Volume 282, number 2, 265–267 FEBS 09665 May 1991

ADONIS 001457939100373H

Evidence for the importance of a positive charge and an α-helical structure of the C-terminus for biological activity of human IL-6

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Received 25 January 1991

Thirteen point mutations of human interleukin-6 at the C-terminus were constructed at the cDNA-level. Two degenerate oligonucleotide primers were used for PCR synthesis of two groups of point mutations at positions 182 and 184. The mutated cDNAs were in vitro transcribed and translated and subsequently assayed for biological activity in the B9 cell proliferation test. Our results confirm our former findings obtained with deletion mutants on the importance of the C-terminus of IL-6 for biological activity. In addition, we now present evidence for the importance of an α-helix at the C-terminus of IL-6 and the presence of the positive charge at position 182 for biological activity.

Interleukin-6; Carboxy-terminus; Biological activity; Point mutant; α-Helix; PCR

1. INTRODUCTION

IL-6 is a multifunctional cytokine involved in the immune defense, hematopoiesis and acute-phase protein regulation and exerts its action via specific surface receptors on the various target cells [1–3]. In order to understand the interaction of IL-6 with its receptor, structure-function studies have been carried out. Brakenhoff et al. [4] first showed that the deletion of 28 amino acids from the N-terminus of IL-6 did not influence the biological activity. Recent results from our laboratory have shown that the three carboxy-terminal amino acids of human IL-6 are essential for its bioactivity [5,6]. From the binding pattern of several monoclonal antibodies Brakenhoff et al. concluded the existence of an epitope composed of closely spaced residues from both the amino- and carboxy-terminals of IL-6 [7].

After the C-terminus of IL-6 had been shown to be important for biological function by deletion studies, we now describe experiments, where point mutations have been introduced into the full-length IL-6-molecule at the positions 182 and 184 crucial for biological activity [5,6].

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Abbreviations: IL-6, Interleukin-6; PCR, polymerase chain reaction

2. MATERIALS AND METHODS

2.1. *Chemicals*

L-[^{35}S]methionine (> 37 TBq/mmol), 6-[^{3}H]thymidine (74 GBq/mmol) and [α-^{35}S]dATP (37 TBq/mmol) were purchased from Amersham International (Amersham, UK). All enzymes were obtained from Boehringer (Mannheim, Germany). For DNA sequencing the T7 DNA polymerase kit of Pharmacia/LKB (Freiburg, Germany) was used. Human IL-6-cDNA and recombinant human IL-6 [8,9] were a generous gift of Drs. T. Hirano and T. Kishimoto (Osaka, Japan); recombinant human IL-6 had a specific activity of 6×10^{6} U/mg protein (B9 cell proliferation assay).

2.2. *Construction of IL-6 mutants*

IL-6 mutants were constructed using PCR techniques [10]. IL-6-cDNA in pGEM4 [8] was amplified by PCR using one primer at the 5'-end (5'-TTG TAC ATA TTG TCG TT-3') and two different primers at the 3'-end (5'-GGATCCTAAGCTTCTACATTTG-C$\underline{XY}_1$AAGAGCCCTCAGGCTGG-3';5'-CCTAAGCTTCTA$\underline{XY}_2$-TTTGCCGAAGAGCCCTCAG-3'). As shown in Fig. 1 $\underline{XY}_1$ and $\underline{XY}_2$ represent degenerate positions of the primers. The obtained IL-6-cDNAs lacked the 3'-non-coding region and contained the translational stop codon TAG. PCR was carried out using 10 ng of plasmid DNA and 200 ng of each primer (annealing, 42°C, 120 s; synthesis, 72°C, 180 s; denaturation, 94°C, 90 s; 35 cycles). PCR products were digested with *Eco*R1 and *Hind*III and subsequently cloned into the transcription vector pGEM3. All constructs were verified by DNA sequencing. In two cases multiple point mutations were obtained inadvertently. In these two cases restriction fragments containing one point mutation were cloned into the wild type IL-6-cDNA. The exchange of Trp-157 to Arg was performed using the gapped duplex DNA technique [11] with the site directed mutagenesis kit from Boehringer (Mannheim, Germany). The linearized plasmids were transcribed with T7 RNA polymerase and subsequently translated in a cell-free system (rabbit reticulocyte lysate) in the presence of [^{35}S]methionine [12]. After SDS-PAGE of the cell-free synthesized polypeptides, bands corresponding to wild-type and mutated IL-6 were excised from the polyacrylamide gel and their radioactivity was

