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EFFECT OF SEMI-RANDOM MUTAGENESIS AT THE C-TERMINAL 4 AMINO ACIDS OF HUMAN INTERLEUKIN-6 ON ITS BIOLOGICAL ACTIVITY

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SUMMARY: The carboxyl(C)-terminus of human interleukin-6 (hIL-6) has a critical role in the expression of the biological activity of this cytokine. To define the structure-function relationships of this region, semi-random mutagenesis of the C-terminal Leu181-Arg182-Qln183-Met184 sequence of hIL-6 was performed. The mutants were produced in Escherichia coli, renatured, and purified. Alterations of the C-terminal 4 amino acids caused a significant reduction of the proliferative effect of the mutants on MH60.BSF2 and KT-3 cells, and also led to a drastic decrease in receptor binding affinity. These results suggest the importance of a positively charged residue at position 182 or 183 and an alpha-helix at position 181 for the biological activity of hIL-6.

Interleukin-6 (IL-6) is a pleiotropic cytokine that is produced by a variety of cells and regulates immune responses, acute phase protein synthesis, neural differentiation, and hematopoiesis among its many functions (1). Human IL-6 (hIL-6) consists of 184 amino acids with a Pro residue at the N-terminus and Met at the C-terminus (2). In a structure-function analysis of hIL-6, Brakenhoff et al. (3) showed that the first 27 amino acids (using our numbering system*) could be removed without significantly affecting its activity. In addition, Krüttgen et

*The numbering system used here is based upon the processed "mature" hIL-6 molecule composed of 184 amino acids (2).

Abbreviations: rhIL, recombinant human interleukin; ORF, open reading frame; PCR, polymerase chain reaction; RP, reversed phase; WT, wild type.

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al. (4) showed that hIL-6 lacking the three C-terminal amino acids was completely inactive. Moreover, Brakenhoff et al. (5) suggested that both the amino- and carboxyl terminals are in close proximity and that they constitute an active site for the molecule. These reports all suggest the importance of the C-terminal portion of hIL-6 for the biological activity of this cytokine, but the details of the structures required to express this activity are not yet understood.

In this communication, we report on semi-random mutagenesis of the C-terminal 4 amino acid segment from Leu181 to Met184 of hIL-6 expressed in Escherichia coli (E.coli). The effect of mutation on the functional activity of hIL-6 was assessed to elucidate structural correlates with the amino acid residues of the C-terminal region.

MATERIALS AND METHODS

Construction of expression plasmids for hIL-6 mutants

Construction of mutant hIL-6 producers was initiated using the high-level expression plasmid for wild type (WT) hIL-6, pBSF2-SD7 (6). This plasmid was digested with PvuII to yield the linear DNA fragment containing hIL-6 with both flanking regions (Fig. 1). A pair of oligonucleotide primers (M4: 5'-GTTTTCCCAGTCACGAC-3' and RV: 5'-CAGGAAACAGCTATGAC-3') was obtained from Takara Shuzou Co., Ltd., and another pair of primers (SD7SacIF: 5'-GTCCAGCCTGAGAGCTCTGCGTC-3' and SD7SacIR: 5'-GACGCAGAGCTCTCAGGCTGGAC-3') was newly synthesized. PCR mutagenesis was used to create a SacI site at residues 179-180 in the hIL-6 coding region. The 5' portion of the hIL-6 gene was generated by PCR using the primer M4 and SD7SacIR with the PvuII fragment as a template, and the 3' portion was similarly produced using the primer RV and SD7SacIF. In the second PCR reaction, a fragment containing a unique SacI site was synthesized by using both fragments mentioned above and a pair of primers (M4 and RV). After the final amplified fragment was digested with ClaI and BamHI, it was cloned into the corresponding sites on pBSF2-SD7 to yield pBSF2-SD7SacI. The SacI recognition site was then used to incorporate the synthesized oligonucleotide fragments for mutagenesis. An oligonucleotide mixture containing C4MIX1F and C4MIX1R was synthesized (Fig. 1C) and was replaced with the natural ORF bounded by the SacI and KpnI sites on pBSF2-SD7SacI to construct a mutant library for the C-terminal 4 amino acids.

