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Original Article

Expression, purification, and functional characterization of recombinant PTD-SARA

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The Smad anchor for receptor activation (SARA) protein is a binding partner for Smad2/3 that plays an important role in the fibrotic promoting signaling pathway initiated by transforming growth factor-β1 (TGF-β1). The C-terminal 665–750 aa of SARA comprises the Smad-binding domain (SBD). By direct interaction through the SBD, SARA inhibits Smad2/3 phosphorylation and blocks the interaction between Smad2/3 and Smad4, thereby restrains the process of fibrosis. In this study, we constructed a SARA peptide aptamer based on the SBD sequence. The recombinant SARA aptamer, fused with a protein transduction domain (PTD-SARA), was cloned, purified from *E. coli*, and characterized for the first time. The full-length *PTD-SARA* coding sequence, created with *E. coli* favored codons, was cloned into a pQE-30 vector, and the recombinant plasmid was transformed into an M15 strain. After Isopropyl β-D-1-Thiogalactopyranoside (IPTG) induction and Ni^{2+} affinity purification, recombinant PTD-SARA was further identified by immunoblotting and protein N-terminal sequencing. Epifluorescence microscopy revealed that the recombinant PTD-SARA was transferred into the cytoplasm and nucleus more efficiently than SARA. Moreover, the recombinant PTD-SARA was found to up-regulate the level of E-cadherin and down-regulate the levels of α-SMA and phospho-Smad3 more efficiently than SARA ($P < 0.05$). Our work explored a method to obtain recombinant PTD-SARA protein. The recombinant PTD-SARA fusion protein could enter HK2 cells (an immortalized proximal tubule epithelial cell line) more efficiently than the SARA protein and reverse the renal epithelial-to-mesenchymal transdifferentiation process that was induced by TGF-β1 more effectively than the SARA protein. Recombinant PDT-SARA is likely to be a potential candidate for clinical prevention and treatment of renal fibrosis.

Keywords PTD-SARA fusion protein; cloning; protein expression and purification

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Introduction

Transforming growth factor-β1 (TGF-β1)-promoted renal fibrosis is one of the most important mechanisms leading to the progression of multiple chronic kidney diseases to end-stage kidney failure. TGF-β1 performs its fibrotic promoting function primarily through the downstream molecules Smad2 and Smad3 [1–3]. The Smad anchor for receptor activation (SARA) protein was initially discovered as a Smad2 binding protein and was found to localize to the early interior serosa through two FYVE zinc finger domains [4]. It can bind to the type I TGF-β receptor and recruit Smad2/3 to the receptor, resulting in their phosphorylation. Phosphorylated Smad2/3 is then released and forms a complex with Smad4 [5,6] to transduce the TGF-β signal and eventually promotes renal fibrosis. Studies have revealed that the C-terminal 665–750 aa of SARA constitutes a Smad-binding domain (SBD). The SBD can bind to Smad2/3 to prevent phosphorylation of Smad2/3 *in vitro* [7] and co-crystallize with the MH2 domain of Smad2. There are three structural motifs of SBD that form direct interactions with the hydrophobic groove of the MH2 domain of Samd2. The SBD specifically interacts with the monomeric form of Smad2/3 and prevents Smad3-Smad4 complex formation. Recently, a peptide aptamer, based on the sequence of the SBD from SARA, was constructed on the *E. coli* thioredoxin A scaffold. The expression of this peptide aptamer significantly inhibited the epithelial-mesenchymal transition of mouse mammary gland cells [8]. Thus, a peptide aptamer of SARA may be a potential candidate for the prevention and treatment of renal fibrosis.

The interaction between the SARA peptide aptamer and Smad2/3 occurs intracellularly, and the prerequisite for its biological function is that the peptide aptamer must be able to enter cells efficiently. The molecular weight of SARA, however, is too large to be used for *in vivo* biological therapy. Conversely, the protein transduction domain

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(PTD) can facilitate the transmembrane transport of covalently conjugated compounds, peptides, nucleic acids or fused full-length proteins into cells in a receptor, transporter, and ATP-independent manner [9]. Conjugation of SARA peptide aptamer with PTD enables the SARA aptamer to enter pathological tissues and cells to counteract fibrotic signaling. Here, we constructed a PTD-SARA peptide aptamer fusion protein, purified the fusion protein, and studied its biological function *in vitro*.

