以 RHOA-TAT 融合蛋白探討 RHOA 在平滑肌的訊息傳遞之角色(1/2)

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During the past ten months (July 2000-May 2001), our experiments have been focused on the construction of plasmids, expression and purification of RhoA mutants, both constitutively active (RhoAV14) and dominant negative (RhoAN19), using the pTAT-HA vector system. The fusion proteins of both RhoAV14 and RhoAN19 have been successfully expressed and purified. In addition, the loading efficiency of the expressed RhoA fusion proteins has been examined in cultured aortic smooth muscle cells and arterial strip preparations. Using immunoblotting method, we have detected RhoA fusion proteins in cultured smooth muscle cells (SMC) while the optimal loading conditions for arterial preparations are currently under going. The experimental progress for different parts of experiments are described as the following:

1. **Construction of pTAT plasmids containing rhoA mutants.** The bacterial expression vector, pTAT-HA, was obtained from Dr. S. F. Dowdy, Howard Hughes Medical Institute and Division of Molecular Oncology, Washington University, St. Louis, Mo, USA. To construct plasmids containing V14rhoA or N19rhoA insert, pTAT plasmid was first cloned into TOP10 competent cells (Invitrogen Corp.), isolated and digested with XhoI and EcoRI restriction endonucleases. V14rhoA and N19rhoA cDNA were obtained by polymerase chain reaction (PCR) using two primers with 5' primer containing XhoI enzyme site and 3' primer containing EcoRI enzyme site. The sequences for 5' and 3' primers are CCCTCGAGATGGCTGATCCTCAGGAAGA (27 mer) and GAATTCACAGACAGGCACACCAG (24 mer), respectively. The mutant rhoA cDNAs were also double digested with XhoI and EcoRI. Following enzymatic digestion, both inserts and plasmid were purified (GFX PCR DNA and gel band purification kit, Pharmacia Inc.). Fig. 1 shows a gel of purified plasmid and insert cDNA which was used to estimate the amount of both. Ligation of pTAT-HA plasmid and cDNA inserts (molar ratio 1:10) were performed in 10 µl reaction mixture containing 150 ng plasmid, 300 ng insert and 0.3 U/ml T4 DNA ligase for 16 h at 16 °C. A representative result from ligation reaction is shown in Fig. 2. Ligation mixtures with or without ligase were transformed into TOP10 competent cells. Transformants resistant to Ampicillin (100 ng/ml) were screened by separating crude bacterial DNA extracted by phenol-chloroform-isoamylalcohol (25:24:1) on agarose gel (0.8%) electrophoresis and further verified by double enzyme digestion of the isolated plasmids. Fig. 3 shows the results from XhoI & EcoRI double digestion of TOP-10 colonies with insert-containing plasmids. The nucleotide sequences of the insert-containing plasmids were determined by automated sequencing (Automated DNA Sequencing Center at NCKU Medical College). We have sequenced six plasmids containing V14rhoA and two plasmids containing N19rhoA. The results
showed that both plasmids containing N19rhoA had correct sequences whereas all of the six V14rhoA cDNA exhibited unexpected changes resulting in point mutation (data not shown). Thus, a hybrid plasmid containing V14rhoA with a predicted mutation sequence of K18R was proceeded for transformation experiments.

2. Transformation of pTAT-HA-rhoAV14 & pTAT-HA-rhoAN19 plasmids into protein-expressing E. coli strain BL-21 and screening of BL-21 containing hybrid plasmids. BL-21 competent cells (20 μl) were mixed gently with 1.5 μl pTAT-HA-rhoAV14 or pTAT-HA-rhoAN19 plasmid (50 ng/μl). The mixture was incubated on ice for 30 min, heat shocked at 42 °C for 65 sec, and incubated on ice for another 2 min. Following the addition of 50 μl LB, the mixture was incubated at 37 °C for 1 hour. The transformation mixtures were spread on 1.5% LB agar plates and incubated at 37 °C overnight.

To screen BL-21 colonies capable of expressing RhoA mutant fusion proteins, BL-21 colonies were cultured in 5 ml LB at 37 °C till an OD$_{600}$ reading of 0.6-0.8 was obtained. IPTG (final concentration = 0.5 mM) was added and culture continued for 4.5 hour at 37 °C. The expression of fusion proteins in BL-21 cells were analyzed by SDS polyacrylamide gel electrophoresis (SDS PAGE). The results from an IPTG induction experiment are shown in Fig. 4. BL-21 colonies whose fusion protein expression was markedly induced by IPTG was cultured, snap frozen and stored in aliquots.

