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Structure-function analysis of human interleukin-6

Evidence for the involvement of the carboxy-terminus in function

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C-terminally deleted analogs of human interleukin-6 (IL-6) have been constructed at the cDNA level, and after cell-free transcription and translation their biological activity was analyzed. Removal of only 4 amino acids resulted in complete loss of biological activity as determined by the B9 cell proliferation assay. Secondary structure prediction of human IL-6 resulted in 58% helix, 14% β-structure, and 28% turn and coil (average of 3 independent methods). The circular dichroism of recombinant human IL-6 was measured in the near and far UV. Evaluation of the latter in terms of secondary structures gave 67% helix, 15% β-structure, and 18% turn and coil.

Interleukin-6; Deletion mutant; Carboxy-terminus; Biological activity; Secondary structure prediction; Circular dichroism

1. INTRODUCTION

Interleukins are a group of signalling molecules involved in the communication between cells. They are produced and secreted by many different cell types, particularly by those of the immune system.

Interleukin-6 (IL-6) elicits a wide spectrum of biological functions. In general, IL-6 represents a growth and differentiation factor acting on B-cells, T-cells, lymphoma cells, hybridoma/plasmacytoma cells, hematopoietic stem cells, and hepatocytes (for reviews see [1,2]).

IL-6 consists of 184 amino acids [3]. It is synthesized as a larger polypeptide precursor with a signal peptide of 28 amino acids. The native molecule is N- and O-glycosylated and may also be phosphorylated [4-6].

It is believed that IL-6 exerts its action via a specific receptor on the surface of the various target cells [7]. The IL-6 receptor has recently been cloned and sequenced [8]. Thus far, nothing is known about the underlying mechanisms for the signal transduction following the binding of IL-6 to its specific plasma membrane receptor. In order to understand the interaction of IL-6 with its receptor, it is important to obtain some information on structural features essential for its biological function. As a first approach we have constructed IL-6 mutants lacking various portions of the carboxy-terminus and evaluated their biological activity. In addition information on the secondary structure of human IL-6 is presented.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

L-[^{35}S]methionine (>37 TBq/mmol) and 6-[^{3}H]thymidine (74 GBq/mmol) were purchased from Amersham International (Amersham, UK). Except for exonuclease III (*E. coli*), which was from AGS (Heidelberg, FRG), enzymes were obtained from Boehringer (Mannheim, FRG).

2.2. *Construction of IL-6 deletion mutants*

pGEM 3 containing the complete coding region of IL-6 was stepwise truncated at the 3'-end using the method described in [9]. After sequencing, deletions were selected, which after in vitro transcription and translation resulted in polypeptides lacking 4, 15, 26, 36 and 48 amino acids at the C-terminus. 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 [10]. After SDS-PAGE the cell-free synthesized polypeptides were excised from the polyacrylamide gel and their radioactivity was determined.

2.3. *IL-6 assay*

The IL-6 assay was performed using the IL-6-dependent murine plasmacytoma cell line B9 kindly provided by L. Aarden (Amsterdam, The Netherlands) [11].

2.4. *Circular dichroism (CD) measurements*

CD measurements were carried out on an AVIV CD spectrometer 62DS prototype. The instrument was calibrated with a 0.1% aqueous solution of d-10-camphorsulphonic acid ($\theta^{1\,g/l;\ 1\,cm}_{290.5\,nm} = 0.308°$). The ratio $|\theta_{192.5\,nm}/|\theta_{290.5\,nm}|$ was 1.90. The temperature was 10°C, the spectral bandwidth 1.5 nm. The protein concentration was based on amino acid analysis. The absorption coefficient calculated with this

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concentration was $A_{278\ nm}^{1\ g/l,\ 1\ cm} = 0.652$. The CD spectrum in the far UV was analyzed with respect to the main chain conformation of IL-6 with the CONTIN program [12].

Secondary structure predictions according to Garnier, Osguthorpe and Robson (GOR) [13] and Chou and Fasman [14] were performed with the computer program package GENMON (version 4.1, 1989; kindly provided by GBF, Braunschweig, FRG). The predictive rules of Chou and Fasman [14] were used in order to decide alternatives in the output of the program. The GGBSM program of Gasquel and Golmard [15] was used independently.

3. RESULTS AND DISCUSSION

The cDNA containing the complete coding region of the human IL-6 was truncated at the 3'-end. After cell-free transcription and translation, 5 mutant polypeptides lacking 4, 15, 26, 36 and 48 amino acids at the C-terminus were obtained. Fig.1 shows the increased electrophoretic mobilities of the [^{35}S]-methionine-labelled IL-6 mutants in comparison to the full-length IL-6 (co), also synthesized by in vitro transcription and translation.