Materials and Methods

Chemicals and reagents

The recombinant plasmid pQE-30 and host strain *E. coli* M15 were stored in our laboratory. The full-length sequence of PTD-SARA was synthesized, and its sequence was verified by Beijing Aoke Biotech Co. (Beijing, China). Restriction enzymes, T4 DNA ligase, and DNA marker DL2000 were purchased from TaKaRa (Shiga, Japan). We obtained lysozyme from Tiangen Biotech (Beijing, China), a plasmid miniprep kit and DNA gel extraction kit from Boda Tech (Beijing, China), a protein marker from Fermentas (Glen Burnie, USA), horseradish peroxidase (HRP)-goat anti-rabbit IgG from Golden Bridge, Inc. (Beijing, China), anti-His monoclonal antibody and Ni^{2+}-nitilotriacetic acid (NTA) slurry from Novagen (Gibbstown, USA), and isopropyl β-D-1-thiogalactopyranoside (IPTG), agar, and agarose from Dingguo, Inc. (Beijing, China). Protein N-terminal sequencing was performed at the Peking University (Beijing, China). Paraformaldehyde (PFA, 4%) and IPTG were purchased from Dingguo Biotech (Beijing, China), DMEM-F12 cell medium was obtained from Hyclone (Logan, USA), and fetal bovine serum (FBS) was obtained from Sijiqing (Hangzhou, China). TGF-β1 was purchased from R&D Company (Minneapolis, USA), mouse anti-SARA monoclonal antibody (McAb) was obtained from Santa Cruz (Santa Cruz, USA), rabbit anti-phospho-Smad3 and anti-E-cadherin McAbs were obtained from Epitomics (Burlingame, USA), rabbit anti-α-SMA, anti-β-actin McAbs, HRP-labeled goat anti-rabbit IgG, and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG were obtained from Boaosen Biotech, Ltd. (Beijing, China).

Construction of the pQE-30-PTD-SARA plasmid

The sequence of the *PTD-SARA* was designed with *E. coli* favored codons based on SARA peptide aptamer sequence [8] and synthesized with a 5′ *Bam*HI site and a 3′ *Sal*I site. The sequences list is as follows: for *PTD-SARA*, 5’-GGATCCTATGCTCGTGCCGCAGCGCGGCAAGCTCGTGCTGGTGGCGGAAGTGGTGGCGGAAGTGGTGGCGGAAGTATGAGCGCGAGCAGCCAGAGCCCGAACCCGAACAACCCGGCGGAATATTGCAGCACCATTCCGCCGCTGCAGCAGGCGCAGGCGAGCGGCGCGCTGAGCAGCCCGCCGCCGACCGTGATGGTGCCGGTGGGCGTGCTGAAACATCCGGGCGCGGAAGTGGCGCAGCCGCGTTAGGTCGAC-3’; for *SARA aptamer*, 5’-GGATCCATGAGCGCGAGCAGCCAGAGCCCGAACCCGAACAACCCGGCGGAATATTGCAGCACCATTCCGCCGCTGCAGCAGGCGCAGGCGAGCGGCGCGCTGAGCAGCCCGCCGCCGACCGTGATGGTGCCGGTGGGCGTGCTGAAACATCCGGGCGCGGAAGTGGCGCAGCCGCGTTAGGTCGAC-3’. These fragments were cloned into pGE-X056G (provided by Aoke, Inc., Beijing, China) and transformed into a DH5 strain. After extraction, this plasmid and the pQE-30 vector were digested by *Bam*HI and *Sal*I. The products were separated using agarose electrophoresis, purified, and ligated. The ligation product was transformed into M15 cells. The pQE-30-PTD-SARA and pQE-30-SARA aptamer plasmids were verified by DNA sequencing. The protein product from this plasmid contained an N-terminal 6 × His tag.

