

Prokaryotic expression and characterization of human AC3-33 protein

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1. ABSTRACT

The transcription factor, AP-1, plays an important role in cellular proliferation, transformation and death. We previously showed that AC3-33 (GenBank name: c3orf33, FLJ31139), significantly inhibited transcriptional activity of AP-1. In this study, we report a method to express and purify AC3-33 in *E. coli* using glutathione-S-transferase (GST) fusion system. A GST-fusion protein was created by insertion of AC3-33 gene into a pGEX-4T-1 vector. The fusion protein, GST-AC3-33, was expressed in BL21 strain, and purified by GSH-affinity chromatography followed by thrombin cleavage. The digested product was further purified in a GSH-affinity column. After cleavage and purification, the recombinant AC3-33 protein exhibited the expected size of 29 kDa by SDS-PAGE and Western blotting and inhibited transcriptional activity of AP-1 in a dual-luciferase reporter assay. The bioactive recombinant GST-AC3-33, can be used to decipher the physiological and biochemical role of this protein.

2. INTRODUCTION

AC3-33 (GenBank name: C3orf33, Accession No. FLJ31139), a novel human gene, also known as chromosome 3 open reading frame 33, encodes 251 amino acids with a predicted molecular mass of 29.3 kDa. AC3-33 protein is located into cytoplasm, which is widely higher expressed in various tissues, such as adrenal glands and cervix, and significantly lower expressed in human leukemia cell line K562 and KG1a (1). To our knowledge, no functional study has been performed on this hypothetical gene.

Using dual-luciferase reporter assay system, we have established high-throughput AP-1 pathway function-related gene screening system based on overexpression in human cell lines, and found that AC3-33 significantly inhibited AP-1 transcriptional activity. The transcription factor AP-1 (activator protein-1) pathway plays important roles in the regulation of cellular proliferation,

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transformation and death (2-4). It is well characterized that AP-1 is a double-edged sword in tumorigenesis, either antioncogenic by inducing apoptosis or oncogenic by signaling cell survival. The outcome of AP-1 activity in tumors seems to depend on AP-1 dimer composition as well as the cellular and genetic contexts (5-7).

A deeper understanding of the relationship between the structure and the function of AC3-33 may provide a new insight into the molecular mechanism of AC3-33 modulating the AP-1 pathway, which might provide a new insight into precise pathological process of tumorigenesis, as well as AC3-33 potential applications in human diseases treatment and diagnosis. However, large quantities of the pure AC3-33 protein are required for such studies. AC3-33 protein is a kind of biomacromolecules with molecular mass of 29.3 kDa, which is hardly to be prepared by a solid phase chemical synthesis, so recombinant methods permit the production of peptides or proteins in microorganisms in a relative convenient and economical way (8). To prevent fibril formation *in vivo*, we attempted to express the AC3-33 protein attached to GST (glutathione-S-transferase), a large soluble fusion partner (9).

In this paper, we cloned and constructed of prokaryotic expression vector of AC3-33, expressed and purified of GST-AC3-33 fusion protein. Moreover, we illustrated that prokaryotic recombinant AC3-33 protein had similar biological activity with eukaryotic over-expressed AC3-33 protein. This study will establish the foundation for further researches of the function of AC3-33.

3. MATERIALS AND METHODS

3.1. Materials

Escherichia coli (*E. coli*) DH5a used for plasmid amplification and *E. coli* BL21 used for the expression of fusion protein were purchased from Qiagen (Germany). Plasmid pGEX-4T-1 was kindly provided by Human Disease Genomics Center (Peking University, China). Glutathione Sepharose 4B (GS4B) was purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden). The Taq DNA polymerase, the restriction enzymes (XhoI, BamHI), T4 DNA Ligase, other PCR reagent and isopropyl- β -D-thiogalactoside (IPTG) were purchased from Takara (Dalian, China). High-Affinity Resin and Phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich (USA). Plasmid mini kit and agarose gel DNA fragment purification kit were purchased from Omega (USA). Rabbit anti GST-tag and Goat anti-Rabbit IgG/HRP were purchased from Beijing Biosynthesis Biotechnology (Beijing, China). Ionomycin was from Santa Cruz Biotechnology (USA).

