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Regular paper

Cloning and purification of functionally active Fas ligand interfering protein (FIP) expressed in *Escherichia coli*

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This report presents purification and characterization of the extracellular domain of rat Fas protein, called FIP (FasL interfering protein), expressed as inclusion bodies in *Escherichia coli*. FIP was extracted from the inclusion bodies, solubilized with 8 M urea, purified by a single-step immobilized metal ion (Ni^{2+}) affinity chromatography and refolded. SDS/PAGE and mass spectrometry analysis of the purified protein verified its purity. Fluorescence spectrum analysis showed that the refolding procedure caused structural changes which presumably might have led to oligomerization. The purified FIP has biological activities: it binds specifically soluble Fas ligand and protects human Jurkat lymphocytes against FasL-dependent apoptosis. This efficient procedure of FIP expression in *E. coli* and renaturation may be useful for production of therapeutically important proteins.

Keywords: recombinant protein purification, Fas, Fas ligand, interfering molecules, apoptosis

INTRODUCTION

The Fas ligand (CD95 ligand) is a 40-kDa type II membrane protein belonging to the tumour necrosis factor (TNF) α family of proteins (Smith *et al.*, 1994; Nagata, 1997). This family consists of trimeric ligands that induce defined cellular responses upon binding to their respective receptors. Fas and other members of the TNF receptor superfamily are type I membrane proteins. They are characterized by the presence of cysteine-rich motifs conferring an elongated structure on their extracellular domains (Nagata, 1997). The interaction of Fas with FasL results in activation of signalling pathways which initiate apoptosis (Scaffidi *et al.*, 1998; Krammer, 2000; Curtin & Cotter, 2003). Activation-induced cell death and Fas–FasL interactions have been shown to play significant roles in immune system homeostasis (Green, 2003) and apoptosis of anti-tumour immune effector cells (Ryan *et al.*, 2005). The Fas/FasL signalling components are expressed in the central nervous system and their expression strongly increases in neurodegenerative diseases (Ethel & Buhler, 2003; Choi & Benveniste, 2004). In addition to its pro-apoptotic functions, accumulating evidence demonstrates that FasL can activate numerous non-apoptotic signalling pathways (Wajant *et al.*, 2003), and that activation of these pathways can result in increased tumorigenicity and metastasis (Barnhart *et al.*, 2004). Thus, antagonizing FasL activity may be beneficial in many pathologic conditions.

A promising way to intervene with biological processes is through the control of protein–protein interactions by means of interfering molecules that modulate the formation of protein–protein complexes. Fas–Fc, which contains the extracellular domain of recombinant human Fas (aa 1–154) fused to the Fc domain of human IgG1 and antagonizes the activa-

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Abbreviations: DMF, dimethylformamide; FasL, Fas ligand; FIP, FasL interfering protein; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani broth; MS, mass spectrometry; MTT, thiazolyl blue tetrazolium bromide; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

tion of Fas by FasL, has been used for many *in vitro* studies (Cheng *et al.,* 1994; Nagata, 1997; Raoul *et al.,* 1999; Ciesielski-Treska *et al.,* 2001). Commonly used recombinant Fas-Fc is produced in HEK 293 cells to preserve the pattern of N-linked glycosylation at several sites in the Fas molecule. However, expression and purification of the recombinant Fas-Fc in human cells, is expensive and not readily available, thus alternative methods are desirable.

In the present study we describe a design, cloning, and purification methods to produce of a large amount of recombinant extracellular domain of rat Fas in *Escherichia coli*. SDS/PAGE and mass spectrometry analysis verified the purity of the recombinant protein preparation. We show that the recombinant protein exhibited biological activities: specific binding of soluble Fas ligand and an efficient blockade of FasL-induced cell death of human lymphoid Jurkat cells.