Expression of PTD-SARA in *E. coli*

The pQE-30-PTD-SARA and pQE-30-SARA aptamer plasmids were transformed into *E. coli* M15 cells. A single clone was inoculated in 2 ml of LB (supplemented with 100 mg/l ampicillin) and cultured overnight at 37°C. The overnight cultured *E. coli* cells were inoculated into LB supplemented with 100 mg/l of ampicillin. When the OD_{600} reached 0.5, IPTG was added at final concentrations of 0.01, 0.05, 0.1, 0.5, 1.0 or 3.0 mM to induce protein expression for 4 h. To explore the optimal induction time, IPTG was added at a final concentration of 0.01 mM, and cells were harvested every 4 h. The expression of target protein was analyzed by SDS-PAGE.

Purification and renaturation of the fusion protein

Cells were harvested by centrifugation at 4000 rpm for 15 min, resuspended and lysed by lysozyme in an ice bath. After centrifugation at 15,000 rpm for 15 min, the cell lysate was collected as the supernatant and the pellet were analyzed using SDS–PAGE. After the PTD-SARA protein was identified in the pellet, the pellet fraction was washed in a buffer containing 20 mM Tris–HCl (pH 8.0), 10 mM EDTA, 2 mM 2-mercaptoethanol, and 0.5% Triton X-100 once, in a buffer containing 20 mM Tris–HCl (pH 8.0) and 2 mM EDTA twice, and in a buffer containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, and 2 mM urea once. After washing, the pellet fraction was resuspended in 8 M urea and incubated at 4°C for 12 h to dissolve. The protein solution was then cleared by centrifugation at 15,000 rpm for 15 min, and the supernatant was applied to a Ni^{2+} bead column. Non-specifically bound proteins were washed away with buffer containing 25 mM imidazole, and the column was eluted with 100 mM imidazole and fractions

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were collected. The purified PTD-SARA protein was diluted 10-fold and further dialyzed to perform protein renaturation. The concentrations of urea were 6, 4, and 2 mM. Finally, the target protein was dialyzed against phosphate-buffered saline (PBS), and all samples were analyzed on SDS–PAGE followed by Coomassie blue staining, then quantitated by densitometric scanning of SDS–PAGE gels and Sygene software analysis [10].

Identification of the PTD-SARA fusion protein

The samples were resolved by SDS–PAGE and transferred electrophoretically to nitrocellulose membranes. The resulting membranes were blocked using 5% non-fat milk in TBST [10 mM Tris–HCl (pH 7.4), 150 mM sodium chloride (NaCl), 0.05% Tween-20], and incubated with the mouse anti-His tag antibody overnight at 4°C. After incubation with the HRP-conjugated secondary antibody, immune complexes were detected using ECL reagents and autoradiography.

Sample preparation and N-terminal sequencing

The purified PTD-SARA protein was resolved by SDS–PAGE and transferred electrophoretically to polyvinylidenedifluoride (PVDF) membranes. The resulting membrane was stained with 0.5% Coomassie blue R-250 at room temperature for 5 min and destained with 50% methanol until the background was clean. The membrane was then dried at 37°C and sent to Peking University. The automated sequencing of PTD-SARA was performed using an Applied Biosystems Procise 494A protein sequencer (PE Biosystems, Warrington, USA) and Edman degradation chemistry.

Cell culture

HK2 cells were cultured in DMEM-F12 medium containing 10% FBS at 37°C with a saturation of 5% CO_2. When the cells grew to 90% confluence, they were digested by trypsin and resuspended. These resuspended cells were then seeded into 6-well plates with a density of 1×10^6/ml and incubated for 24 h. The literature [11] revealed that 10 ng/ml TGF-β1 and 24 h of incubation time were the optimal dose and induction time for cell transdifferentiation. Cells were thus treated by adding the following reagents: (i) no TGF-β1 treatment: no proteins or TGF-β1 except cell medium; (ii) TGF-β1 treatment: cell medium plus TGF-β1 (10 ng/ml); (iii) SARA contrast: cell medium plus SARA (5 or 10 μg/ml) followed by TGF-β1 (10 ng/ml) 45 min later; and (iv) PTD-SARA trial: cell medium plus PTD-SARA (5 or 10 μg/ml) followed by TGF-β1 (10 ng/ml) 45 min later. The cells were then incubated for 24 h, after that the cell morphologies were observed using a microscope and photographed.

