The three carboxy-terminal amino acids of human interleukin-6 are essential for its biological activity

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Received 3 September 1990

We have constructed on the cDNA level deletion mutants of human interleukin-6 lacking one, two, three or four amino acids from the carboxy-terminus of the molecule. After in vitro transcription and translation the biological activity of these deletion mutants was determined by two independent bioassays. Both, the mouse B9 cell proliferation assay and the fibrinogen induction assay with the human hepatoma cell line HepG2 led to the following result: already the removal of the last amino acid resulted in a five-fold loss of biological activity. An additional slight reduction was seen when two amino acids were removed from the carboxy-terminus. Interleukin-6 lacking three or four C-terminal amino acids were completely inactive. The presented results emphasize the extreme importance of the carboxy-terminus of interleukin-6 for its biological function.

Interleukin-6; Deletion mutant; PCR; Carboxy-terminus; Biological activity; Acute phase protein induction

1. INTRODUCTION

Interleukin-6 (IL-6) is produced and secreted by many different cell types, particularly by those of the immune system. It elicits a wide spectrum of biological functions. In general, IL-6 acts as a growth and differentiation factor on B-cells, T-cells, lymphoma cells, hybridoma/plasmacytoma cells, hematopoietic stem cells, and hepatocytes (for reviews see [1,2]). More recently the involvement of IL-6 in the oncogenesis of plasma cell neoplasias has been discussed [3].

IL-6 consists of 184 amino acids [4]. It is synthesized as a larger polypeptide precursor with a signal peptide of 28 amino acids. IL-6 exerts its action via a specific receptor on the surface of various target cells [5]. A human IL-6-receptor-cDNA has recently been cloned from natural killer cells [6]. 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. Recently we have constructed a whole set of C-terminal truncations of the IL-6 protein and could show that the removal of only four amino acids is sufficient to completely abrogate its biological activity [7].

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Abbreviations: IL-6, interleukin-6; PCR, polymerase chain reaction; rh, recombinant human

In the present study we present evidence that residues 182 and 184 are of particular importance for the biological function of IL-6.

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, FRG). Anti-human fibrinogen was obtained from Dako (Hamburg, FRG). Human IL-6-cDNA and rhIL-6 [8] were a generous gift of Drs T. Hirano and T. Kishimoto (Osaka, Japan); rhIL-6 had a specific activity of 6×10^8 units/mg protein (B9 cell proliferation assay).

2.2. *Construction of IL-6 deletion mutants*

IL-6 deletion mutants were constructed using PCR techniques [9]. IL-6-cDNA in pGEM4 [4] was amplified by PCR using one primer at the 5'-end (5'TTG TAC ATA TTG TCG TT 3') and 3 different primers at the 3'-end (5'TCT AGA ATT CTA TTG CCG AAG AGC CCT CAG 3'; 5'TCT AGA ATT CTA CCG AAG AGC CCT CAG GCT 3'; 5'TCT AGA ATT CTA AAG AGC CCT CAG GCT GGA 3'). The obtained IL-6-cDNAs lacked the 3'-non-coding region and contained the translational stop codon TAG after leucine, arginine and glutamine, at positions 181, 182 and 183, respectively. 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 cloned into the transcription vector pGEM3. All constructs were verified by DNA sequencing. The construction of the IL-6-cDNA lacking the last 12 nucleotides of the coding region ($\triangle$4) has been recently described [7]. The linearized plasmids were transcribed with T7 RNA

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00145793/90/$3.50

polymerase and subsequently translated in a cell-free system (rabbit reticulocyte lysate) in the presence of [^{35}S]methionine [10]. After SDS-PAGE of the cell-free synthesized polypeptides IL-6 bands were excised from the polyacrylamide gel and their radioactivity was determined. The values were normalized taking into account the number of methionine residues present in full length IL-6 and deletion 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) [11]. One μl of the translation mixture and serial dilutions (1:2) were used in the B9 cell proliferation assay.

2.4. *Fibrinogen induction assay*

IL-6 activity was measured by fibrinogen induction in human HepG2 cells [12]. 10^4 cells were incubated in a total volume of 300 μl medium with serial dilutions (1:2) of cell-free synthesized full length IL-6 and deletion mutants. After 18 h the medium was removed and replaced by medium containing 167 kBq [^{35}S]methionine. 4 h later, newly synthesized and secreted radiolabeled fibrinogen was immunoprecipitated, separated by SDS-PAGE and subjected to fluorography [13].