MATERIALS AND METHODS

Cloning and expression. Total RNA was isolated from rat brain as described (Zawadzka & Kaminska, 2005) using an RNA isolation kit (Qiagen). cDNA corresponding to the extracellular domain of mature rat Fas (spanning aa 21–176) was synthesized by extension of specific primers with 200 units of SuperScript II reverse transcriptase (Gibco BRL) and dNTP mix (final concentration 1 μM each). Primer sequences were as follows: 5′-TAGGTCGACAACTGCTTTCTCTTTCTGC-3′ and 5′-ACTGGATCCTCTCTTCGTGGCTGGAACCG-3′. The resulting cDNA was amplified by PCR with the second pair of specific primers (0.15 μM each) containing restriction sites (underlined) for *Nde*I 5′-CTGTCGACCATGCATATGCAAGGGACTGAT-AGC-3′ and for *Bam*HI 5′-GATGGATCCTATCAT-ATCAGCAGCCAAAGGAGC-3′. The resulting PCR product was digested with the restriction enzymes, purified using spun columns, cloned into pET28a (+) expression vector (Novagene) and sequenced. The cloning procedure shifted the open reading frame and the transcription termination site, which resulted in an additional stretch of 41 hydrophilic amino acids constituting a "solubilization enhancing domain".

Escherichia coli (Rosetta strain DE3, Novagen) were transformed by electroporation and 200 mL of LB medium supplemented with 30 μg/mL of kanamycin was inoculated in a 1 : 20 ratio. The bacteria were cultured at 37°C, 150 r.p.m. until OD_{600} reached 0.6. FasL interfering protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a 3 h incubation bacteria were collected by centrifugation.

Isolation of inclusion bodies and protein purification. Cell pellet was suspended in 10 mL of cold 50 mM Tris/HCl buffer (pH 8.0) containing 10 mM β-mercaptoethanol, 1 mM EDTA and 100 000 U/mL lysozyme, and kept for 30 min on ice. Genomic DNA was fragmented by sonication for 5 × 20 s (Digital Sonifier 250, Branson). Crude extract was centrifuged at 14 500 × *g*, 20 min at 4°C. Pellet was dissolved at room temperature in 100 mM phosphate buffer, pH 7.5, supplemented with 8 M urea. Denatured preparation was centrifuged at 14 500 × *g*, 20 min, 4°C and the supernatant was subjected to IMAC chromatography using 1.25 mL of a nickel resin (His-Select, Sigma). The column was equilibrated with 100 mM phosphate buffer, pH 7.5, containing 8 M urea. Proteins were eluted in a linear gradient of imidazole (0–250 mM) and 33 fractions of 2 mL were collected. Fractions characterized by the highest amount of protein (A_{280} > 0.5) were pooled.

Renaturation, fluorometric assay and mass spectrometry analysis. The FIP preparation was diluted to a concentration of 70 μg/mL, and subsequently dialyzed overnight at 4°C against 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA and 10 mM β-mercaptoethanol. The obtained preparation was dialyzed against phosphate-buffered saline (PBS) under the same conditions. Precipitated proteins were removed by centrifugation at 170 × *g* (1000 r.p.m. in MPW 370 centrifuge, rotator Nr. 12108), 5 min at 4°C.

Fluorometric assay was performed using Fluorog 2. Tryptophan fluorescence was excited at 295 nm and fluorescence spectra were measured in the range of 315–450 nm.

Protein samples were analyzed by liquid chromatography-electrospray mass spectrometry with collision fragmentation using an Electrospray Ionization–Quadrupole-Time Of Flight (ESI-QUAD-TOF) instrument (Micromass, Manchester, UK). The NCBInr database was searched with the obtained results using the Mascot software. Semitrypsin digestion with a maximum of one missed enzymatic cleavage, Cys and Lys carbamidomethyl and Met oxidation were covered. One microgram of final preparation was visualized on polyacrylamide gel by a silver staining as described elsewhere (Blum *et al.,* 1987).

Native polyacrylamide gel electrophoresis. Separation gel contained 12% glycerol, 50 mM Tris/glycine buffer, pH 9.1, 7.5% polyacrylamide/bisacrylamide (29 : 1), 600 μg/mL ammonium persulfate and 0.06% TEMED. Stacking gel contained 12% glycerol, 50 mM Tris/glycine buffer, pH 9.1, 4% polyacrylamide/bisacrylamide (29 : 1), 800 μg/mL ammonium persulfate and 0.12% TEMED. The protein preparation was mixed with glycerol (1 : 10) and loaded

onto the gel. Electrophoresis was performed at 12.5 V/cm, 4°C.

