Journal of Huazhong University of Science and Technology [Med Sci]
DOI 10.1007/s 11596-007-0102-5
27 (1): 5-8, 2007

Optimal Expression Condition of Recombinant RAP*

ZHANG Jie (章 洁)[1], ZHANG Hong (张 红)[1], BI Hao (毕 昊)[1], LIU Zhiguo (刘志国)[2], GUO Jianli (过建莉)[1], QU Shen (屈 伸)[1#]

[1]*Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

[2]*Department of Biotechnology and Chemical Engineering, Wuhan Polytechnic University, Wuhan 430023, China*

Summary: In order to construct the expression recombinant of human receptor associated protein (RAP), optimize its expression condition and obtain the recombinant protein after expression with high efficiency, two prokaryotic expression vectors—pT7-PL and pET-28a(+) were used to construct the expression recombinant containing RAP cDNA, and the expression efficiency of two kinds of expression *E. coli* of BL21 strains was compared. The effect of different induction conditions on the expression of recombinant RAP was observed. After recombinant protein was purified with Ni^{+}-nitrilotriacetic acid (Ni^{+}-NTA) affinity chromatogram, its binding ability with microphage was observed. The results showed that two recombinant plasmids both obtained high expression of RAP. The expression levels of RAP in plasmid pT7-PL-RAP in BL21 (DE3, plysS) strain were significantly higher than in BL21 (DE3) strain. The expression of pT7-PL-RAP in the presence of chloramphenicol was higher than in the absence of chloramphenicol, and most of the inducible expressed RAP was soluble. The RAP which was purified by Ni^{+}-NTA resin could strongly bind with the RAW264.7 cells rich in low density lipoprotein receptor (LDLR) family receptors. It was concluded that the expression condition of recombinant RAP was optimized and functional RAP was obtained, which offered a good foundation for the further production of RAP as research tool.

Key words: receptor associated protein; induced expression; purification

Receptor associated protein (RAP) is a kind of resident protein in endoplasmic reticulum with molecular weight of 39 ku. It has been verified that RAP serves as a molecular chaperone for low density lipoprotein receptor (LDLR) family and plays important roles in transporting receptors from endoplasmic reticulum to membrane, folding of receptor proteins correctly and preventing receptors from bound by other ligands in the process of becoming mature[1]. In addition, if RAP is overexpressed, it can be excreted out of membrane and bind to LDLR family, competing with other ligands (such as Apo E, ApoE abundant lipoprotein, uPA-PA1 complex, some coagulation factors and many proteases)[2]. So, RAP has becoming a useful tool to study LDLR and correlated diseases[3]. Because RAP can be used as an antagonist of Apo E4 in the binding to low density lipoprotein receptor associated protein (LRP), it has been widely used in the research of Alzheimer's disease (AD) in recent years[4,5].

Our research showed that human RAP cDNA got high expression from the expression plasmids pT7-PL-RAP and pET-28a(+)-RAP. We optimized the expression conditions of pET-28a(+) in many aspects such as host bacteria, induction time, and got human RAP with high purity after purified by Ni^{2+}-NTA affinity chromatography. In our study, biological activity of RAP was studied by the binding test of RAP to murine macrophage RAW264.7, which endowed a good foundation for the RAP production.

1 MATERIALS AND METHODS

1.1 Materials

Plasmid containing human RAP cDNA was kindly provided by Doctor Morten Nielsen in Denmark. BL21 (DE3, plyaS) and BL21 (DE3) strains were stored in our laboratory. Murine macrophage strain RAW264.7 was purchased from Shanghai Cell Biochemistry Institute (China). Ldl-A7 not expressing LRP and VLDLR but expressing LDLR in trace was kindly presented by Professor Monty Krieger. Restriction endonucleases were from Sino-American Biotechnology Co. (China). Isopropylthiogalactoside (IPTG) was purchased from Sigma Biotechnology Co. (USA). Medium PRMI-1640, DMEM-F12 and DMEM were all from Gibco Biotechnology Co. (USA). Ni^{2+}-NTA affinity chromatography and fluorescein isothiacyanate (FITC) were from Shanghai Huasun Co. (China).

1.2 Methods

1.2.1 Construction of Prokaryotic Expression Plasmids of RAP and Expression of RAP Protein Recombinants, pT7-PL- RAP and pET-28a(+)-RAP containing human RAP cDNA in correct reading frame were transformed into BL21 (DE3, plyaS) and BL21 (DE3) expression strains respectively. Positive clones were selected and cultured in LB medium with ampicillin (pT7-PL-RAP) and kanamycin (pET-28a(+)-RAP) at 37

ZHANG Jie, female, born in 1964, Technician
E-mail:tjzj121@sina.com
#Corresponding author
*This project was supported by a grant from National Natural Sciences Foundation of China (No. 39970307).