Immunofluorescence assay

HK2 cells with a density of 1×10^6/well were seeded into 24-well plates that were inlaid with glass coverslips at the bottom. After 24 h, recombinant PTD-SARA was added to each well with a final concentration of 10 μg/ml. After incubation at 37°C for 4 h, the cell medium was discarded on an ice bath, and then the cells were rinsed with pre-cooled PBS (4°C, pH 8.0) three times for 5 min each time. Then, the cells on glass coverslips were treated as follows. PFA (4%) was added to each well and incubated for 30 min for fixation. Triton X-100 (0.3%) was added dropwise onto cells and incubated for 30 min. Goat blocking serum (10 g/l) was added at 37°C for 30 min, and one drop of mouse anti-SARA McAb at a dilution of 1:50 was added at 4°C overnight. FITC-labeled goat anti-mouse IgG at a dilution of 1:100 was added in darkness at 37°C for 1 h. DAPI (4',6-diamidino-2-phenylindole, 1%)-containing PBS was added to each well for 10 min. Rinses were repeated three times after each step. Glycerol, at a concentration of 500 ml/l, was used to seal the glass cover slips. Finally, cells were observed under a fluorescence microscope, and the FITC-dyed and DAPI-dyed images were captured. MERGGE Version 2.0 software was used to merge the two types of fluorescence-dyed images. The parallel trials of both the SARA contrast and blank control followed the same procedures as those used for the PTD-SARA group except for the supplementation of SARA and no protein, respectively, instead of PTD-SARA.

Protein extraction and western blotting

Protein was extracted from cells (2×10^6) with lysis buffer as described [12]. Protein concentration was measured using a protein assay kit (Bio-Rad). Protein concentration was determined using the Bradford method. The total protein extraction was resolved by SDS–PAGE. After SDS–PAGE, the gel and PVDF membranes were immersed in precooled 1× transfer buffer for 10 min. Then, the proteins in the gels were transferred electrophoretically to PVDF membranes at a constant voltage of 100 V for 1 h. The PVDF membranes were blocked with 5% BSA for 30 min, incubated in primary antibody at 4°C overnight, incubated in secondary antibody at room temperature for 1 h, and rinsed as described above. Thereafter, an ECL chromophore was used to develop the film. The software Quantity One 4.62 (Bio-Rad) was used to measure the grayscales of the proteins of interest and the internal protein control.

Statistical analysis

Data were presented as the means ± SD. A *t*-test was used to test differences in means between two groups. All statistical analyses were conducted using the SPSS 15.0 software package. Significance levels were set at $P < 0.05$.

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Results

Construction of the PTD-SARA expression plasmid

The recombinant expression vector pQE-30-PTD-SARA was transformed into M15 cells. The plasmid was then extracted after amplification. The plasmid was identified by digestion with *Bam*HI and *Sal*I. After digestion, a 255-bp fragment was observed on the agarose gel, as expected. Sequencing results confirmed the correct DNA sequence (data not shown).

Expression of PTD-SARA

The concentration of IPTG used to induce protein expression was first optimized. After 4 h of induction, SDS–PAGE showed a specific protein band that appeared at the expected size (10 kDa). Furthermore, there was no significant difference among the different IPTG concentrations [**Fig. 1(A)**]. Next, we optimized the duration of induction; samples were collected every hour and analyzed on whole lysate SDS–PAGE. After 4 h induction, the expression of the PTD-SARA fusion protein was stabilized [**Fig. 1(B)**]. The protein product was ~10 kDa and accounted for ~25% of the total amount of cellular protein by gradation, which were determined by densitometric scanning of SDS–PAGE gels and Sygene software analysis.

Purification and renaturation of PTD-SARA fusion protein

Most of the PTD-SARA protein was found to form an inclusion body (data not shown), so we employed the denaturing purification method. The inclusion body was washed, solubilized in 8 M urea, and affinity purified using Ni^{2+} chromatography (**Fig. 2**). After serial dialysis, the protein was analyzed using SDS–PAGE; the purity reached more than 90%, the final yields were 1.2 mg purified PTD-SARA per gram of cell. The protein concentration was determined to be 0.2 mg/ml using the Lowry method.

