Expression and Purification of a Human Tumor-Associated Protein Annexin V

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Abstract: Annexin V is a phospholipase A2 and protein kinase C inhibitory protein with calcium channel activity and an undefined role in cellular growth and differentiation. The product of *annexin* V gene is necessary for doing experiments to determine whether the tumor-associated *annexin* V protein can be used as a new molecular marker for diagnosis and prognosis of gastric carcinoma. This study describes the procedures which were used for cloning and expressing the human *annexin* V as a maltose binding protein (MBP) fusion polypeptide in bacteria. The expression plasmid for *annexin* V was constructed by ligation of the *annexin* V cDNA into the expression vector pMAL-c2x. The protein was expressed by *E. coli* strain TB1 cells and purified by amylose affinity column chromatography. The purity of the protein was assessed by SDS-PAGE and Western blot. The results showed that the molecular weight of the recombinant MBP-*annexin* V polypeptide was consistent with the calculated molecular weight, and that the purified protein appeared as an apparent single band of 77 kDa on the gel filtration column by SDS-PAGE. Our expression system allows the expression and purification of *annexin* V with MBP in high yield with no need of removal of the tag and gives pure protein in one purification step, and also makes it possible for the structural and functional studies of these proteins. [Life Science Journal. 2005;2(1):22 - 26] (ISSN: 1097 - 8135).

Keywords: *annexin* V; expression; purification; pMAL-c2x

1 Introduction

Annexins are Ca^{2+} and phospholipid-binding proteins forming an evolutionary conserved multigene family. Each member of this protein family contains a conserved protein core characterized by high alpha-helix content that includes the calcium and phospholipids binding sites, and a variable N-terminal domain that is specific in sequence and length for each *annexin*. Although the structure of the protein core is highly conserved, different *annexins* exhibit a wide biochemical and functional diversity, e. g., inflammatory response, membrane fusion and exocytosis, ion channel regulation, and inhibition of blood coagulation[1]. Despite an abundance of experimental evidence suggesting that *annexins* are associated with a plethora of biological processes, the exact physiological function of *annexins* remains to be investigated. Annexin V is a phospholipase A2 and protein kinase C inhibitory protein with calcium channel activity and an undefined role in cellular signal transduction, inflammation, growth and differentiation[2]. It has been previously isolated as placental anticoagulant protein[3]. Annexin V may be used to assess tumor response to chemotherapy[4]. We have previously described the overexpression of *annexin* V in gastric carcinoma tissues using proteome-based approach[5]. In this study we have made an *annexin* V expression construct, purified the protein and further determined the identity of the protein for the purpose of studying its role in tumor cells[6].

2 Materials and Methods

Construction of pMAL-Annexin V: Total RNA extracted from human placental by Rnasey Midi Kit (Qiagen, Valencia, CA, USA). The mRNA was then reverse transcribed to cDNA using AMV reverse transcriptase (Promega Co, USA) with 9-mers random primer. The cDNA encoding for human *annexin* V (Accession # J03745) was amplified by PCR using the upstream primer 5′-GC GAATTCATGGCACAGGTTCTCAGAGGCA-3′ designed with an upstream *EcoR* I restriction site (underlined), and the downstream primer 5′-GCGCTGCAGTTAGTCATCTTCTCCACA-3′ designed with a down-stream *Pst* I restriction site (underlined). Both PCR products contain an 'A' at the 3′ end, the 965-bp DNA fragments from the PCRs were directly cloned into pMD T vector

(TAKARA Bio Inc.) following standard procedures[7]. pMAL-c2x plasmid (New England Biolabs) contains an Ampr gene, tac promoter, the *lac Iq* gene, a polylinker, and the *mal* E gene, which encodes for maltose binding protein (MBP). pMD-*annexin* V was digested with the restriction enzymes *EcoR* I and *Pst* I (Promega Co., America), and pMAL was digested with *EcoR* I and *Pst* I with manufacturer's buffers at 37°C for 2 hours. The fragments from these restriction digests were gel-purified using the Qiaex II Gel Extraction Kit (Qiagen, Valencia, CA, USA). The cut pMAL vector was then ligated with the *annexin* V gene using T_4 DNA ligase (Promega Co., USA). All ligations were transformed into MAX efficiency *E. coli* strain TB1 cells. Bacterial cells were plated onto LB-Amp plates and incubated overnight at 37°C. A colony containing each of the constructs were inoculated into LB-Amp culture medium overnight and then midi-prepped using a Plasmid Midiprep Kit (Vitgene, China).

