Expression, renaturation and purification of recombinant human interleukin 4 from *Escherichia coli*

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The lymphokine human interleukin 4 (IL-4) has been expressed from a plasmid in the cytoplasm of *Escherichia coli*. Advantage has been taken of insolubility of the human IL-4 in *E. coli* for rapid purification of this protein in only a few steps. We describe extraction and renaturation procedures which solubilize human IL-4 yielding biologically active protein. The protein was purified to homogeneity by one passage over a gel-filtration column. The refolded human IL-4 was characterized by N-terminal sequence analysis, amino acid analysis and bioassays. The refolded *E. coli*-derived human IL-4 has biological activity on T and B cells and binds to the human IL-4 receptor, comparable to mammalian expressed human IL-4, indicating that the protein is folded correctly.

Interleukin 4 (IL-4), or B-cell-stimulatory factor 1 is a lymphokine initially identified in the supernatant of cells of the mouse thymoma line EL-4 stimulated with phorbol myristate acetate [1]. It is also produced by several T cell lines upon stimulation with concanavalin A or with antigen and antigen-presenting cells [2]. In murine systems, IL-4 has been shown to act on T and B lymphocytes, mast cells and several other hematopoietic lineage cells. It affects the activation, growth, and program of gene expression in these cells [2]. The human homologue of IL-4 has not been found in natural systems but recently has been cloned and characterized based on homology with the mouse cDNA [3]. The human IL-4 stimulates proliferation of anti-IgM-preactivated human tonsillar B cell [3, 4] as well as the proliferation of human T cells and helper T cells clones [3, 5]. It also induces the low-affinity receptor for IgE (FceRL/CD23) [6]. The human IL-4 consists of 129 amino acids and contains six cysteine residues, with the potential to form three disulfide bonds. The lack of natural human IL-4 requires a recombinant source for further study of its role in the immune system. To obtain a large quantity of this lymphokine, we have cloned and expressed the cDNA in *Escherichia coli*.

Frequently, over-expression of mammalian proteins in bacterial cells leads to the accumulation of aggregated insoluble protein complexes (inclusion bodies) [7–10]. The protein in these complexes is present in scattered, partially oxidized forms as well as in largely reduced forms [9, 11]. To solubilize and activate these aggregates, the protein often has to be refolded in *vitro*.

Some groups have been successful in refolding recombinant mammalian proteins expressed in *E. coli*; for example, insulin [12], prochymosin [13], urokinase [10] and transforming growth factor α [14]. The success of refolding depends critically on the nature of the expressed polypeptide, the selection of the chaotropic and reducing reagents used to solubilize the aggregates and the conditions under which the solubilized and denatured polypeptide is refolded into the biologically active form [15, 16].

In this paper we describe (a) the expression of human IL-4 in *E. coli*, produced in an aggregated insoluble form; (b) the extraction and refolding of the aggregates; and (c) the purification of the refolded biologically active human IL-4. The purified protein has a specific activity of $5 \times 10^7$ U/mg measured by an assay for T cell growth factor (TCGF) and has been characterized by N-terminal sequence analysis, amino acid analysis, and biological activity on T and B cells.

**MATERIALS AND METHODS**

**Recombinant DNA technology**

All restriction enzymes, DNA polymerase, kinase and ligase reactions were performed as described by Maniatis et al. [17]. Enzymes were purchased from New England Biolabs and Boehringer Mannheim. Plasmids were isolated according to the alkaline method (small scale [17]) or a modification of this method (large scale [18]).

DNA oligonucleotides were synthesized by phosphoramidite chemistry using an Applied Biosystems 380A synthesizer. DNA sequence analysis was performed using standard dideoxy procedures [19].

**Bacterial strain and plasmid construction**

*E. coli* AB1899 (thr1, leuB6, thi1, argE3, hisG4, proA2, lon1, lacY1, galK2, mtl1, xyl5, ara14, strA31, tss33, x-, supE44) was obtained from Schering Research.

The EcoRV–BamHI fragment from the human IL-4 cDNA clone (pcD-hIL-4) [3] was ligated together with the synthetic linkers 5'-d(CGATGCACAAGTGCGAT)-3' (+ strand) and 5'-d(ATCGCAGCTTGGCAT)-3' (− strand).
and ClaI/BamHI-digested pTrpC11-mIL2 [18] (Fig. 1). Thus, the mIL-2 sequence present in the TrpC11-mIL2 plasmid was replaced by the human IL-4 sequence. The identity of the final construct (pTrpC11-hIL-4) was confirmed by DNA sequence analysis.

