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Identification and characterization of a C-terminally extended form of recombinant murine IL-6

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Murine interleukin-6 (mIL-6) was expressed in *Escherichia coli* in the insoluble fraction of cell lysates. Approximately equal amounts of two polypeptide species, reactive with anti-IL-6 antibodies, were produced. The two forms of mIL-6 were isolated and found to have identical N-terminal sequences initiated by Met-Phe-Pro-Thr-Ser-Gln-. Peptide mapping after endoproteinase glu-C digestion led to isolation and characterization of the C-terminal peptides from each of the two forms and allowed the source of the heterogeneity to be identified as a C-terminal addition of three amino acids, Gln-Lys-Leu, to authentic mIL-6. Inspection of the nucleotide sequence of the plasmid containing the mIL-6 gene and expression of the plasmid in other strains suggested that the addition of three amino acids was caused by a readthrough of the termination codon arising from an unexpected suppressor mutation in the original host strain. Although the C-terminus of IL-6 is critical for the activity of this cytokine, the IL-6 variant with extended C-terminus was fully active in two separate bioassays. This suggests that the additional amino acids do not disrupt the structure or function of this important region of the molecule.

Recombinant mIL-6; C-terminal extension; Bioactivity

1. INTRODUCTION

Interleukin-6 (IL-6) is a cytokine whose wide variety of biological functions suggests a central role in the host defense against infection and inflammation. Among the various activities originally described separately and later attributed to this single polypeptide are the final differentiation of B-cells into IgG-secreting cells [1], plasmacytoma and B-cell hybridoma growth induction [2], and induction of acute-phase proteins in hepatocytes [3]. The mature murine IL-6 molecule is a 187 amino acid polypeptide with two disulfide bridges. Human and murine IL-6 are 42% homologous at the amino acid level with highest homology restricted to the middle and the C-terminal regions [4,5]. Studies with deletion mutants have shown that the C-terminus of the polypeptide is critical for bioactivity of this cytokine [16,18]. In order to obtain sufficient amounts of pure murine IL-6 for structure elucidation and for studies with a mouse model of inflammation, we expressed the mature form of mIL-6 in *E. coli*.

Proteins expressed in *E. coli* by recombinant DNA technology are occasionally found to contain structural modifications which lead to heterogeneity of the desired product. Some of the well recognized modifications leading to heterogeneity are: incomplete removal of initiator methionine from the N-terminus [6], proteolytic truncation of either the N- or C-terminus [7,8] and deamidation [9,10] or oxidation [10] of sensitive amino acid residues. Here we present an additional form of modification leading to heterogeneity; the addition of amino acids to the C-terminus due to partial readthrough of the termination codon. The variant, containing three additional amino acids (Gln-Lys-Leu) at the C-terminus, accounted for 50–60% of the total IL-6 expressed and had activity equal to the wild-type in several different assays. As recent studies of IL-6 had demonstrated that the C-terminal tetrapeptide is absolutely required for biological activity, the question of whether the C-terminal extension might have biological activity was of considerable interest. We found equivalent biological activity and suggest that maintenance of an α-helical structure at the C-terminus is important for activity.

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2. MATERIALS AND METHODS

2.1. *Cloning, expression and purification of mIL6*

The murine IL-6 gene was cloned from a cDNA library prepared from mouse J774.1 monocyte/macrophage cells [11]. An expression plasmid was constructed and used to transform *E. coli* strain GE81 as described elsewhere [12]. Mature IL-6 was expressed at about 5–10% of total cell protein in an insoluble form and was identified on SDS-PAGE as a close doublet of bands both of which reacted with anti-murine and anti-human IL-6. The purification of the correctly ter-

minated and C-terminally extended forms of mIL-6 is described elsewhere [12].

2.2. *N-terminal sequence analysis and amino acid analysis*

IL-6 samples for N-terminal sequence or amino acid analysis were desalted by C4 reverse phase chromatography (Vydac C4) in a gradient of 0–70% acetonitrile with 0.1% TFA and sequenced on an ABI Model 470A gas-phase sequencer equipped with a Model 120A PTH analyzer. Amino acid analysis was by pre-column phenylisothiocyanate derivatization (Waters Pico-Tag).

