

# Expression, purification and identification of LBD domain of human PPAR $\delta$ in *E. coli*<sup>☆</sup>

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## Abstract

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors known to play a pivotal role in regulations of metabolism. In order to yield soluble ligand binding domain of PPAR $\delta$  (PPAR $\delta$ LBD) for screening ligands, the cDNA was amplified using total RNA from HepG2 cells by RT-PCR. Then the enzyme-digested product was inserted downstream of the *malE* gene in the vector *pMAL-p2x*, which encoded maltose-binding protein (MBP), resulting in the expression of an MBP-PPAR $\delta$ LBD fusion protein. The recombinant plasmid was transformed into *E. coli* TB1 that was cultured shakily at 30 °C, 200 r/min and induced by 0.4 mmol/L IPTG for 6 h. The cells were harvested by centrifugation and broken by sonication. The expressed fusion protein was soluble and accounted for 0.31 of the total protein in the supernatant. Western blot analysis showed that the expressed MBP-PPAR $\delta$ LBD could bind to anti-MBP-antibody. The MBP-PPAR $\delta$ LBD fusion protein of 77 kDa and the PPAR $\delta$ LBD protein of 34 kDa were obtained by amylose-resin affinity chromatography without or with digestion of Factor Xa. They were both homogeneity, judged by SDS-PAGE. The recombinant MBP-PPAR $\delta$ LBD and PPAR $\delta$ LBD protein with high purity is obtained, which provides the necessary material for screening and researching PPAR $\delta$  ligands.

**Keywords:** PPAR $\delta$ LBD; Maltose-binding protein; Soluble expression; Purification; Affinity chromatography

## 1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the superfamily of nuclear receptors [1]. PPARs have three isoforms designated PPAR $\alpha$ ,

PPAR $\beta/\delta$ , and PPAR $\gamma$ , each of which has identified ligand binding domain (LBD) recognizing special ligands. PPARs will be activated once LBD of them is bound to a variety of agonist-like ligands, such as some of fatty acids and prostaglandins, hypolipidemic drugs from fibric acid derivatives, as well as thiazolidinediones-like insulin-sensitizing drugs. PPARs participate in a series of biological processes, including cell differentiation, energy balance, lipid

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metabolism, insulin sensitivity, bone formation, inflammation, and tissue remodeling [2]. Several studies have demonstrated that there exists a cross talk between PPARs signaling and other nuclear receptor pathways. This convergence and interaction of different pathways can occur at multiple levels, including competition between PPARs and other nuclear receptors [3]. The diverse biological effects of PPARs indicate that both agonists and antagonists of PPARs may provide new therapeutic options for a variety of human diseases. Therefore, seeking ligands of PPARs has a great significance for us to face the challenge from many diseases and research into the correlation among the nuclear receptor signaling pathways. However, to date, the ligand screening assays were carried out mainly on the PPAR $\alpha$  and  $\gamma$ , but rarely PPAR $\delta$ . Therefore, it is necessary to develop a series of the gene engineering technique that are used to conveniently express and purify PPAR $\delta$ LBD for the identification of ligands of PPAR $\delta$ .

*E. coli* maltose-binding protein (MBP), is a large-affinity tag for fusion of a protein, which is advantageous in terms of increasing expression, enhancing solubility, preventing proteolysis, improving folding, and purifying protein via affinity chromatography [4, 5]. Several studies showed that MBP was the best N-terminal and C-terminal protein fusions to promote soluble expression for 125 mammalian proteins [6]. However, the MBP fusion protein expression system has not been used to express PPAR $\delta$  proteins as yet.

In this study, an effort was done to create PPAR $\delta$ LBD using the MBP fusion protein expression vector, *pMAL-p2x*, through the gene engineering technique. The harvested PPAR $\delta$ LBD could be used to seek new PPAR $\delta$  ligands and investigate correlation between PPAR $\delta$  and other nuclear receptors. The protocol taken in this experiment may be suitable for preparation of other nuclear receptors.