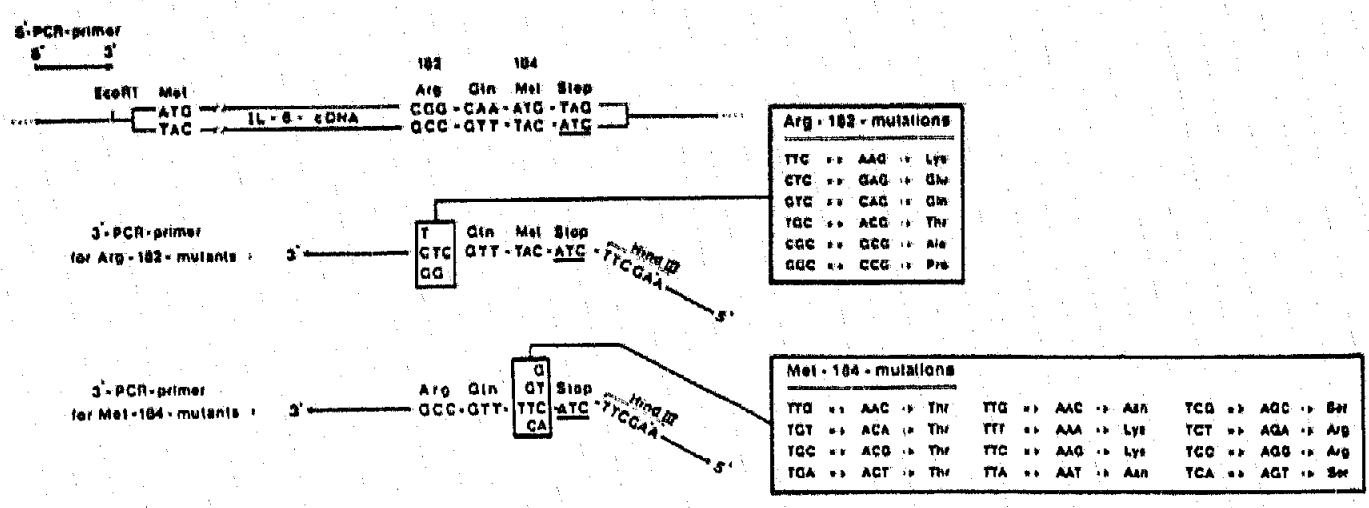

Fig. 1. Schematic representation of PCR primers used for the construction of point mutations in human IL-6-cDNA. The possible amino acid exchanges are boxed.

determined. The values were normalized taking into account the number of methionine residues present in the respective mutants.

2.3. *B9 cell proliferation assay*

The IL-6 assay was carried out with the IL-6-dependent murine plasmacytoma cell line B9, kindly provided by L. Aarden (Amsterdam, The Netherlands) [13]. One μl of the translation mixture and serial dilutions (1:2) were used in the B9 cell proliferation assay.

3. RESULTS AND DISCUSSION

To define the precise structural requirements for the biological function of IL-6, it was desirable to have a series of point mutations introduced into the C-terminal part of human IL-6. In previous studies [5] we have used the classical 'gapped duplex' method for site-directed mutagenesis of IL-6-cDNA [11] to exchange tryptophan-157 by arginine. This method is not very suitable for the preparation of numerous point mutations, because it is time-consuming and requires the synthesis of a new oligonucleotide for each construct.