**Analytical methods**

Guanidine · HCl (sequanal grade) was obtained from Pierce, urea (ultra pure, enzyme grade) from BRL. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [20]. Proteins were visualized with Coomassie brilliant blue R-250 or by silver stain [21, 22]. Densitometric scanning of stained gels was carried out using an LKB Ultrascan XL laser densitometer.

Protein concentrations were measured by the method of Bradford [23] using bovine serum albumin fraction V (Miles Scientific) as a standard. In a later stage of this study the protein concentration of a sample of pure human IL-4 was determined by amino acid analysis and this sample was subsequently used as a standard for Bradford assay. The use of pure human IL-4 as a standard measured a protein concentration 2.9 times lower than using bovine serum albumin as a standard. The protein concentration necessary for the determination of the specific activity was measured against the human IL-4 standard. Other Bradford assays used bovine serum albumin as a reference.

Purified human IL-4 was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) on a column (4.6 x 75 mm) of Ultrapore C3 (Beckman) eluted with a gradient of 25-60% acetonitrile in 0.1% trifluoroacetic acid/water.

Amino acid composition was determined by pre-column derivatization with o-phthalaldehyde followed by RP-HPLC [24].

Amino-terminal sequence analysis was performed by automated Edman degradation on an Applied Biosystems 470A sequenator [25]. Resultant phenylthiohydantoin derivatives were analyzed by RP-HPLC [26].

Analysis for free thiol groups in the purified protein was done by the method of Ellman [27]. The concentration of human IL-4 in the assay was 0.18 mg/ml.

**Immunological methods**

Antiserum 12-MYO was derived from a rat immunized with a synthetic peptide corresponding to 16 N-terminal amino acids of human IL-4. The monoclonal antibody 11B4 was raised against mammalian-derived recombinant human IL-4 (Chrétien et al., unpublished results).

Western blots were performed as described [18], except that 12-MYO was used at a 100-fold dilution and 11B4, obtained from culture supernatants, was used at a 10-fold dilution.

Human IL-4 obtained by gel filtration chromatography (described below) was assayed by an indirect enzyme-linked immunosorbent assay (ELISA) [29]. Aliquots (1%) of individual column fractions were used to coat the assay plates and 11B4 was used as the primary antibody.

**Biological assays**

Human IL-4 was tested for T cell growth factor (TCGF) activity on human peripheral blood lymphocytes using a colorimetric assay [3] at a starting dilution of 1:40 of the SDS extracts (described below). The assay medium contained 50 μg/ml gentamicin to prevent growth of E. coli cells that survived the extraction procedure. One unit is the amount of human IL-4 that provides a signal 50% of that elicited by saturating amounts of lymphokine.

**Extraction of human IL-4 with SDS**

E. coli AB1899 cells carrying the TrpC11-hIL4 plasmid were grown in L-broth (1:50 dilution of an overnight culture) to a total absorbance at 560 nm of 2; 1 ml of this culture was centrifuged for 5 min and the cells were resuspended in 500 μl phosphate-buffered saline. Cells were then lysed by sonication (30-50% pulses, 40 W) using a Branson cell disruptor 200. After centrifugation, the pellet was resuspended in 100 μl phosphate-buffered saline containing 1% SDS and incubated for 10 min at 37°C. The proteins solubilized with 1% SDS were separated from the insoluble fraction by centrifugation.
for 10 min at 4°C. Centrifugations were performed at 15000 × g.

**Extraction and renaturation procedure**

One liter of *E. coli* AB1899 cells harboring the TrpC11-hIL4 plasmid were grown (1:50 dilution of an overnight culture) in a 2-l conical flask at 37°C in L-broth for 10 h. Cells were harvested by centrifugation at 4500 × g for 15 min at 4°C and stored at −20°C overnight.

To lyse the cells, the pellets were resuspended in 30 ml 50 mM Tris/HC1 pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (buffer A), and sonicated (50 50% pulses, 70 W). The cell lysates were centrifuged at 25000 × g for 15 min at 4°C. The resulting pellet containing the insoluble human IL-4 protein was resuspended in buffer A supplemented with 5 M guanidine · HCl and 2 mM reduced and 0.2 mM oxidized glutathione. For every 1 g wet pellet, 9 ml buffer was added to a maximum protein concentration of 2.5 mg/ml. After 1 h at room temperature, this solution was added slowly to 9 vol. buffer A (without phenylmethylsulfonyl fluoride) supplemented with 2 mM reduced and 0.2 mM oxidized glutathione, and incubated at room temperature for 1.5—4 h.

