Mol Biotechnol (2015) 57:160–171
DOI 10.1007/s12033-014-9812-7

RESEARCH

New Strategy for High-Level Expression and Purification of Biologically Active Monomeric TGF-β1/C77S in *Escherichia coli*

Yana V. Kim · Marine E. Gasparian · Eduard V. Bocharov · Rita V. Chertkova · Elena N. Tkach · Dmitry A. Dolgikh · Mikhail P. Kirpichnikov

Published online: 5 November 2014

Abstract Mature transforming growth factor beta1 (TGF-β1) is a homodimeric protein with a single disulfide bridge between Cys77 on the respective monomers. The synthetic DNA sequence encoding the mature human TGF-β1/C77S (further termed TGF-β1m) was cloned into plasmid pET-32a downstream to the gene of fusion partner thioredoxin (Trx) immediately after the DNA sequence encoding enteropeptidase recognition site. High-level expression (~1.5 g l^{-1}) of Trx/TGF-β1m fusion was achieved in *Escherichia coli* BL21(DE3) strain mainly in insoluble form. The fusion was solubilized and refolded in glutathione redox system in the presence of zwitterionic detergent CHAPS. After refolding, Trx/TGF-β1m fusion was cleaved by enteropeptidase, and the carrier protein of TGF-β1m was separated from thioredoxin on Ni-NTA agarose. Separation of monomeric molecules from the noncovalently bounded oligomers was done using cation-exchange chromatography. The structure of purified TGF-β1m was confirmed by circular dichroism analysis. The developed technology allowed purifying biologically active tag-free monomeric TGF-β1m from bacteria with a yield of about 2.8 mg from 100 ml cell culture. The low-cost and easy purification steps allow considering that our proposed preparation of recombinant monomeric TGF-β1 could be employed for in vitro and in vivo experiments as well as for therapeutic intervention.

Keywords TGF-β1 · Monomeric TGF-β1 · TGF-β1/C77S · Thioredoxin-tag fusion protein · *Escherichia coli* · Refolding · CD spectrum

Introduction

Transforming growth factor beta 1 (TGF-β1) is a member of the transforming growth factor beta superfamily of cytokines that control many cellular processes including cell proliferation and differentiation, apoptosis, cellular homeostasis, wound repair, and other cellular functions [1]. Members of TGF-β family are synthesized as homodimeric proproteins with large amino-terminal prodomains, which are required for the proper folding and dimerization of the carboxy-terminal growth-factor domain [2]. The TGF-β1 precursor contains 390 amino acids with an N-terminal signal peptide of 29 amino acids required for secretion from a cell, a 249 amino acid pro-region (latency-associated peptide or LAP), and a 112 amino acid C-terminal region that becomes the active TGF-β1 upon activation. The TGF-βs are secreted from the cell as homodimers in their latent precursor form, where the LAP remains

Electronic supplementary material The online version of this article (doi:10.1007/s12033-014-9812-7) contains supplementary material, which is available to authorized users.

Y. V. Kim · M. E. Gasparian (✉) · R. V. Chertkova · E. N. Tkach · D. A. Dolgikh · M. P. Kirpichnikov
Department of Bioengineering, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, RAS, 16/10 Miklukho-Maklaya, 117997 Moscow, Russia
e-mail: marine_gasparian@yahoo.com

E. V. Bocharov
Department of Structural Biology, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry RAS, str. Miklukho-Maklaya 16/10, 117997 Moscow, Russia

E. V. Bocharov
NBIC Centre of NRC "Kurchatov Institute", Akademika Kurchatova pl. 1, 123182 Moscow, Russia

D. A. Dolgikh · M. P. Kirpichnikov
Biological Department, Lomonosov Moscow State University, 119991 Moscow, Russia

noncovalently associated with the C-terminal bioactive dimer and prevents the binding of TGF-β to its specific cell-surface receptor. Processing of active mature TGF-β dimers is regulated by many factors including enzymes, integrins, and low or high pH [3].

TGFβ signals through the sequential activation of two cell-surface receptors, termed type I (TβRI) and type II (TβRII), both of which have intrinsic serine/threonine protein kinase activity [4]. TβRII is a constitutively active kinase regulated by auto-phosphorylation. TβRII can bind ligands but requires TβRI for signaling, while TβRI alone is incapable of ligand binding [5]. Binding of TGFβ induces formation of a complex of the type I and type II receptors. Ligand binding allows for phosphorylation and activation of the type I receptor by the type II receptor kinase, followed by the phosphorylation of SMAD signaling proteins, which are the key intracellular signal transducers in the pathway downstream from the TGF-β receptors through the cytoplasm and into the nucleus [6].

Three isoforms of TGF-β, namely TGF-β1, TGF-β2, and TGF-β3, have been identified in mammals with highly similar sequences (homologies about 70–80 %) and peptide structures [7, 8]. The crystal structure of the TGF-β2 revealed a unique protein fold where eight cysteine residues form four intrachain disulfide bonds, creating the characteristic knotted arrangement. The dimer is stabilized by hydrophobic interactions and usually further strengthened by an intersubunit disulfide bridge between cysteine 77 residues of monomers [9, 10]. The solution structure of human TGF-β1 was determined using multinuclear magnetic resonance spectroscopy with hybrid distance geometry/simulated annealing [11]. The structure showed a high degree of similarity to that of TGF-β2, but with notable differences in structure and flexibility.

In early studies, TGF-β1 had been purified from human and porcine blood platelets, which are the richest source of TGF-β1 (20 mg/kg) or human placenta [12, 13]. After cloning of cDNA in 1985 [14], the recombinant mature dimeric TGF-β1 protein is purified mainly in CHO cells [15–18]. The major proteins secreted by these cells consisted of latent dimeric TGF-β complex comprising noncovalently associate precursor and mature forms of TGF-β with molecular weight of 90–110 kDa. Active 25 kDa TGF-β was produced by acid-induced dissociation of LAP complexes. These technologies allowed purifying about 1–5 mg of mature active dimeric TGF-β from 1 l of cell culture.

Existence of four intrachain and one interchain disulfide bonds in homodimers of mature TGF-β and extremely low solubility in neutral pH (30–50 ng/ml) created difficulties for correct folding of the dimeric molecules from mature monomeric protein expressed in bacterial strains. Several attempts were described (mainly in patent documents) for production of TGF-β2 and TGF-β3 isoforms in *E. coli* strains with direct expression of mature monomeric protein and further refolding and purification of active dimeric molecules from monomers using three-step purification and ion-exchange chromatography for separation monomeric and dimeric forms [19, 20]. Despite detailed description of refolding technology, no information about the refolding efficiency and final yield of carrier protein was elucidated in these studies.

The recombinant monomeric TGF-β1 and TGF-β3 preparations, where the cysteine residue C77 which normally forms the covalent link between the two TGF-β monomers was substituted to a serine, demonstrated the same binding affinity to the type II receptor [21, 22]. However, the monomeric TGF-β1 and TGF-β3 were less potent in the classic assay for growth inhibition in mink lung epithelial cells. On the other hand, it was shown that monomeric TGF-β3 showed greater effectiveness than did the dimeric form in in vivo wound-healing experiments [23].

In this work, we report a novel and effective approach for expression, refolding, and purification of human monomeric TGF-β1 in *Escherichia coli* based on expression and refolding of thioredoxin/TGF-β1 fusion which allows us to obtain 2.8 mg of highly purified, biologically active tag-free protein from 100 ml cell culture. This approach with relatively easier purification steps and the low cost of developed technology may be recommended for the production of TGF-β family proteins for in vitro studies and applications in medicine.