Therefore, we designed a strategy involving the use of a degenerate oligonucleotide combined with PCR-technique for the construction of a series of point-mutants. We have shown in previous studies [5,6] that methionine-184 and arginine-182 are of particular importance for the biological activity of IL-6. Fig. 1 shows two PCR-primers for the synthesis of 11 possible IL-6 point mutants. PCR-products were ligated into the transcription-vector pGEM3 and analyzed by DNA-sequencing. We obtained 10 of the potential 11 point mutations (Fig. 2). In addition, we detected two unexpected double mutations at positions 145/182 and 176/184. The occurrence of unexpected mutations might be connected to the use of the degenerate oligonucleotides in the PCR, since no such mutations were observed, when perfect-matching oligonucleotides were used as primers.

The constructed IL-6-cDNA mutants were cell-free transcribed and translated into the corresponding polypeptides. The biological activity of the synthesized IL-6 mutants was measured in the B9 cell proliferation assay. The values obtained were normalized as detailed in section 2.2. It is of no importance that the cell-free synthesized IL-6-polypeptides carry a signal peptide of 28 amino acids, since IL-6 obtained after cell-free transcription/translation shows the same biological activity regardless of the presence of a signal peptide (data not shown).

Fig. 3 shows a comparison of the biological activities of all single point mutations. It is evident that the mutations beyond amino acid 182 do not affect biological activity of IL-6 (left) with the exception of the mutant, where serine-176 is exchanged by proline. The low biological activity of this mutant (13% of the control) may be explained by the helix-breaking property of the amino acid proline. An α-helical structure at the

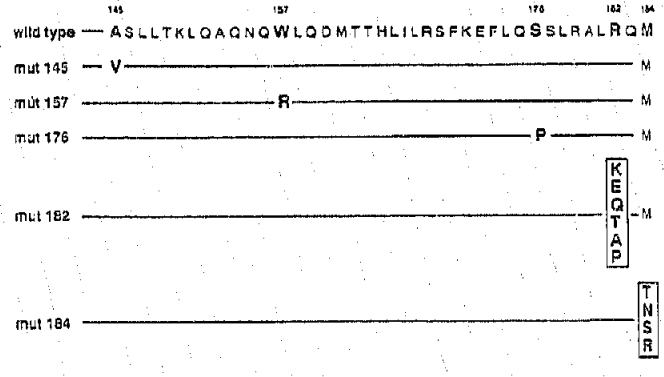

Fig. 2. C-terminal portion of human IL-6 and point mutations introduced. Bold letters in the wild-type IL-6-sequences indicate positions, where point mutations have been introduced.

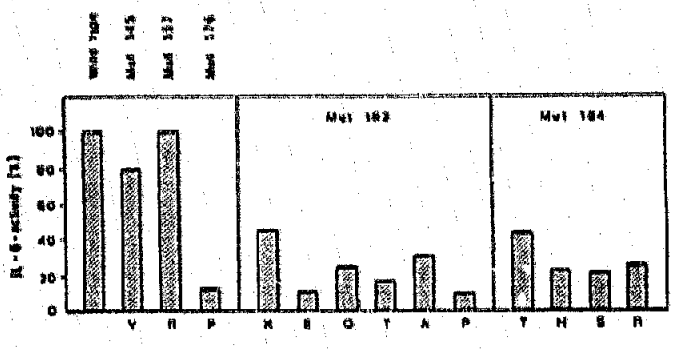

Fig. 3. Comparison of the biological activities of IL-6 mutants depicted in Fig. 2 as measured in the B9 cell proliferation assay. Values normalized for different amounts of IL-6 synthesized, were compared using the dilution leading to half-maximal stimulation in the respective test. The biological activity exerted by wild type IL-6 was set to 100%. The bars represent the average of 3 determinations.

carboxy-terminus of IL-6 has been predicted on the base of its primary structure [5].

All IL-6-species with altered amino acids at positions 182 and 184 exhibit biological activities of <45% of controls (middle and right parts of Fig. 3). Similar to the mutant 176 the helix-breaking amino acid proline at position 182 severely impairs the biological activity of IL-6. Thus, it can be concluded that an α-helical structure at the C-terminus of IL-6 is essential for its biological activity.