Verification of constructs: The recombinant plasmid was detected by restriction endonucleases and specific PCR. The midi-prepped constructs were sequenced on an ABI 3700 Sequencer to verify correct splicing of the gene into the plasmid. Sequence analysis was done with the program Sequencher, software available from Gene Codes Corporation (Ann Arbor, MI).

Expression of fusion proteins: TB1 competent bacterial cells were transformed according to manufacturer's instructions with the sequenced MBP-Annexin V[8]. Cells were plated onto LB-Amp plates and grown overnight at 37°C. The overnight culture was added to 900 ul 50% glycerol to make glycerol stocks. Overnight cultures were diluted 1:100 with pre-warmed LB-Amp media. The protein expression was induced by addition of Isopropyl-Beta-D-thiogalactoside (IPTG) to a final concentration of 0.2 mM when OD_{600} reached 0.4 − 0.5. After a 6 h post-induction period, the cells were harvested by centrifugation at 6,000 g at 4°C for 20 min. One ml of both uninduced and induced cultures were spun down and resuspended in 100 ul 2 × SDS-PAGE sample buffer and analyzed by 12% SDS-PAGE gel.

Purification of MBP fusion proteins: The fusion proteins were purified based on a protocol[9,10] as described (New England Biolabs, USA). One liter of cells transformed with the fusion construct was induced to produce protein as described above. The cultures were spun down and resuspended in 50 ml column buffer (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). Resuspended cultures were frozen at −20°C until purification. Frozen cultures were defrosted slowly in ice-cold water. 0.5 M DTT, 250 mM PMSF, and 100 × protease inhibitor cocktail from Sigma were added to the defrosted cultures to final concentrations of 5 mM, 0.1 mM respectively. The mixtures were sonicated in short bursts of 10 seconds for 20 minutes total[11,12]. Sonicates were spun down, and the supernatants were collected. 1.5 ml amylose resin beads (New England Biolabs, USA) were added to the supernatants and the protein was allowed to bind to the beads overnight on a rotary shaker at 4°C. The beads were washed with column buffer and protein was eluted with 100 mM maltose in column buffer overnight at 4°C. Samples for SDS-PAGE analysis were taken from induced culture, post-sonicate pellet, post-sonicate supernatant, supernatant after binding to amylase resin beads, and eluted protein was analyzed using a 12.5% SDS-PAGE.

Detection of recombinant proteins by western blot: Electrophoretically separated proteins were transferred to an Immobilon PVDF transfer membrane (pore size 0.45 μm, Millipore) by electrophoresis in transfer buffer (PBS, pH 7.4, 200 mM glycine, 20% methanol, 0.1% SDS) using a Transblot cell (Biorad, USA) at 275 mA for 30 min. After transfer the non-specific binding sites on the membrane were pre-blocked by incubating with blocking buffer overnight at 4°C. The primary rabbit anti-*annexin* V antibody (Abcam Ltd, USA) was diluted 1:300 in PBS and incubated with the pre-blocked membrane for 60 min at room temperature with constant shaking. Non-bound antibodies were removed by washing three times in wash buffer (0.05% Tween-20 in PBS) for a total of 60 min. The antibody antigen complex on the membrane was detected by incubating the membrane with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1:5,000) for 1 hour at room temperature. The membrane was then washed as before and rinsed with PBS[13-15]. Reactivity was visualized according to the manufacturer's instructions.