3.2. cDNA cloning and vector construction

The full-length coding region of AC3-33 was amplified and clone to pcDNA3.1B-TOPO-AC3-33 plasmid as our previous report (1). To construct pGEX-4T-1-AC3-33 expression vector, using pcDNA3.1B-TOPO-AC3-33 plasmid as template, AC3-33 cDNAs were PCR-

amplified using a forward primer that contains a BamHI site (5'- CGC GGA TCC ATG GCC ATA GCT GGA ATA ATG TTA -3'; BamHI site underlined) and a reverse primer that contains a XhoI site after the stop codon TAG (5'- CCG CTC GAG TCA CCC TTT TCT ACG AAA GTT TAT G -3'; XhoI site underlined). Amplification conditions were 25 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C. The amplification products were purified from agarose gel by using gel purification kit and then ligated into the pGEX-4T-1 plasmid at the BamHI and XhoI sites. *E. coli* DH5a were transformed with this recombinant plasmid and selected on Luria-Bertani (LB) broth containing ampicillin (100 μ g/ml). The plasmid was confirmed by sequencing using an ABI Prism[®] 3100 Genetic Analyzer (Applied Biosystems, USA). After confirmation, the pGEX-4T-1 vector or positive pGEX-4T-1-AC3-33 recombinant plasmid was transformed into *E. coli* BL21 to produce GST or GST-AC3-33 fusion protein, respectively.

3.3. Expression of GST-AC3-33 fusion protein

E. coli BL21 cells transformed with pGEX-4T-1 vector or pGEX-4T-1-AC3-33 plasmid were cultured, followed by the treatment with IPTG to induce protein production. To optimize the expression conditions, the effects of different culture mediums (LB, 2 \times YT, SOB, Nutrient broth), the concentration of NaCl (0 g/L, 2 g/L, 4 g/L, 6 g/L, 8 g/L, 10 g/L, 12g/L), pH values (pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0, pH 10.0), the concentration of IPTG (0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM, 1.2 mM, 1.4 mM, 1.6 mM), the temperature of induction (25°C, 30°C, 37°C, 42°C) and the time course of induction (0h, 1h, 2h, 3h, 4h, 5h, 6h, 7h) were evaluated. In each condition, aliquots of the culture were collected and analyzed by 10% SDS-PAGE.

When the optimized expression conditions were determined, the fusion protein was expressed on a large-scale and purified as follows. Briefly, the cells were inoculated into 4 ml of 2 \times YT cultural medium containing 100 μ g/ml ampicillin and grown overnight (12 h) at 37°C with gentle shaking. The following morning, 1 ml of the overnight culture was inoculated into 250 ml of 2 \times YT cultural medium with pH 7.0, and incubated at 37°C for about 2 h, at which time the culture was in the mid-log phase (OD₆₀₀=1.0). Protein expression was then induced by adding IPTG to a final concentration of 0.2 mM. The cells were cultured at 37°C for a further 5 h and then harvested by centrifugation at 10,000g at 4°C for 15 min. The pellet was washed and resuspended in 50 ml of cold phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and pH 7.4).

3.4. Affinity purification of GST-AC3-33 fusion protein

The suspended cells in cold PBS buffer were lysed by sonication at 300 W 50 times for 10 s, each with a 10 s pause between sonication intervals on ice (Thermo Electron Corporation, China). The suspensions were then centrifuged at 12,000g for 30 min at 4 °C. The supernatant (soluble fraction) or the pellet (insoluble fraction) were analyzed by 10% SDS-PAGE. The GST-AC3-33 fusion protein was affinity purified using GS4B following a batch

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method (8). Briefly, the supernatant containing the fusion protein was transferred to 10 ml volume of 50% GS4B beads suspension that had been pre-equilibrated three times with PBS, and incubated with gently agitation at room temperature for 30 min. After being centrifuged at 500g for 5 min, the sediment matrix was packed into a column and washed with 3× bed volume of PBS. Then GST-AC3-33 was eluted with 10 mM reduced glutathione in 50 mM Tris-Cl (pH 7.4) and collected as 1-ml fractions. To evaluate the purity of the protein, eluted proteins were analyzed by 10% SDS-PAGE.

3.5. Cleavage of GST-AC3-33 fusion protein

The GST-tag was cleaved from GST-AC3-33 fusion protein using thrombin protease. Briefly, after GSH-affinity purification, free reduced glutathione in eluted proteins was removed using a PD-10 desalting column. Then the fusion protein was treated with thrombin (2.5 U/mg fusion protein) in 1× PBS at 25°C for 2 h and applied to an additional GSH-affinity column to remove non-cleaved proteins and free GST-tags. The remaining thrombin in cleaved protein was removed using 1-ml column of Benzamidine Sepharose Fast Flow. The purified AC3-33 was confirmed via SDS-PAGE and Western blot analysis.