Western blotting. Native polyacrylamide gel electrophoresis was followed by electrotransfer (400 mA, 90 min, 4°C) onto nitrocellulose membrane (Hybond, Amersham Biosciences). The membrane was incubated with anti-His-tag monoclonal antibody (Sigma) and subsequently with anti-mouse antibody linked to horseradish peroxidase. Immunocomplexes were visualized using the enhanced chemiluminescence detection system (ECL, Amersham Biosciences).

Cell viability (MTT metabolism assay). Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (50 units/mL penicillin, 50 μg/mL streptomycin). Cells were sieved at a concentration of 6×10^4 cells/mL in 24-well culture plates in a total volume of 0.5 mL. Cells grew in a humidified atmosphere of CO_2/air (5%/95%) at 37°C. Twenty-four hours after plating the cells were treated with 400 ng of recombinant rat FasL (R&D) alone or in combination with 1.6 μg/mL of FIP or PBS. Twenty-four hours after the addition of FasL, MTT stock solution was added to a final concentration of 0.5 mg/mL. After 2 h of incubation at 37°C formazan crystals formed in actively metabolizing cells were dissolved in lysis buffer containing 20% SDS, 2.5% HCl, 2.5% acetic acid and 50% DMF. Absorbance was measured at 570 nm using a scanning multi-well spectrophotometer. All measurements were carried out in triplicates.

RESULTS

Purification of FasL interfering protein under denaturing conditions followed by refolding

Recombinant FIP protein was produced in *E. coli* Rosetta strain DE3 transformed with pET28a(+) plasmid coding for the extracellular domain of Fas receptor (ΔFas) fused to a histidine tag and a solubilization enhancing domain (Fig. 1A and B). The expression of FIP was induced by addition of 1 mM IPTG for 3 h at 37°C. Bacterial cells were lyzed by lysozyme digestion and sonication, and the extracts were separated into soluble and insoluble fractions by centrifugation. When aliquots of those fractions were analyzed by staining of SDS/PAGE gels with Coomassie Brilliant Blue (Fig. 1D, lane 3 and 4), a prominent protein band (25 kDa) was found in the insoluble fraction of the crude extract (Fig. 1D, lane 4).

Recombinant FIP was solubilized from inclusion bodies with 8 M urea, purified on an affinity columns and refolded by dialysis. Typically, 40 mg of total solubilized proteins from 200 mL of induced *E. coli* culture was loaded onto a Ni^{2+}-column and the bound proteins were eluted with 250 mM imidazole. The presence of recombinant protein in elution fractions was confirmed by immunoblotting with specific anti-6xHis antibody (Fig. 3). This step gave a relatively good result ending up with a highly purified preparation (Fig. 1D, lane 6). At this stage the determined yield was 14 mg of recombinant protein per 1 L of culture. The molecular mass of the recombinant FIP protein was about 25 kDa, as determined by SDS/PAGE, in agreement with the predicted molecular mass.

The purified preparation was dialyzed overnight against phosphate buffer supplemented with EDTA (to avoid protein aggregation) and β-mercaptoethanol. The removal of urea resulted in protein precipitation. The final protein concentration was 20 μg/mL. The total yield was 4 mg of native protein per 1 L of culture.

A fluorometric assay based on tryptophan spectra shifts, was performed in order to evaluate protein folding after renaturation. The spectrum of tryptophan fluorescence in the preparation subjected to dialysis had a maximum at 347 nm, shifted by 20 nm as compared to the preparation containing 8 M urea (with a maximum at 367 nm), showing conformational changes placing tryptophan residues into a more hydrophobic environment. In contrast, FIP preparation rapidly diluted 100-fold in dialyzing buffer had a fluorescence with a maximum at 375 nm (Fig. 2) and exhibited no biological activity (not shown).

In order to estimate the protein purity, 1 μg of the final preparation was run on SDS/PAGE followed by silver staining. FIP constituted about 80% of total proteins (Fig. 1E). The remaining 20% was represented by two major additional bands with molecular masses of about 18.8 kDa and 15.9 kDa. The final preparation was subjected to MS/MS analysis using an Electrospray Ionization–Quadrupole–Time Of Flight (ESI-QUAD-TOF) instrument. The obtained results identified additional proteins as *E. coli* glutamate synthase (GI 551808, corresponding to 18.8 kDa), β-galactosidase α peptide (GI 3559832, corresponding to 15.9 kDa) and transposition regulator protein (GI 10955466, corresponding to 50 kDa). This analysis confirmed a lack of potentially toxic bacteria-derived proteins which could affect mammalian cells.