Since the reticulocyte lysate at higher concentrations interferes with both IL-6 assays, we always used equal and subtoxic amounts of translation mixtures to evaluate the biological activity of the IL-6 mutants.

3. RESULTS AND DISCUSSION

Previous results had demonstrated the crucial role of the C-terminus of IL-6 for its biological activity [7]. Already the elimination of the 4 carboxy-terminal amino acid residues led to a complete loss of activity [7]. For a more detailed analysis of this finding we have now synthesized deletion mutants of IL-6 lacking 1, 2, or 3 amino acids of the C-terminal end of the polypeptide. These mutants constructed by PCR technology were cell-free transcribed and subsequently translated into proteins using a reticulocyte lysate. As shown in Fig. 1 aliquots of the translation mixtures were separated by SDS-PAGE. A slight increase in electrophoretic mobility can be observed when the deletions are compared to the full length IL-6 (Δ0).

In Fig. 2 we show the IL-6 dependent proliferation of B9 cells as measured by [^{3}H]thymidine uptake. A dose-dependent proliferation of B9 cells is detected, when full length IL-6 (Δ0) is added to the cells. No cell growth is observed without IL-6 addition ('no IL-6'). The removal of the last amino acid (compare Fig. 5) led to a significant decrease in the biological activity of this IL-6 mutant. A further slight reduction was observed for an IL-6 polypeptide lacking 2 amino acids of its carboxy-terminus. Elimination of a third amino acid completely abolished IL-6 activity (Fig. 2). As controls we used rhIL-6 in concentrations, which gave a complete response profile in the B9 proliferation assay (co A). Addition of one μl of reticulocyte lysate did not change the activity of the IL-6 standard (co B).

In the experiments described above we used murine plasmacytoma cells (B9) to measure the biological activity of human IL-6. Therefore, we chose to verify our results in a homologous system using the fibrinogen induction assay with human hepatoma cells (HepG2) [12]. These results are shown in Fig. 3. Full length IL-6 induced the synthesis of all three fibrinogen polypeptide chains in a dose-dependent manner. Again, removal of one or two amino acids resulted in a substantial reduction of biological activity, whereas truncation of 3 amino acid residues led to a completely inactive IL-6 molecule. In both assay systems mutants lacking 4 carboxy-terminal amino acids were inactive, too. Controls were the same as described for Fig. 2. Fig. 4 shows a comparison of data obtained in the B9 proliferation and the fibrinogen induction test. The fact that major decreases in IL-6 activity occur upon deletion of Met-184 (Δ1) and Arg-182 (Δ2) indicates the particular importance of these residues. It should be noted that the effect of the respective mutations is the same in the murine and the human assay system. This is especially interesting in view of the fact that murine IL-6 only binds to the murine IL-6-receptor, whereas human IL-6 recognizes both, the human and murine receptor [14], pointing to important structural differences between the two receptors and ligands.

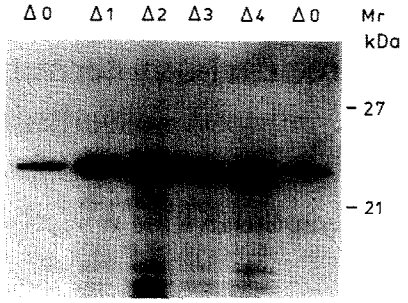

Fig. 1. SDS-PAGE of cell-free synthesized IL-6 mutants deleted at the carboxy-terminus. Full length IL-6 and deleted forms obtained after in vitro transcription and translation were separated by SDS-polyacrylamide gel (12.5%) electrophoresis and visualized by fluorography [13]. Δ0 represents the full length IL-6 control; Δ1, Δ2, Δ3, Δ4 denote the IL-6 polypeptides lacking 1, 2, 3, 4 amino acids from the carboxy-terminus, respectively.

Having shown the importance of the carboxy-terminus of IL-6 for its function, we examined the possibility that a synthetic peptide, consisting of the 15 carboxy-terminal amino acids of mouse IL-6 [15], could exert IL-6 activity. This peptide had neither an inhibitory nor stimulatory effect on the proliferation of B9 cells if compared to rhIL-6 (data not shown).