The exchange of the positively charged amino acid arginine by glutamic acid, which bears a negative charge, also abrogates biological activity. The positive charge at position 182 is likely to be essential for a functional IL-6-molecule, since the replacement of arginine-182 by the positively charged lysine results in a rather active IL-6-polypeptide (45% of controls). In spite of the fact that arginine and lysine carry a positive charge, their side chains differ. This may explain why an IL-6-polypeptide carrying a lysine at position 182 does not reach full biological activity. Interestingly, the arginine residue is conserved in human, mouse and rat IL-6.

The exchange of the C-terminal methionine by asparagine, serine or arginine results in IL-6-molecules with biological activities around 20% of controls. However, when threonine, which is the C-terminal amino acid of murine and rat IL-6, is introduced at this position, a mutant IL-6 of higher biological acitivity is created.

It is an intriguing question, whether the IL-6 mutants, which are biologically inactive, still bind to the IL-6-receptor. Preliminary data (not shown) using cross-linking techniques suggest that an IL-6 molecule lacking the four C-terminal amino acids does not bind to its receptor. Accordingly, this mutant does not show biological activity.

Using molecular modelling Bazan [14] recently proposed a tertiary structure common for growth hormone, prolactine, granulocyte colony stimulating factor (G-CSF), erythropoeitin and IL-6. This structure is characterized by 4 helices forming a bundle. The C-terminal α-helix is believed to establish receptor-ligand recognition [14] by exposure of hydrophilic amino acids. In the case of IL-6, serine-176 and arginine-182 are proposed to be localized at the surface. From our findings the involvement of an α-helical structure at the C-terminus of IL-6 as well as the presence of a positive charge (arginine-182) are essential for biological function. We suggest that the C-terminal α-helix of IL-6 together with the correctly positioned positive charge fits to a rigid groove of the IL-6 receptor.

Acknowledgements: We are grateful to Dr. P. Freyer for the synthesis of oligonucleotides. The authors like to thank B. Wroblowski for helpful discussion, M. Robberitz for his assistance in the preparation of the figures, and B. Minnich for her help with the preparation of the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft, Bonn and the Fonds der Chemischen Industrie, Frankfurt.

REFERENCES

[1] Kishimoto, T. (1989) Blood 74, 1-10.
[2] Heinrich, P.C., Castell, J.V. and Andus, T. (1990) Biochem. J. 265, 621-636.
[3] van Snick, J. (1990) Annu. Rev. Immunol. 8, 253-278.
[4] Brakenhoff, J.P.J., Hart, M. and Aarden, L.A. (1989) J. Immunol. 143, 1175-1182.
[5] Krüttgen, A., Rose-John, S., Möller, C., Wroblowski, B., Wollmer, A., Müllberg, J., Hirano, T., Kishimoto, T. and Heinrich, P.C. (1990) FEBS Lett. 262, 323-326.
[6] Krüttgen, A., Rose-John, S., Dufhues, G., Bender, S., Lütticken, C., Freyer, P. and Heinrich, P.C. (1990) FEBS Lett. 273, 95-98.
[7] Brakenhoff, J.P.J., Hart, M., de Groot, E.R., Di Padova, F. and Aarden, L.A. (1990) J. Immunol. 145, 561-568.
[8] Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S.-I., Nakajiama, K., Koyama, K., Iwamatsu, A., Stunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T. and Kishimoto, T. (1986) Nature 324, 73-76.
[9] Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimizu, K., Nakajiama, K., Pyun, K.H. and Kishimoto, T. (1985) Proc. Natl. Acad. Sci. USA 82, 5490-5494.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.
[11] Kramer, W., Drutsa, V., Jansen, H.-W., Kramer, B., Pflugfelder, M. and Fritz, H.-J. (1984) Nucleic Acids Res. 12, 9441-9456.
[12] Geiger, T., Andus, T., Bauer, J., Northoff, H., Ganter, U., Hirano, T., Kishimoto, T. and Heinrich, P.C. (1988) Eur. J. Biochem. 175, 181-186.
[13] Aarden, L.A., de Groot, E.R., Schaap, O.L. and Lansdorp, P.M. (1987) Eur. J. Immunol. 17, 1411-1416.
[14] Bazan, J.F. (1990) Immunol. Today 11, 350-354.