

Site-specific mutagenesis of human interleukin-6 and its biological activity

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Amino acid substitutions of human interleukin-6 (IL-6) were performed. Single substitution Met¹⁶²→Ala and double substitutions Leu^{159,166}→Val resulted in a significant decrease of IL-6 activity in the production of immunoglobulin (Ig) from B-cells. Single substitution Leu¹⁶⁶→Val or Leu¹⁵⁹→Val gave a slight or no significant decrease in the Ig-induction activity, respectively. The receptor-binding activity of each IL-6 mutant was also examined. It was observed that the decrease of the receptor-binding activity was generally in parallel with that of the Ig-induction activity. We therefore suggest that hydrophobic side-chains existing in Met¹⁶², Leu¹⁵⁹, and Leu¹⁶⁶ are significantly involved in the receptor-binding of IL-6.

Interleukin-6; Site-specific mutagenesis; B-cell stimulatory activity; Receptor-binding activity; Hydrophobic side-chain; Human

1. INTRODUCTION

Interleukin-6 (IL-6) plays an important role in the growth and differentiation of various cells [1]. The induction of IL-6 production has been associated with a variety of diseases such as rheumatoid arthritis [2,3], severe burns [4], and acquired immunodeficiency syndrome [5-7]. Human IL-6 consists of 185 amino acids ($M_r = 21\,000$) [8], and recombinant human IL-6 has been expressed at a high level in *Escherichia coli* [9]. Human IL-6 receptor [10] associates with a non-ligand-binding membrane glycoprotein, gp130, in the presence of IL-6 and mediates its function [11].

Although biological functions of IL-6 in vitro and in vivo have been examined extensively, only a few studies concerning the structure/function relationship of IL-6 have been reported. Brakenhoff et al. [12] showed that the 28 amino-terminal amino acid residues can be removed without significantly affecting the biological activity of IL-6. Krüttgen et al. [13] showed that the three carboxy-terminal amino acids of IL-6 are essential for its biological function. No study has so far been reported concerning the substitution of amino acids in the IL-6 molecule.

In previous studies, we have partially assigned the ¹H nuclear magnetic resonance (NMR) signals of the

aromatic residues in human IL-6 [14], and, on the basis of the chemical modification and ¹H NMR data, we have concluded that Trp¹⁵⁸, Met¹⁶², His¹⁶⁵, and Met¹⁸⁵ are in spatial proximity, comprising the receptor-binding region [15]. In the present study, on the basis of our previous findings, we have prepared several mutant IL-6 cDNAs by site-directed mutagenesis and examined the effect of the substitution of amino acid(s) on the expression of the IL-6 activity.

2. MATERIALS AND METHODS

2.1. Construction of plasmids

pBSF2.38-1 cDNA [9], containing the entire coding region of human IL-6, was digested with *Kpn*I and *Sal*I, and the resulting ca. 1000 bp cDNA fragment coding IL-6 was inserted into M13mp19 (Takara). For the insertion of IL-6 cDNA into the expression plasmid pSVL (Pharmacia) after site-specific oligonucleotide mutagenesis, *Bam*HI site was introduced by destroying its *Hind*III site in M13mp19. Mutant IL-6 cDNA was prepared using an in vitro Mutagenesis System (Amersham) with synthetic nucleotide oligomers, (i) 5'-ACCACTGGGTCCAGGACA-3' (Leu¹⁵⁹→Val); (ii) 5'-ACAACACGTGATTCTGCG-3' (Leu¹⁶⁶→Val); (iii) 5'-TGCAGGATGCGCAACT-3' (Met¹⁶²→Ala). For the COS1 cell expression, native and mutant IL-6 cDNAs were digested with *Sac*I and *Bam*HI, and inserted in the expression plasmid pSVL. The nucleotide sequences of mutant clones were verified by the dideoxynucleotide chain termination procedure [16].

2.2. Transfection of COS1 cells and analysis of expressed proteins

Each plasmid (10 µg) was transfected into 1×10^6 COS1 cells by the calcium phosphate method [17]. The transfected cells were cultured in Dulbecco's modified Eagle's medium without serum in 10 cm dishes for 3 days. The culture supernatant was collected and concentrated (25×) with an Amicon Centriprep 10 cartridge for the analysis of biological activity. IL-6 and mutant IL-6 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using recombinant human IL-6 [9] as a standard. Anti-human IL-6 polyclonal an-

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Abbreviations: IL-6, interleukin-6; NMR, nuclear magnetic resonance; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin

tibody (rabbit) was used for ELISA. A mock control was prepared using pSVL vector without the insert.