The various IL-6 forms were tested for their biological activity in the B9 cell assay. Since higher concentrations of the reticulocyte lysate interfered with the IL-6 assay, equal and subtoxic volumes of the translation mixture were used. The measured IL-6 activities were compared by taking into account the radioactivity incorporated and the number of methionine residues present in the mutant polypeptides (see insert of fig.2).

It is clearly seen in fig.2 that the removal of only 4 amino acids is sufficient to abolish essentially all biological activity. As expected, the IL-6 mutants Δ15, Δ26, Δ36 and Δ48 did not exert any biological activity either under our assay conditions.

In order to obtain a first idea of the molecule's structural make-up, 3 different methods were used to predict secondary structure from the amino acid sequence [3]. The average fractions obtained are 58% helix, 14% β-structure and 28% turn and coil. As can be seen in fig.3, there are 8 regions where a helical structure is predicted conformably by all 3 methods: positions 37–41, 53–62, 67–68, 92–101, 111–120, 127–133, 156–160, 174–181. It should be noted that all 3 methods predict an α-helix at the C-terminus of human IL-6.

In order to determine the α-helix content of rhIL-6, CD measurements were performed. The CD spectrum of rhIL-6 in the far UV (fig.4A) is characteristic of that of a protein with a high helix content. The analysis with the CONTIN program [12] results in 67% α-helix, 15% β-structure and 18% turn and coil. Fig.4b shows the CD spectrum in the near UV. The main band is due to tyrosine and the long region tailing off towards longer wavelengths seems to represent a background from the disulphide chromophore. The latter may also be responsible for the hesitantly positive ellipticity at 253 nm [17].

Agreement of experiment and prediction with respect to the percentage of helix is best for the GOR method

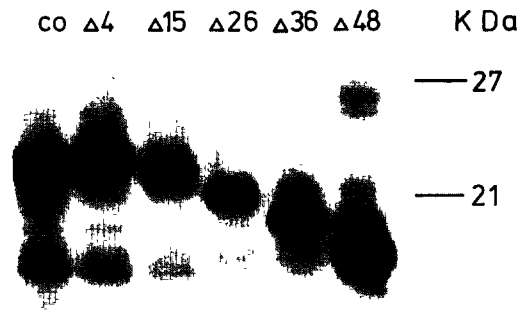

Fig.1. SDS-PAGE of cell-free synthesized IL-6 mutants truncated at the carboxy-terminus. Full length IL-6 and truncated forms of it obtained after in vitro transcription and translation were separated by SDS-polyacrylamide gel (12.5%) electrophoresis and visualized by fluorography [16]. 'co' represents the full length IL-6 control; Δ4, Δ15, Δ26, Δ36, Δ48 denote the IL-6 polypeptides lacking 4, 15, 26, 36, 48 amino acids from the carboxy-terminus, respectively.

(67% vs 69%). On the other hand, helices of some 40 residues in length as predicted by this method (see fig.3) are unusual. Their continuity is also questionable in view of the two other predictions and of the incompatibility with the 44–50 cystine bridge.

The high helix content of human IL-6 is in contrast to the structures of IL-1β [18] and TNFα [19]. IL-1β is composed of 12 β-strands forming a tetrahedron core structure, where all 6 edges consist of 2 antiparallel β-strands [18]. Tumor necrosis factor α (TNFα) is a trimeric molecule, each subunit of which consists of an antiparallel β-sandwich [19]. From our experiments presented in fig.2, it is clear that the 4 carboxy-terminal amino acids of the human IL-6 are essential for the biological activity of this cytokine. Comparison of these 4 amino acids with the amino acid sequences at the

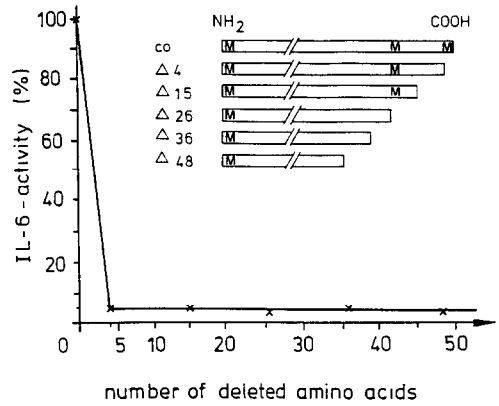

Fig.2. Biological activity of IL-6 mutant polypeptides. One μl of each translation mixture was used for IL-6 activity determinations. The obtained values were normalized on the basis of the incorporated radioactivity and the number of methionine residues present in the mutant polypeptides as shown in the schematic representation in the insert. For the full-length IL-6, maximal incorporation of [^{3}H]thymidine into DNA was 8000 cpm = 100% per well containing 5×10^3 cells.