Materials and Methods

Materials

Host strain *E. coli* BL21(DE3) and plasmid pET-32a were obtained from Novagen (USA). Restriction enzymes and Pfu DNA polymerase were from MBI Fermentas (Lithuania). Ni-NTA agarose was from Qiagen (Germany). Recombinant human enteropeptidase light chain (L-HEP) and recombinant human TGF-β1 type II receptor-extracellular domain (TβRII-ED) were prepared in our laboratory as described before [24, 25]. Recombinant human TGF-β1 and recombinant mouse IL-2 were from R&D systems (USA). Cell culture medium was from PanBiotech (Germany), and fetal bovine serum was from Hyclone (USA). Other reagents used in this study were purchased from AppliChem (Germany).

Design of Oligonucleotides, PCR Amplification, and Cloning

The DNA sequence encoding the mature human TGF-β1 (336 bp) with 112 residues was synthesized from 14

overlapping oligonucleotides (35–40 nucleotides each) by polymerase chain reaction (PCR). The PCR reaction with Pfu DNA polymerase was performed in two steps. In the first step, two halves of the gene were synthesized in separate reactions. In both PCR reactions, 50 pmol of the first and the last oligonucleotides and 2.5 pmol of all the others in a final volume of 100 µl were mixed. PCR was performed by heating the mixtures for 5 min at 95 °C followed by 20 cycles of 95 °C for 30 s, 47 °C for 30 s, and 72 °C for 45 s. The PCR products were eluted from a 1.5 % agarose gel, and 14 ng of both, with 50 pmol of the first and the last oligonucleotides, were used to produce the complete DNA sequence of TGF-β1 gene. The prokaryotic codon usage table was used to substitute the codons, which have low expression level in prokaryotes (Fig. S1). The mixture was heated for 5 min at 95 °C followed by 33 cycles of 95 °C for 1 min and 72 °C for 2 min. The amplified DNA sequence encoding the gene of human TGF-β1 was cloned into pET-32a expression vector with restriction sites *Bgl*II and *Hin*dIII downstream to the gene of fusion partner thioredoxin (Trx) immediately after the DNA sequence encoding enteropeptidase recognition site. To obtain the monomeric form of TGF-β1m (TGF-β1/C77S), the cysteine 77 forming intermolecular disulfide bond in mature dimer was substituted to serine by site-directed mutagenesis using appropriate oligonucleotides. TGF-β1 gene was confirmed by DNA sequencing.

Expression, Refolding, and Purification of TGF-β1m

E. coli BL21(DE3) cells were transformed by plasmid pET-32a/TGF-β1m. One colony was inoculated into 10 ml LB supplemented with 100 µg/ml ampicillin and grown overnight at 37 °C. The culture was diluted in 1:100 ratio by adding TB culture media with 100 g/ml ampicillin and grown for 2.5–3 h shaking at 37 °C up to OD_{600} nm = 0.6. Protein expression was induced in the presence of 0.05 mM IPTG. Cells cultivation was continued for 24 h with stirring at 28 °C. The cells were collected by centrifugation at 4 °C at 5,000×*g* for 10 min, resuspended in 37 ml of lysis buffer containing 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, and1 % Triton X-100, and disrupted using French pressure at 2000 psi. The inclusion bodies were collected by centrifugation at 75,000×*g* for 30 min at 4 °C, washed with lysis buffer twice and solubilized in the buffer containing 0.1 M Tris–HCl (pH 8.6), 6 M guanidine-HCl, 50 mM DTT, and 1.0 mM EDTA. After incubation at room temperature for 4 h by gently stirring with the magnetic stirrer, the insoluble materials were removed by centrifugation at 75,000×*g* for 30 min at 4 °C. The solubilized protein was dialyzed at room temperature against 3 M guanidine-HCl (pH 2.5), mixed with equal volumes of oxidation buffer containing 50 mM Tris–HCl (pH 8.3), 6 M guanidine-HCl, and 0.1 M oxidized glutathione. The mixture was dialyzed against 3 M guanidine-HCl (pH 8.0) overnight at room temperature. To initiate disulfide exchange and refolding, the protein solution was dropwise diluted in buffer containing 100 mM Tris–HCl (pH 8.5), 1 M NaCl, 1 % zwitterionic detergent CHAPS, and 5 mM reduced glutathione. The protein concentration in refolding buffer was no more than 0.2 mg/ml. After incubation for 4 days at 4 °C, the protein was concentrated up to 0.6 mg/ml using Amicon Ultrafiltration Stirred Cell 8400 (Millipore, USA) fitted by ultrafiltration membrane YM with a nominal cut-off of 30 kDa (Millipore, USA). The Trx/TGF-β1m fusion was purified by metal affinity chromatography on Ni-NTA agarose in the presence of 0.5 % CHAPS in all buffers and dialyzed against 5 l of enteropeptidase cleavage buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.05 % CHAPS. After dialysis, aggregates were removed by filtration using Millex-GV syringe filter unit with pore size of 0.22 µm. CHAPS was added up to 0.5 %, and the fusion was cleaved by recombinant human enteropeptidase light chain at substrate concentration 0.2 mg/ml for 6–8 h at room temperature. To improve the efficiency of fusion cleavage by enteropeptidase, the lysine residue in specific cleavage sequence D_4K was substituted by arginine by site-directed mutagenesis, as was described before [26]. Cleaved TGF-β1 was separated from thioredoxin on Ni-NTA agarose. After dialyzing against a buffer containing 20 mM sodium acetate (pH 4.0) and 30 % isopropanol, the final purification step of TGF-β1m was performed by cation-exchange chromatography on a Source 15S column (GE Healthcare, Sweden) in 0–400 mM linear NaCl gradient using the ÄKTA-FPLC system (GE Healthcare, Sweden). After purification, the protein was dialyzed against 100 mM acetic acid overnight, lyophilized, and stored at −70 °C for further investigation. For future experiments, the lyophilized TGF-β1m was dissolved in 4 mM HCl at concentration range of 20–50 µg/ml. The N-terminal amino acid sequence of TGF-β1m was determined after SDS-PAGE and electroblotting of protein sample onto polyvinylidene difluoride (PVDF) membrane by means of Edman's N-terminal automatic sequencing on Beckman LF3000 Protein Sequencer. Spectrophotometric determination of protein concentration was done at 280 nm using the absorbance value 2.21 as the extinction coefficient for a 0.1 % (1 mg/ml) calculated by the PC GENE computer analysis program of protein sequences (IntelliGenetics). The quality and purity of the protein samples were evaluated by Tricine-SDS-PAGE in the presence or absence of 2-mercaptoethanol (βME) using the Coomassie Brilliant Blue R-250 staining or Silver-staining method for visualization according to the manufacturer's specifications.

Growth Inhibition Assay

Biological activity of purified TGF-β1m was analyzed on HT-2 (clone A5E-Murine IL-2 dependent T lymphocyte, ATCC® CRL-1841™) and Mv1Lu (NBL-7, Mink lung epithelial cells) cells. HT-2 cells were maintained in RPMI 1640 medium with 2 mM L-glutamine supplemented with 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 5 ng/ml mouse IL-2, and 10 % fetal bovine serum. For cell growth inhibition assay [27], 100 μl of HT-2 cells 7×10^4 cells/ml was seeded in 96-well plates, and 100 μl of medium containing serially diluted TGF-β1 or TGF-β1m was added. In neutralization experiments, TGF-β1 and TGF-β1m were preincubated in complete medium containing TBRII-ED (1:10,000 ratio) for 1 h before being added to the cells. The plates were incubated at 37 °C with 5 % CO_2 for 48 h. Cell viability was quantified by WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay as described before [28]. Briefly, equal volumes of stock solutions of 10 mM WST-1 (dissolved in 20 mM HEPES, pH 7.4) and 0.2 mM PMS (1-Methoxy-5-methylphenazinium methyl sulfate, dissolved in water) were mixed and 20 μl of mixture was added to each well. The plates were incubated for 4 h, and the absorbance was measured at 450 nm with the reference wavelength at 655 nm using microplate reader (Bio-Rad 680, USA).