Expression

E.coli HB101 cells transformed with the plasmid expressing mutated hIL-6 were cultured in flasks at 37^{o}C in modified M9 casamino acids medium supplemented with ampicillin, as described previously (6). The expression of hIL-6 mutants was induced with IAA, and then culture was continued for a further 16 hrs.

Protein purification

The cells producing hIL-6 mutants were disrupted by lysozyme treatment followed by sonication, and then the inclusion body

fractions containing hIL-6 mutants were collected by a low-speed centrifugation. The denatured mutant hIL-6 proteins were solubilized and refolded as described previously (6), and the fractions were desalted on a Sephadex G-25 column (Pharmacia). Refolded mutants were then further purified by RP-HPLC (Vydac C4-column).

MH60.BSF2 cell proliferation assay

An IL-6-dependent cell proliferation assay was carried out using the murine hybridoma clone, MH60.BSF2 (7). Various mutant hIL-6 proteins were serially diluted 1:3 in RPMI 1640 medium containing 10% FCS and were mixed with MH60.BSF2 cells ($5X10^4$ cells). Cultures were incubated at 37°C in 5% CO_2 for 72 h. Then an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) colorimetric assay was done (8). The activity of each mutant protein was expressed by comparison with that of standard intact hIL-6 at 1 unit/ml, a concentration which induces half-maximal stimulation of cell proliferation in this assay system.

KT-3 cell proliferation assay

A human T lymphoma cell line, KT-3 (9), was used. Cells ($1X10^4$) were suspended in RPMI 1640 medium with 10% FCS and cultured with hIL-6 mutants for 48 h in a 5% CO_2 incubator at 37°C. The MTT assay was then carried out in the same manner as described for MH60.BSF2 cells.

Receptor competition assay

Human U266 myeloma cells ($5X10^5$) were suspended in RPMI 1640 medium containing 0.5% BSA and 0.02% NaN_3, and were treated with test samples (100 µl) of hIL-6 at 4°C for 30 min. Then [^{125}I]rhIL-6 (0.2 ng = 20,000 c.p.m./50 µl/well) was added and incubation was continued at 4°C for a further 1.5 h. The cell suspension was then layered onto a cushion mixture of olive oil and di-n-butyl phthalate (1:4, v:v) in a polyethylene tube and centrifuged. The cell pellet thus obtained was tested for cell-bound radioactivity using a gamma counter (Packard, Multiprias-4)(10).

RESULTS AND DISCUSSION

Semi-random mutagenesis of the coding region for the C-terminal 4 amino acids of hIL-6

To define the structure-function relationships for the C-terminus of the hIL-6 molecule, amino acid substitutions at the Leu181-Met184 region were introduced by cassette mutagenesis using an oligonucleotide mixture (Fig. 1(C)). The divergence of amino acid substitution was intended to produce semi-random mutations including the following: 1) Pro, a breaker of the alpha-helix structure (11), was substituted at positions 181 and/or 183. 2) Positively charged amino acids were incorporated at every one of the 4 positions (181-184) to elucidate their importance. Accordingly, the oligonucleotides shown in Fig. 1 (C) were designed and expression plasmids encoding various hIL-6