2.3. Assay for B-cell stimulatory activity

B-cell stimulatory activity was assayed as described by Hirano et al. [18]. Four thousand SKW6-CL4 cells, EBV-transformed B-cell lines, were cultured in 200 μ l of culture medium in the presence of the test sample. After three days, the concentration of immunoglobulin (Ig) M, which is secreted into the culture medium by SKW-CL4 cells, was determined by ELISA.

2.4. Assay for the receptor-binding activity

Ninety-six-well microplate was coated with 100 μ l of anti-IL-6 receptor antibody MT18 [19] (2 μ g/ml) in 0.1 M sodium-hydrogen carbonate buffer (pH 9.6) at 4°C overnight. After this coating, all experiments were performed at room temperature. The wells were blocked with 100 μ l of 1% bovine serum albumin in 10 mM phosphate, 150 mM NaCl/H₂O (pH 7.4) for 2 h, then washed with 10 mM phosphate, 150 mM NaCl/H₂O (pH 7.4) containing 0.05% Tween 20, and 100 μ l of soluble IL-6 receptor (50 ng/ml) [20] was incubated for 2 h. After washing, 100 μ l of test sample was added to the wells and incubated for 2 h. The wells were washed, and 100 μ l of anti-human-IL-6 polyclonal antibody (rabbit) (5 μ g/ml) was added. After a 2-h incubation, the wells were washed, and 100 μ l of alkaline phosphatase-conjugated anti-rabbit-IgG polyclonal antibody (Cappel) ($\times$ 1/5000) was added. After a 2-h incubation, the wells were rinsed, and 100 μ l of alkaline phosphatase substrate (*p*-nitrophenyl phosphate) (1 mg/ml) was added. The enzyme activity was measured spectrophotometrically at 405 nm.

3. RESULTS AND DISCUSSION

In the previous study [15], effects of oxidation of the Met residues of human IL-6 molecule on its receptor-binding activity have been examined. Of five Met residues, Met¹⁶² was the only residue in which the receptor-binding activity decreases in parallel with the degree of the oxidation reaction. In this report, we have focused our attention not only on Met¹⁶² but also on Leu¹⁵⁹ and Leu¹⁶⁶. We have previously shown that

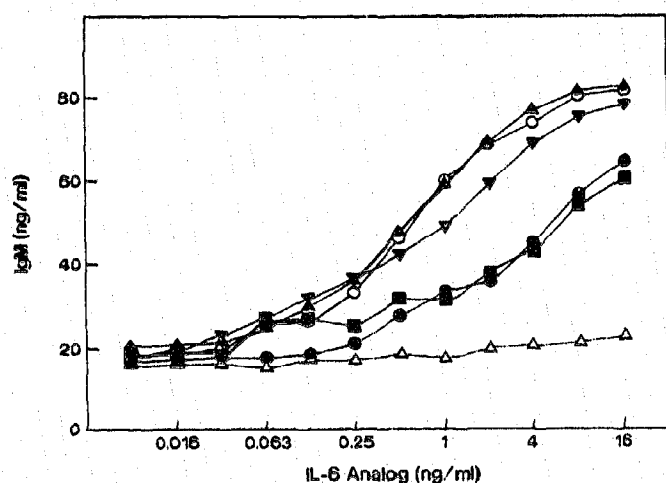


Fig. 1. B-Cell stimulatory assay of native IL-6 (○), Met¹⁶² → Ala (●), Leu¹⁵⁹ → Val (▲), Leu¹⁶⁶ → Val (▼), and Leu^{159,166} → Val (■) mutants, and mock control (△). The concentration of IgM, which is produced in the culture medium of SKW6-CL4 cells, was determined by ELISA. Data represent averages of triplicate determinations.

Trp¹⁵⁸ and His¹⁶⁵ are in close spatial proximity to Met¹⁶² [15]. Trp¹⁵⁸ or His¹⁶⁵ is sequential to Leu¹⁵⁹ or Leu¹⁶⁶, respectively. Leu¹⁵⁹ and Leu¹⁶⁶ are highly conserved among mammalian IL-6 species [21]. Four mutants (Met¹⁶² → Ala, Leu¹⁵⁹ → Val, Leu¹⁶⁶ → Val, and Leu^{159,166} → Val) were established by site-directed mutagenesis of a human IL-6 coding sequence cloned in M13mp19. Native IL-6 and mutant IL-6 species were expressed in COS-1 cells and secreted into the culture medium. The amounts of IL-6 proteins detected varied between 2 and 10 ng/ml. We have tested the biological activity of IL-6 proteins in Ig-induction and receptor-binding assays.