Identification of the PTD-SARA fusion protein

The affinity-purified protein fraction was subjected to western blotting using the anti-His tag antibody. The results showed that the protein fraction was recognized by the antibody against the 6× His tag (**Fig. 3**), suggesting that the protein at 10 kDa was PTD-SARA, encoded by the recombinant plasmid.

Protein transfer to PVDF membrane and N-terminal sequencing

The purified protein, corresponding to the second peak [**Fig. 2(B)**], was separated using SDS–PAGE and subsequently transferred to a PVDF membrane. The protein was loaded in nine different lanes to obtain the maximum amount of target protein. After staining, clear bands were visible on the membrane. The protein samples were then N-terminally sequenced at the Peking University. The sequencing result showed that the N-terminal 14-aa fragment of PTD-SARA was Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-Tyr-Ala. It conformed that the N-terminal sequence of PTD-SARA was in agreement with the sequence that we designed.

Identification of transmembrane transduction of recombinant PTD-SARA

Cytological immunofluorescence assay showed that PTD-SARA localized in the cytoplasm and nuclei with strong green fluorescence, while SARA only localized in

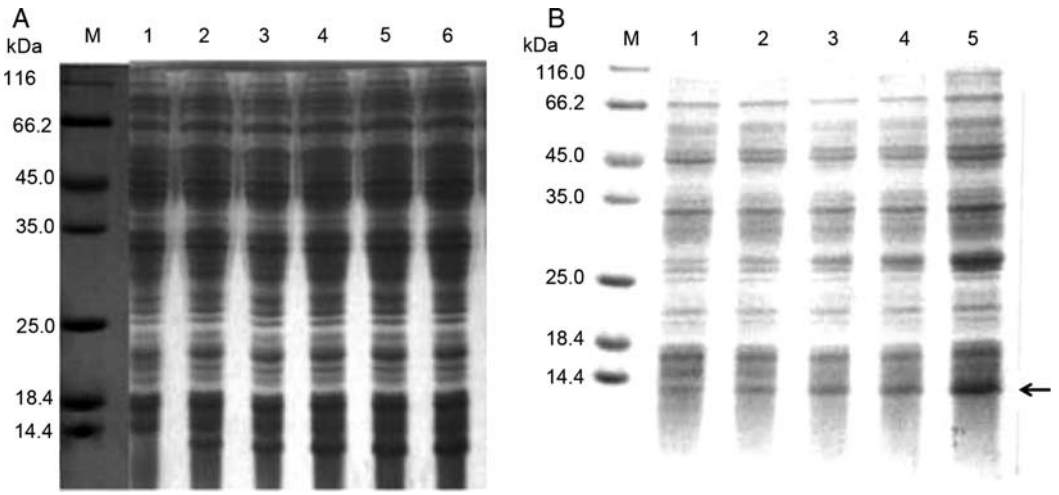

Figure 1 Optimization of PTD-SARA expression conditions (A) SDS–PAGE analysis of PTD-SARA expression after induction with different concentrations of IPTG. M, protein marker; 1, lysate before induction; 2–7, lysates after induction with 0.01, 0.05, 0.1, 0.5, 1.0, and 3.0 mM of IPTG. (B) SDS–PAGE analysis of PTD-SARA expression at different time points after induction. M, protein marker; 1–5, lysates after 1, 2, 3, 4, and 5 h of induction.

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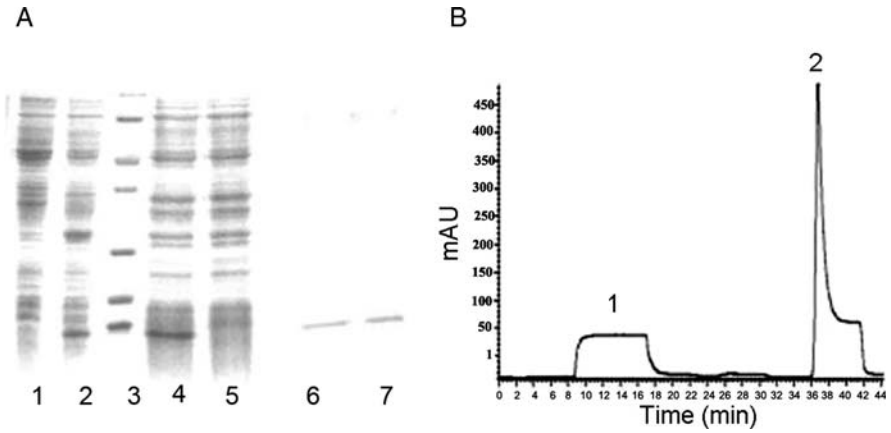