3.6. SDS-PAGE Electrophoresis

SDS-PAGE analysis was performed in order to assess the purity of recombinant GST-AC3-33 and AC3-33 protein. The samples were treated as above, dissolved in 1 M Tris-HCl SDS lysis buffer (containing 2.7 M glycerol, 0.15 M sodium dodecyl sulfate and 0.15 mM bromophenol blue, pH 6.8) for 30 min at 4°C, and protein concentration of the lysates was determined using the BCA protein assay reagent (Pierce, USA) using bovine serum albumin as the standard (10). The samples (30 µg total protein per well) were separated with 10% acrylamide separating gel using a Minigel Apparatus (BioRad Laboratories, Hertfordshire, UK). After running for 1.5 h at 80 V, the gel was stained with Coomassie Brilliant Blue R250 for 30 min, washed in washing buffer (45% methanol, 10% acetic acid, 45% H₂O, v/v) for 30 min and analyzed.

3.7. Polyclonal anti-AC3-33 antibody preparation

Antibodies against AC3-33 were generated by immunization of rabbits with KLH-coupled peptide (YFSVNLNNEILRRGLGKTVLVKGLKYDSKIYWTV HRNLLK), which were synthesized by solid phase synthesis and purified by HPLC to 95% purity (Chinese Peptide, China). Rabbit polyclonal antibodies were purified using CNBR Sepharose 4B coupled with specific AC3-33 peptide. The antibodies were validated by ELISA and Western blot analysis according to standard procedures as above (data not shown).

3.8. Western blot analysis

Immunoblot analysis was conducted as previously described (11) with a slight modification. Briefly, cells were lysed in 1 M Tris-HCl SDS lysis buffer. Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes

(Amersham Pharmacia, UK). Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% nonfat milk for 2 hours, and incubated overnight at 4°C with the appropriate primary antibody. After washing in TBST buffer, membranes were incubated for 1 hour in the dark with the appropriate IRDyeTM 800-conjugated secondary antibodies. Signals were detected on an Odyssey Infrared Imager (LI-COR Bioscience, USA).

3.9. Cell culture and transient transfection with plasmids

HEK 293T (human embryonic kidney cell line) and HeLa (human cervical carcinoma cell line) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and L-glutamine (2 mM) at 37°C in a humidified 5% CO₂. Plasmids were transfected into HEK 293T and HeLa cells using VigoFect (Vigorous, USA), a non-liposomal cationic formula, according to the manufacturer's instructions.

3.10. Expression of mammalian recombinant protein

PCDB-AC3-33 plasmid (20 mg) containing a c-myc-tag at the C terminus was transiently transfected into HeLa cells (3×10^6) by VigoFect as previously described. The transfected cells were cultured in the media without serum (Gibco, USA) for 24 hours, and then harvested for purification. The transfected cell supernatant was combined with 3 µl anti-c-myc antibody and protein G beads at 4°C, for overnight, followed by three washes with wash buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl₂, 0.4% Nonidet P-40). Bound proteins were analyzed by Western blot (data not shown), and used as the positive control for dual-luciferase reporter assay.

3.11. Dual-luciferase reporter assay

AP-1 luciferase activity was measured using cis-reporting system. Approximately 1.0×10^4 293T or HeLa cells per well were seeded into a 96-well culture plate. After 24 hours, the cells in each well were co-transfected with 50 ng of the pAP-1-Luc plasmids containing the firefly luciferase reporter gene (PathDetect, Stratagene), and 4 ng of the pRL-TK plasmids as the internal control containing the Renilla luciferase gene (Promega, Madison, WI).

At 24 hours after transfection, the cell culture were supplemented with the prokaryotic recombinant AC3-33 protein (20 µM), with eukaryotic immunoprecipitated AC3-33 protein (20 µM) as positive control and recombinant GST protein (20 µM) as negative control. 2 hours later, the cells were stimulated with PMA (12-O-Tetradecanoylphorbol-13-acetate) (50 mM, sigma) and Ionomycin (1 mM, Calbiochem, USA) for 6 hours, and then lysed in standard lysis buffer. Using a GENios Pro reader (Tecan, Switzerland) the cell lysates were assayed for both firefly and renilla luciferase activities according to the manufacturer's instructions. Each independent experiment was performed three times.