Fig. 1 shows the effect of substitution of amino acid(s) on B-cell stimulatory activity. The substitution Met¹⁶² → Ala resulted in a decrease in the production of IgM from SKW6-CL4 cells. Single substitution Leu¹⁶⁶ → Val or Leu¹⁵⁹ → Val gave a slight or no significant decrease in the Ig-induction activity, respectively. However, double substitutions Leu^{159,166} → Val showed a significant decrease in the Ig-induction activity. The relative activity to native IL-6 estimated at half-maximal stimulation of Ig-induction activity (IgM = 50 ng/ml) was 11%, 58%, and 10% for Met¹⁶² → Ala, Leu¹⁶⁶ → Val, and Leu^{159,166} → Val mutants, respectively.

Fig. 2 also shows the effect of amino acid substitution(s) on receptor-binding activity. A significant decrease in receptor-binding activity was observed for the Met¹⁶² → Ala and Leu^{159,166} → Val mutants. Single substitution Leu¹⁶⁶ → Val slightly decreased the activity and Leu¹⁵⁹ → Val to a lesser extent. The decrease of the receptor-binding activity induced by amino acid

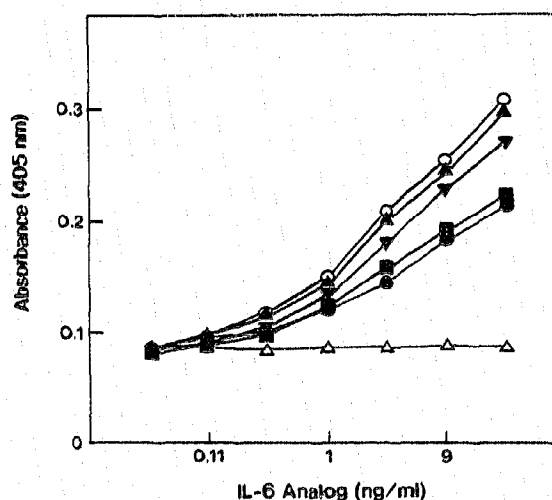


Fig. 2. Receptor-binding assay of native IL-6 (○), Met¹⁶² → Ala (●), Leu¹⁵⁹ → Val (▲), Val (▼), Leu¹⁶⁶ → Val (■), and Leu^{159,166} → Val (◆) mutants, and mock control (△). The quantity of native or mutant IL-6, which bound to soluble IL-6 receptor, was spectrophotometrically measured. Data represent averages of duplicate determinations.

substitution(s) was generally consistent with that of the Ig-induction activity. We therefore suggest that the decrease of biological activity is due to that of the receptor-binding affinity.

Chemical modification specific to Met residues led us to suggest that Met¹⁶² plays an important role in the receptor-binding activity of IL-6 [15]. This possibility has been confirmed by the present study showing the decrease in biological activity which was induced by Met¹⁶² → Ala substitution. It is likely that the hydrophobic side-chain of Met¹⁶² is required for the biological activity of IL-6. Moreover, the importance for receptor-binding of the hydrophobic side-chain of Leu¹⁵⁹ and Leu¹⁶⁶ has been shown by double substitutions Leu^{159,166} → Val. These results are consistent with the conclusion in the previous report that the region between Trp¹⁵⁸ and His¹⁶⁵ comprises the receptor-binding region. Since Met¹⁶² → Ala or Leu^{159,166} → Val mutant has not completely abolished the biological activity, we cannot exclude the possibility that another residue(s) is (are) also involved, along with Met¹⁶², Leu¹⁵⁹, and Leu¹⁶⁶, in the receptor-binding. The single substitution Leu¹⁵⁹ → Val or Leu¹⁶⁶ → Val resulted in only a small change in the activity. This is probably due to a conserved substitution of Leu residue with Val residue.

The previous circular dichroism and NMR studies [14,15,22] have shown that (i) IL-6 molecule is rich in α -helical structure, and (ii) the side-chains of Trp¹⁵⁸, Met¹⁵⁸, Met¹⁶² and His¹⁶⁵ are in spatial proximity. If the Leu¹⁵²-Leu¹⁶⁶ segment takes on an α -helix conformation, the side chains of three Leu residues, Leu¹⁵², Leu¹⁵⁹, and Leu¹⁶⁶, can be aligned in the tertiary structure. In the present studies, double substitutions Leu^{159,166} → Val resulted in a significant decrease of the IL-6 activity. This is reminiscent of a significant decrease in DNA-binding activity, which was previously reported for the double substitutions of Leu residues at 7-amino acid interval with Val residues that exist in the leucine zipper region of DNA-binding proteins [23-25]. An intriguing possibility is that this region in the IL-6 molecule takes a structure like the leucine zipper motif [26]. Further NMR analyses are being carried out in our laboratory for a better understanding of the structure in this region of the IL-6 molecule.

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