Mv1Lu cells were cultured as a monolayer in 25-cm^2 flask at 37 °C and atmosphere of 5 % CO_2 in the air. The cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) of fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. For growth inhibition assay [29], cells were trypsinized, washed with PBS, and seeded in 96-well plates at 7×10^3 cells per well in DMEM medium supplemented with 10 % FCS and antibiotics. After cells had attached (4 h), the medium was replaced, and TGF-β1 or TGF-β1m in serial dilution was added. The plates were incubated in a 5 % CO_2 atmosphere at 37 °C for 48 h. Cell growth inhibition was analyzed by MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) test measuring the absorbance of formazan solution in wells at a wavelength 540 nm with background subtraction at 655 nm using microplate reader (Bio-Rad 680, USA).

Circular Dichroism (CD) Spectroscopy

CD spectrum of 40 μM solution of TGF-β1m in 4 mM HCl (pH 3.0) was measured with a JASCO-810 spectropolarimeter (Jasco, Tokio, Japan) at 20 °C using a quartz cell of 0.1-mm path length. The experimental data were expressed as mean molar ellipticity. The contribution of the buffer was subtracted. The analysis of the CD spectrum was performed using the program CONTINLL [30].

Smad3 Phosphorylation Assays

Mv1Lu mink lung epithelial cells were treated with different concentrations of TGF-β1 and TGF-β1m for 30 min. The cells were washed twice with ice-cold PBS and lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 % NP-40, 0.1 % SDS, and 0.5 % Na-deoxycolate supplemented with protease inhibitor cocktail. Samples containing 20 g of total protein were separated by 14 % SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked at 4 °C overnight with TBST buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1 % (v/v) Tween-20) containing 5 % non-fat dried milk. Afterward, the membrane was incubated with a rabbit polyclonal anti-phospho-Smad3 antibody (Millipore, USA) or a mouse anti-actin antibody (Sigma-Aldrich, USA) for 2 h at room temperature. After washes with TBST, the membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch, USA) for 1 h at room temperature, washed again, and developed with ECL prime western blotting detection reagent (GE Healthcare, Sweden).

Limulus Amebocyte Lysate (LAL) Assay

The gel-clot technique was used for detecting and quantifying endotoxins based on clotting of the LAL solution (Charles River Endosafe, USA) in the presence of endotoxin. The lyophilized samples of TGF-β1m were dissolved in 4 mM HCl at concentration 0.4 mg/ml and a serial dilution of the protein was carried out using the LAL reagent water. Diluted samples (100 μl) were mixed with 100 μl of reconstituted Endosafe LAL reagent in depyrogenated tubes. Solutions were mixed gently and incubated for 1 h at 37 °C. A negative control containing only LAL reagent water, positive controls containing endotoxin standard, and positive specimen controls containing diluted samples with an amount of endotoxin in the sample that exceed the reagent's labeled sensitivity by twofolds were performed concurrently. After incubation, each tube was inverted by 180°. A negative test was characterized in the absence of gel or by the formation of a viscous mass, which does not hold when the tube is inverted.

Results

Expression, Refolding, and Purification of TGF-β1m

The fusion protein Trx/TGF-β1m containing 6-His tag and enteropeptidase cleavage site (Fig. 1) was largely

expressed (approximately 1.5 g from 1 l cell culture) in *E. coli* strain BL21(DE3) transformed with pET-32a/TGF-β1m plasmid. Almost the entire 29.5 kDa fusion (17 kDa thioredoxin with a linkers and 12.5 kDa TGF-β1m protein) was in insoluble form forming inclusion bodies (Fig. 2). The Trx/TGF-β1m was purified from inclusion bodies by solubilization and refolding in glutathione redox system in the presence of 1 % CHAPS, as was described in "Materials and Methods" section. The refolded protein was concentrated up to 0.6 mg/ml, purified on the Ni-NTA agarose at pH 8.0 in the presence of 0.5 % CHAPS, and cleaved by recombinant human enteropeptidase catalytic subunit (Fig. 3a). The solubility of Trx/TGF-β1m fusion was quite high (up to 0.6 mg/ml), but we performed the cleavage at concentration no more than 0.2 mg/ml as TGF-β1m formed insoluble aggregates after cleavage of fusion at concentration range higher than 0.1 mg/ml at pH 8.0, even in the presence of detergent (data not shown). Earlier it was shown that TGF-β3 strongly aggregated at pH range of 6.5–8.5 at concentration 0.1 mg/ml [31]. The substitution of the lysine residue to arginine in the enteropeptidase cleavage sequence D_4K significantly improved the efficiency of fusion cleavage, which allowed saving the enzyme (enzyme-substrate molar ratio 1:1,43,000 for Trx-D_4R-TGF-β1m instead of 1: 53,200 for Trx-D4 K-TGF-β1m) and improving the yield of the desired product (Fig. 3a). After cleavage of fusion, two major bands were detected in SDS-PAGE gel with molecular weights of 17 and 12.5 kDa corresponding to thioredoxin and TGF-β1m, respectively (Fig. 3b). An additional weak band of 15.4 kDa corresponded to the nonspecific cleavage of carrier protein thioredoxin after arginine 143 residues at the $KFER^{143}\downarrow QHM$ site (determined by amino acid sequence analysis of gel bands) [26]. About 90 % of pure preparation of TGF-β1m was obtained after separation of the carrier protein from thioredoxin on the Ni-NTA agarose (Fig. 3b, lane 3). After SDS-PAGE and electroblotting of purified TGF-β1m on PVDF membrane, the N-terminal amino acid sequence (ALDTNYCF) was determined. This sequence coincides with 1–8 amino acid sequence of human TGF-β1 confirming the identity of the recombinant protein. The final purification of TGF-β1m by cation exchange chromatography revealed the existence of dimeric, trimeric, and other oligomeric molecules, which probably were formed during purification procedure (Fig. 4a, b). Only purified monomeric form was capable to phosphorylate the Smad3 protein in Mv1Lu cells at concentration range of 2–10 ng/ml (Fig. 4c).

The developed technology allowed us to purify 2.8 mg of TGF-β1m from 100 ml cell culture (Table 1). However, described refolding procedure is not good enough for production sufficient quantities of active dimeric TGF-β1

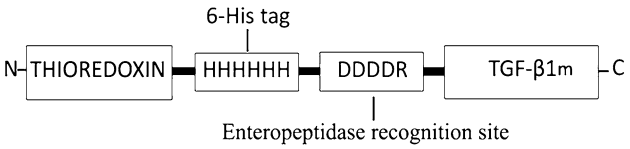

Fig. 1 The schematic representation of Trx/TGF-β1m fusion protein

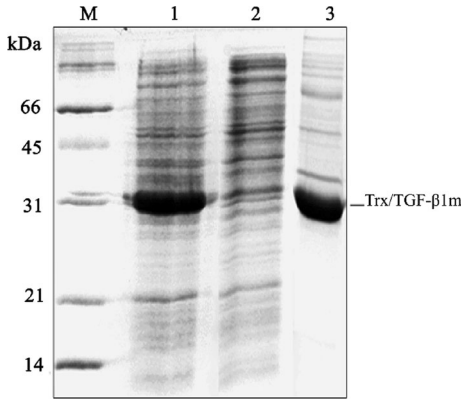

Fig. 2 Analyses of solubility of expressed Trx/TGF-β1m fusion. The expression of Trx/TGF-β1m fusion was induced by 0.05 mM IPTG in *E. coli* strain BL21(DE3)/pET32a/TGF-β1m. The samples containing 10 g protein were analyzed in 14 % SDS-PAGE followed by protein staining with Coomassie Brilliant Blue R-250. *M* molecular weight markers. *Lane 1* total cell lysate, *lane 2* fraction of soluble cytoplasmic proteins, *lane 3* fraction of insoluble proteins solubilized in 6 M guanidine

from native monomers due to formation of large amount noncovalently bound dimers and oligomers (data not shown).

It should be mentioned that the level of LPS determined by LAL test was quite low (<1 EU/mg protein) which allows applying TGF-β1m preparation in medical applications.