## 2. Materials and methods

### 2.1. Materials

*E. coli* TBI, *pMAL-p2x*, amylase-resin, Go3S-PCR product purification kit, and rabbit anti-MBP serum were purchased from New England Biolabs. DNA ladder was from Sangon (China). Primer synthesis and DNA sequencing were performed by Shanghai Shenergy Biocolor Bioscience & Technology Company (China). T4 DNA ligase, isopropylthio- $\beta$ -D-galactopyranoside (IPTG), and Xgal were products of Takara (China). Pfu DNA polymerase was from TIANGEN BIOTECH (China). Trizol Reagent was obtained from Invitrogen (USA). SuperScript II first-strand system was from Invitrogen. HRP color development reagent (DAB) was from Sino-American Biotechnology Company. DEAE-52 anion exchange chromatography column was purchased from Amersham-Pharmacia. Goat anti-rabbit IgG conjugated with HRP and other agents generally used were provided by Beyotime Institute of Biotechnology (China). HepG2 cells were obtained from the Cell Bank of Chinese Academy of Sciences.

### 2.2. Methods

#### 2.2.1. Cell culture

HepG2 cells were cultured under humidified 50 ml/L CO<sub>2</sub> and 950 ml/L air at 37 °C with Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum, 100 unit/ml penicillin G, and 100  $\mu$ g/ml streptomycin sulfate.

#### 2.2.2. Preparation of recombinant plasmid containing *hPPAR $\delta$* cDNA segment

Total RNA was extracted with Trizol Reagent from HepG2 cells and RT-PCR primers were designed

based on the mRNA sequence of human PPAR $\delta$ LBD published online.

First-strand cDNA synthesis was performed using 1  $\mu$ g total RNA, as described in the SuperScript II First-Strand System User Manual. One-tenth of the first-strand synthesis reaction was used as cDNA template for PCR, with hPPAR $\delta$ LBD (138–440aa, GenBank accession No. NM006229, mRNA sequence) primers: sense (5'-ACGTCTAGAATGTCACACAA-CGC TATCCG-3') and antisense (5'-TCTAAGCTT-GTGCCGCCGTTAGTACATG-3'). The temperature profile was: 95 °C for 2 min prior to amplification, then 10 cycles, 94 °C for 10 s, 60 °C for 10 s, 72 °C for 1 min; 26 cycles, 94 °C for 10 s, 55 °C for 10 s, 72 °C for 1 min; 1 cycle, 72 °C for 5 min and 4 °C for 1 h. PCR was implemented in a 50  $\mu$ l reaction volume containing 1  $\mu$ l Pfu DNA polymerase in the manufacturer reaction buffer (with 1.5 mmol/L MgCl<sub>2</sub>) in the presence of 0.5 mol/L PCR primers and 1 mmol/L dNTP. The RT-PCR products were cloned into the *pMAL-p2X* expression vector according to the manufacturer instructions. Primer synthesis and DNA sequencing were performed by Shanghai Shenergy Biocolor Bioscience & Technology Company (China). The recombinant plasmid containing hPPAR $\delta$  cDNA was identified by restriction enzyme digestion and DNA sequencing, and then transformed into *E. coli* TB1. Fig. 1 showed the construction scheme of expression vectors for hPPAR $\delta$ LBD.

### 2.2.3. Optimization of protein expression in *E. coli*

Single colony on the LB plate was inoculated into 5 ml LB broth with 0.2% glucose and 100  $\mu$ g/mL ampicillin and incubated overnight at 37 °C in a bacterial shaker. 100 ml of fresh engineering bacterium preparation was added to 20 ml antibiotic-containing LB broth. The bacteria were incubated in bacterial shaker for an additional 2 h at 37 °C after induced with 0.3 mmol/L IPTG when the optical

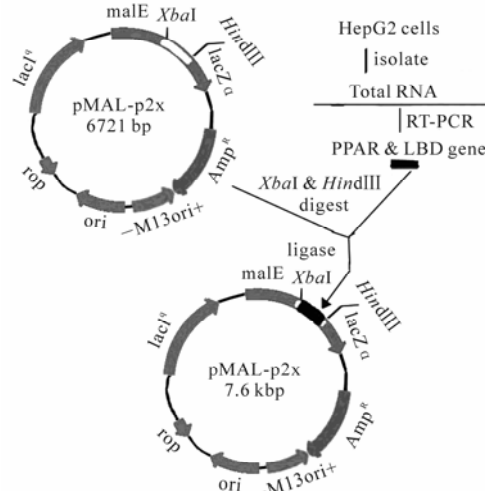


Fig. 1. Schematic illustration of the construction of expression vector of *pMAL-p2x-PPAR $\delta$ LBD*.

density of the bacteria reached 0.5 *A* at 600 nm.