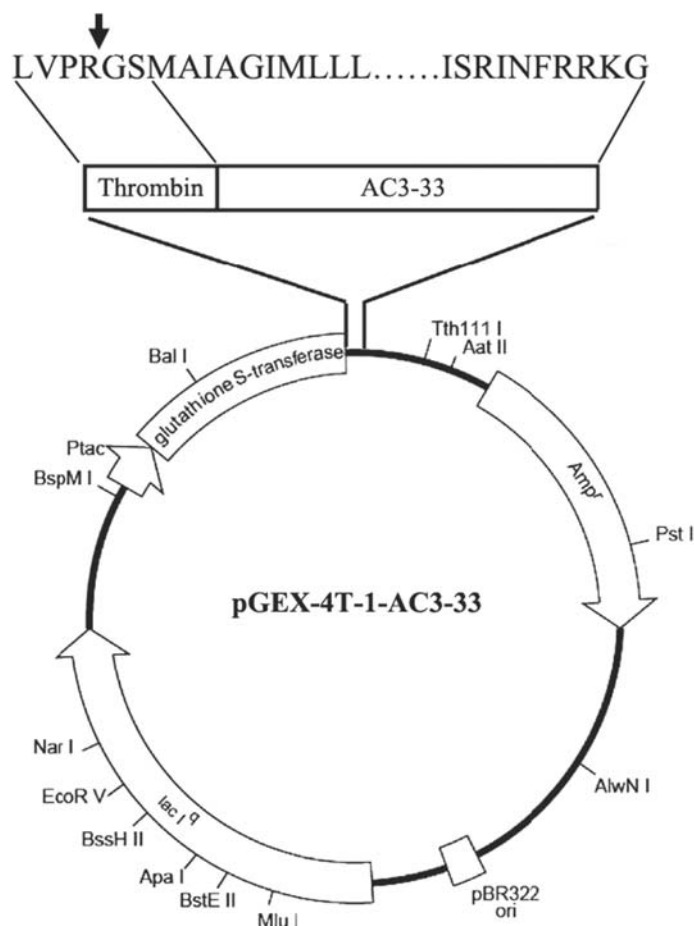


Figure 1. Construction of pGEX-4T-1-AC3-33 expression vector. The cDNA encoding the AC3-33 Protein was inserted into BamHI and XhoI sites of pGEX-4T-1. Arrow indicates the cleavage site of thrombin.

4. RESULTS

4.1. Construction and identification of prokaryotic expression vector pGEX-4T-1-AC3-33

The constructed plasmid pGEX-4T-1-AC3-33 for the expression of the GST-AC3-33 fusion protein was shown in Figure 1. The AC3-33 gene was inserted downstream of GST-encoding DNA in the same open reading frame at the BamHI and XhoI restriction sites in the multiple cloning sites of the pGEX-4T-1. Between GST and AC3-33 there was a site-specific recognition sequence of thrombin for the release of intact AC3-33. The insertion was confirmed by PCR (Figure 2A), as well as digestion with restriction endonuclease BamHI and XhoI (Figure 2B). The results showed that the fragment was about 750bp. Then the clone was confirmed by sequencing using an ABI Prism[®] 3100 Genetic Analyzer (Applied Biosystems, USA). After confirmation, the plasmid was extracted and purified using EndoFree Plasmid Mini Kit (Qiagen, USA).

4.2. Optimization of expression condition of GST-AC3-33 fusion protein in *E. coli* BL21

It is well known that *Escherichia coli* BL21 transformed with pGEX-4T-1 vector produced a protein of

26 kDa (GST protein) after IPTG induction. In our study, *E. coli* BL21 transformed with pGEX-4T-1-AC3-33 plasmid produced a protein of around 55 kDa after IPTG induction (GST-AC3-33; Figure 3A, lanes 1-2). The migration of this protein corresponds to a fusion of AC3-33 (MW of 29.3 kDa) and GST (MW of 26 kDa). The result showed that the protein band existed in both the lysed supernatant and the precipitation (pellet), which indicated that recombinant GST-AC3-33 protein could be partially dissoluble into cytoplasm, although some amount of the fusion protein was found in the pellet (the inclusion body).

To optimize the expression conditions, *E. coli* BL21 cells transformed with pGEX-4T-1 vector or pGEX-4T-1-AC3-33 plasmid were cultured, followed by the treatment with IPTG to induce protein production in different condition as described in Materials and methods. Regarding culture mediums, the expression level of GST-AC3-33 were highest in 2×YT (Figure 4A, 2×YT > SOB > LB > Nutrient broth). Regarding the concentration of NaCl, there were no difference in the expression level of GST-AC3-33 when the concentrations of NaCl were within 0~12g/L (Figure 4B). Regarding pH values, the expression level of GST-AC3-33 were highest when pH values were within 5.0~7.0, but significantly lower in strong acidic or