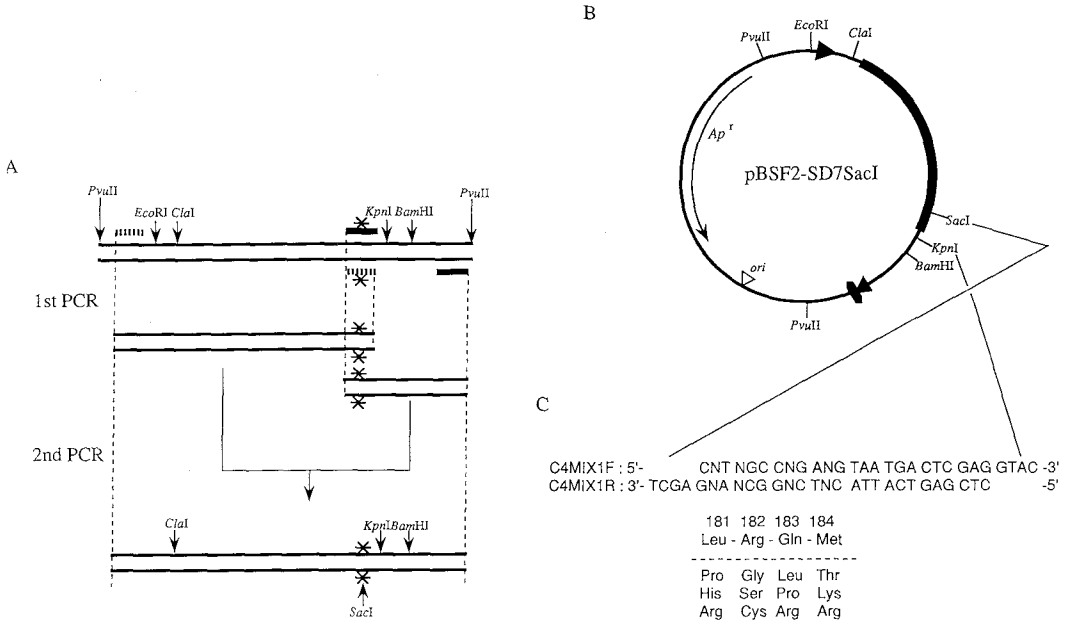
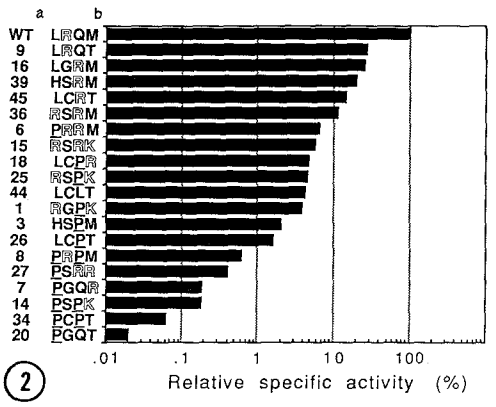


Figure 1. A) Diagram of the creation of a unique SacI restriction site within the hIL-6 coding region by the PCR. The PvuII-PvuII DNA fragment (double parallel lines) is derived from pBSF2-SD7. The bold dashed lines represent the M4 and SD7SacIR primers and the bold lines indicate the SD7SacIF and RV primers. Asterisks indicate the position of the SacI site. B) Structure of pBSF2-SD7SacI. It was obtained by inserting a ClaI-BamHI segment containing the SacI site into the corresponding site of the pBSF2-SD7 plasmid. C) DNA structure of the oligonucleotide mixture (C4MIX1F and C4MIX1R) used for cassette mutagenesis. N represents the unspecified bases. The amino acid substitutions are indicated beneath the WT amino acid sequence of the C-terminus (positions 181-184).

mutants were constructed (Fig. 1). From the transformant, 19 individual clones defined by DNA sequencing analysis were obtained. The amino acid substitutions of the mutant hIL-6 proteins and their reference numbers are summarized in Fig. 2.

Biological activities of the purified hIL-6 mutants

Each hIL-6 mutant was refolded with disulfide bond formation (6) and was purified to greater than 95% as determined by RP-HPLC analysis. Figure 2 shows the response of MH60.BSF2 cells to stimulation by each of the mutants in comparison with the effect of WT hIL-6, which had a specific activity of 5.8 X 10^6 units/mg in the assay system used. The mutants showed a moderate (Class A; 10% - 30% of the activity of WT hIL-6), considerable (Class



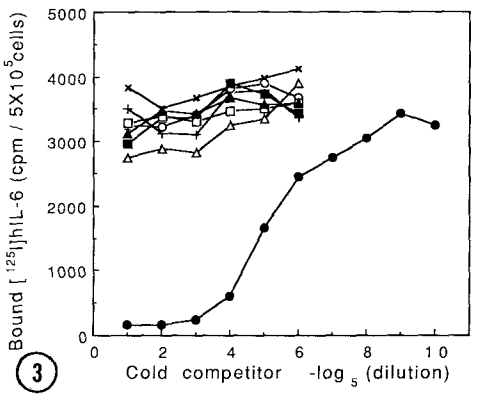


Figure 2. The specific biological activity of each mutant was determined from its effect on the proliferation of MH60.BSF2 cells. Bars indicate the biological activity of each mutant relative to that of WT hIL-6. Data represent means of triplicate determinations. [a] Shows mutant number, except in the case of intact hIL-6 (WT). [b] Indicates the C-terminal 4 amino acids. Proline (P) residues are underlined, and the positively charged residues (arginine (R) and lysine (K)) are shown as white letter characters.