To observe the biological behaviors of the expressed fusion proteins, six samples from cultured engineering bacteria received different treatments were obtained: un-induced and induced cells, total cell crude extract, suspension of the insoluble material from the crude extract, a fraction containing protein that binds to the amylose resin, and periplasmic fraction prepared by the cold osmotic shock procedure. All these samples were analyzed by SDS-PAGE.

To optimize the inducing conditions, the host bacteria were induced with 0.1–1.6 mmol/L IPTG when the optical density reached 0.5 *A* at 600 nm, then incubated in bacterial shaker for an additional 1–8 h at 22–37 °C. 1 ml of the bacteria collected under variously inducing conditions was centrifuged and lysed. Then cell lysate, after centrifuging the soluble and insoluble fragments was electrophoresed on a SDS-PAGE for evaluation of protein expression, and standard protocols were followed for Western analysis where MBP-hPPAR $\delta$ LBD was probed with polyclonal anti-MBP IgG.

#### 2.2.4. Purification of MBP-hPPAR $\delta$ LBD and hPPAR $\delta$ LBD proteins

The *E. coli* TBI pellet from the 1 L culture was washed with 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L sodium EDTA, and 200 g/L sucrose. The pellet was resuspended with sonication buffer (20 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) at the ratio of 1:40. The suspension were sonicated at the ultrasonic powers of 50 Watts for periods of 5 min. During sonication, the temperature of the suspension was maintained at 4 °C by controlling the temperature of cooling water. The lysate was centrifuged at 12 000 g for 50 min at 4 °C to separate out the debris. The supernatant containing the MBP-hPPAR $\delta$ LBD fusion proteins was loaded on preequilibrated amylose columns. The columns were washed with 12 volumes of lysis buffer and the MBP-hPPAR $\delta$ LBD proteins eluted with 10 mmol/L maltose. The protein fractions were analyzed by SDS-PAGE.

In order to obtain hPPAR $\delta$ LBD proteins, the fusion proteins were cleaved with Factor Xa (the ratio of enzyme to substrate was 1:35) in 20 mmol/L Tris buffer (pH 8.0), containing 100 mmol/L NaCl, 2 mmol/L CaCl<sub>2</sub>. The digestion was performed at 23 °C for 3 h. To remove the MBP, the cleavage mixture was loaded on preequilibrated amylose columns and washed with lysis buffer. The eluates containing hPPAR $\delta$ LBD and Factor Xa, were applied to DEAE-52 anion exchange chromatography column in an equilibration buffer (20 mmol/L Tris, 20 mmol/L NaCl, pH 6.0) and eluted from DEAE-52 by the elution buffer containing 20 mmol/L Tris, 2 mol/L NaCl, pH 6.0. The fractions containing hPPAR $\delta$ LBD were collected and stored at -80 °C until use. The procedures above were mainly performed at 4 °C. All protein fractions were analyzed by SDS-PAGE and protein concentrations were measured by Bradford assay.

### 3. Results

#### 3.1. Construction of pMAL-p2x-PPAR $\delta$ LBD

After the agarose electrophoresis of RNA extracted from HepG2 cells, the appearances of 5S band, clear 28S and clear 18S suggested that RNA was complete (Fig. 2A). PCR products were electrophoresed on the agarose gel and the targeted DNA fragments, approximately 900 bp (Fig. 2B), were excised from the gel and cloned into the pMAL-p2X expression vector to construct pMAL-p2x-PPAR $\delta$ LBD. The recombinant plasmids were confirmed by releasing 900 bp DNA fragments after restriction enzyme digestion (Fig. 2C) as well as PCR and sequencing. The cloned sequences were entirely identical to the sequences registered in the GenBank database (the data not shown).

