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Expression, purification and renaturation of truncated human integrin β1 from inclusion bodies of *Escherichia coli*

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ABSTRACT

Integrins are a family of transmembrane receptors and among their members, integrin β1 is one of the best known. It plays a very important role in cell adhesion/migration and in cancer metastasis. Preparation of integrin β1 has a great potential value especially in studies focused on its function. To this end, recombinant plasmids were constructed containing DNA segments representing 454 amino acids of the N-terminal of integrin β1. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) cells and after induction by isopropyl-β-D-thiogalactopyranoside (IPTG), the recombinant protein (molecular weight: 53 kD) was expressed, mainly in the form of inclusion bodies. The inclusion bodies were solubilized by 8 M urea solution then purified by nickel affinity chromatography. The recombinant protein was renatured by a stepwise dialysis and finally dissolved in phosphate buffered saline. The final yield was approximately 5.4 mg/L of culture and the purity of the renatured recombinant protein was greater than 98% as assessed by SDS–PAGE. The integrity of the protein was shown by Western blot using monoclonal antibodies against his-tag and integrin β. Its secondary structure was verified as native by circular dichroism spectra and the bioactivity of the recombinant protein was displayed through the conformation switch under Mn^{2+} stimulation.

Introduction

The integrin family consists of 24 members [1]. Their expression in cells, when triggered, relates to various functions of embryonic development, angiogenesis, cell proliferation, adhesion and migration etc [2,3]. Among these, integrin β1 is one of the most studied subunits. Integrin β1 is expressed in a cell- and tissue-specific manner, but almost universally expressed in tumor cells [4]. Its high expression results in the progression of some types of cancers [5]. Moreover, α2β1 [6], α3β1 [7,8], α5β1 [9,10], or α6β1 [11] have been identified as predictors for cancer metastasis.

Integrins exist in two distinct conformations, one of which is "closed" (bent) and the other "open". These correspond to the inactive and activated forms of integrin, respectively [12]. How these two conformations switch is still unknown although research suggests that the relationship between the ligands and integrin activation [12], or the signal transduction involved in this process [13] are critical. Being able to obtain enough purified integrin β1 protein is a prerequisite to carrying out these types of studies, especially for *in vitro* experiments.

There have been a few studies focusing on the expression of the extracelluar region of integrin β1 [14], and to the best of our knowledge, none of them utilized a prokaryotic expression system. In present study, an efficient process was shown to produce highly purified, soluble and active integrin β1 ectodomain using *Escherichia coli*. A gene segment that represented 454 amino acids of the N-terminal of integrin β1 was cloned into pET-28a-c (+) (abbreviated as pET-28a) vector and transformed into *E. coli* BL21 (DE3) cells. The recombinant polypeptides (53 kD) were expressed as inclusion bodies. The inclusion bodies were purified by nickel affinity resin and refolded through stepwise dialysis. The refolded protein was successfully dissolved in phosphate buffered saline (PBS)[1], and the yield was about 5.4 mg/L of culture. We proved that

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[1] *Abbreviations used:* PBS, phosphate buffered saline; FBS, fetal bovine serum; IPTG, isopropyl-β-D-thiogalactopyranoside; PVDF, polyvinylidene difluoride.

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the renatured polypeptides had native structure and the correct bioactivities.

Materials and methods

Cell culture and cDNA synthesis

NCM460 normal human colon mucosa epithelial cells were cultured in DMEM/F12 (1:1) culture medium (Hyclone) plus 10% fetal bovine serum (FBS, Sangon biotech, Shanghai, China), and grown in humidified air with 5% CO_2. Cells were harvested using a cell scraper. Total RNA was isolated using RNAiso Plus (Takara Biotechnology) and purified according to the standard protocol. The cDNA was synthesized using PrimeScript 1st strand cDNA synthesis kit (Takara) according to the user manual.

Construction of recombinant plasmid

The PCR primers used for cloning the ectodomain of integrin β1 were designed (Table 1) according to the protein sequence included in PDB: 3VI3 (chain B) [15]. Restriction sites of *Bam* HI and *Xho* I were separately introduced to the forward or reverse primers in order to facilitate downstream operation. The specific target DNA segment was amplified on a thermocycler (Mastercycler personal, Eppendorf) using the following program: denaturation at 98 °C for 5 min, followed by 98 °C for 10 s, 15 s at 55 °C, 72 °C for 2 min for 35 cycles. Products underwent a final extension at 72 °C for 10 min. PrimeSTAR HS DNA Polymerase (Takara) was used to ensure high fidelity and accuracy during amplification. The PCR products were resolved using 1% agarose gel and extracted. Purified segments were ligated into *pEASY*-Blunt Zero Cloning vector (Transgen, Beijing, China) and transformed into Trans5α competent cells (Transgen). Cells were spread on LB plates containing 0.05 mg/mL kanamycin and cultured at 37 °C overnight. Positive clone was selected and grown in 5 mL LB liquid medium containing kanamycin. The recombinant plasmid DNA was extracted (Sangon, Shanghai, China) and digested using *Bam* HI and *Xho* I in buffer K (Takara) at 30 °C for 2 h. After gel extraction and purification, inserts were ligated into pET-28a vector (Novagen), that had been digested with the same restriction enzymes as mentioned above using DNA Ligation Kit Ver. 2.1 (Takara) with a ratio of insert: vector = 10:1 (mol/mol) as per the user manual. Recombinant pET-28a was transformed into BL21 (DE3) *E. coli* cells. Cells were grown overnight at 37 °C on LB plates with kanamycin. Positive colonies were identified by colony PCR, restriction digestion, and verified by DNA sequencing (Sangon, Shanghai, China).