2.3. *Peptide mapping and determination of the C-terminus*

Samples were dialyzed against 25 mM ammonium bicarbonate, pH 8 overnight. Each sample was made 2 M in guanidine hydrochloride (GnCl) and endoproteinase Glu-C in water was added to get a 1:25 ratio by weight of Glu-C:mIL-6. After 17 h, additional proteinase was added (1:50) and the incubation continued for 24 more h. Aliquots of the digests to be compared before and after reduction with DTT were first brought to 5 M GnCl then treated with either 100 mM DTT or water for 30 min at 45°C. Peptide mapping and isolation were done on a C18 reverse phase column (Pep-RPC HR5/5, Pharmacia) in a gradient of acetonitrile with 0.1% TFA. Peptides to be analyzed were brought almost to dryness in a Savant Speed-Vac prior to N-terminal sequence or amino acid composition analysis.

2.4. *Bioassays*

The B9 cell stimulation assay was performed according to Aarden et al. [14]. IL-6 activity was also measured by its ability to stimulate production by primary hepatocytes as previously described [15].

3. RESULTS

3.1. *Purification and initial characterization of mIL-6*

The mIL-6 was expressed in the insoluble fraction of the *E. coli* cell lysate producing a closely spaced doublet at about 23 000 Da on SDS-PAGE (Fig. 1A, lane 4). Each of the forms was approximately equally represented in the doublet and both reacted with anti-IL-6 antibody (Fig. 1B, lane 4).

A washed inclusion body preparation (Fig. 2, lane 4) was prepared and was dissolved in 4 M GnCl at pH 7.5–8.0 prior to chromatography on Sephacryl S-200. An IL-6 fraction was obtained which upon dialysis resulted in a soluble, active mIL-6 preparation. Optimal refolding was from 25 mM Na acetate, pH 5.5 and attempts to refold from material solubilized with reducing agents present or without the gel filtration step resulted in reduced recovery of IL-6. The IL-6 preparation at this stage contained both forms of the protein (Fig. 2, lane 5). Two-dimensional gel electrophoresis of the preparation at this point revealed that the two forms had different isoelectric points as well as apparent molecular mass (data not shown). The form with the higher pI (7.8) had the higher molecular mass while the species with lower pI (7.1) had the lower mass.

In order to characterize further the two IL-6 forms, they were separated using cation exchange chromatography. Based upon elution order from a Bakerbond CBX column, they were designated CBX1 and CBX2. N-terminal sequencing of the separated IL-6 species verified that each had methionine at its N-terminus and amino acid analysis suggested no gross differences between the compositions of the two molecules.

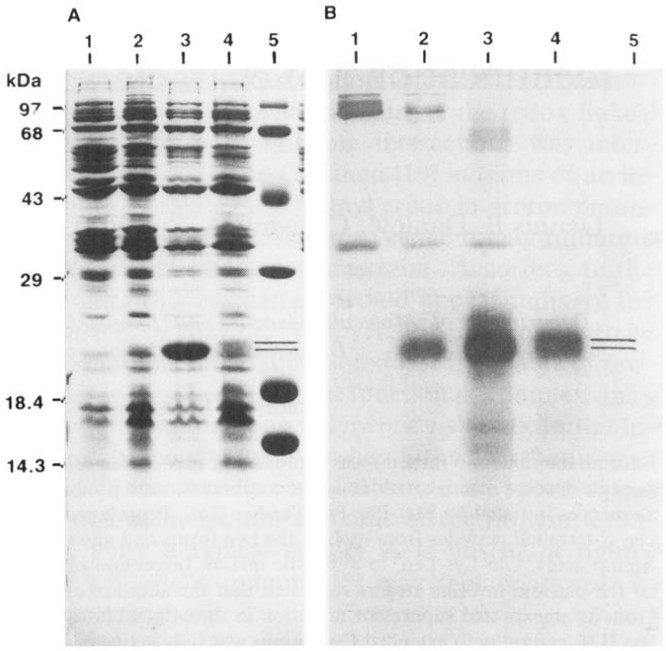

Fig. 1. Expression of mIL-6 in *E. coli* strains. Total cell lysates from various expression strains were evaluated by (A) SDS-PAGE [13] and (B) Western blot analysis. Lane 1, GE81/pBR322; 2, GE196/pNB217; 3, W3110/pNB217; 4, GE81/pNB217; 5, M.W. standards.