#### 3.2. Optimization of fusion protein expression and solubility analysis

The results of MBP-PPAR $\delta$ LBD expression were displayed in Fig. 3. As shown in lane 3 of Fig. 3A, high expression level of an induced protein with about

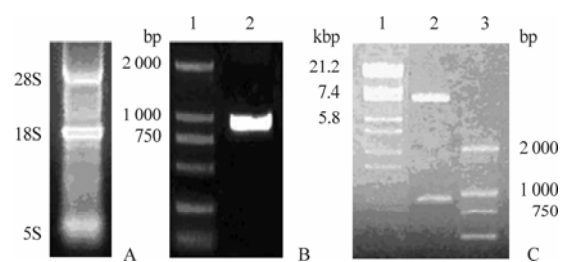


Fig. 2. Electrophoretic results from (A) total RNA, (B) RT-PCR products, and (C) double digested products of recombinant pMAL-p2x-PPAR $\delta$ LBD by agarose. B: lane 1, DNA markers; lane 2, RT-PCR products of hPPAR $\delta$ LBD. C: lane 1 and 3, DNA markers; lane 2, double digested products of recombinant pMAL-p2x-PPAR $\delta$ LBD with *Xba* I and *Hind*III.

77 kDa was achieved in the *E. coli* harbouring expression plasmid *pMAL-p2x/hPPAR $\delta$ LBD*, which was consistent with the expected molecular mass of the fusion MBP (45 kDa) and hPPAR $\delta$ LBD (34 kDa). Lane 6 of Fig. 3A and Lane 2 of Fig. 3B showed that the protein of 77 kDa could bind to the amylose resin and react with anti-MBP polyclonal antibody, suggesting that the MBP was fused into the target protein. The quantitative analysis of the gel bands gray showed that these fusion proteins accounted for about 0.22 of the total bacterial proteins, while the soluble parts (ultrasonic supernatant) accounted for only 0.12. More than 0.95 of the fusion proteins existed in the cytoplasm, only few retained in the periplasm. The results indicated that MBP-hPPAR $\delta$ LBD proteins were expressed successfully in the cytoplasm, part of which existed in an insoluble form.

In order to increase the production of soluble fusion proteins, the induction conditions were optimized (Fig. 4). Fig. 4A showed that when inducing temperature was 37 °C, about 0.55 of the recombinant proteins was soluble, when 30 °C, about 0.76 was soluble, but 22 °C, the total fusion protein was significantly reduced. So, 30 °C was favorable. As shown in Fig. 4B, the expression level kept changing markedly when a variety of induction time was taken (from 1 h to 6 h). Fig. 4C presented that no prominent difference was found in expression of MBP-hPPAR $\delta$ LBD using IPTG of 0.4–1.0 mmol/L. But when IPTG concentrations used was less than 0.2 mmol/L or more than 1.4 mmol/L, the expression level could be sharply decreased. Under reasonable conditions for induction, the amount of the soluble MBP-hPPAR $\delta$ LBD products could reach as high as 0.31 of the total cellular proteins and one liter cultures could yield approximately 20 mg soluble recombinant proteins.

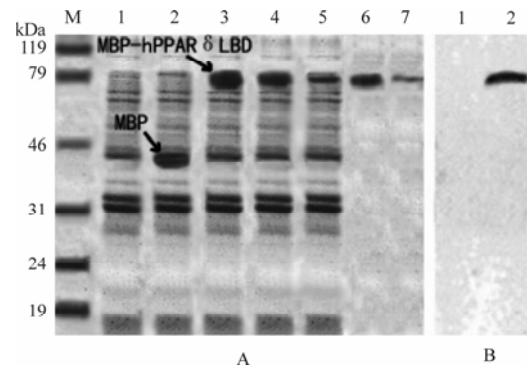


Fig. 3. SDS-PAGE (A) and Western blot analysis (B) of MBP-hPPAR $\delta$ LBD. A: lane M, protein molecule marker; lane 1, total protein of *pMAL-p2x/hPPAR $\delta$ LBD*/TB1 before induction; lane 2, total protein of *pMAL-p2x*/TB1 after induction; lane 3, total protein of *pMAL-p2x/hPPAR $\delta$ LBD*/TB1 with induction; lane 4, supernatant obtained after sonication of *pMAL-p2x/hPPAR $\delta$ LBD*/TB1 with induction; lane 5, deposit obtained after sonication of *pMAL-p2x/hPPAR $\delta$ LBD*/TB1 with induction; lane 6, deposit of the mixture of the amylose resin and ultrasound supernatant of *pMAL-PPAR $\delta$ LBD*/TB1 after induction; lane 7, osmotic-shock fluid of *pMAL-p2x/hPPAR $\delta$ LBD*/TB1 after induction. B: lane 1, total protein of *pMAL-p2x/hPPAR $\delta$ LBD*/TB1 before induction; lane 2, ultrasound supernatant of *pMAL-p2x/hPPAR $\delta$ LBD*/TB1 after induction.