3. The expression and purification of mutant RhoA fusion proteins. Protein purification was performed according to the procedure provided by Dr. S. F. Dowdy's laboratory with modifications whenever necessary. This procedure applies high concentration of urea to produce partially denatured proteins that are crucial for successful loading into cells. E. coli strain BL21 containing pTAT-HA-rhoAV14 or pTAT-HA-rhoAN19 plasmid was cultured in 200 ml LB and treated with IPTG to induce fusion protein expression. Cell pellets from centrifugation were washed and suspended in 30 ml buffer A (20 mM HEPES, 100 mM NaCl, pH 8.0) and homogenized with French Press (1500 lb, 3 times). Homogenate was centrifuged (3000 x g, 10 min) to collect the inclusion bodies. The pellet was washed with buffer A, re-suspended in 15 ml buffer Z (8 M urea, 100 mM NaCl, 20 mM HEPES, pH 8.0) containing 25 mM imidazole and mixed by stirring. The mixture was added into a column of 5 ml Ni$^{2+}$-NTA (Pharmacia) pre-equilibrated in buffer Z plus 25 mM imidazole, mixed for 30 min and settled for 20 min. The column was washed twice with 5 volumes of the same buffer and eluted with 5 volumes of 500 mM imidazole in buffer Z. Column fractions containing fusion protein were pooled, diluted with equal volume of buffer B (20 mM Tris-HCl, pH 8.8) containing 10 mM dithiothreitol (DTT) and 1 mM EDTA,
and mixed at 4 °C for 30 min. The pool of Ni²⁺-NTA column was mixed with 5 ml DEAE Sephacel pre-equilibrated in buffer B at 4 °C for 30 min and packed into a column. The DEAE column was washed with 8 volumes of buffer B containing 2 mM DTT and 1 mM EDTA and eluted with 5 volumes of 1 M NaCl in the same buffer. Eluted protein fractions were pooled and loaded onto a Sephadex G25 column (1.5 cm in diameter and 12 cm in height) in 20 mM HEPES buffer, pH 7.4 (buffer C). The column was eluted with 2 volumes of buffer C containing 2 mM DTT and 1 mM EDTA. Eluted fractions containing protein were mixed with glycerol (final concentration of 10%) and flash frozen in aliquots of ~250 µl in liquid nitrogen and stored at −80 °C. In order to establish the optimal conditions for the purification by Ni²⁺-NTA column, the concentration of imidazole in the washing solution was first tested between 10 and 80 mM whereas that for column elution was tested with 100, 250, 500 mM and 1 M steps. To minimize the purification time to prevent protein from renaturing, protein elution profiles for Ni²⁺-NTA, DEAE and Sephadex columns were examined by SDS PAGE to determine the fraction volume required for protein elution. A representative purification profile of TAT-HA-RhoAV14 fusion protein is shown in Fig. 5.

4. The evaluation of loading efficiency of TAT-HA-RhoA mutant fusion proteins into cultured SMC. To examine the loading efficiency of mutant RhoA-TAT fusion proteins into cultured SMC, fusion proteins with concentration ranging from 100 to 800 nM were incubated with confluent A7r5 SMC for 1 or 2 h. Following the incubation, cells were washed three times and lysed in homogenization buffer (120 µl for 3.5-cm well) containing 20 mM Tris, pH 7.4, 2 mM EGTA, 2% Triton X-100, 0.2% SDS, 2 mM sodium orthovanadate, 2 mM sodium pyrophosphate with freshly added aprotinin and leupeptin at 1 µM each. Cell extracts were separated by SDS PAGE, transferred to nitrocellulose membranes and detected with immunoblotting. Briefly, filter membranes were incubated sequentially with anti-hemaglutinin (HA) monoclonal antibody and horseradish peroxidase-conjugated horse anti-mouse secondary antibody to detect TAT-HA fusion protein loaded. As shown in Fig. 6, a positive correlation was observed between the incubation time period or the concentration of fusion proteins and the amount of protein detected in the cells. Currently, we are trying to establish the optimal conditions for loading fusion proteins into intact tail artery and aortic strips.
Figure 1. Purified pTAT-HA and rhoA V14/N19