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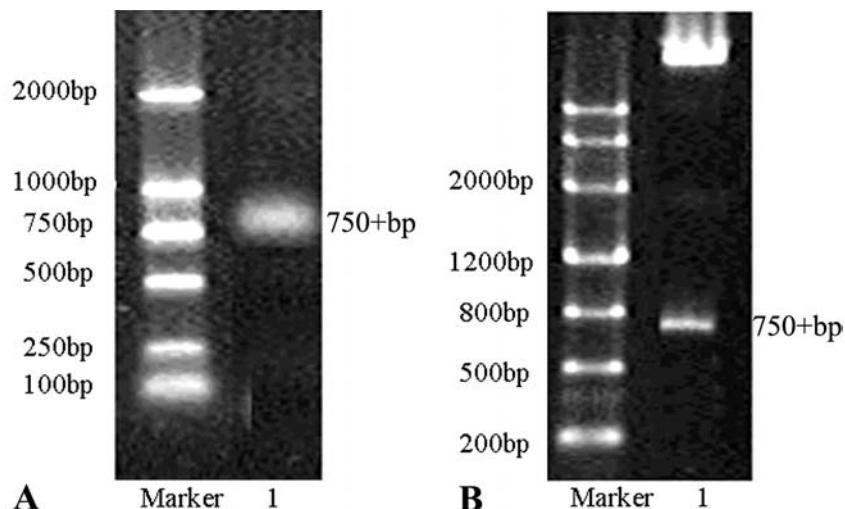


Figure 2. Construction and identification of recombinant expression vector pGEX-4T-1- AC3-33. (A) PCR amplification and purification of AC3-33 cDNAs. Using pcDNA3.1B-TOPO-AC3-33 plasmid as template, AC3-33 cDNAs were PCR-amplified using a forward primer containing a BamHI site and a reverse primer containing a XhoI site after the stop codon TAG. The amplification products were purified by using gel purification kit. Lane 1, purified AC3-33 cDNAs. (B) Restriction enzyme analysis of the recombinant plasmid. Lane 1, pGEX-4T-1-AC3-33 expression plasmid digested by BamHI/XhoI.

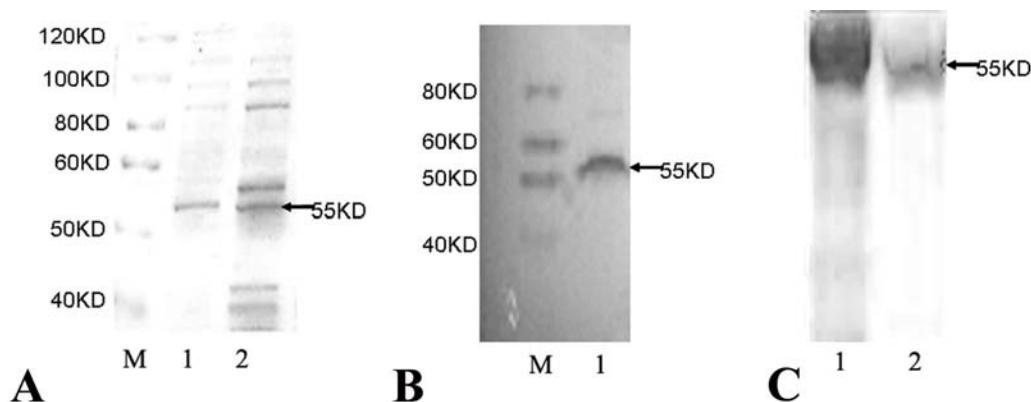


Figure 3. Expression, purification and identification of GST-AC3-33 fusion protein in *E. coli* BL21. M, protein molecular weight marker; Arrow indicates recombinant GST-AC3-33 protein. (A) SDS-PAGE analysis of the lysates obtained from *E. coli* BL21 after IPTG induction with 0.2 mM IPTG at 37°C. Lane 1, total protein of the lysed supernatant; lane 2, total protein of the lysed precipitation (pellet). (B) SDS-PAGE analysis of eluted proteins from supernatant by affinity chromatography on GS4B. Lane 1, the purified GST-AC3-33 fusion protein. (C) Western blot analysis of the lysates. Lane 1, total protein of the lysed precipitation (pellet); lane 2, total protein of the lysed supernatant.

basic condition (Figure 4C). Regarding the concentration of IPTG, there were no difference in the expression level of GST-AC3-33 when the concentration of IPTG were within 0.2~1.6 mM (Figure 4D). Regarding the temperature of induction, the expression level of GST-AC3-33 were higher when the temperature were 30°C and 37°C, whereas lower in 25°C and 42°C (Figure 4E). Regarding the time course of induction, the expression level of the GST-AC3-33 increased with the induction time. The highest amount of fusion protein was produced at 5~7h, whereas lower at overnight (Figure 4F). In summary, the optimal condition to gain the highest amount of fusion protein were using 2×YT cultural medium, with 2g/L NaCl and pH 7.0, induced with 0.2 mM IPTG in 37°C for 5~7h.