### 3.3. Purification of MBP-hPPAR $\delta$ LBD and hPPAR $\delta$ LBD proteins

MBP was used as a fusion partner to provide a “tag”, which could be used for the subsequent purification of hPPAR $\delta$ LBD. The purified fusion protein showed a single protein band of approximately 77 kDa on SDS-PAGE (Fig. 5, lane 3). The recovery rates and the yields of all three purified fusion proteins were about 0.70 and 13.5 mg per liter of culture.

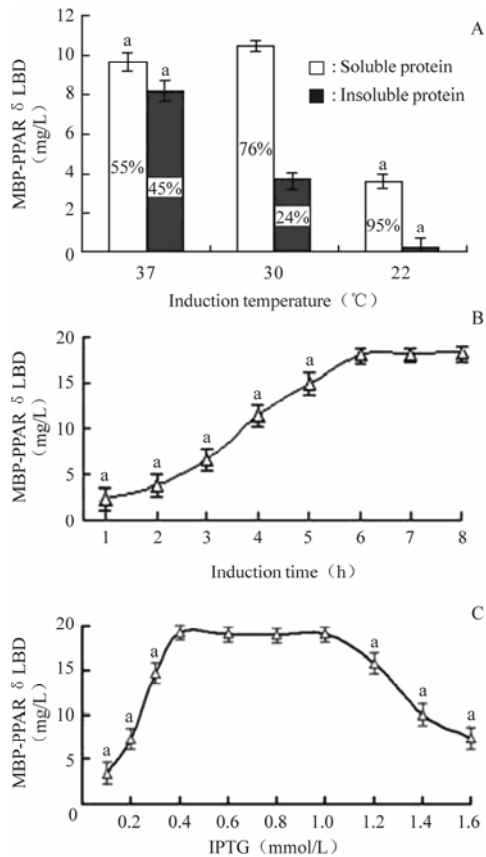


Fig. 4. Optimization of inducing conditions of MBP-hPPAR $\delta$ LBD expression. A: influence of induction temperature; B: Influence of induction time; C: Influence of concentration of IPTG. Values are the *Mean*±*SE* for three samples of the fusion proteins performed in triplicate. <sup>a</sup>*P*<0.05 vs 30 °C in A, 6 h in B, and 0.4 mmol/L in C, respectively.

The results from SDS-PAGE analysis also showed that the fusion protein could be cleaved by Factor Xa and bands with a apparent molecular weight of 77, 42, 34, 30 and 20 kDa for MBP-hPPAR $\delta$ LBD (not been cut), MBP, hPPAR $\delta$ LBD, large subunit and small subunit of factor Xa were observed respectively (Fig. 5, lane 4 and 7). The target proteins existed in the cleavage mixtures were harvested through amylose resin affinity chromatography (Fig. 5, lane 5) and DEAE-52 anion exchange chromatography (Fig. 5,

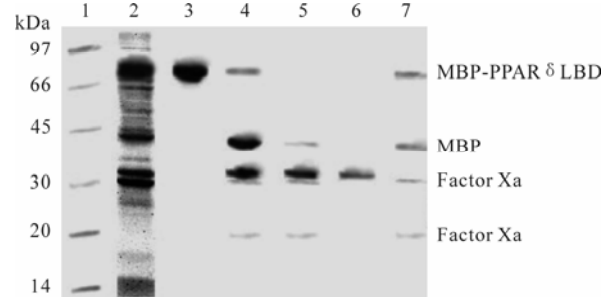


Fig. 5. Purification analysis of MBP-hPPAR $\delta$ LBD and hPPAR $\delta$ LBD by SDS-PAGE. Lane 1: The low-molecular weight marker; Lane 2: Ultrasound supernatant of *pMAL-p2x/hPPAR $\delta$ LBD/TB1* after induction; Lane 3: Purified MBP-hPPAR $\delta$ LBD; Lane 4: Purified MBP-hPPAR $\delta$ LBD cleaved with Factor Xa; Lane 5: hPPAR $\delta$ LBD isolated by amylose resin affinity chromatography; Lane 6: Purified hPPAR $\delta$ LBD isolated by amylose resin affinity chromatography and DEAE-52 anion exchange chromatography; Lane 7: Negative control.

lane 6), and showed one band of hPPAR $\delta$ LBD. The recovery rates and yields of the purified hPPAR $\delta$ LBD were approximately 45% and 5 mg, respectively.