Evaluation of biological activity of recombinant FIP

To detect direct protein–protein interactions, FIP and recombinant rat FasL, as well as appropriate controls, were incubated overnight at 4°C. Subsequently, the preparations were run on a native

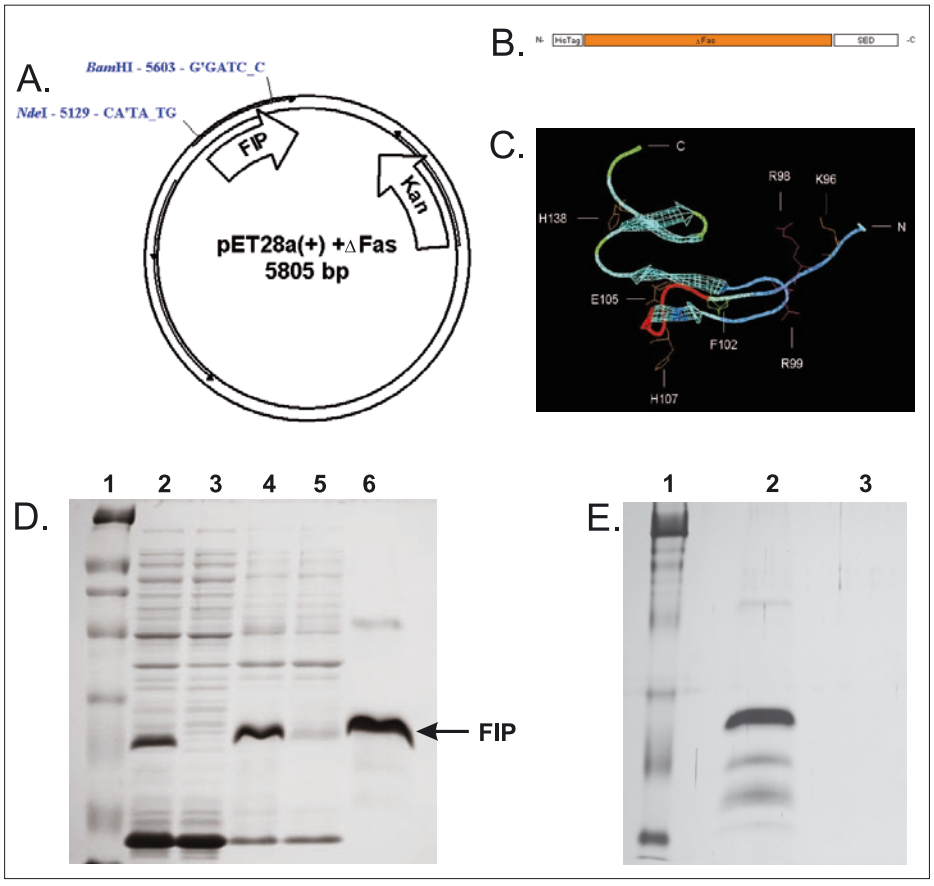

Figure 1. Schematic representation of plasmid encoding FIP, predicted FIP tertiary structure and SDS/PAGE of affinity-purified recombinant FIP.
A–B. A schematic view of the construct: cDNA encoding a truncated extracellular domain of rat Fas was cloned into pET28a (+) vector (A) and the expressed recombinant protein (FIP) consisting of Histidine Tag (HisTag), extracellular domain of rat Fas receptor (ΔFas) and a solubilization enhancing domain (SED) (B). **C.** Tertiary structure of FIP was predicted using First Approach mode at Automated Comparative Protein Modelling Server (Swiss-Model) by means of the following PDB templates: 1tnrR, 1ncfB, 1extA, 1ft4B. Amino acids potentially involved in binding of FasL are indicated. **D.** Polyacrylamide gel stained with Coomassie Brilliant Blue. Lane description: 1. Molecular mass standards (Sigma C1992-1VL); 2. *E. coli* crude extract; 3. Supernatant collected after crude extract centrifugation; 4. Pellet collected after crude extract centrifugation; 5. Flow-through collected during metal affinity chromatography; 6. Fraction eluted from nickel resin. **E.** One μg of FIP final preparation was run on SDS/PAGE and silver stained. Lanes: 1. Molecular weight standards (SIGMA C1992–1VL), 2. FIP final preparation, 3. Control buffer.