Analysis of Circular Dichroism (CD) Spectrum of TGFβ1 Monomer

The analysis of the CD spectrum (having a broad minimum at 210 nm) of the monomeric TGFβ1m mutant reveals the presence of about 42, 22 and 32 % of β-sheet, turn and coil structure, respectively, but only 4 % of α-helix (Fig. 5a). Such amount of β-structure is almost consisting with the NMR spatial structure of the TGF-β1 dimer (PDB ID: 1KLA) calculated with the program MOLMOL [32], while the amount of α-helices was three fold less (Fig. 5b, c; Table 2). The finding that the TGF-βs α-helices can be rather flexible [33] would explain the small amount of α-helix structure observed for the TGF-β1m. In addition, the distortion of the dimer by the Cys77Ser mutation breaking

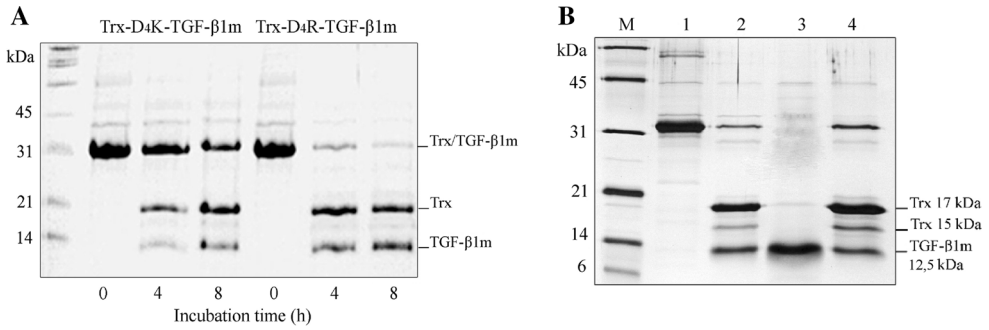

Fig. 3 Cleavage of Trx/TGF-β1m enteropeptidase and purification of TGF-β1m on Ni-NTA agarose. **a** Lys/Arg substitution in enteropeptidase cleavage site D_4K improved the efficiency of Trx/TGF-β1m fusion cleavage. Samples containing 10 g of Trx-D_4K-TGF-β1m or Trx-D_4R-TGF-β1m fusions were incubated with recombinant human enteropeptidase catalytic subunit at enzyme–substrate molar ratio of 1:1,43,000 for different times at 25 °C and analyzed in 14 % SDS-PAGE followed by protein staining with Coomassie Brilliant Blue R-250. **b** Purification of TGF-β1m after cleavage by enteropeptidase on Ni-NTA agarose. Samples containing 0.5–1 g protein were analyzed on 14 % SDS-PAGE by silver staining. *M* molecular weight markers. Purified Trx/TGF-β1m fusion before (*lane 1)* and after cleavage by enteropeptidase. *Lane 3* and *lane 4* protein fractions eluted from Ni-NTA agarose column with 20 and 250 mM imidazole, respectively

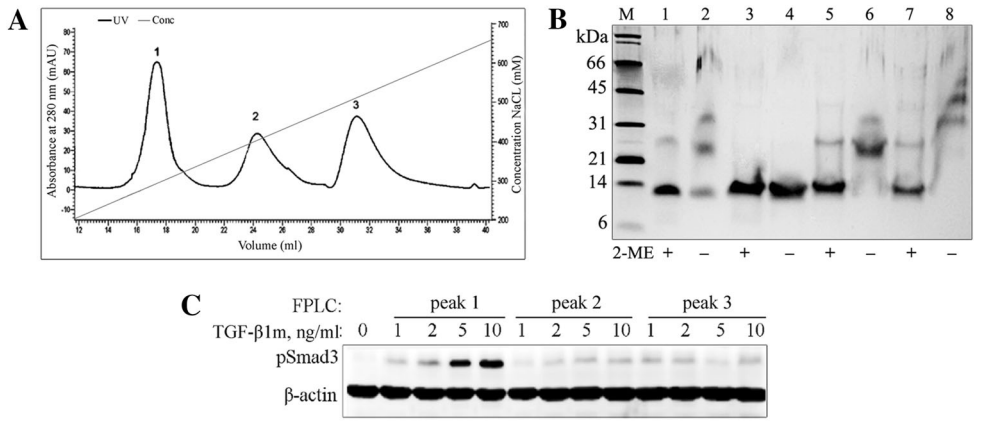

Fig. 4 Purification of TGF-β1m by cation-exchange chromatography on Source 15S column. **a** FPLC cation exchange elution profile of purification of TGF-β1m. Three major peaks (indicated as *1*, *2*, *3*) are eluted as a function of increasing concentration of NaCl. **b** The proteins fractions eluted from Source 15S column (*panel a*) were analyzed on Tris-Tricine SDS–PAGE by silver staining under both reducing (+2-ME) and non-reducing (−2-ME) conditions. *M* molecular weight markers. *Lanes 1, 2* TGF-β1m before purification. *Lanes 3, 4* samples corresponded to *peak 1*, *lanes 5, 6* samples corresponded to *peak 2* and *lanes 7, 8* samples corresponded to *peak 3*. **c** Western blot analysis of SMAD3 phosphorylation induced by purified fractions of TGF-β1m in Mv1Lu cells. Control for equal loading (β-actin) is aligned below

intermolecular S–S bond can also destabilize the α-helices situated in the TGF-β1 dimer interface.

Biological Activity of Recombinant TGF-β1m

As the commercial monomeric TGF-β1 is not available, we compared the biological activity of the TGF-β1m with TGF-β1 dimer expressed and purified from CHO cells (R&D systems). Mv1Lu mink lung epithelial cells were treated with different concentrations of TGF-β1 and TGF-β1m for 30 min, and the cell lysates were analyzed by western blotting with phospho-Smad3 antibodies (Fig. 6a). The results indicated that more than 100-fold higher amount of TGF-β1m was needed for phosphorylation of Smad3 in comparison to the dimeric TGF-β1. Similar results were obtained for preparation of recombinant monomeric TGF-β3 expressed and purified in *E. coli* [34].

The biological activity of TGF-β1m was also characterized in classical growth-inhibition assays on T lymphocyte HT-2 and Mv1Lu cells (Fig. 6b, c). Both cells were treated with the TGF-β1m and dimeric TGF-β1 over a range of concentrations for 48 h. The dose–response curves showed that the monomeric TGF-β1m was less potent inhibitor in both cells when comparing dimer with an ED_{50}

Table 1 Summary of TGF-β1m purification from 100-ml cell culture

Purification step	Protein (mg)	Overall protein recovery (%)	
		Trx/TGF-β1m	TGF-β1m
Washed inclusion bodies	Trx/TGF-β1—150 mg (TGF-β1—63 mg)	100	42[a]
After refolding	120	80	
Ni-NTA agarose purification	80	53	
Fusion cleavage by enteropeptidase	55	37	
Ni-NTA purified TGF-β1m	8.7		13.8
FPLC purified TGF-β1m	2.8		4.4

[a] The percentage of TGF-β1m as a part of Trx/TGF-β1m

Table 2 The structural analysis of the monomeric TGF-β1m mutant based on CD spectrum

Sample	α-helix (%)	β-sheet (%)	Turn (%)	Coil (%)	NRMSD[a]
Recombinant TGFβ1m from *E. coli*	4	42	22	32	0.109
TGFβ1m calculated from PDB ID: 1KLA[b]	12	40	21	27	

[a] NRMSD (normalized root-mean-square deviation) shouldn't exceed 0.1 [30]