#### 4. Discussion

Sufficient receptor protein is necessary for the ligand binding assay, a basic method to confirm a ligand of a receptor. However, it is difficult to obtain enough PPAR $\delta$  protein directly from tissues because of their trace expression. Therefore, it is necessary to develop a set of molecular biological technique to conveniently prepare PPAR $\delta$  proteins with high purity. For this purpose much effort was done through the glutathione S-transferase (GST) and polyhistidine affinity tag fusion prokaryotic expression systems fusing the GST or His6 gene to produce fusion proteins containing hPPAR $\delta$ LBD [7, 8]. However, the two expression systems mentioned above have several drawbacks that may limit the subsequent use of the

fusion proteins. First, the GST is not only involved in the interactive call of PPARs with other signal transduction pathways [9], but also can bind to some PPARs ligands or hamper the binding of ligands to PPARs [10]. Second, the His-tag and GST fusion proteins are often expressed in the form of inclusion bodies, which often results in insoluble protein aggregates and refolding of the proteins (denaturation and renaturation) as well as purification cost much effort and money [11]. Third, the final yield of fragments is only a small part of the protein that initially exists in the inclusion bodies, even though purification steps are nowadays facilitated by affinity chromatography using C-terminal polypeptide tails, like poly-His6 or GST [12, 13]. Forth, His-tag and GST-tag may inhibit PPARs to combine with PPARs antibody [14], which seriously affected the rapid identification of the fusion protein.

To obtain a substantial quantity of the functional PPAR $\delta$ LBD, the *pMAL-p2X* was used in the study because of its many advantages, compared with the vectors containing GST and His tag. First, the MBP tag can increase the solubility of recombinant protein [15] and the fusion protein can be easily identified by SDS-PAGE owing to its large molecular weight (45 kDa) as well as by Western blot with anti-MBP serum. Second, the function of protein is retained effectively in affinity chromatography because of the moderate elution by maltose. Finally, MBP does not influence on PPARs-mediated signal transduction system. Using the *pMAL-p2X*, PPAR $\delta$ LBD was expressed successfully as MBP fusion proteins in the cytoplasm, but partly in insoluble form.

The solubility of recombinant proteins generally increased with the decreasing temperature in cell culture [16]. However, if the temperature was very low the total fusion protein was significantly reduced. Therefore, the induction conditions were optimized. The results showed that 30 °C incubation temperature

with 6 h incubation time and 0.4–1.0 mmol/L IPTG was the best condition; the ratio of the soluble MBP-PPAR $\delta$ LBD to the total cytoplasm protein was 0.31 and one liter culture could yield approximately 20 mg soluble proteins.

In these experiments fusion protein was conveniently purified with one-step affinity chromatography with maltose and the MBP tag was easily removed by digestion of Xa and two-step chromatography. About 13.5 mg MBP-hPPAR $\delta$ LBD fusion protein and 5 mg target protein per liter engineering bacterial culture medium were obtained under the optimized conditions and purification procedures.

Whether the harvested MBP-hPPAR $\delta$ LBD fusion protein had biological function and MBP tag affected hPPAR $\delta$ LBD activity was very important. Unfortunately, the function of MBP-hPPAR $\delta$ LBD and hPPAR $\delta$ LBD failed to be identified in this study. There is a remote possibility that their biological function could be kept because MBP does not hinder the spatial structure of some target proteins by crystal structure analysis [17]. Nevertheless, a standard radioligand binding assay would be used to confirm the characteristics of two receptor protein in future studies.

In conclusion, a substantial quantity of MBP-PPAR $\delta$ LBD and PPAR $\delta$ LBD was obtained by the *pMAL-p2X* vector, which laid a foundation for screening PPAR $\delta$  ligands and will also offer a new strategy for expression and research of the other nuclear receptors.

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