Figure 3. Competitive inhibition of the binding of [^{125}I]hIL-6 to U266 cells by WT hIL-6 or various mutants (●, WT hIL-6; x, #3; ○, #6; ■, #8; □, #14; ▲, #25; △, #39; +, #45). The initial concentration of the cold competitor was 2 μM.

B; 1% - 10%), or severe (Class C; < 1%) reduction of proliferative activity and no mutants expressing a more potent biological activity than that of WT hIL-6 were obtained. It was thus reconfirmed that the C-terminal region of hIL-6 is essential to the activity of this cytokine, as was previously suggested by the results of C-terminal deletion analysis (4).

At position 181, the mutation study revealed a preference for Leu followed by His in the expression of biological activity (Class A), and demonstrated that mutants in which Leu181 was replaced by Pro (Class C, and mutant #6) had a marked decrease in activity. At the C-terminus region of hIL-6 (positions 154 to 184), an alpha-helical conformation has been predicted from the primary sequence (13). Leu or His favors an alpha-helix, and Arg has no effect on the secondary structure. However, Pro substitution imposes constraints on the peptide bond that are unfavorable for alpha-helix formation (11). Thus, our findings strongly suggested that a C-terminal alpha-helix conformation at

Leu181 of hIL-6 is critical for the biological activity of this cytokine.

With regard to positive charge, its importance within the C-terminal segment was indicated by comparison of WT(LRQM) and mutants retaining Leu181, i.e., #9(LRQT), #16(LGRM), #45(LCRT), #18(LCPR), #44(LCLT), and #26(LCPT). The hIL-6 mutants carrying charged residues at positions 182 or 183 exhibited reasonable bioactivity (15% - 30%), while those bearing uncharged and/or positively charged residues at 184 had a reduced level of activity (1.5% - 5%). The class C mutants (#8, #27, #7, #14, #34 and #20) and mutant #6 showed a similar tendency concerning the effect of a positive charge on activity. Thus, these results suggest that a positively charged amino acid in the C-terminal region (182/183) plays an important role in the expression of bioactivity. This conclusion is consistent with the recent findings of Lütticken et al. (12), who indicated the importance of Arg182 by a point mutagenesis study, although they used a cell-free synthetic hIL-6 bearing a signal peptide extention of 28 amino acids.

Seven of the mutants (#3, #6, #8, #14, #25, #39, and #45) were examined further to assess their proliferative effect on a human cell line (KT-3). The activity expressed by #39, #45, and the other mutants was respectively 5%, 4%, and <1% of the activity of WT hIL-6. Thus, all the mutants tested had a weaker proliferative effect on KT-3 cells as compared with NH60-BSF2 cells, but the relative order of their activities remained unchanged.

Competitive receptor binding activities of purified hIL-6 mutants

To investigate the correlation between the biological activity and the receptor binding affinity of the mutants, we examined their ability to compete with the specific binding of [^{125}I]-labeled rhIL-6 to U266 cells. The 50% inhibitory concentration (IC_{50}) for receptor binding by WT hIL-6 was approximately 30 ng/ml, and the mutants did not compete significantly or at all under our experimental conditions (Fig.

3), even though mutants #39 and #45 respectively exhibited 5% and 4% of the activity of WT hIL-6. This result indicates that slight alterations at the C-terminal region induced a drastic reduction in the affinity for the receptors expressed by U266 cells, and suggests that this region might participate in interactions with the receptor.

Recently, Lütticken et al. (12) reported the importance of the alpha-helix structure at Ser176 and of a positive charge at Arg182 in the bioactivity of hIL-6. In this study, we focused on the C-terminal 4 amino acids, and further defined the potentially critical structural elements by semi-random mutagenesis, a bioactivity assay, and a receptor binding assay. As a result, the importance of Leu181 and a positive charge at position 182 (and even 183) in the activity of hIL-6 was shown. These findings suggest that Leu181 might also be a critical residue for setting the positively charged C-terminal residue in the proper position. It was also shown that the C-terminal 4 amino acids of hIL-6 play an essential role in receptor binding.

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