Figure 2 Purification of the PTD-SARA fusion protein (A) SDS−PAGE analysis of fractions from the Ni purification of the PTD-SARA fusion protein. 1, lysate before induction; 2, lysate after induction; 3, protein marker (order from top to bottom: 116,000, 66,200, 45,000, 35,000, 25,000, 18,400, and 14,400 Da); 4, pellet of cell lysate; 5, supernatant of cell lysate; 6 and 7, purified PTD-SARA [corresponding to the second peak in (B)]. (B) Elution profile from Ni^{2+} chelate affinity column. The first peak is flow through; the second peak is the target protein.

the cytoplasm, and in the parallel control cells only weak fluorescence staining was observed (**Fig. 4**). These results indicated that recombinant PTD-SARA was transferred to the cytoplasm and nuclei of HK2 cells.

Western blot

It was shown that no TGF-β1 treatment cells expressed α-SMA but expressed the highest levels of E-cadherin, while the TGF-β1 treatment cells expressed the highest level of α-SMA and the lowest level of E-cadherin. Cells of the PTD-SARA trial expressed lower levels of α-SMA and higher levels of E-cadherin compared with the SARA trial. The differences between the PTD-SARA trial and the control trials (TGF-β1 treated and SARA trial) were significant ($P < 0.05$ for all) (**Fig. 5**). The western blot results showed that cells of the no TGF-β1 treatment had trace amounts of phospho-Smad3 (P-Smad3) expression, cells of the TGF-β1 treatment expressed the highest levels of phospho-Smad3 among all groups, and cells of the PTD-SARA trial expressed lower levels of phospho-Smad3 compared with the SARA trial. The differences between the PTD-SARA trial and the control trials (TGF-β1 treated and SARA trial) were significant ($P < 0.05$ for all) (**Fig. 6**), while there were no different for the expression of total Smad3 (T-Smad3) among all of them. These results indicated that recombinant PTD-SARA inhibited TGF-β1-induced Smad3 phosphorylation more significantly than SARA.

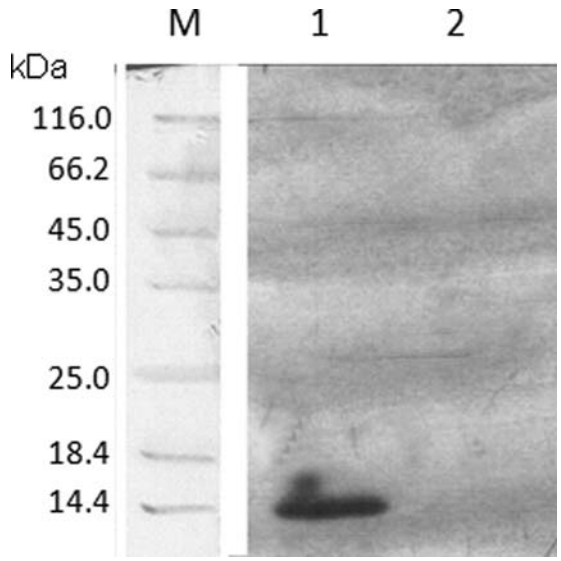

Figure 3 Western blotting of the purified PTD-SARA M, molecular weight standards (kDa); 1, purified protein labeled with anti-His-tag antibody; 2, negative control lane.