4.3. Affinity purification of GST-AC3-33 fusion protein

Bacterial lysates obtained from cultures grown at 37°C and induced with IPTG for 5 h were separated into soluble (supernatant) and insoluble (pellet) fractions by centrifugation. Upon IPTG induction, there was some amount fusion protein with higher purity localized in the supernatant (Figure 3A, lane 1), although a quite quantity of the fusion protein was found in the pellet (Figure 3A, lane 2). Therefore, the GST-AC3-33 fusion protein was purified from supernatant by affinity chromatography on GS4B. Proteins bound to GS4B beads were eluted from the matrix by the addition of reduced glutathione and analyzed on SDS-PAGE. As shown in Figure 3B, a major band at the expected size of the GST-AC3-33 protein (55 kDa) was

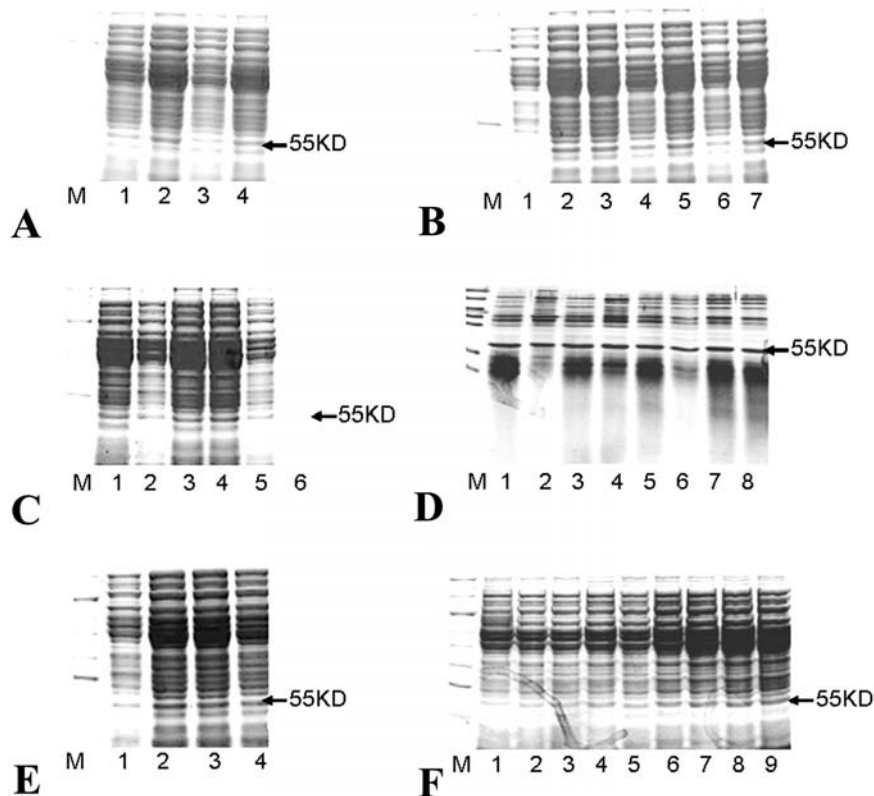


Figure 4. Optimization of expression condition of GST-AC3-33 fusion protein in *E. coli* BL21. In each condition, aliquots of the culture were collected and lysed in 1×SDS sample buffer for 5 min at 100 °C. The lysates were analyzed by 15% SDS-PAGE. (A) The effects of different culture mediums were evaluated. 1, LB; 2, 2×YT; 3, SOB; 4, Nutrient broth. (B) The effects of different concentrations of NaCl were evaluated. 1, 0 g/L; 2, 2 g/L; 3, 4 g/L; 4, 6 g/L; 5, 8 g/L; 6, 10 g/L; 7, 12g/L. (C) The effects of different pH values were evaluated. 1, pH 5.0; 2, pH 6.0; 3, pH 7.0; 4, pH 8.0; 5, pH 9.0; 6, pH 10.0. (D) The effects of different concentrations of IPTG were evaluated. 1, 0.2 mM; 2, 0.4 mM; 3, 0.6 mM; 4, 0.8 mM; 5, 1.0 mM; 6, 1.2 mM; 7, 1.4 mM; 8, 1.6 mM. (E) The effects of different temperatures of induction were evaluated. 1, 25°C; 2, 30°C; 3, 37°C; 4, 42°C. (F) The effects of different time course of induction were evaluated. 1, 0h; 2, 1h; 3, 2h; 4, 3h; 5, 4h; 6, 5h; 7, 6h; 8, 7h; 9, overnight.

observed at >95% purity. The recombinant protein was further confirmed by Western blot using AC3-33 polyclonal antibodies raised from rabbit as shown in Figure 3C. The results showed that strong bands at a molecular weight of 55 kDa were recognized by antisera either in the lysed supernatant or in the precipitation (pellet), confirming that GST-fusion protein was purified successfully.