Expression of recombinant protein

Positive colony was selected and used to inoculate 1 L of LB medium containing 50 μg/mL kanamycin. Cells were grown in a shaker at 37 °C until OD_{600} reached 0.9. Following induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) being added into the culture medium for 3 h at 37 °C with 200 rpm shaking, cells were harvested by centrifugation at 8000*g*, 4 °C for 10 min and washed with cold phosphate buffered saline (PBS, 0.2 g/L KCl, 3.63 g/L Na_2HPO_4-$12H_2O$, 0.24 g/L KH_2PO_4, and 8 g/L NaCl, pH = 8.0). The pellets were resuspended in lysis buffer (containing 5 mM β-mercaptoethanol, 1% (w/v) sodium deoxycholate and 1 mM PMSF in PBS, pH = 8.0) then lysed using ultrasound. Intracellular compounds were isolated by 15,000*g* centrifugation at 4 °C for 15 min. Pellets were washed three times in wash buffer (2 M urea, 1% (v/v) Triton X-100 in PBS, pH = 8.0), then resuspended in extraction buffer (8 M urea, 0.5 M NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol in PBS, pH = 8.0) and allowed to dissolve overnight at 4 °C.

Table 1
Primers used for cloning the coding sequence of integrin β1 ectodomain. Underlined characters indicated the restriction sites, italic characters represented the termination signal of transcription.

Primer name	Sequences	Restriction enzyme
ITGB1-head-F	5′-CGC GGA TCC CAA ACA GAT GAA AAT AGA TG	*Bam* HI
ITGB1-head-R	5′-CCG CTC GAG *TTA* TTC ACA TTC ACA GAT GTA C	*Xho* I

ITGB1-head purification and refolding

Extracted inclusion bodies were centrifuged at 4500*g* (swing-bucket rotor, Eppendorf) for 15 min to remove any insoluble debris. The supernatant was transferred to a new falcon tube. Protein concentration was assessed using the BCA method (Beyotime Institute of Biotechnology, Jiangsu, China), and was regulated to be lower than 0.2 mg/mL by extraction buffer to avoid protein aggregation. The protein solution was mixed with binding buffer (same as the above extraction buffer)-equilibrated Ni Sepharose 6 fast flow (GE healthcare) slurry and shaken gently for 1 h at room temperature. After centrifugation at 800*g* for 5 min, pellets were mixed with binding buffer at a ratio of 1:1 (v/v). The slurry was loaded on a PD-10 column (GE healthcare). Bound fractions were washed and eluted under denaturing conditions according to manufacturer's instructions.

The purified recombinant polypeptide was stepwise dialyzed at 4 °C with the following refolding buffers: (1) refolding buffer A (4 M urea, 0.1 mM glutathione, 0.01 mM glutathione disulfide, 1 mM EDTA, 5% (v/v) glycerol in PBS, pH = 8.0) for 12 h; (2) refolding buffer B (2 M urea, 0.1 mM glutathione, 0.01 mM glutathione disulfide, 1 mM EDTA, 5% (v/v) glycerol, 0.15 M L-arginine in PBS, pH = 8.0) for 12 h; (3) refolding buffer C (1 M urea, 0.1 mM glutathione, 0.01 mM glutathione disulfide, 1 mM EDTA, 5% (v/v) glycerol, in PBS, pH = 8.0) for 12 h; and (4) refolding buffer D (PBS, pH = 8.0) for 12 h, twice. Concentration of refolded ITGB1-head was determined by BCA protein assay kit.

Purity and bioactivity assay

The purity of recombinant ITGB1-head polypeptide was determined by SDS–PAGE. Briefly, 7.5 μg of each refolded protein sample was boiled for 10 min in loading buffer (50 mM Tris–HCl pH6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol and 1% (v/v) β-mercaptoethanol). The samples were loaded onto a 10% SDS–polyacrylamide gel, electrophoresed at a constant current of 15 mA for 2 h. Gel was stained using 0.1% (w/v) coomassie brilliant blue R250 for 2 h then destained using 45% (v/v) methanol with 10% (v/v) acetic acid for 2 h.

The bioactivity of refolded ITGB1-head was assessed under Mn^{2+} stimulation and shown by native-PAGE. Briefly, refolded protein or BSA (as negative control, BSA Standard, Beyotime Institute of Biotechnology) was treated with 3 mM $MnCl_2$ at 37 °C for 1 h. Treated samples and their controls were mixed with native-PAGE loading buffer (50 mM Tris–HCl pH6.8, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol), then loaded onto the 10% native polyacrylamide gel to separate at 4 °C for 2 h. Specific ITGB1-head bands were shown by Western blotting as described below. The BSA bands were shown using coomassie brilliant blue R250 stain.

Western blotting

For Western blotting, after the samples had been separated, the proteins on the gel were transferred onto a polyvinylidene

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