Discussion

Protein transduction technology is the utilization of the protein transduction domain to deliver a covalently bonded compound, peptide, nucleic acid, or protein into cells in a receptor, transporter, or ATP-independent way [13,14]. Multiple proteins have been found to contain PTD, such as the HIV-1 TAT protein, HSV-1 VP22 protein, and *Drosophila antennapedia* homeoprotein (pANTP) [15−19]. Here, we constructed a PTD-SARA fusion protein with an N-terminal 6×His tag for easy Ni^{2+} affinity purification. Among the three commonly used PTD sequences, RRRRRRRRR and YARAAARQARA have been shown to have higher protein transduction activity than RKKRRQRRRPP from the HIV-1 TAT protein. The RRRRRRRRR sequence, however, has been reported to impair the expression levels of recombinant fusion proteins. Therefore, we chose to use the YARAAARQARA sequence in PTD-SARA.

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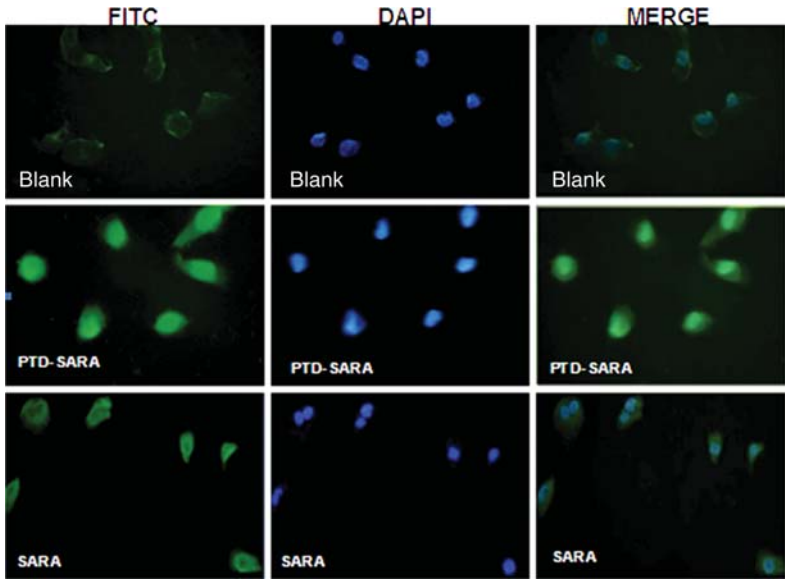

Figure 4 Detecting the transmembrane function of recombinant PTD-SARA fusion protein.

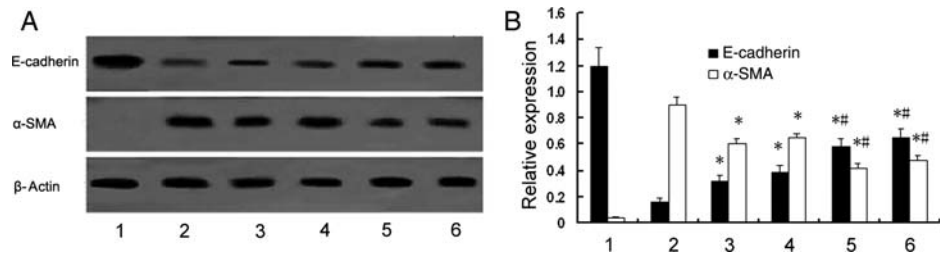

Figure 5 The expression levels of E-cadherin and α-SMA protein were detected by western blotting 1, no TGF-β1 treatment; 2, TGF-β1 treated; 3, 10 μg SARA; 4, 5 μg SARA; 5, 10 μg/ml PTD-SARA; 6, 5 μg/ml PTD-SARA. $^{*}P < 0.05$ compared with TGF-β1 treated; $^{\#}P < 0.05$ compared with SARA contrast (10 μg/ml).

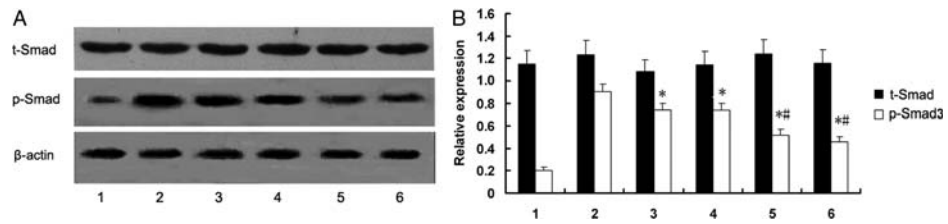

Figure 6 The expression level of Smad3 protein was detected by western blotting 1, no TGF-β1 treatment; 2, TGF-β1 treated; 3, 10 μg SARA; 4, 5 μg SARA; 5, 10 μg/ml PTD-SARA; 6, 5 μg/ml PTD-SARA. $^{*}P < 0.05$ compared with TGF-β1 treated; $^{\#}P < 0.05$ compared with SARA contrast (10 μg/ml).