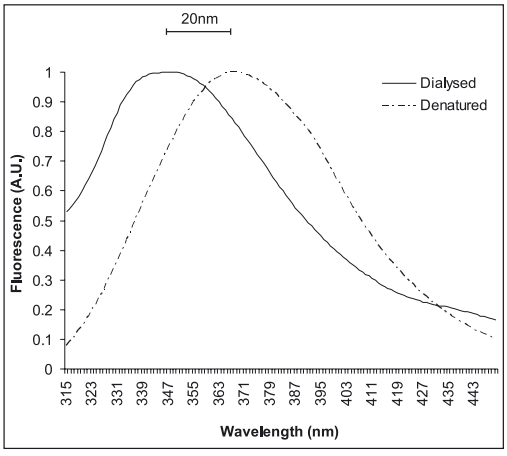

Figure 2. Verification of protein folding after renaturation. Fluorescence spectra of protein preparations: denatured, rapidly diluted in renaturation buffer, and subjected to renaturation process. The protein concentration was 20 μg/ml.

polyacrylamide gel at 4°C in order to preserve the preformed FIP-FasL complexes. Figure 3A presents the result of a Western blot analysis, which reveals a shift of the band representing FIP–FasL complexes as compared to FasL alone.

The biological, cytotoxic activity of recombinant rat FasL can be measured by evaluating its ability to induce apoptosis of Jurkat lymphoid cells. Therefore, to evaluate recombinant FIP activity in a biological system, Jurkat cells were cultured in the presence of FasL alone or together with FIP in a molar ratio of 1 : 4 (FasL/FIP). The cell viability, determined by the MTT metabolism test, was evaluated after 24 h. The obtained results show an approx. 30% decrease in cell viability in cultures treated with FasL alone, whereas the viability of cells treated with FasL/FIP was unaffected and remained at the