3 Results

Verification of constructs of MBP-Annexin V: As shown in Figure 1, the recombinant plasmid of pMAL-*annexin* V was digested into two fragments by *EcoR* I and *Pst* I which are 6.7 kb pMAL vector fragment and 0.96 kb *annexin* V gene fragment[15], and there was a single 7.0 kb fragment by the digestion of *EcoR* I alone. The 0.96 kb *annexin* V gene fragment could be ampli-

fied by specific PCR. It was demonstrated that recombinant plasmid of pMAL- *annexin* V was constructed successfully. Sequencing analysis showed that the construct, which was used for protein purification contains the full-length 965 bp *annexin* V gene. The cDNA opening reading frame of *annexin* V gene contained 965 bp which coded for a protein with 319 amino acids and a calculated molecular mass of 35,805 Da.

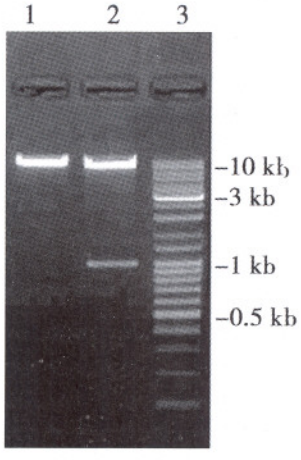

Figure 1. The recombinant plasmid was verified by restriction endonucleases. Lane 1, recombinant plasmid DNA was digested by *EcoR* I and *Pst* I; lane 2, recombinant plasmid DNA was digested by *EcoR*I alone; lane 3, DNA molecular markers (10 kb).

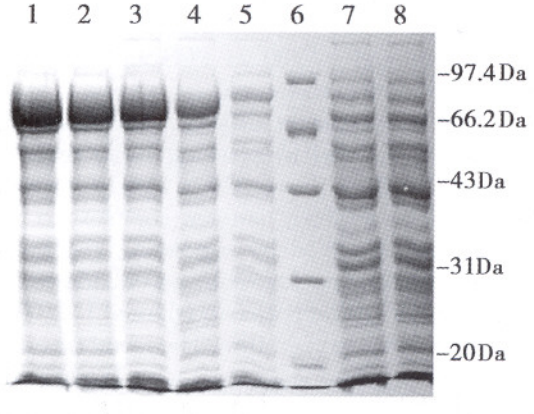

Figure 2. Expression of MBP-*Annexin* V fusion constructs. Lane 1, TB1 (pMA) 0.2 mM IPTG induced 5 hours; lane 2, TB1 (pMA) 0.2 mM IPTG induced 4 hours; lane 3, TB1 (pMA) 0.2 mM IPTG induced 3 hours; lane 4, TB1 (pMA) 0.2 mM IPTG induced 2 hours; lane 5, l TB1 (pMA) 0.2 mM IPTG induced 1 hours; lane 6, protein marker; lane 7, TB1 induced 4 hours; lane 8, TB1 (pMAL－c2x) un-induced 4 hours.

Expression of recombinant MBP-Annexin V fusion protein: Annexin V was cloned into the pMAL-c2x expression vector, as described in materials and methods. MBP-Annexin V constructs were readily expressed in TB1 cells. To obtain high Annexin V expression, Figure 2 shows the TB1 cellular content of Annexin V expressed in the different conditions. After SDS-PAGE analysis, the MBP-Annexin V protein was detected at 77 kDa (42 kDa MBP + 35 kDa Annexin V = 77 kDa) by Coomassie blue staining. As shown in Figure 3, the level of expressed Annexin V was apparently affected. Annexin V constituted approximately 39.9% of total cellular protein after IPTG induction for 4 hours.

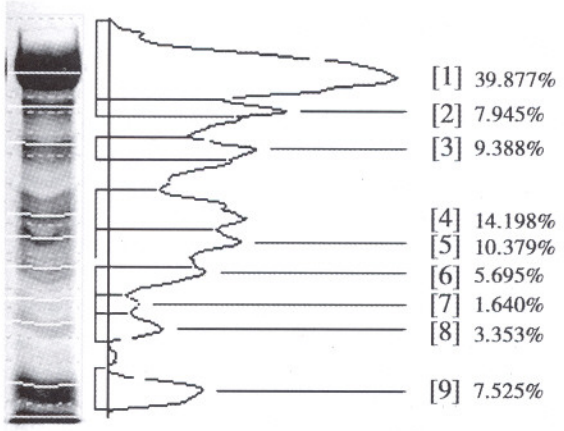

Figure 3. Contents analysis of the MBP- Annexin V fusion protein. Percentage of the each protein was showed in the figure. Annexin V constituted approximately 39.9% of total cellular protein after IPTG induction for 4 hours.