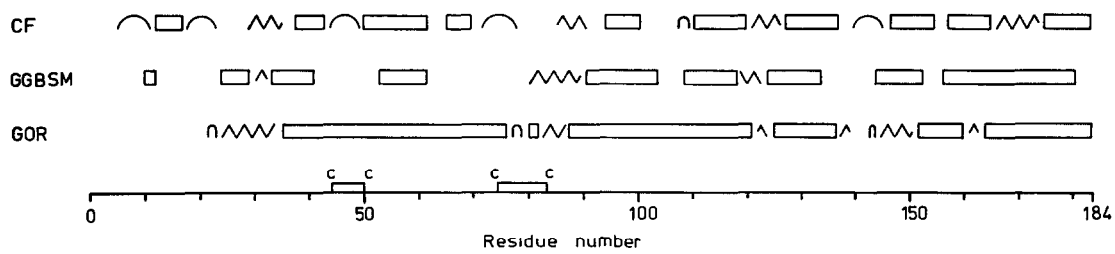

Fig.3. Secondary structure of human IL-6 as predicted by the methods of Chou and Fasmann (CF), Gasquel and Golmard (GGBSM), and Garnier, Osguthorpe and Robson (GOR). Helical segments are indicated by ▭, β-sheet by ⩘ and turn by ∩. The positions of the cysteine residues are indicated (c).

C-termini of mouse [20] and rat [21] IL-6 shows that 2 residues are completely conserved: human = Leu-Arg-Gln-Met, and mouse = rat = Thr-Arg-Gln-Thr. It is conceivable that these 4 amino acid residues either form the binding site or part of it, or are important for the maintenance of structural requirements for receptor-ligand interaction. In particular, the positive charge of arginine could be essential. Our observations are in accordance with recent results of Ida et al. [22]. These authors described a neutralizing monoclonal antibody, which binds to the carboxy-terminal part of human IL-6 (Ala 153–Thr 162). Interestingly the exchange of Trp 157 to Arg by site-directed mutagenesis had no effect on the biological activity (Möller et al., unpublished results). Brackenhoff et al. [23] described the first 28 amino acids of human IL-6 as not being essential for biological activity. A detailed understanding of the structure-function relationship of IL-6 has to await the elucidation of the 3-dimensional structure by X-ray analysis.

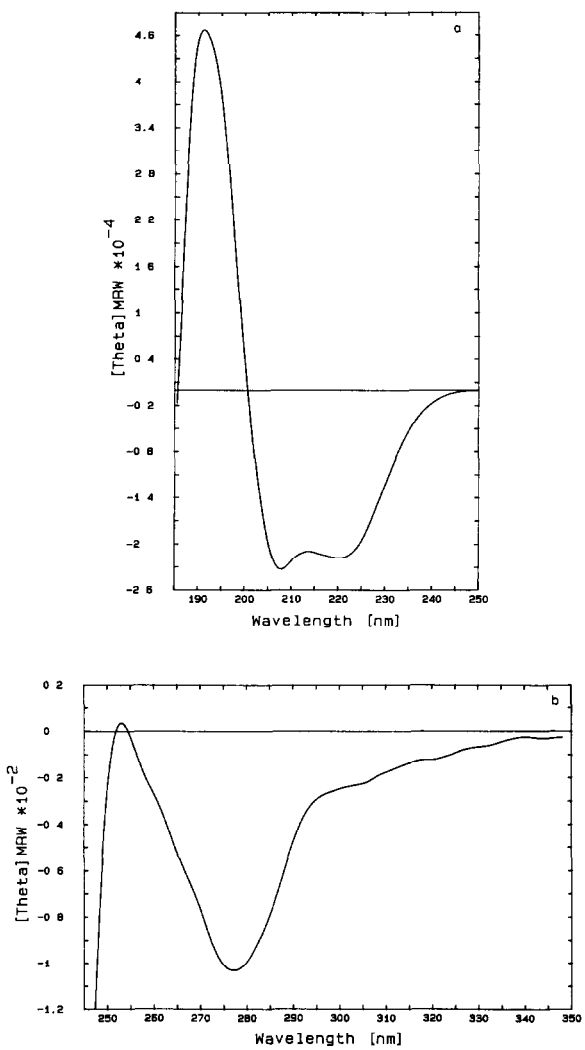

Fig.4. CD spectra or recombinant human IL-6 in the far (a) and near (b) UV. Concentration: 0.34 g/l; solvent: 10 mM phosphate buffer, pH 7; pathlengths: 0.02, 0.1 and 2 cm.

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