The concentration of IPTG had little effect on the expression level of PTD-SARA. However, the expression levels increased with longer induction times. The expression level of recombinant PTD-SARA peaked ~4–5 h after induction and started to decrease after 6 h. We also tested different host *E. coli* strains, including M15, BL21, and DH5, and found that M15 had the highest expression level. The M15 strain is the recommended host for the pQE-30 vector in our study.

When an exogenous gene is expressed in prokaryotic cells, especially when that gene is highly expressed in an *E. coli* system, the expressed protein can form an inclusion body and aggregate in cells. The major component in the inclusion body is the recombinant protein, but there are also some bacterial components, such as membrane proteins and plasmid DNA. Usually, <1% detergent, including Tween, Triton, Lubel, and NP40, can be used together with EDTA, dithiothreitol, or

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β-mercaptoethanol to wash away the bacterial components. NaCl can also be added at 50 mM to increase the strength of the detergent. By these means, the purity of the inclusion body can be as high as 50%, with preservation of structure. Insoluble contaminated proteins can be removed by washing with low concentrations of guanidine hydrochloride or urea, neutral detergent, EDTA, and a reducing agent. Because the PTD-SARA protein was present in an inclusion body, we first performed the wash step with Triton to remove lipids and a low concentration of urea to remove contaminated proteins. Finally, PTD-SARA was purified using Ni^{2+}-NTA beads, and the purity reached more than 90%.

The protein aggregated in an inclusion body cannot fold correctly, failing to show biological activity. It is therefore necessary for these proteins to be renatured to achieve their expected biological function. There are many methods to facilitate protein refolding, such as dilution, dialysis, ultrafiltration, chromatography, bed absorption, and the temperature jump method. We used a simple and traditional dilution [20] and dialysis [21] method to renature PTD-SARA. The denatured protein solution was diluted 10 fold and maintained at a pH of 8.0. The protein was then dialyzed against solutions with gradually decreasing urea concentrations. Under the appropriate conditions, the PTD-SARA protein gradually refolded.

A common problem in N-terminal protein sequencing is the blocking of the N-terminus [22–24]. To prevent N-terminal blockage during SDS–PAGE, the gel was pre-run for 2 h. N-terminal sequencing requires a protein purity of >90%, a protein concentration of >10 pM, a salt concentration of <50 mM, and no detergent. We minimized the protein handling time and electrotransferred the protein samples onto a PVDF membrane as soon as the SDS–PAGE was finished to prevent samples from diffusing. The sequence we obtained from N-terminal sequencing was consistent with the designed protein sequence. These results indicate that the purity and protein amount are ideal for further experimentation.

There are very few reports regarding the application of a recombinant SARA peptide aptamer in the prevention and treatment of renal fibrosis. We used cytological immunofluorescence to identify the biological function of the transmembrane transduction of PTD-SARA; our results confirmed that the PTD sequence allowed the SARA protein to enter cells efficiently, and PTD-SARA localized to the cytoplasm and nucleus. The results of western blotting analysis indicated that the recombinant PTD-SARA fusion protein inhibited TGF-β1-induced transdifferentiation from renal tubular epithelial cells to interstitial cells more significantly than SARA. The introduced PTD sequence in our trial did not reduce the biological activity of the recombinant PTD-SARA fusion protein, which presented with superior biological functions compared with the SAPA peptide aptamer. Our pilot trial also indicated that the target for PTD-SARA in blocking HK2 fibrosis was the phosphorylation level of Smad3. However, this finding still needs further study. Our trial conducted a pilot study regarding the biological function of a recombinant PTD-SARA fusion protein at the cytological level. Our studies provide a potentially effective protein drug to relieve renal fibrosis, which might serve as a replacement for gene therapy and BMP7 treatment.

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