Purification of recombinant MBP-Annexin V fusion protein: The efficiency of the purification was dependent on appropriate amounts of equilibrated amylose resin. Due to the relatively binding capacity of the resin, we found that 1 ml crude extract (approximately 10 mg protein) needs 1 ml of equilibrated gel slurry. Prolonged incubation times of more than 15 min at 4℃ had to be used in order to achieve optimal recovery of bound protein. We observed that the first washing steps contained large amounts of MBP-Annexin V complexes. Most of the MBP-Annexin V protein produced by IPTG induction was found in the post-sonicate pellets, and not in the post-sonicate supernatants. Almost all of the protein contained in the supernatants was able to bind to the amylose resin and could be eluted from the beads with a high efficiency. After purification and SDS-PAGE analysis, the MBP-Annexin V protein was detected as a single band of 77 kDa, and it also immunoreacted with anti-Annexin V polycolonal antibody, The result showed that this protein was expressed and purified as a single chain MBP-Annexin V fusion protein. The final yield was 1.9 mg of purified protein per liter

culture cells and the purity of the preparation was estimated to be over 95% as judged by SDS-PAGE analysis.

4 Discussion

This study describes the process of bacterial expression and purification of a MBP-Annexin V fusion protein. The results indicate that fusion protein using the MBP purification system from New England Biolabs can be readily produced in bacteria and purified with high yield. The experiments which have been done in this study showed that the post-sonicate supernatant contains cellular soluble fractions from the rupture of cells. The appearance of more protein in this component of the sonicate implies that the number of cells that remained ruptured after sonication is greater than the number of cells that released their protein during sonication. The anti-Annexin V polyclonal antibody was used to assess the specificity of *annexin* V protein by Western blot analysis. The results demonstrated that the purified Annexin V protein had strong reactivity with the polyclonal anti-Annexin V antibody, suggesting that it could be used in the subsequent experiments to characterize the gene *annexin V* in terms of its function.

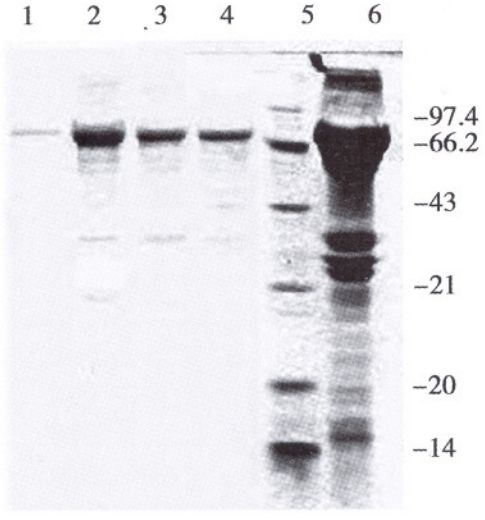

Figure 4. Purification of recombinant MBP-Annexin V fusion protein. Lanes 1 – 4, Purification of MBP-Annexin V protein from tubes 1 – 4; lane 5, Molecular marker; lane 6, TB1 (pMAL-annexinV) post-sonicate supernatants.

Some other experiments are currently underway to further investigate the function of Annexin V. Its probable role in regulation of the expression of the tumor-specific protein may make it as an intriguing subject of research[16]. This study only describes the procedure of expression and purification of recombinant Annexin V, which will be useful for further investigations of Annexin V. The purified Annexin V protein could be used as important experimental material for the studies that will be performed to gain insight into the functioning of Annexin V.

In summary, we reported a robust method to express, isolate and purify recombinant MBP-Annexin V fusion protein in bacterial cells. Due to its high soluble content, the Ca^{2+}-dependent phospholipid binding proteins have proven readily to express and isolate in sufficient quantities for structural and functional studies. The protocol which was used in this study allows the protein production of at least 2 mg per liter of culture, and also makes it possible for the structural studies of these proteins.

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