The lysate was centrifuged at 2500 × g for 15 min at 4°C to remove the precipitate formed during the dilution step. The clear supernatant was dialyzed against phosphate-buffered saline pH 7.4 with three changes of buffer (total dilution 1000-fold) at 4°C to remove the refolding reagents. After removal by centrifugation of the precipitate formed during dialysis, the protein concentration of the dialyzed fraction was increased to 8 mg/ml using an Amicon concentrator with an YM5 filter. Again precipitates formed which were removed by centrifugation.

**Gel filtration chromatography**

The refolded and concentrated dialyze was loaded onto a Bio-Gel P-30 (Bio-Rad) column (1.5 × 90 cm) equilibrated with phosphate-buffered saline and eluted at a flow rate of 8 ml/h; the eluate was collected in 2-ml fractions. The column was calibrated with the following molecular mass markers from Pharmacia: ribonuclease (*M*ₐ 13700), chymotrypsinogen (*M*ₐ 25000), ovalbumin (*M*ₐ 43000) and bovine serum albumin (*M*ₐ 67000).

**RESULTS**

**Expression and solubility of human IL-4**

To confirm the expression of human IL-4, lysates of *E. coli* AB1899 cells transformed with plasmid TrpC11-hIL4 were analyzed by PAGE and Western blotting using 12-MYO (Fig. 2). These lysates (lane 2) contained a band at an *M*ₐ of 14500 representing the human IL-4 molecule which was absent in the control (lane 1). The expressed human IL-4 was found in the pellet fraction after sonication and centrifugation and represented the major protein in this fraction (Fig. 2A lane 4). These results indicate that the human IL-4 is expressed as insoluble aggregates.

The aggregates were solubilized with 1% SDS but the specific activity of the extracted human IL-4 as measured by the TCGF assay was low. Partial removal of the SDS by cold precipitation followed by centrifugation a 4°C failed to yield a more active protein (data not shown). These results indicated that such measures were insufficient to renature the molecule fully.

**Renaturation and purification of human IL-4**

To optimize existing extraction and renaturation procedures [10, 13] for the human IL-4 protein, different denaturants (guanidine and urea) with or without the addition of reduced and oxidized glutathione were tested and the refolded extracts were assayed for TCGF activity. The highest specific activity of human IL-4 was obtained using 5M guanidine as denaturant with the addition of 2 mM reduced and 0.2 mM oxidized glutathione (results not shown). The specific activity...
Fig. 4. Bio-Gel P-30 gel filtration of refolded protein extract. (A) The column (1.5 × 90 cm) was run at 4°C in phosphate-buffered saline; 2-ml fractions were collected at a flow rate of 8 ml/h. (---) \( A_{280} \); (○---○) ELISA; (●—●) biological activity. (B) Silver-stained SDS/polyacrylamide gel electrophoresis of column fractions 14–28 in A. Sample loaded into each well contained the equivalent of 10 µl of the corresponding fraction.

of human IL-4 was comparable to mammalian expressed human IL-4, indicating that the refolding is complete at this step.

The refolding during the extraction and renaturation procedure was followed. The toxic reagents in the samples during the refolding interfere with the bioassay so that the extent of refolding could not be measured by the specific activity. Therefore another approach was taken to analyze the samples. The degree of reoxidation of human IL-4 was inferred from its migration in SDS-PAGE under non-reducing conditions and compared with the native (oxidized) human IL-4. Formation of intramolecular disulfide bonds, occurring in the oxidized molecule, presumably results in a more compact structure with a faster mobility than the reduced molecule. The samples were analyzed by Western blotting using 12-MYO (Fig. 3A). In the total cell lysate human IL-4 was present as aggregates (lane 2), which were solubilized with guanidine (lanes 4 & 5). This resulted in a molecule with a slower mobility than the native human IL-4 (lanes 6 & 7), indicating that this form is still incompletely oxidized. Only after dialysis was native (oxidized) human IL-4 present. This result was supported by the results of a Western blot of the same samples treated with 11B4. Under non-reducing conditions described above 11B4 recognizes only native human IL-4 (data not shown) and reacted with the human IL-4 only after the dialysis step (data not shown).