℃, shaking until A_{600}=014-110, and IPTG was added for induction and treated for another 16 h. Then, collected bacteria were mixed with agarose gel electrophoresis loading buffer, boiled at 100 ℃ for 5 min and loaded on SDS-PAGE electrophoresis gel with un-induced bacteria as control group. The concentration of separation gel and concentration gel were 5% and 10% respectively.

BL21 (DE3, plysS) transformed by plasmid pT7-PL-RAP were cultured in large amount for the preparation of RAP. The supernatant of ruptured bacteria was purified following Ni-NTA His•Bind Resin manufacturer's instructions. SDS-PAGE was adopted for the verification of purity.

1.2.2 Optimization of RAP Expression and Detection of Solubility Comparison of expression levels between pT7-PL-RAP and pET-28a(+)-RAP was performed between transforming BL21 (DE3, plyaS) and BL21 (DE3). The expression of RAP in BL21 (DE3, plyaS) transformed by plasmid pT7-PL-RAP was detected in the presence or absence of chloramphenicol. Bacteria, after induction with IPTG for 5 h and 16 h were collected and ruptured by supersonic wave (2 mW, 20 kHz with intermission of 10—15 s) and 1% Triton 2100 and centrifuged at 4 ℃, 13 500 r/min for 25 min. Supernatant and deposit were collected separately for SDS-PAGE. The comparison of amount of RAP was performed between supernatant and deposit.

1.2.3 Preparation of VLDL and Labeling of FITC Very low density lipoprotein (VLDL) was isolated from human fresh blood serum[6], stored at 4 ℃ in dark and used within 2 weeks. VLDL, RAP and BSA were labeled with FITC following the instructions offered by Roche Biotechnology Co. (Switzerland). Labeled protein and lipoprotein were used for cell binding test.

1.2.4 Cell Culture and Binding Test of RAP with RAW264.7 Murine macrophage strain RAW264.7 was cultured in PRMI 1640 medium containing 10% new born calf serum, 2 mmol/L glutamine at 37 ℃ in 5% CO_2. ldl-A7 strain was cultured in DMEM-F12 medium under the same culture condition of RAW264.7.

Murine macrophage strain RAW264.7 and ldl-A7 strains were cultured in the DEMEM without serum for climbing slide culture. Before cell binding test, culture medium was sucked off and cells were washed by PBS for 2—3 times, and then DEMEM containing 6 mg/mL BSA, ligands labeled with FITC and 5 mmol/L $CaCl_2$ were added and placed in dark at 4 ℃ for 3 h. Before testing, solution in the sample well was sucked off and washed with 1 mL PBS containing 2 mg/mL BSA 2 times. Each time lasted 5 min with shaking in order to get rid of unbinding ligands and then all were ready for observation under fluorescence microscopy[3]. Binding of ligands with ldl-A7 cells and binding of BSA with RAW264.7 cells served as negative group, and binding of VLDL and RAW264.7 cells as positive group.

2 RESULTS

2.1 Induced Expression of RAP

To get high expression of exogenous gene, two prokaryotic expression vectors, pT7-PL-RAP and pET-28a(+)-RAP were constructed and transformed into BL21 (DE3, plyaS) and BL21 (DE3). The expression level with or without induction of IPTG was detected (fig. 1). As shown in fig. 1, both expression vectors obtained high expression of RAP. RAP expressed by pET-28a(+)-RAP was at position of 43 kD and RAP expressed by pT7-PL-RAP was at position of 40 kD. GIS analysis showed that the expression of pT7-PL-RAP in BL21 (DE3, plyaS) was significantly higher than that in BL21 (DE3).

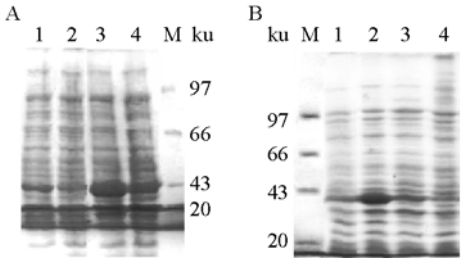

Fig. 1 Effect of expression vector and host bacteria strains on RAP expression

A: Expression of pET-28a(+)-RAP in different host bacteria strains. M: Protein marker in low molecular weight; 1: BL21 (DE3, plyaS) transformed by pET-28a(+)-RAP with induction of IPTG for 16 h; 2: BL21 (DE3, plyaS) transformed by pET-28a(+)-RAP without induction of IPTG for 16 h; 3: BL21 (DE3) transformed by pET-28a(+)-RAP with induction of IPTG for 16 h; 4: BL21(DE3) transformed by pET-28a(+)-RAP without induction of IPTG for 16 h.