We find it puzzling that the last amino acid, which seems to be important for function is not conserved between mouse and man (Fig. 5). Site-directed mutagenesis studies of the carboxy-terminus of IL-6 will be needed to define the precise structural requirements of this part of the molecule for biological activity.

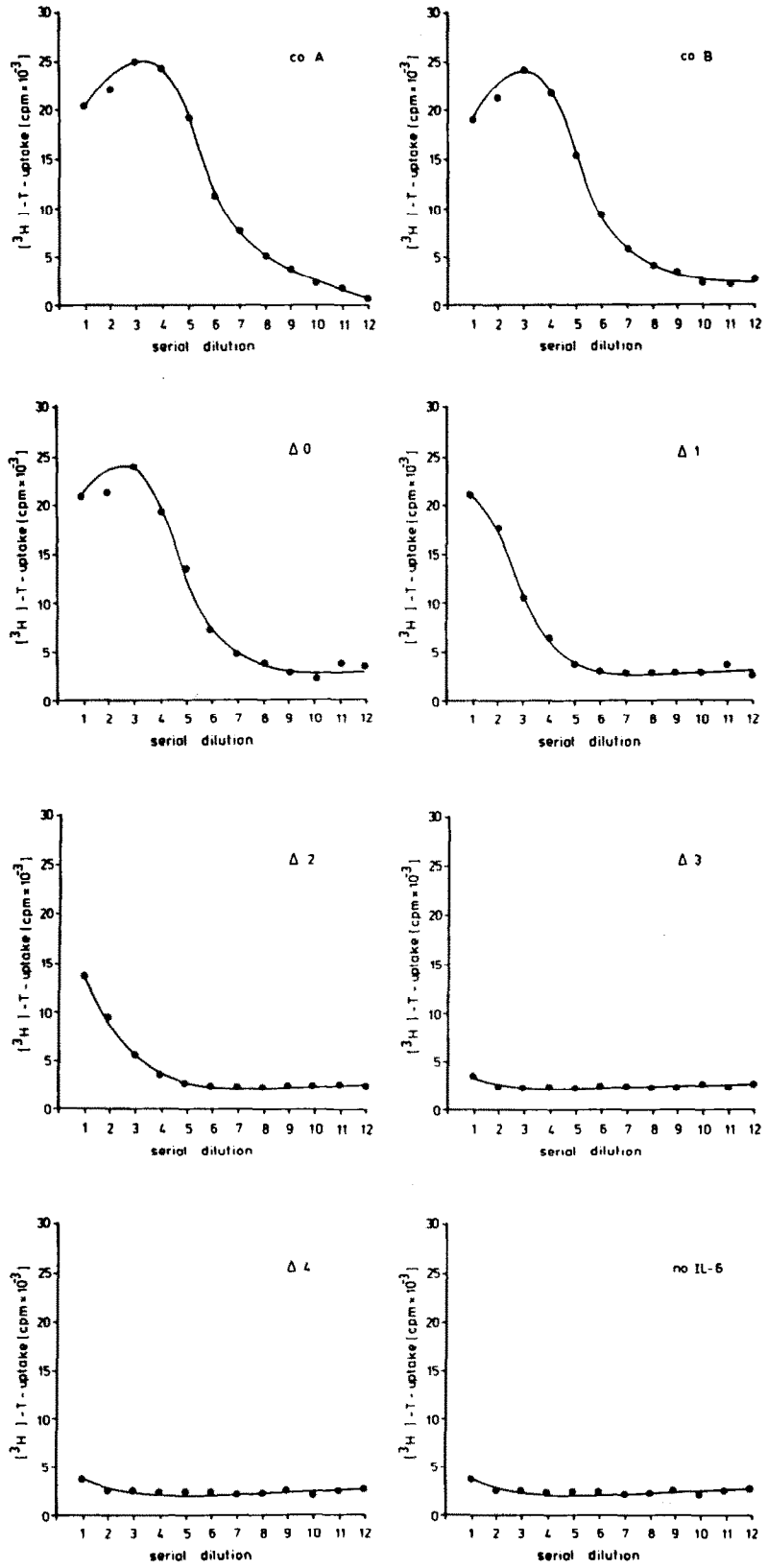

Fig. 2. Growth factor activity of full length and carboxy-terminally deleted IL-6 polypeptides. Aliquots of the translation cocktails containing comparable amounts of cell-free synthesized IL-6 were used in the B9 cell proliferation assay as measured by [^{3}H]thymidine uptake. 'co A' represents the dilution of a rhIL-6 standard (activity of the first dilution: 20 U/ml), 'co B' is identical to 'co A' except for the addition of 1 μl of a mock translation mixture; 'no IL-6' represents a control, where proliferation of unstimulated B9 cells was measured.