Insert cDNA(rhoa V14/ N19) were amplified by polymerase chain reaction. After purification, pTAT-HA and insert cDNA were double digested by Xho I and EcoRI. This 1% agarose gel shows that the size of pTAT-HA plasmid is ~3.0 kb, and that of insert cDNA is ~0.6 kb. (lane P: pTAT-HA, lane V: rhoA V14, lane N: rhoA N19)
Figure 2. Ligation of insert cDNA and pTAT-HA
line 1: pTAT-HA + rhoA V14 cDNA + T4 DNA ligase. line 2: pTAT-HA + rhoA N19 cDNA + T4 DNA ligase. line 3: pTAT-HA + T4 DNA ligase. line 4: pTAT-HA (linear). line 5: purified circular pTAT-HA. line 6: purified circular pTAT-HA-rhoA V14. line 7: purified circular pTAT-HA-rhoA N19. This 1% agarose gel shows that rhoA V14/N19 cDNA had been inserted into pTAT-HA (hybrid plasmid formation)
Figure 3. Isolated hybrid plasmids identified by XhoI and EcoRI double digestion.

Ligation mixture I and II were transformed into TOP-10 *E.coli* strain and screened for colonies containing hybrid plasmids. Hybrid plasmids were isolated and identified by XhoI and EcoRI double digestion. This 1% agarose gel shows that insert cDNA had been inserted into pTAT-HA in all samples examined.
Figure 4: Screening of fusion protein-expressing BL-21 colonies by isopropyl-β-thiogalactoside (IPTG) induction.

BL-21 containing pTAT-HA-rhoA V14/N19 hybrid plasmids were cultured to a density of O.D = 0.6–0.9 and screened by IPTG induction. These SDS-PAGE images show that whole extract of V4 and N4 BL-21 colonies contained more fusion proteins (30–35 kd) following IPTG induction.
**Figure 5**: Purification of TAT-HA-RhoA V14 fusion protein.

BL-21 *E. coli* (200 ml culture) containing pTAT-HA-rhoA V14 plasmids were treated as described in the text. Homogenate containing 8 M urea was mixed with 5 ml Ni\(^{2+}\)-IDA gel and packed into column. After washing with same buffer containing 25 mM imidazole, the fusion protein was eluted with 0.5 M imidazole in the same buffer. Pool of Ni\(^{2+}\)-IDA column elution fractions was diluted (1:1) and mixed with 5 ml DEAE-sephacel before packed into column. After washing, fusion protein was eluted with 20 mM Tris-HCl (pH 8.8) with 1 M NaCl. Pooled fractions of DEAE column was passed through a sephadex G-25 column to remove salt. **lane WH**: BL-21 *E. coli* whole homogenate (WH). **lane SWH**: supernatent of WH. **lane WP**: washing of pellet. **lane RP**: resuspended pellet. **lane NFT**: Ni\(^{2+}\)-IDA column flow-through. **lane NWI~NWII**: washing of Ni\(^{2+}\)-IDA column. **lane N1~N11**: elution fraction (EF) no. of Ni\(^{2+}\)-IDA column. **lane NP**: EF no. 1~10 pool of Ni\(^{2+}\)-IDA column. **lane DNP**: 1:1 diluted NP. **lane DFT**: DEAE-sephacel column flow-through. **lane DWI~DWDII**: washing of DEAE-sephacel column. **lane D2~D8**: EF no. of DEAE column. **lane DP**: EF no. 2~5 pool of DEAE-sephacel column. **lane S3~S23**: EF no. of sephadex G-25 column.
Figure 6: Effects of concentration and incubation time on the loading of TAT-HA-RhoA N19 into A7r5 cells

Confluent A7r5 cells were cultured in serum-free DMEM with or without TAT-HA-RhoA N19 for 1 hr (A) or 2 hr (B). After washing with HBSS, cells were lysed and cell lysates were processed with SDS-polyacrylamide gel electrophoresis, gel transferred to nitrocellulose membrane and detected by western blotting. **lane 1:** untreated A7r5. **lane 2 ~ lane 5:** A7r5 cells treated with 100 nM, 200 nM, 400 nM, and 800 nM TAT-HA-RhoA N19, respectively for 1 hr (A) or 2 hr (B). **lane 6:** purified TAT-HA-RhoA N19 only. The result of western blotting shows that TAT-HA-RhoA N19 can be loaded into cells and the amount of protein detected is correlated to the time and the dose of treatment.