4.4. Cleavage of GST-AC3-33 fusion protein and purification of recombinant AC3-33

Thrombin, a protease recognizing -Leu-Val-Pro-Arg-Gly-Ser and cleaving specifically the peptide bond between Arg and Gly, was used to release AC3-33 from GST-fusion protein. The recombinant AC3-33 was cleaved from its GST-tag with various concentrations of thrombin. After digestion with 2.5 U of thrombin/mg of protein at 25 °C for 2 h, the fusion protein GST-AC3-33 with the molecular weight of 55 kDa was mainly split into two parts, GST of 26 kDa and a fragment of about 29.3 kDa. Then the suspension was passed through GS4B. The flow-through fraction contained free AC3-33 peptide and thrombin, while the released GST-tag or non-cleaved

fusion protein remained bound to GSH-affinity column. Thrombin in the cleaved product was further removed as previous report using Benzamidine Sepharose Fast Flow, a purification medium with a high specificity for serine proteases. As shown in Figure 5A, the purified AC3-33 protein showed only one single band at SDS-PAGE (Figure 5A line 1). The cleaved fusion proteins were further confirmed by Western blot as illustrated in Figure 5 A line 2.

4.5. Prokaryotic over-expression of AC3-33 suppresses AP-1 activity

Using dual-luciferase cis-reporter assay system, we found that in the cell culture medium, supplementation with the prokaryotic over-expressed recombinant AC3-33 protein significantly suppressed AP-1 reporter gene activity in the absence or presence of AP-1 stimulus, which was similar to the results of supplementation with the eukaryotic over-expressed AC3-33 protein. PMA plus ionomycin was the potent activator of AP-1 signaling (12-13). As shown in Figure 5B, PMA plus ionomycin treatment enhanced AP-1 luciferase activity markedly. The

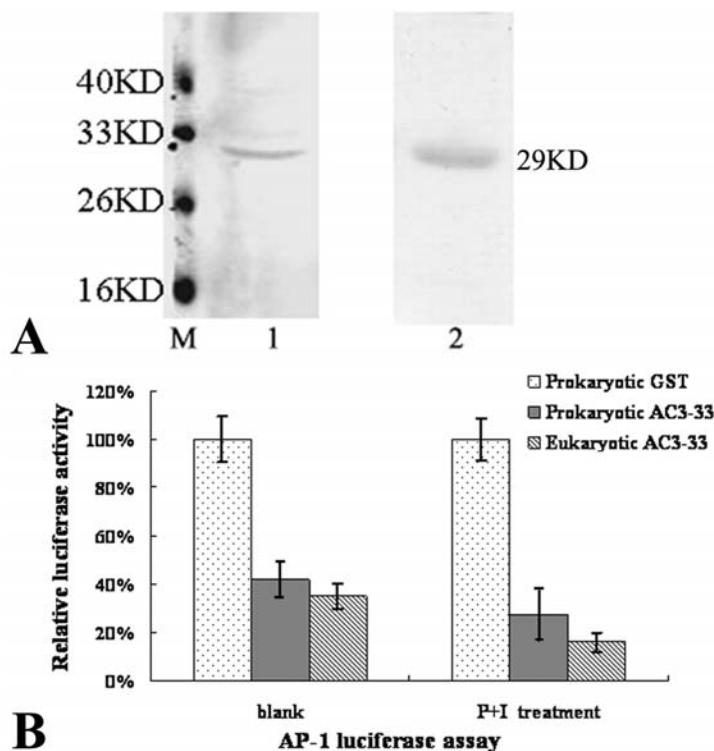


Figure 5. Purified prokaryotic recombinant AC3-33 suppresses AP-1 activity. (A) Purification and identification of recombinant AC3-33 protein. The Cleavage of GST-AC3-33 fusion protein was cleaved by using Thrombin. Then the suspension was passed through GS4B and Benzamidine Sepharose. Line 1, SDS–PAGE analysis of the purified protein; Line 2, the purified AC3-33 protein was confirmed by Western blot. (B) Prokaryotic over-expression of AC3-33 suppresses AP-1 activity in the absence or presence of AP-1 stimulus. AP-1 luciferase activity was measured using cis-reporting AP-1 system. Cells were transiently cotransfected with the pAP-1-Luc plasmids and pRL-TK plasmid. Relative luciferase activity was normalized by co-transfection with pRL-TK plasmid (internal control). At 24 hours after transfection, the cell culture were supplemented with the prokaryotic recombinant AC3-33 protein (20μM), with eukaryotic immuno-precipitated AC3-33 protein (20μM) as positive control and recombinant GST protein (20μM) as negative control. 2 hours later, the cells were stimulated with PMA (50 mM, sigma) and Ionomycin (1 mM) for 6 hours, and then lysates were assayed for both firefly and renilla luciferase activities according to the manufacturer’s instructions.