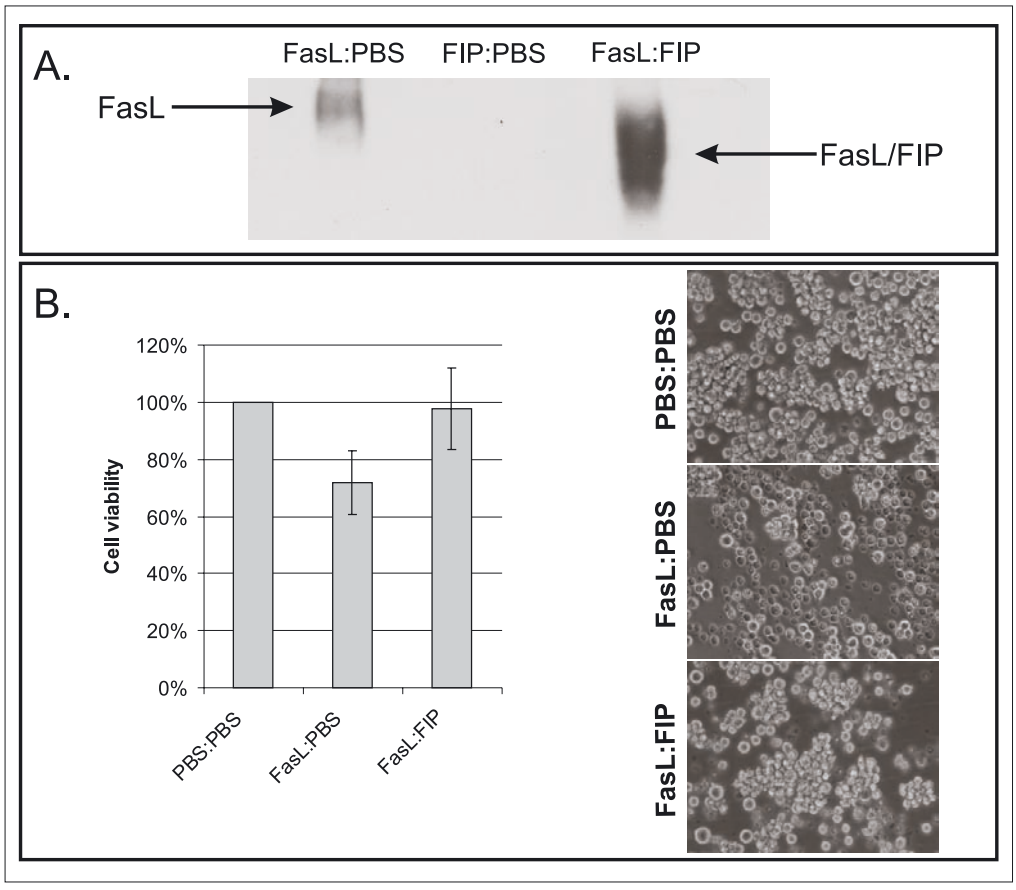

Figure 3. Evaluation of FIP biological activity.
A. Recombinant rat FasL or FIP alone or a combination of FasL with FIP in a molar ratio of 1 : 1 were incubated overnight. Subsequently, preparations were run on native PAGE, transferred onto a nitrocellulose membrane and probed with an anti-His-tag monoclonal antibody. **B.** Jurkat lymphoid cells were treated with recombinant rat FasL or with FasL and FIP in a molar ratio of 1 : 4. Cell viability was measured by MTT metabolism test 24 h after treatment (left panel). Right panel shows phase-contrast microscopy pictures of treated cells.

level of untreated cells (Fig. 3B). Phase-contrast microscopy shows a reduction of cell proliferation and colony formation in FasL-treated Jurkat cell cultures, while co-treatment with recombinant FIP blocked those changes.

DISCUSSION

A major cell surface receptor involved in the induction of apoptosis is Fas that is activated by binding Fas ligand (Curtin & Cotter, 2003). Fas-Fc, which contains the extracellular domain of recombinant human Fas (aa 1–154) fused to the Fc domain of human IgG1, antagonizes the activation of Fas by FasL. Fas-Fc peptide has been extensively used for many *in vitro* studies, mostly to verify if stimulus-induced apoptosis was mediated through Fas–FasL interactions (Cheng *et al.,* 1994; Nagata, 1997; Raoul *et al.,* 1999). Commonly used recombinant Fas–Fc is produced in human HEK 293 cells to preserve the pattern of N-linked glycosylation at several sites in the Fas molecule. Although commercially available, Fas-Fc is very expensive and used mostly for cell culture studies. It is not suitable for studies in rat models of disease.

In the present study we demonstrate the purification of the extracellular domain of rat Fas protein, called FIP (FasL interfering protein), expressed as inclusion bodies in *E. coli*. FIP was solubilized from inclusion bodies through denaturation, affinity chromatography and refolding. The final preparation was highly purified, 80% homogenous and MS/MS analysis revealed the presence of only traces of three bacterial proteins. The fluorometric assay based on tryptophan spectra shifts confirmed the protein folding after renaturation.

Domain deletion and mutational analysis of amino-acid residues in the Fas extracellular domain demonstrated critical residues in two domains that correspond to positions critical for ligand binding (Itoh & Nagata, 1993; Starling *et al.,* 1997; 1998). Two

amino acids, R86 and R87 in domain 2, are critical for the ability of Fas to interact with its ligand (Starling *et al.*, 1997).

Direct interactions between recombinant FIP and recombinant rat FasL protein were demonstrated by detection with anti-His-tag antibody preformed complexes of FIP–FasL separated by native gel electrophoresis. Purified FIP exhibited a biological activity and blocked FasL-induced apoptosis of Jurkat lymphoid cells. Recombinant rat FIP used in this study efficiently blocked the decrease in cell viability of human lymphoid cells induced by recombinant rat FasL.

It has been widely accepted that preservation of N-linked glycosylation pattern in the Fas molecule is necessary for its biological activity and secretion, thus it has to be expressed in mammalian cells (Li *et al.*, 2007). Our studies demonstrate that the extracellular domain of Fas expressed in *E. coli* is biologically active and retains the ability to interfere with FasL signalling. Although the recombinant FIP emerged as inclusion bodies, it could efficiently be solubilized with 8 M urea and purified to almost electrophoretic homogeneity by a single-step immobilized metal ion (Ni^{2+}) affinity chromatography and refolded by simple dialysis. This efficient procedure of FIP purification and renaturation may be useful for production of large quantities of this therapeutically important protein. It offers a simple alternative to the costly procedure of recombinant protein purification from human cells.

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