[b] The distribution of the secondary structure elements for the TGFβ1 dimer (PDB ID:1KLA) was estimated with the program MOLMOL [32]

close to 5.7 ± 1.1 ng/ml versus 0.04 ± 0.1 ng/ml for TGF-β1 dimer. TGF-β1m only at concentrations of 40 ng/ml elicited a maximal growth-inhibitory response, compared to 0.25 ng/ml for native TGF-β1 dimer. Earlier it was demonstrated that monomeric TGF-β1/C77S obtained from CHO cells elicited a maximal growth inhibitory response on MvlLu cells at concentrations of 26–40 ng/ml, compared to 0.4–0.6 ng/ml for native TGF-β1 [21]. Preincubation of TGF-β1 as well as TGF-β1m with type II receptor extracellular domain TβRII-ED completely

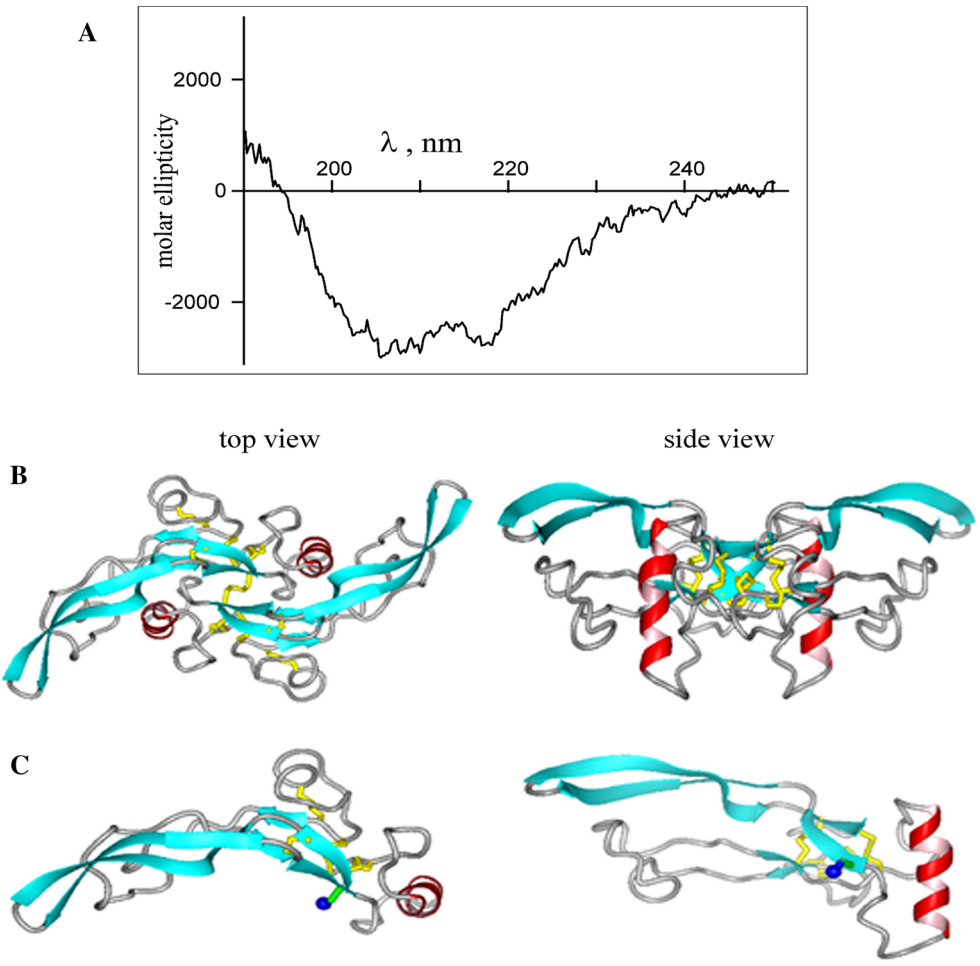

Fig. 5 Structural analysis of TGFβ1m. **a** CD spectrum of monomeric TGFβ1m mutant. **b** Ribbon representation of the NMR structure of the TGFβ1 dimer (PDB ID: 1KLA). The β-sheets, α-helices and S–S bonds are highlighted in *cyan*, *red*, and *yellow*, respectively. **c** Ribbon representation of the TGFβ1 monomer subunit with inserted Cys77Ser mutation breaking intermolecular S–S bond and dimer interface is shown as *blue* ball. The spatial structures were visualized with the program MOLMOL [31]

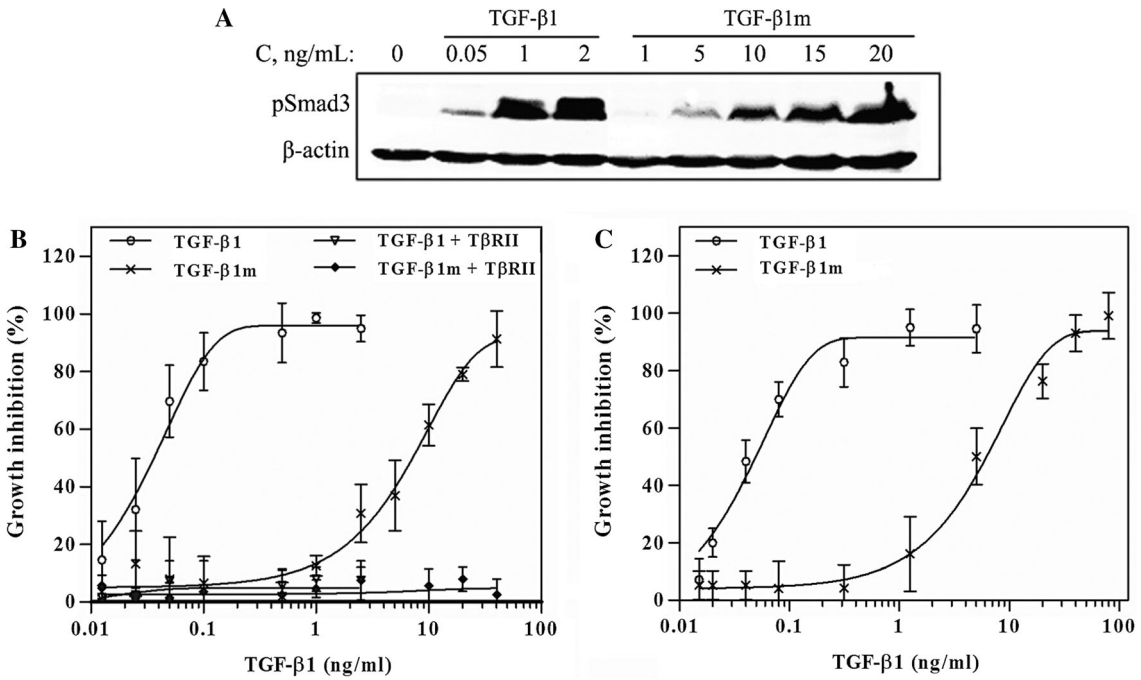

Fig. 6 Comparison of biological activities of TGF-β1m purified from *E. coli* and recombinant TGF-β1 (dimer, R&D system) purified from CHO cells. **a** Induction of SMAD3 phosphorylation in Mv1Lu cells by monomeric and dimeric TGF-β1. Control for equal loading (β-actin) is aligned below. **b** Growth inhibition of HT-2 cells induced by recombinant preparations of TGF-β1 in the presence and the absence of type II receptor (TβRII) was performed using WST-assay. **c** Growth-inhibition activities of TGF-β1 dimer and TGF-β1m were performed on Mv1Lu cell using MTT-assay. The scale on the ordinate in **b** and **c** represents absorbance as a percentage of values from control cells

neutralized the growth inhibitions of both preparations in HT-2 cell demonstrating the specific action of monomeric TGF-β1. Our results indicated that the TGF-β1 monomers could be successfully refolded from recombinant bacterial protein to the full biological active form.