3.2. *Verification of disulfide bond homogeneity*

In order to determine whether the two IL-6 variants had different disulfide bonding patterns, the digested peptides were compared on peptide maps with and without first treating the digest with 100 mM dithio-

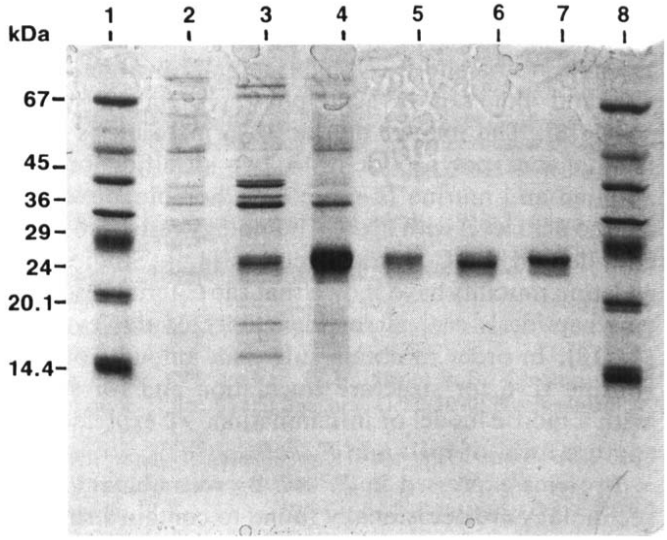

Fig. 2. SDS-PAGE analysis of mIL-6 purification steps. Lane 1, molecular weight markers; 2, soluble fraction of *E. coli* cell lysate; 3, insoluble pellet from *E. coli* (GE81) cell lysate; 4, washed inclusion body preparation; 5, mIL-6 fraction from Sephacryl S-200 chromatography; 6, purified, correctly terminated mIL-6 peak from cation exchange chromatography (CBX1); 7, purified, extended C-terminal variant of mIL-6 peak from cation exchange (CBX2).

threitol (DTT) in the presence of 5 M GnCl (Fig. 3). In each case, two peptides disappeared from the chromatogram representing the unreduced digest and four new peptides appeared in each DTT reduced digest. One of the two peptides which disappeared after reduction was identified by amino acid analysis as peptides 70-84 and 85-96, one of the disulfide linked peptide pairs expected in mIL-6. The second expected disulfide linked peptide pair was not identified, but identification of one of the two correct pairs combined with the identical change in peptide maps between the reduced and unreduced digests suggests that both CBX1 and CBX2 have the same, correct S-S bond pattern.

2.3. *Identification of C-terminal heterogeneity*

The glu-C peptide maps of CBX1 and CBX2 revealed only one unique peptide for each form (Fig. 4); peptide 3 was present in the CBX1 peptide map but absent in the CBX2 map and peptide 4 was present in the CBX2 map but missing in the CBX1 map. These two peptides were identified by amino acid analysis and automated sequencing to be the respective C-terminal peptides for CBX1 and CBX2. Peptide 3 from the CBX1 digest had the correct C-terminal sequence for mIL-6, (FLKVTLRSTRQT). Peptide 4, recovered from the CBX2 digest, had the sequence FLKVTLRSTRQTQKL, revealing three additional amino acids at the C-terminus of CBX2. Inspection of the nucleotide sequence in the plasmid at the 3′ end of mIL-6 cDNA suggested a mechanism for this result.

In the DNA sequence of TAG stop codon and an adjacent *Hin*dIII site (AAGCTT) were apparently read through resulting in a glutamine (Q) insertion for the TAG codon, and lysine (K), leucine (L) for the AAGCTT (Fig. 5). A TAA codon following the *Hin*dIII site caused termination. Such a readthrough of a UAG codon is consistent with an amber suppressor mutation in *E. coli* strain GE81. In support of this hypothesis, this plasmid results in the expression of a single mIL-6 immunoreactive band corresponding in size to unmodified mature IL-6 when transformed into two other strains without the suppressor mutation, GE196 and WC3110 (Fig. 1).