B: Expression of pT7-PL-RAP in different host bacteria strains. M: Protein marker in low molecular weight; 1: BL21 (DE3, plyaS) transformed by pT7-PL-RAP with induction of IPTG for 16 h; 2: BL21 (DE3, plyaS) transformed by pT7-PL-RAP without induction of IPTG for 16 h; 3: BL21 (DE3) transformed by pT7-PL-RAP with induction of IPTG for 16 h; 4: BL21 (DE3) transformed by pT7-PL-RAP without induction of IPTG for 16 h

2.2 Effects of Chloramphenicol and Glucose on the Expression of RAP

In the process of expression of pT7-PL-RAP in BL21 (DE3, plyaS), resistance of chloramphenicol was added for the screening of plasimid plysS. It was found that chloramphenicol could improve the IPTG-induced RAP expression and the RAP expression decreased in the absence of IPTG (fig. 2).

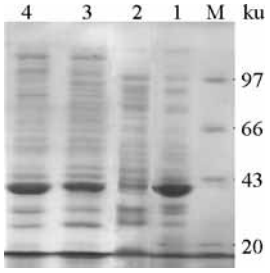

Fig. 2 Effect of chloramphenicol on the expression of RAP

M: Protein marker in low molecular weight. 1: RAP expression without chloramphenicol selection and without induction of IPTG.; 2: RAP expression without chloramphenicol selection and with induction of IPTG.;

3: RAP expression with chloramphenicol selection and without induction of IPTG; 4: RAP expression with chloramphenicol selection and with induction of IPTG.

2.3 Assay of Solubility of RAP and Effect by Induction time

In the process of expression of pT7-PL-RAP in BL21 (DE3, plyaS), most expressed RAP were soluble and resided in the supernatant of ruptured cells. RAP expression level with induction of IPTG for 16 h was significantly higher than that for 5 h (fig. 3). With Optimization of RAP expression conditions, production of RAP could reach 76% of total bacteria proteins, which was verified by GIS analysis.

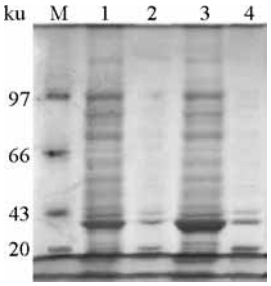

Fig. 3 Effect of induction time on RAP solubility

M: Protein marker in low molecular weight; 1: Supernatant of ruptured bacteria with induction of IPTG for 5 h; 2: Deposit of ruptured bacteria with induction of IPTG for 5 h; 3: Supernatant of ruptured bacteria with induction of IPTG for 16 h; 4: Deposit of ruptured bacteria with induction of IPTG for 16 h

2.4 Electrophoresis Results of Purified RAP

Only single band was found in SDS-PAGE gel after purification of RAP, which indicated that purity of RAP reached SDS-PAGE purity (fig. 4).

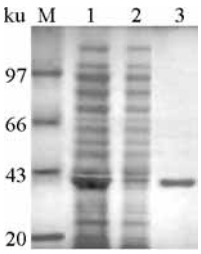

Fig. 4 Purification results of RAP

M: Protein marker in low molecular weight; 1: Supernatant of ruptured bacteria with induction of IPTG overnight; 2: Elution after binding of supernatant of ruptured bacteria with affinity chromatography; 3: Purified protein

2.5 Observation on Binding Test of RAP and RAW264.7

Under fluorescence microscopy, no fluorescence was seen in the negative group and the result of binding of BSA and RAW264.7 was the same. In the positive group, strong fluorescence was observed. And strong fluorescence was also seen in the binding between RAP and RAW264.7 (fig. 5). The results above showed that RAP had higher binding ability with RAW264.7, while no binding ability with ldl-A7 lacking VLDLR, LRP, LDLR. BSA could not bind to the two kinds of bacteria, which could exclude possibility of unspecific binding. All above showed that RAP could bind to VLDLR, LRP, and LDLR, which was in agreement with the data reported in reference[2].