During the extraction and renaturation procedure the purity of human IL-4 was increased from 8% to 32% (Table 1 and Fig. 3B). The step during which most of the contaminating proteins were removed was the sonication and centrifugation step separating the soluble proteins from the insoluble human IL-4 (lanes 1 and 2). During the dialysis to remove
the refolding reagents, precipitation of some contaminating proteins occurred (lane 5).

For further purification, the refolded protein was concentrated and passed over a Bio-Gel P-30 gel filtration column (separation range of 2500–40000 Da). The absorbance at 280 nm ($A_{280}$). ELISA and bioactivity profiles of the human IL-4 from this column are shown in Fig. 4A. There were two major peaks in the $A_{280}$ profile, the first of which was the void volume of the column and the second contained proteins eluting with the $M_r$ of human IL-4 (14500). Further characterization of the protein peaks with an ELISA using 11B4 showed the antibody reacting mainly with the proteins eluting in the second peak and to some extent with column fractions from the first protein peak. Bioassays localized activity mainly to the second peak.

Protein in the column fractions was analyzed by silver-stained PAGE (Fig. 4B). Fractions 22–28 (peak two) contained only human IL-4 and no contaminating proteins. The void volume peak (lanes 14–19) contained mainly high-molecular-mass proteins with some human IL-4 accounting for the ELISA reactions described.

Further characterization of the isolated protein was pursued on pooled column fractions 23–27.

**Characterization of the human IL-4**

To estimate the purity of human IL-4, an aliquot from the gel filtration step was analyzed on a RP-HPLC column. The $A_{280}$ profile gave a major symmetrical peak with some minor contaminants (data not shown). Based on peak integration, the human IL-4 purity was 95%.

The amino acid composition of the purified protein was determined to be the same as human IL-4 [3] with the addition of one mole methionine/mole protein (Table 2). This methionine was added to the mature sequence for the initiation of translation.

The identification of the N-terminal amino acid as methionine indicates that the amino acid specified by the initiation codon is not removed. The results of a further 19 sequenator cycles were consistent with the known N-terminal sequence of human IL-4 [3]. The yield of the polypeptide at the first step was identical to the amount determined by amino acid analysis on a parallel sample.

Ellman’s reagent did not react with the purified human IL-4 indicating that all the cysteine residues (six) are disulfide-linked. The sensitivity of the assay for thiol groups was 1 nmol. The amount of human IL-4 present was 10 nmol.

**DISCUSSION**

In this report we have demonstrated the expression of human IL-4 in the cytoplasm of *E. coli*. Human IL-4 was expressed at high levels and was found as aggregates in the low-speed pellet fraction after cell lysis. Denaturation and refolding of these aggregates was necessary to activate the expressed protein. The human IL-4 can be renatured by extraction and refolding procedures [10, 13] using guanidine and a mixture of reduced and oxidized glutathione. The use of urea in the extraction procedure did not yield active human IL-4 (result not shown), indicating that the choice of denaturant is important.

The protein concentration during the refolding procedure is critical. Too low a protein concentration can result in insta-
The human IL-4 protein has six cysteine residues with the potential to form three intramolecular disulfide bonds. It can be concluded from the results described here that the fully oxidized form represents the active molecule. Dialysis against a buffer containing 2 mM reduced glutathione caused a substantial decrease of activity (result not shown), reflecting the importance of the disulfide bonds for activity.

During separation of refolded monomeric human IL-4 from contaminants, an important observation was made regarding the choice of buffer in the recovery of monomeric human IL-4. Equilibration and elution with Tris buffer containing 2 mM reduced glutathione caused a substantial decrease of activity (result not shown), reflecting the importance of the disulfide bonds for activity.

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In conclusion, we have described here the expression of human IL-4 by denaturation and refolding of the protein, and the carbohydrates attached to mammalian-expressed human IL-4 are not necessary for the biological activity of the protein in the assays examined.

In conclusion, we have described here the expression of human IL-4 by denaturation and refolding of the protein, and the binding to the human IL-4 receptor are comparable for the E. coli and mammalian-derived recombinant human IL-4. This indicates that (a) the human IL-4 derived from E. coli is folded correctly and (b) the carbohydrates attached to mammalian-expressed human IL-4 are not necessary for the biological activity of the protein in the assays examined.

In conclusion, we have described here the expression of human IL-4 in E. coli, the activation of the aggregated human IL-4 by denaturation and refolding of the protein, and the purification of biologically active human IL-4 by gel filtration. The expression of human IL-4 in E. coli means that large quantities of this lymphokine can be produced. Our ability to refold this molecule correctly and recover its biological activities will facilitate further studies of its role in the immune system.

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