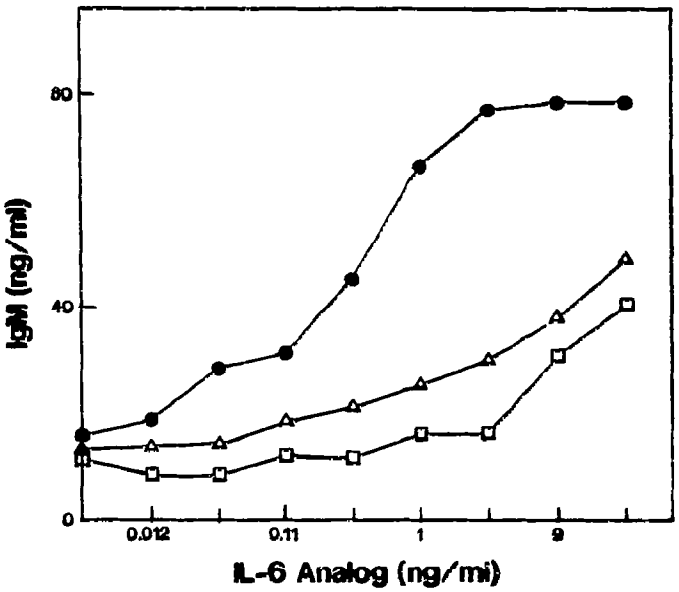

Fig. 3. The Ig-induction activity of wild-type (●), mutant Leu^{152,159,166}→Val (△), and mutant Leu^{168,175,182}→Val (□). The assay was performed as described under Materials and Methods. The amount of IgM produced from SKW6-CL4 cells was measured by ELISA. Data represent means of triplicate assays.

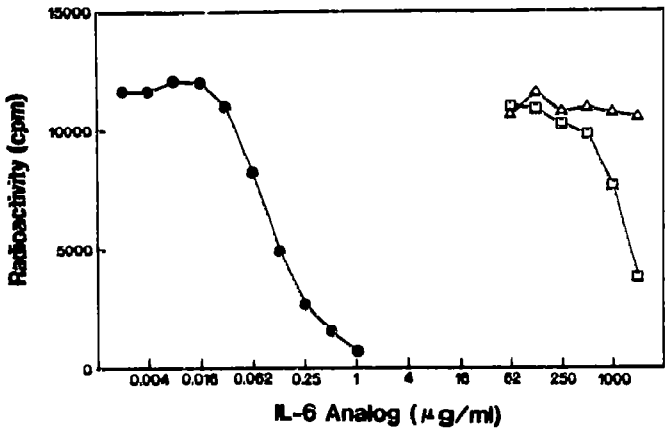

Fig. 4. The competitive binding activity to the soluble IL-6 receptor of intact IL-6 (●), and synthetic peptides, Leu[152]-Arg[169] (△) and Leu[168]-Met[185] (□). The immunosorbent assay was performed as described under Materials and Methods. Radioactivities of [^{125}I]IL-6 binding to soluble IL-6 receptor were measured by a γ-counter. Data represent means of triplicate assays.

No receptor-binding activity was observed for the peptide Leu[152]-Arg[169].

4. DISCUSSION

On the basis of the crystal structure of hGH [18], Bazan proposed a model of IL-6 folding comprising bundles of four antiparallel helices A, B, C, and D [19]. Helix D (Gln[155]-Met[185]) in the C-terminal region of IL-6 was suggested to play an important role in the receptor-binding [11,20]. In the C-terminal region of human IL-6, there are two sets of three periodic Leu residues which appear at every seventh position. This suggests an intriguing possibility that helix D composes one side of the leucine zipper structure [21]. A significant decrease in DNA-binding activity has previously been reported for substitutions with Val residues of Leu residues that exist at every seventh position in the leucine zipper region [22–24]. On the basis of these results, we tried substitutions of Leu[152], Leu[159], Leu[166], Leu[175], and Leu[182] with Val residues of IL-6. The receptor-binding activity of mutant Leu[168,175,182]→Val is only 1% compared with that of wild-type IL-6 and 13-fold less than that of mutant Leu[152,159,166]→Val. This suggests that the hydrophobic side chains of Leu residues in the C-terminal region, especially Leu[168], Leu[175], and Leu[182], play an important role in the receptor-binding. Among three single mutations, the single substitution of Leu[175]→Val was most effective in reducing the receptor-binding activity. This is consistent with the following two results. (i) Leu[175] in IL-6 molecule is all conserved among other helical cytokines, such as GH, prolactin, erythropoietin, granulocyte-colony-stimulating factor, and myelomonocytic growth factor. (ii) It was concluded by alanine-scanning mutagenesis of hGH [20] that mutation of Thr[175] or Phe[176], which is near Leu[177] corresponding to Leu[175] in IL-6, is most effective on the receptor-binding. On the basis of the mutagenesis studies of human IL-6, we propose that helix D composes the leucine zipper structure. The dimer formation of IL-6 molecule is suggested by Rose-John et al. [25]. However, it is still unclear whether the hydrophobic interaction between two helices which participate in the formation of the leucine zipper structure would be intermolecular or intramolecular.

The Ig-induction activity is observed to be more susceptible to the substitutions of Leu[152,159,166]→Val than the receptor-binding activity, whereas both activities are shown to be equally susceptible to the single, double, and triple substitutions of Leu[168], Leu[175], and Leu[182]. These results suggest that Leu[152], Leu[159], and Leu[166] play a role not only in the receptor-binding but also in other mechanisms involved in the Ig-induction activity. It is possible that some residues existing in the Leu[152]-Leu[166] region are involved in the interaction of IL-6 with a signal transducer, gp130. By uncoupling the receptor-binding and biological activities, it should be possible, in principle, to design IL-6 antagonists.

We have concluded that Leu[168], Leu[175], and Leu[182] play a more important role in the receptor-binding activity than Leu[152], Leu[159], and Leu[166]. In order to obtain more direct information on the receptor-binding region, we prepared two peptides, Leu[152]-Arg[169] and Leu[168]-Met[185]. A significant activity of receptor-binding was observed for the peptide Leu[168]-Met[185], but not for the peptide Leu[152]-Arg[169]. These results not only support that Leu[168], Leu[175], and Leu[182] existing in the peptide Leu[168]-Met[185] are important in binding of IL-6 to its receptor, but also directly show that the peptide Leu[168]-Met[185] comprises a part of the receptor-binding region. The peptide Leu[168]-Met[185] has a much lower activity of receptor-binding than intact IL-6, probably because the peptide fragment cannot take the same tertiary structure as the corresponding region which exists in intact IL-6.

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