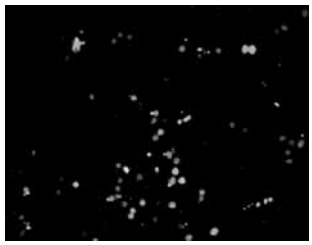

Fig. 5 Binding of RAP with RAW264.7 under fluorescence microscopy

3 DISCUSSION

After human RAP cDNA was inserted into expression vector pT7-PL and pET-28a(+) and expressed in *E. coli*, large amount of fusion proteins could be produced and their molecular weight were 40 ku and 43 ku respectively. The electrophoresis analysis by comparing with protein marker supported that the molecular weight of expressed protein was accorded with protein coded by insertion. Two kinds of fusion protein contain 6-His in the N terminal, which is coded in expression vector. Because the functional domain of RAP is centralized in C terminal reported in reference[7], 6 amino acids in the N terminal of fusion protein expressed by pT7-PL RAP are not cut off, considering their little effect on size and function of protein.

In the prokaryotic expression of proteins, many factors can affect the amount and solubility of expressed proteins. In our study, the effects of many factors such as vector, host bacteria and antibody on the amount of expressed RAP were studied. And effect of different induction time on the solubility of expressed RAP was also detected. The results showed that no obvious difference in expression was observed by transforming different vectors, but high expression level could only be obtained in the special host bacteria. For expression of PAP in the BL21 (DE3, plyaS) transformed by pT7-PL-RAP, the induced expression was decreased in the absence of chloramphenicol. The results also suggested that accumulation of RAP had no toxicity on bacteria, and both expression level and solubility of prokaryotic expression RAP could be increased by extending induction time.

Our research showed that high expression of soluble RAP could be obtained by optimal condition. For fusion protein containing 6-His, Ni^{+}-NTA resin was used to separate and purify RAP under un-denaturation condition. The result of purification could obtained single band in SDS-PAGE. The result of protein quantitation showed that 10 μg purified RAP could be obtained in 1 mL bacterial culture medium.

After purification, His-RAP was labeled with FITC for the binding test to RAW264.7. It was found that purified RAP in our study could bind to RAW264.7 which was rich in LDLR family such as LRP, VLDLR, LDLR

and so on. Because 77% homogeneity existed between human RAP and murine RAP[8], in our study, human RAP still showed high binding ability with murine macrophage strain.

RAP as the chaperone and many kinds of lipoprotein ligand, plays an important role in the research on the structure and function of receptor. Meanwhile it is correlated with the development of many diseases, so it can be used for the researches on membranous nephropathy and Alzheimer's disease. In our study, a new method for the preparation of RAP keeping biological function with high production and purity was successfully established, which provides a good foundation for the RAP production.

REFERENCES

1 Bu G J, Marzolo M P. Role of RAP in the biogenesis of lipoprotein receptors. Trends Cardiovasc Med, 2000,10: 148-155

2 Melman L, Cao Z F, Rennke S *et al*. High affinity binding of receptor-associated protein to heparin and low density lipoprotein receptor-related protein requires similar basic amino acid sequence motifs. J Biol Chem, 2001,276:29 338-29 346

3 Narita M, Holtzman D M, Fagan A M *et al*. Cellular catabolism of lipid poor apolipoprotein E via cell surface LDL receptor-related protein. J Biochem (Tokyo), 2002,132(5):743-749

4 Gylys K H, Fein J A, Tan A M *et al*. Apolipoprotein E enhances uptake of soluble but not aggregated amyloid-beta protein into synaptic terminals. J Neurochem, 2003,84:1442-1451

5 Nathan B P, Jiang Y, Wong G K *et al*. Apolipoprotein E4 inhibits, and apolipoprotein E3 promotes neurite outgrowth in cultured adult mouse cortical neurons through the low-density lipoprotein receptor-related protein. Brain Res, 2002,928: 96-105

6 Feng Z C, Deng Y Z. Rapid isolation of large amount of plasma VLDL and LDL by a two step ultracentrifugation. J Tongji Med Univ (Chinese), 1995(24),169-171

7 Savonen R, Lynn M O, Julie S T *et al*. The carboxyl-terminal domain of receptor-associated protein facilitates proper folding and trafficking of the very low density lipoprotein receptor by interaction with the three amino-terminal ligand-binding repeats of the receptor. J Biol Chem, 1999,274:25 877-882

8 Striekland D K, Ashcom J D, Williams S *et al*. Structure of alpha 2-macroglobulin receptor-associated protein. J Biol Chem, 1991,266:13 364-369

(Received Sept.19, 2005)