AP-1 activity of supplementation with either prokaryotic or eukaryotic AC3-33 protein under stimulation was significantly inhibited compared with the GST control (approximately 27% and 16% of that in the empty vector, respectively). The same luciferase results were observed in both HeLa and 293T cells (data not shown), which suggested the bioactivity of prokaryotic AC3-33 protein was not cell-line specific. Taken together, these data indicated that the prokaryotic recombinant AC3-33 protein had AP-1 pathway suppressed activity *in vitro*.

5. DISCUSSION

Using dual-luciferase reporter assay system, we found AC3-33 significantly inhibited AP-1 transcriptional activity (1). To our knowledge, no functional study has been performed on this hypothetical gene. Though the amino acid sequence of AC3-33 was highly conserved in different species, AC3-33 had no homology to other known proteins. The highly efficient production of biologically active AC3-33, with large quantity and low cost, is absolute for revealing the relationship between structure and

function of AC3-33, which will benefit both basic research and potential clinical applications. Due to the need for a large quantity of AC3-33 for these studies, we developed a production method of AC3-33 using the pGEX-4T-1 expression vector in *E. coli*.

It is well known that GST, a soluble cytoplasmic protein, is a useful partner for the expression of highly soluble and stable fusion proteins (14). Here, human AC3-33 cDNA was cloned into pGEX-4T-1, an *E. coli* expression vector containing the GST coding region followed by a thrombin cleavage site, to generate recombinant plasmid pGEX-4T-1-AC3-33 (Figures. 1 and 2). *Escherichia coli* BL21 transformed with plasmid pGEX-4T-1-AC3-33 and induced with IPTG expressed a protein with 55 kDa, in accordance with expected molecular weight for a fusion protein containing GST and AC3-33. The recombinant protein was confirmed by Western blot (Figure 3).

To optimize the expression conditions, the effects of different culture mediums, the concentration of NaCl,

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pH values, the concentration of IPTG, the temperature of induction and the time course of induction were evaluated. The results showed that a relative high amount of GST-AC3-33 fusion protein were acquired using 2×YT cultural medium, with 2g/L NaCl and pH 7.0, induced with 0.2 mM IPTG in 37°C for 5~7h, which would provide reference for related research. It is worth noting that there were no difference within in the expression level of GST-AC3-33 when the concentration of IPTG were within 0.2~1.6 mM in our study, which suggested that maybe 0.2 mM be enough for the induction of recombinant protein expression. The dose was obviously lower than that of previous related reports (15-16).

Though the GST expression system is designed for inducible, high level intracellular expression of genes as soluble proteins (8), many factors influenced the yield of the fusion protein. In our study, some of GST-AC3-33 fusion protein was in soluble form, but a relative high amount was also found in inclusion body (Figure 4A). In order to reduce the formation of insoluble aggregates, different expression conditions such as shorter expression period, lower expression temperature and decreased induction time were tried but none of these conditions resulted in an increased solubility of the fusion protein (data not shown). This phenomenon may partly due to the hydrophobic nature of AC3-33 protein and/or PGEX expression system (17-18).

Using GSH-affinity chromatography for purification, a major band at the expected size of the GST-AC3-33 protein was recovered (>95% purity, Figure 4B and C). Recombinant fusion proteins are usually cleaved to release the target protein of interest for biological and clinical studies. The results showed that the best cleavage condition for the fusion protein was 2.5 U of thrombin/mg of protein for 2 h at 25°C (Figure 5A). The product digested under this condition was further purified with a GSH affinity column and a Benzamidine chromatography column. One single band corresponding to approximately 29 kDa, the molecular weight of the chemically synthesized AC3-33, was observed on SDS-PAGE and confirmed by Western blot (Figure 5A).

To investigate the bioactivity of our purified recombinant AC3-33, AP-1 dual-luciferase cis-reporter assays were performed. The results illustrated that supplementation with prokaryotic AC3-33 recombinant protein significantly suppressed AP-1 reporter gene activity in the absence or presence of AP-1 stimulus, which was similar to supplementation with eukaryotic AC3-33 protein (Figure 5B). These data indicated that the prokaryotic recombinant AC3-33 protein had AP-1 pathway suppressed activity *in vitro*, and therefore suitable for the further functional research.

In summary, we have succeeded in expressing and purifying the functional recombinant AC3-33 protein by biological engineering. To our knowledge, it was the first report on the effective expression and purification of intact and bioactive AC3-33. It provides us with a biochemical tool to obtain AC3-33 recombinant protein,

which will certainly facilitate its structural and functional studies, as well as clinical investigations.

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