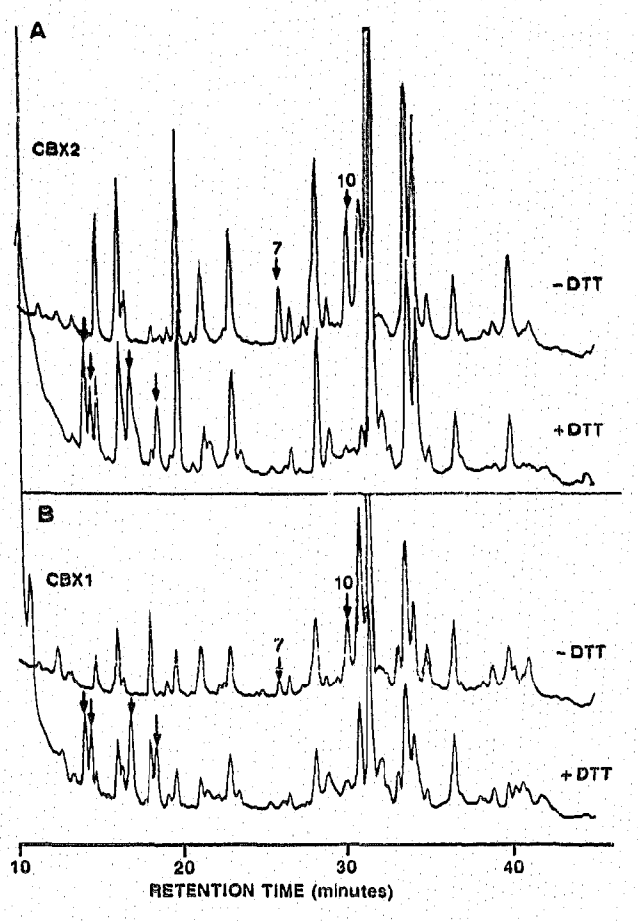

Fig. 3. Endoproteinase Glu-C peptide maps of reduced and non-reduced digests. CBX2(A) and CBX1(B) were each digested as described in Section 2, and aliquots compared before and after reduction with DTT. Peptide 10 was identified as peptide 70-84 disulfide linked to peptide 85-96 (numbered according to [4]). Peptide 7 was not conclusively identified. The same four new peptides (arrows) appeared in each digest upon reduction with DTT.

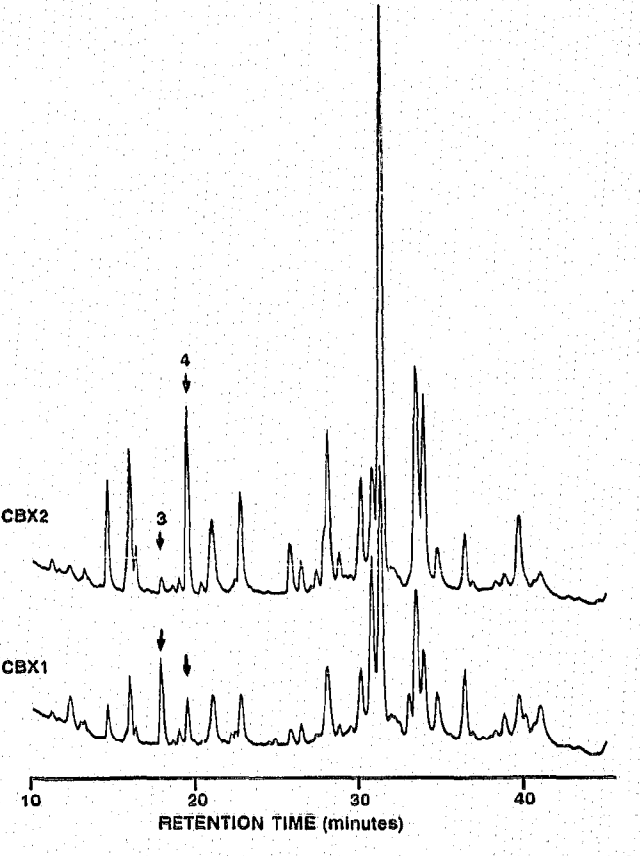

Fig. 4. Endoproteinase Glu-C peptide maps of CBX1 and CBX2. CBX1 and CBX2 were digested and chromatographed as described in Materials and Methods. Peptides 3 and 4 (arrows) were identified as the C-terminal peptides respectively of CBX1 and CBX2.

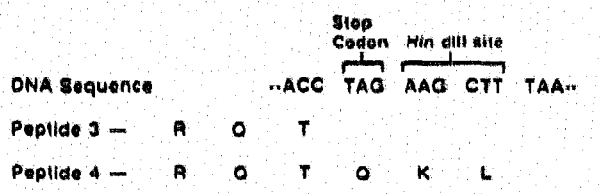

Fig. 5. Alignment of nucleotide and amino acid sequences at the C-terminus. Readthrough of the TAG stop codon into the *Hin*dIII site resulted in C-terminal extension Q-K-L. An adjacent TAA stop codon in the plasmid DNA terminated translation as described in the text.