Discussion

One of the disadvantages of using TGF-βs as therapeutic agents is to produce biologically active protein dimers using a prokaryotic host, when intensive renaturation to the biologically active dimeric form is required. Finding that monomeric TGF-βs are able to exert biological activities makes possible, the usage of monomeric TGF-βs as effective medicaments capable of utilizing the biological properties of a selected TGF-β. The synthesis, refolding, and secretion of TGF-βs isoforms are quite complicated processes. The three TGF-βs are synthesized as proproteins (pro-TGF-βs) with large amino-terminal pro-domains—LAPs, which are required for proper folding and dimerization of carboxy-terminal growth-factor domain (mature peptide) [2]. After folding and dimerization, TGF-β dimer is cleaved from its propeptides, but it remains associated with its pro-peptide through noncovalent interactions creating "large latent complex" which then is released into extracellular matrix. In mammalian expression systems, isoforms of TGF-β are expressed and secreted with LAP in inactive form. After purification, active mature protein can be dissociated from LAP under extreme pH, by heating to high temperature, or in the presence of proteases and glycosidases [35].

Complicated processes of synthesis, folding, and activation create difficulties in obtaining active TGF-βs from bacterial expression systems. The only recourse describing expression, refolding, and purification of active dimeric TGF-β2 and TGF-β3 from bacteria was described in 2000, but the yield of target protein was not indicated [19]. Also couple of works concerning expression, refolding, and purification of TGF-β1 and TGF-β2 isoforms fused with collagen-binding protein are described recording yield of about 40–80 μg from 50 ml cell culture [36, 37]. However, in these studies, the monomeric molecules were not separated from dimers after refolding procedure, and it is not clear if the obtained activity is concerned with dimers. Direct expression, refolding, and purification of monomeric TGF-β isoforms were described recently in patented document where the yield of purified target proteins has not been indicated [23]. It should be mentioned that in case of direct expression of mature TGF-β isoforms in *E. coli*, additional N-terminal methionine residue is required.

In this work, we have described a novel strategy for high-level expression and purification of monomeric

tag- and N-terminal methionine-free TGF-β1 in *E. coli*. Since all the members of TGF-β family proteins are highly hydrophobic and insoluble at neutral pH ranges, which creates difficulties for refolding and purification, we have used fusion expression system choosing thioredoxin as a carrier partner. *E. coli* thioredoxin is a compact, highly soluble, highly translated, and thermally stable protein with robust folding characteristics. We chose Trx mainly for three reasons. First, in our experience, it is particularly easy to express proteins as Trx fusions in *E. coli* to high expression levels (up to 40 % of the total cellular protein) [38]. This extraordinary level of expression suggests that thioredoxin is translated very efficiently. This property is often conferred to heterologous proteins fused to thioredoxin, especially when thioredoxin is positioned at the amino acid terminus of the fusion where protein translation initiated [39, 40]. Second, thioredoxin enhanced the solubility of targeted protein and facilitated the protein folding [41]. In addition, thioredoxin is small (12 kDa) and therefore constitutes only a minor proportion of the total mass of most protein fusion. Using the thioredoxin as a carrier protein allowed us to obtain highly rich expression level of Trx/TGF-β1m fusion (~1.5 g l^{-1}) from bacterial strain BL21(DE3); however, almost the whole expressed protein was in insoluble form. Direct or indirect expression of TGF-β isoforms in *E. coli* always resulted in the accumulation of recombinant protein in inclusion bodies [19, 35]. The solubility of refolded fusion Trx/TGF-β1m was 0.6 mg/ml at pH 7.5–8.0, while under physiological conditions (pH 7.4), TGF-β isoforms have almost the highest tendency to aggregate and remain in solution only at nanomolar concentrations (25–100 ng/ml) [32]. For this reason, usually the purification of recombinant TGF-βs has been performed at low pH (2.5–4) [12, 13, 20, 23]. In our case, we had the problem to cleave the purified Trx/TGF-β1m protein by enteropeptidase as the working range of enzyme is at pH 6.5–8.5 [42]. Cleavage of fusion at neutral pH resulted in aggregation and precipitation of 90 % of TGF-β1m protein (data not shown). To escape the aggregation of TGF-β1m at neutral pH, the cleavage of fusion by enteropeptidase was carried out at low concentration of Trx/TGF-β1m (less than 0.2 mg/ml) in the presence of 0.5 % of CHAPS detergent. In these conditions, only 16 % of protein was precipitated (Table 1). After separation of thioredoxin molecules on Ni-NTA agarose, the yield of purified TGF-β1m was 8–9 mg from 100-ml cell culture. However, the cation-exchange chromatography purification of this preparation revealed that only about 30 % were monomeric molecules, and more than 60 % of the protein was in inactive dimeric, trimeric, and higher oligomeric forms (Fig. 4). These results may explain the difficulties in obtaining active dimeric molecules upon monomers refolding, as even the monomers with substituted cysteine 77 to serine formed noncovalently linked inactive dimers. In fact, described refolding procedure was not good enough for production of sufficient quantities of active dimeric TGF-β1 from native monomers due to formation of large amounts of noncovalently bounded inactive dimers (data not shown). It should be mentioned that the level of LPS was quite low in purified preparation of TGF-β1m (<1 EU/mg protein), which allows applying TGF-β1m preparation in medical applications. The low-level expression of endotoxins in our preparation can be attributed to the purification procedure. First, we have purified the Trx/TGF-β1fusion from inclusion bodies after disruption of the cells in 1 % Triton X-100 following two washing steps in Triton X-100 (1 %) and water [43, 44]. Second, the existence of zwitterionic detergent CHAPS in the refolding and purification steps interferes with the interaction of LPS with protein and probably partially removes the Ni-NTA bounded LPS during washing steps of column [45]. In addition, the last step of purification was carried out at low pH in the presence of 30 % isopropanol on the strong cation-exchanger Source 15S (GE Healthcare) which also prevents the binding of LPS to the protein and partially washes out LPS from the column. It was shown that the isopropanol was effective in the separation of LPS from protein–LPS complexes, while the separation of LPS from protein–LPS complexes was more efficient on cation exchangers than on anion exchangers [46].

As was expected, the cell growth inhibition of Mv1Lu cells as well as SMAD3 phosphorylation by purified TGF-β1m was impaired more than 100-folds in comparison with the native dimer [21, 34]. Monomeric TGF-β3/C77S binds to TβRII-ED with the same affinity as the wild-type homodimer, but is impaired nearly 100-folds in its ability to bind and recruit TβRI-ED [5]. It is generally believed that the initial receptor dimerization is an essential event for receptor activation. Based on crosslinking experiments, it was shown that monomeric TGF-β3 possesses an intrinsic propensity to dimerize, and such dimerization is enhanced by cooperative bindings of TβRI and TβRII [47]. On the other hand, recent single-molecule fluorescence imaging studies demonstrated that both TβRI and TβRII are predominantly monomeric in the absence of ligand and TβRII dimerized upon TGF-β stimulation [48, 49]. It was demonstrated also that TGF-β signaling is mediated by two autonomously functioning TβRI:TβRII pairs forming heterodimers, and it was suggested that dimerization is required to increase potency by enhancing the apparent affinity for binding TGF-β via membrane-localization effects [34]. Based on the literature data, we suggested that the purified TGF-β1m can transmit a signal, either forming autonomously functioning TβRI:TβRII heterodimeric complex or by dimerization during the formation of the heterotetrameric complex. This does not exclude the fact

that TGF-β1m may exist in more than one conformation within cells by reversible formation and breaking of the disulfide bond at Cys77 as this bond exists in an aqueous microenvironment [9]. Naturally occurring monomeric activin has been isolated from follicular fluid (40–60 % of the total activin from this source was monomeric) and has been reported to have 19 % the activity of dimeric activin A in the inhibition of growth of rat thymocytes [50]. It will be important to explore the possibility that monomeric TGF-β occurs naturally. Whether such native monomeric TGF-β might exist with a free thiol at Cys77 remains to be determined.