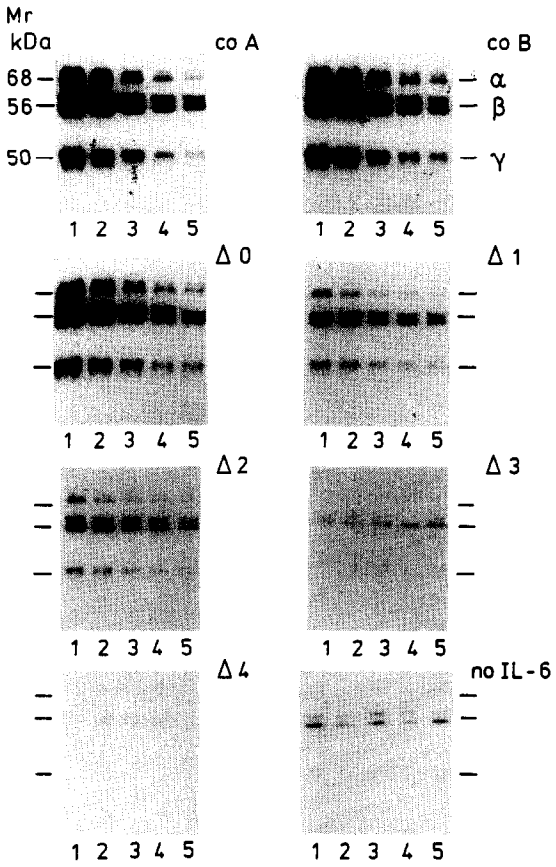

Fig. 3. Fibrinogen induction of full length and carboxy-terminally deleted IL-6 polypeptides. 15 μl aliquots and serial dilutions (1:2) of the translation mixtures were used to induce fibrinogen secretion in HepG2 cells. Immunoprecipitated α-, β- and γ-fibrinogen was counted and separated on 10% SDS-PAA-gels and subjected to fluorography. 'co A' is the dilution of rhIL-6 (330 U/ml), 'co B' is the same as 'co A' except for the addition of 15 μl of a mock translation mixture; 'no IL-6' represents a control, where fibrinogen secretion of unstimulated HepG2 cells was measured.

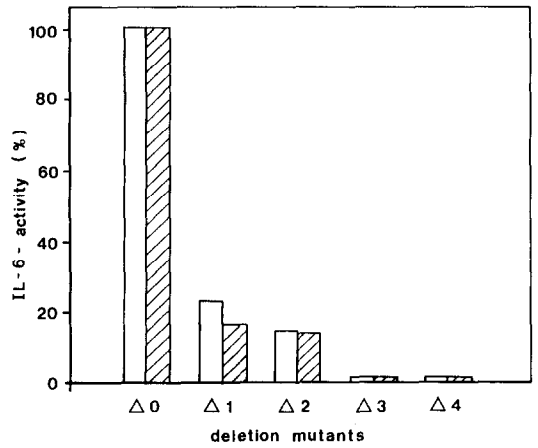

Fig. 4. Comparison of the biological activity of IL-6 mutants as measured in the B9 and HepG2 cell assay. Values, normalized for different amounts of IL-6 synthesized, were compared using the dilution leading to half-maximal stimulation in the respective test. The biological activity exerted by full-length IL-6 (Δ0) was set to 100%. Open bars: B9 cell proliferation assay, average of 3 determinations; hatched bars; HepG2 cell fibrinogen assay, average of 2 determinations.

- leu - arg - gln - met	human
- thr - arg - gln - thr	mouse

Fig. 5. Sequence comparison of the carboxy-terminus of human and murine IL-6.

Acknowledgements: We would like to thank Drs K.-H. Wiesmüller (Tübingen) and W. Bessler (Freiburg) for providing us with the synthetic oligopeptide corresponding to the C-terminus of murine IL-6. We also are indebted to M. Robbertz for his excellent assistance in the preparation of the figures and A. Stammschulte for her help with the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

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