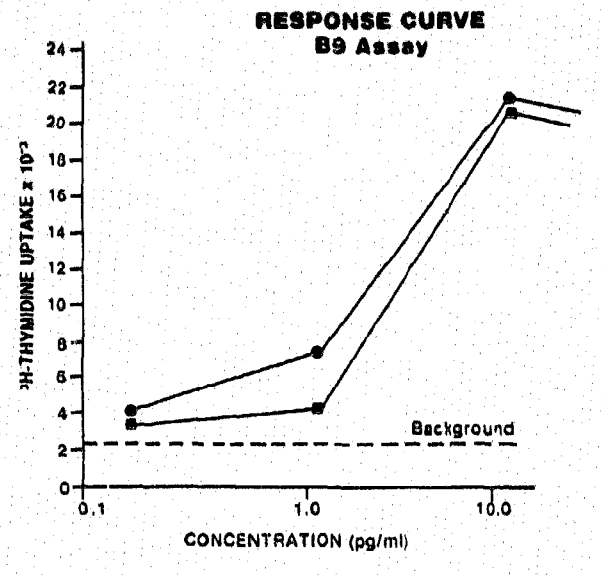

Fig. 6. Comparison of the bioactivities of CBX1 and CBX2 in the B9 cell stimulation assay. 5000 B9 cells maintained in Iscove's Modified Dulbecco's Medium (IMDM), 5% FCS, 5×10^{-5} M mercaptoethanol, penicillin and streptomycin (100 μl) were placed in 100 μl of concentrations of IL-6. After 54 h, cells were labeled with 1 μCi/well [^{3}H]thymidine. Closed circles (J) represent CBX1 and closed squares (B) represent CBX2. One unit was the amount of IL-6 required to induce 50% maximal stimulation.

3.4. *Activities of the native and extended mIL-6*

Two assays, based on different cell types, were used to evaluate the relative activities of the native and C-terminally extended forms of mIL-6. Using B9 hybridoma cells, the specific activity of rmIL-6 in this assay ranged between 4.3×10^7 and 2.8×10^8 units per milligram and was dependent upon the age of the B9 culture not the preparation of IL-6. In comparative assays ($n=4$), no significant difference was seen between the two forms. A representative assay is shown in Fig. 6. In the hepatocyte stimulating factor assay, the ability of IL-6 to specifically stimulate the production of fibrinogen by primary hepatocytes was measured and a specific activity of 1.2×10^8 units/mg was found. Again in comparative assays ($n=4$), no difference in activity was seen.

4. DISCUSSION

The expression of proteins by recombinant DNA technology has provided a major improvement in the availability of these important biomolecules as reagents, therapeutics and drug targets as well as for structure-function studies. While mutations in the gene sequence and post-translational modifications are widely recognized as potential causes of recombinant protein heterogeneity, this instance of a translational error points out the less obvious possibility of C-terminal extension when expressing and isolating recombinant proteins.

This novel mIL-6 variant with a C-terminal addition provided us with the opportunity to determine the effect of this structural modification on the biological activity of this cytokine. Recently, it has become clear that residues in the C-terminal region including those very close to the C-terminus of human IL-6 are required for the activity of this cytokine [16–18]. Antibodies which neutralize IL-6 activity bind an epitope near the C-terminus [17] and deletion mutants with as few as four of the C-terminal amino acids missing completely lose activity [16]. The latter finding suggests that the extreme C-terminal residues are either directly involved in a function required for activity or that they are necessary to maintain some structure required for activity. Bioassays comparing the native and C-terminally extended variant of mIL-6 revealed that the specific activity was unaffected by addition of the tripeptide, Gln-Lys-Leu. The finding that this addition does not alter the biological activity of IL-6, argues that this modification has not caused disruption of the structure at the C-terminus nor the overall fold of the protein and suggests that the charged C-terminal carboxyl is not directly involved in receptor binding. In addition, the bulk of the extra three amino acids, including a charged residue, must introduce neither steric nor electrostatic constraints on the ability of IL-6 to bind to its receptor.

The IL-6 receptor belongs to a structurally homologous family to cytokine receptors [19,20]. The protein fold of the cytokine ligands for this receptor family is known [21] or predicted to be four helix bundles [19,22]. In a model of binding for these receptor-ligand pairs, Bazan [22] has proposed that there is interaction of a recognition helix of the cytokine with a 'rigid groove' in the receptor. Mutation analysis of one of these ligands, human growth hormone (hGH), identified residues on the face of the C-terminal helix, helix D, which form the primary receptor binding structure [23]. Alignment of conserved sequence patterns in helix D of helical cytokine structures with hGH, identified exposed residues in IL-6 which might be important in receptor binding [22]. Of the four C-terminal residues of rat [24], murine [25] and human [1,5] IL-6 (X-Arg-Gln-X), arginine is conserved and aligns with one of these exposed residues. It might therefore be respon-

sible for the loss of activity upon deletion of the four C-terminal residues. Alternatively, maintenance of a complete recognition helix including all four C-terminal residues could be the important requirement for function. Several models of secondary structure predict an α-helix at the C-terminus of IL-6 [16,19]. The additional residues, Gln-Lys-Leu, of the fully active variant reported here are each amino acids found most frequently in the α-helix [26] and therefore are unlikely to disrupt an important α-helical structure at the C-terminus.

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