The structure analysis by resolution of CD spectrum showed that the amount of β-sheets, turns, and coil structures in purified TGF-β1m coincided with those calculated from NMR spatial structure of the TGF-β1 dimer, while the amount of α-helices was decreased (Table 2). However, the biological activity of refolded TGF-β1m was comparable to the TGF-β1/C77S monomeric recombinant preparation secreted in CHO cell [21], indicating that the distortion of α-helices is not the result of an improper folding and probably dimerization is necessary for its stabilization. The resolution of crystal structure of the ternary complex between human TGF-β1 and the extracellular domains of its type I and type II receptors revealed that the dimeric interface of TGF-β1 is required for binding to the type I but not to type II receptor [51]. Both monomers of TGF-β1 generated two primarily hydrophobic patches of the interface one of which included amino acid residues located in 1α and 3α helices of TGF-β1. We suggested that stabilization of α helices of monomers could progress during formation of tetramer complexes on the cell surface by dimerization of ligand. The fact that more than 100-fold excess of monomeric preparations of TGF-β1 are required to reach maximal biological effect of dimeric forms could support our assumption. The preparation of TGF-β1m can be used for further structural and biological studies. Certainly, the TGF-β dimeric form is more appropriate for the studies of the ligand–receptor signaling complex in the membrane. Unfortunately, at the present stage of development of the structural biology methods, obtaining high-resolution structure of the dimeric TGF-β ligand in complex with full-length TβRI and TβRII receptors is a scientific challenge. Issues with crystallization of flexible multiple-domain transmembrane proteins are inherent to X-ray techniques, whereas NMR cannot effectively handle large supramolecular complexes. However, the parts of the ligand–receptor complex can be studied separately. Indeed, there is an example of a successful usage of the monomeric TGF-β3 in the complex with TβRII ectodomain [22]. Moreover, the monomeric TGF-β can be potentially used as co-crystallizer at the structural studies of the TβR receptors and initial steps of the ligand–receptor recognition.

The technique developed in this study allows us to obtain highly purified biologically active monomeric TGF-β1 with relatively high yield (about 28 mg of protein from 1 liter cell culture) in *E. coli*. The recombinant LAP (latent-associated peptide)-associated TGF-β1 was expressed in CHO cells in inactive form with the yieldin the range of 0.04–30 mg from 1 l culture [15–18]. LAP is necessary for secretion and proper folding of TGF-βs. The expression of latent TGF-β have been described also in HEK293T cells were the matrix metalloproteinase (MMP) cleavage site was inserted between LAP domain and TGF-β for LAP-MMP-TGF-β fusion cleavage and activation of mature TGF-β [52]. However, the expression level of latent TGF-β has not been indicated in this study.

In all mammalian expression systems, the major secreted protein by these cells consisted of latent dimeric TGF-β complex comprising noncovalently associate precursor and mature forms of TGF-β with molecular weight of 90–110 kDa were TGF-β (25 kDa) is about 25 %. These significantly decrease the yield of mature active TGF-β after activation and removal of LAP protein. In fact, the best expression levels of LAP-TGF-β1 fusion in CHO cells described were from 30 mg of fusion, and about 5 mg mature active dimeric TGF-β1 can be purified from 1 l of cell culture [18]. The technology we have used in this work allowed obtaining 28 mg of monomeric TGF-β1m from 1 l bacterial cell culture. There is no other study for prokaryotic expression of active monomer or dimer TGF-β1 to compare with. Even if the biological activity of TGF-β1m is highly reduced in comparison to dimeric form, the production of recombinant proteins in bacterial systems requires relatively lower costs compared with the eukaryotic expression. The developed in this work technology is good enough to create the monomeric mutant forms, which can block the TGF-β1 signaling for the therapy of advanced cancers. The preparation also can be applied for different therapeutic contexts, particularly for treatment of chronic non-healing wounds and bone regeneration or for diagnostic applications for determination of the concentration of TGF-β1 in blood. The fact that the monomeric TGF-β3 showed greater effectiveness than did the dimeric form in in vivo wound healing experiments [23] provides reason to suggest that in some biological processes low signaling of TGF-βs is preferable.

Acknowledgments This work was supported by the program of Molecular and Cellular Biology, Russian Academy of Sciences.

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Massagué, J. (2000). How cells read TGF-β signals. *Nature Reviews Molecular Cell Biology, 1*, 169–178.
2. Annes, J. P., Munger, J. S., & Rifkin, D. B. (2003). Making sense of latent TGFbeta activation. *Journal of Cell Science, 116*, 217–224.

3. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., et al. (2011). Latent TGF-β structure and activation. *Nature, 474*, 343–349.
4. Kang, J. S., Liu, C., & Derynck, R. (2009). New regulatory mechanisms of TGF-beta receptor function. *Trends Cell Biology, 198*, 385–394.
5. Groppe, J., Hinck, C. S., Samavarchi-Tehrani, P., Zubieta, C., Schuermann, J. P., Taylor, A. B., et al. (2008). Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. *Molecular Cell, 29*, 157–168.
6. Shi, Y., & Massagué, J. (2003). Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell, 113*, 685–700.
7. Massagué, J. (1998). TGF-β signal transduction. *The Annual Review of Biochemistry, 67*, 753–791.
8. Denrynck, R., & Miyazono, K. (2008). TGF-β and the TGF-β family, In Derynck, R., (Ed), *The TGF-β Family*, chapter 2 (pp. 29–44). NY: Cold Spring Harbor Press.
9. Daopin, S., Piez, K. A., Ogawa, Y., & Davies, D. R. (1992). Crystal structure of transforming growth factor-beta 2: An unusual fold for the superfamily. *Science, 257*, 369–373.
10. Schlunegger, M. P., & Grutter, M. G. (1992). An unusual feature revealed by the crystal structure at 2.2 Å resolution of human transforming growth factor-beta 2. *Nature, 358*, 430–434.
11. Hinck, A. P., Archer, S. J., Qian, S. W., Roberts, A. B., Sporn, M. B., Weatherbee, J. A., et al. (1996). Transforming growth factor beta 1: Three-dimensional structure in solution and comparison with the X-ray structure of transforming growth factor beta 2. *Biochemistry, 35*, 8517–8534.
12. Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M., & Sporn, M. B. (1983). Transforming growth factor-beta in human platelets. *The Journal of biological chemistry, 258*, 7155–7160.
13. Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M., & Sporn, M. B. (1983). Purification and initial characterization of a type beta transforming growth factor from human placenta. *Proceedings of the National Academy of Sciences USA, 80*, 3676–3680.
14. Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., et al. (1985). Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature, 316*, 701–705.
15. Gentry, L. E., Webb, N. R., Lim, G. J., Brunner, A. M., Ranchalis, J. E., Twardzik, D. R., et al. (1987). Type 1 transforming growth factor beta: Amplified expression and secretion of mature and precursor polypeptides in Chinese hamster ovary cells. *Molecular and Cellular Biology, 7*, 3418–3427.
16. Brunner, A. M., Marquardt, H., Malacko, A. R., Lioubin, M. N., & Purchio, A. F. (1989). Site-directed mutagenesis of cysteine residues in the proregion of the transforming growth factor beta 1 precursor. Expression and characterization of mutant proteins. *The Journal of Biological Chemistry, 264*, 13660–13664.
17. Archer, S. J., Bax, A., Roberts, A. B., Sporn, M. B., Ogawa, Y., Piez, K. A., et al. (1993). Transforming growth factor beta 1: Secondary structure as determined by heteronuclear magnetic resonance spectroscopy. *Biochemistry, 32*, 1152–1163.
18. Zou, Z., & Sun, P. D. (2004). Overexpression of human transforming growth factor-β1using a recombinant CHO cell expression system. *Protein Expression and Purification, 37*, 265–272.
19. Cerletti, N. (2000). Process for the production of biologically active dimeric protein. *US Patent*, 6057430.
20. Cerletti, N., Cox, D., Schmitz, A., & McMaster, G. (1991). Process for the production of biologically active protein. *European Patent Application,* 0433225.
21. Amatayakul-Chantler, S., Qian, S. W., Gakenheimer, K., Böttinger, E. P., Roberts, A. B., & Sporn, M. B. (1994). [Ser77] transforming growth factor-beta 1. Selective biological activity and receptor binding in mink lung epithelial cells. *The Journal of biological chemistry, 269*, 27687–27691.
22. Ilangovan, U., Deep, S., Hinck, C. S., & Hinck, A. P. (2004). Sequential resonance assignments of the extracellular domain of the human TGFbeta type II receptor in complex with monomeric TGFbeta3. *Journal of Biomolecular NMR, 29*, 103–104.
23. Ferguson, M. W. J., Mellors, P., Laverty, H. G., Occleston, N., O'Kane, S., & Atkinson, E. (2011). Medicaments and proteins based on TGF-β monomers for the treatment of wounds. *US patent,* 7902150.
24. Gasparian, M. E., Ostapchenco, V. G., Schulga, A. A., Dolgikh, D. A., & Kirpichnikov, M. P. (2003). Expression, purification, and characterization of human enteropeptidase catalytic subunit in *Escherichia coli*. *Protein Expression and Purification, 31*, 133–139.
25. Gasparian, M. E., Elistratov, P. A., Yakimov, S. A., Dolgikh, D. A., & Kirpichnikov, M. P. (2010). An efficient method for expression in *Escherichia coli* and purification of the extracellular ligand binding domain of the human TGFβ type II receptor. *Journal of Biotechnology, 148*, 1113–1118.
26. Gasparian, M. E., Bychkov, M. L., Dolgikh, D. A., & Kirpichnikov, M. P. (2011). Strategy for improvement of enteropeptidase efficiency in tag removal processes. *Protein Expression and Purification, 79*, 191–196.
27. Ruegemer, J. J., Ho, S. N., Augustine, J. A., Schlager, J. W., Bell, M. P., McKean, D. J., et al. (1990). Regulatory effect of transforming growth factor-β on IL-2- and IL-4-dependent T cell-cycle progression. *The Journal of Immunology, 144*, 1767–1776.
28. Ishiyama, M., Shiga, M., Sasamoto, K., Mizoguchi, M., & He, P. (1993). A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. *Chemical & Pharmaceutical Bulletin, 41*, 1118–1122.
29. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., & Massague, J. (1990). Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. *Cell, 62*, 175–185.
30. Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta, 1751*, 119–139.
31. Pellaud, J., Schote, U., Arvinte, T., & Seelig, J. (1999). Conformation and self-association of human recombinant transforming growth factor-beta3 in aqueous solutions. *The Journal of Biological Chemistry, 19*, 7699–7704.
32. Koradi, R., Billeter, M., & Wüthrich, K. (1996). MOLMOL: A program for display and analysis of macromolecular structures. *Journal of Molecular Graphics, 14*, 51–55.
33. Bocharov, E. V., Korzhnev, D. M., Blommers, M. J. J., Arvinte, T., Orekhov, V Yu., Billeter, M., et al. (2002). Dynamics-modulated biological activity of transforming growth factor beta3. *The Journal of Biological Chemistry, 277*, 46273–46279.
34. Huang, T., David, L., Mendoza, V., Yang, Y., Villarreal, M., De, K., et al. (2011). TGF-β signalling is mediated by two autonomously functioning TβRI:TβRII pairs. *The EMBO Journal, 6*, 1263–1276.
35. Zou, Z., & Sun, P. D. (2006). An improved recombinant mammalian cell expression system for human transforming growth factor-beta2 and -beta3 preparations. *Protein Expression and Purification, 50*, 9–17.
36. Han, B., Hall, F. L., & Nimni, M. E. (1997). Refolding of a recombinant collagen-targeted TGF-beta2 fusion protein expressed in *Escherichia coli*. *Protein Expression and Purification, 11*, 169–178.
37. Tuan, T. L., Cheung, D. T., Wu, L. T., Yee, A., Gabriel, S., Han, B., et al. (1996). Engineering, expression and renaturation of targeted TGF-beta fusion proteins. *Connect Tissue Research, 34*, 1–9.

38. Lunn, C. A., Kathju, S., Wallace, B. J., Kushner, S. R., & Pigiet, V. (1984). Amplification and purification of plasmid-encoded thioredoxin from *Escherichia coli* K12. *The Journal of Biological Chemistry, 259*, 10469–10474.
39. LaVallie, E. R., DiBlasio-Smith, E. A., Collins-Racie, L. A., Lu, Z., & McCoy, J. M. (2003). Thioredoxin and related proteins as multifunctional fusion tags for soluble expression in *E. coli*. *Methods in Molecular Biology, 205*, 119–140.
40. Katti, S. K., LeMaster, D. M., & Eklund, H. (1990). Crystal structure of thioredoxin from *Escherichia coli* at 1.68 A resolution. *Journal of Molecular Biology, 212*, 167–184.
41. LaVallie, E. R., Lu, Z., Diblasio-Smith, E. A., Collins-Racie, L. A., & McCoy, J. M. (2000). Thioredoxin as a fusion partner for production of soluble recombinant proteins in *Escherichia coli*. *Methods in Enzymology, 326*, 322–340.
42. Gasparian, M. E., Ostapchenko, V. G., Dolgikh, D. A., & Kirpichnikov, M. P. (2006). Biochemical characterization of human enteropeptidase light chain. *Biochemistry (Moscow), 71*, 113–119.
43. Jang, H., Kim, H. S., Moon, S. C., Lee, Y. R., Yu, K. Y., Lee, B. K., et al. (2009). Effects of protein concentration and detergent on endotoxin reduction by ultrafiltration. *BMB Reports, 42*, 462–466.
44. Liu, S., Tobias, R., McClure, S., Styba, G., Shi, Q., & Jackowski, G. (1997). Removal of endotoxin from recombinant protein preparations. *Clinical Biochemistry, 30*, 455–463.
45. Franken, K. L., Hiemstra, H. S., van Meijgaarden, K. E., Subronto, Y., den Hartigh, J., Ottenhoff, T. H., et al. (2000). Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expression and Purification, 18*, 95–99.
46. Lin, M. F., Williams, C., Murray, M. V., & Ropp, P. A. (2005). Removal of lipopolysaccharides from protein-lipopolysaccharide complexes by nonflammable solvents. *Journal of Chromatography, B, 816*, 167–174.
47. Zúñiga, J. E., Groppe, J. C., Cui, Y., Hinck, C. S., Contreras-Shannon, V., Pakhomova, O. N., et al. (2005). Assembly of TbetaRI:TbetaRII:TGFbeta ternary complex in vitro with receptor extracellular domains is cooperative and isoform-dependent. *Journal of Molecular Biology, 354*, 1052–1068.
48. Zhang, W., Jiang, Y., Wang, Q., Ma, X., Xiao, Z., Zuo, W., et al. (2009). Single-molecule imaging reveals transforming growth factor-beta-induced type II receptor dimerization. *Proceedings of the National Academy of Sciences USA, 106*, 15679–15683.
49. Zhang, W., Yuan, J., Yang, Y., Xu, L., Wang, Q., Zuo, W., et al. (2010). Monomeric type I and type III transforming growth factor beta receptors and their dimerization imaging. *Cell Research, 20*, 1216–1223.
50. Robertson, D. M., Foulds, L. M., Prisk, M., & Hedger, M. P. (1992). Inhibin/activin beta-subunit monomer: Isolation and characterization. *Endocrinology, 130*, 1680–1687.
51. Radaev, S., Zou, Z., Huang, T., Lafer, E. M., Hinck, A. P., & Sun, P. D. (2010). Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily. *The Journal of Biological Chemistry, 285*, 14806–14814.
52. Mullen, L., Rigby, A., Sclanders, M., Adams, G., Mittal, G., Colston, J., et al. (2014). Latency can be conferred to a variety of cytokines by fusion with latency-associated peptide from TGF-β. *Expert Opinion on Drug Delivery, 11*, 5–16.