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Communication

Soluble Expression in *Escherichia coli* of Active Human Cyclic Nucleotide Phosphodiesterase Isoform 4B2 in Fusion with Maltose-Binding Protein

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Recombinant expression in *Escherichia coli* of human cyclic nucleotide phosphodiesterase 4B2 (hPDE4B2) fused to maltose-binding-protein (MBP-hPDE4B2) was investigated. hPDE4B2 DNA amplified *via* nested RT-PCR with total RNAs from U937 cells was ligated with pMAL-p2x. After induction at 18 °C for 16 h, soluble MBP-hPDE4B2 was produced in *E. coli*. MBP-hPDE4B2 after amylose-resin chromatography showed 35% homogeneity, and its Michaelis-Menten constant was $10 \pm 2\,\mu$M ($n = 3$). Rolipram had a dissociation constant of 9 ± 2 nM ($n = 2$), and zinc ion was a potent inhibitor. Hence, MBP-hPDE4B2 was expressed in *E. coli* as a soluble active protein.

Key words: cyclic nucleotide phosphodiesterase 4; recombinant expression; maltose-binding-protein; kinetic properties; rolipram

Adenosine 3′,5′-cyclic monophosphate (cAMP) is a key signal suppressing functions of immune cells involved in inflammation reactions,[1–3] and its action is terminated *via* hydrolysis to adenosine 5′-monophosphate (AMP) by cyclic nucleotide phosphodiesterase (PDE). Among PDE isozymes, cAMP-specific PDE (PDE4) predominates in inflammation reaction cells. Therefore, PDE4 is a target in anti-inflammation therapy,[3–8] and some selective PDE4 inhibitors have been proven to have promising efficiency to treat inflammatory respiratory diseases.[8–10]

For screening of human PDE4 inhibitors, active PDE4 is required. Human PDE4 can be produced *via* recombinant expression in eukaryotic cells, but it shows high cost and low efficiency.[11–14] Alternatively, it can be produced *via* recombinant expression in *E. coli* at low cost, but it suffers from insolubility and laborious renaturation steps.[14] The expression in *E. coli* of eukaryotic genes in fusion with maltose-binding-protein (MBP) is effective to produce soluble active proteins after an easy purification step.[15–18] Herein, we report the soluble expression in *E. coli* of active human PDE4 isoform B2 in fusion with MBP (MBP-hPDE4B2).

U937 cells were cultured in RPMI1640 medium containing 10% fetal beef serum and 5% CO_2 at 37 °C for 2 d to extract total RNAs with reagent kits from Takara (all materials and reagent kits for operating DNA and the vector were from Takara, Dalian, China). According to the coding sequence of hPDE4B2 (Genebank accession no. M97515), three primers, CTBF1 (5′-GAATTCATGAAGGAGCACGGGGCAC-3′), CTBF2 (5′-AAGAGACCTCCTAAAGACAT-3′), and CTBR (5′-AAGCTTTTATGTATCCACGGGGG-ACTTGT-3′), were used to amplify human PDE4B2 (hPDE4B2) *via* nested RT-PCR with standard protocols. Then the PCR product was digested with EcoR I and Hind III, purified by agarose electrophoresis, and inserted into pMD19-T for amplification in *E. coli* JM109.

After full-length sequencing, however, two point mutations were identified in the inserted DNA fragment, of which one coded a different amino acid. Hence, site-directed mutation was used to correct the DNA sequence to give the amino acid sequence for the native hPDE4B2 protein. Afterwards, hPDE4B2 DNA fragment was released from pMD19-T by digestion with EcoR I and Hind III, and ligated with pMAL-p2x after digestion with the same two enzymes, to give pMAL-p2x-hPDE4B2. Then competent *E. coli* BL21 cells were transformed with pMAL-p2x-hPDE4B2, and positive clones were verified by full sequencing. Finally, a selected positive clone was cultured at 37 °C for 3 h, and induced by 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 18 °C to produce MBP-hPDE4B2. *E. coli* BL21 transformed with the blank vector was also induced with IPTG to produce endogenous PDEs.

After induction with IPTG, the *E. coli* cells were harvested, diluted with a lysis buffer (50.0 mM sodium citrate buffer at pH 6.4 containing 10.0 mM $MgCl_2$, 5.0 mM EDTA, 2.0 mM *p*-aminobenzamidine, 1.0 mM

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Abbreviations: EDTA, ethylenediamine-tetraacetic acid; hPDE4B2, human PDE4 isoform B2; IEC, ion-exchange chromatography; K_m, Michaelis-Menten constant; MBP, maltose-binding-protein; MBP-hPDE4B2, human PDE4B2 fused to the C-terminal of MBP; PDE, cyclic nucleotide phosphodiesterase

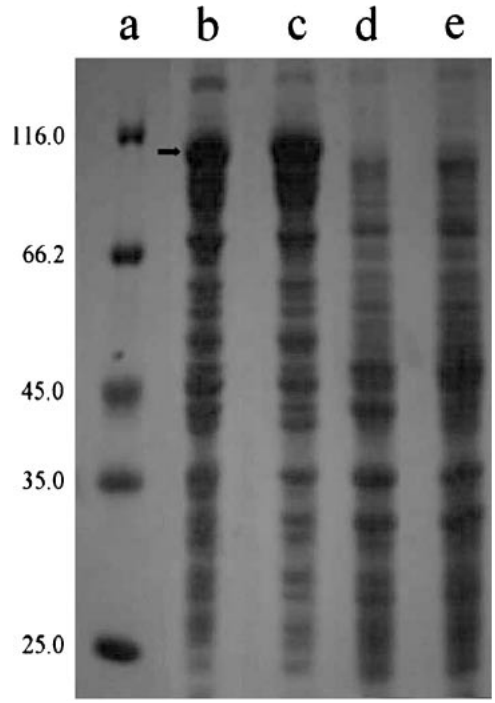

Fig. 1. Induction of MBP-hPDE4B2 in *E. coli* with 1.0 mM IPTG.
The amounts of proteins in various lanes were approximately the same. An arrow indicate the band for the desired MBP-hPDE4B2. a, Molecular weight marker as labeled; b, pellets of cell lysate dissolved in the sampling buffer for SDS–PAGE; c, the supernatant of cell lysate after supersonic treatment; d, cell lysate from the same positive clone of *E. coli* without induction by IPTG; e, cell lysate from that transformed with the blank vector and induced with IPTG.

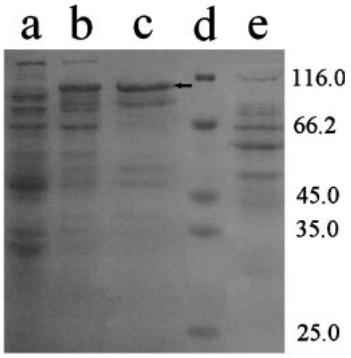

Fig. 2. Purification of MBP-hPDE4B2 from Cell Lysate.
The amounts of proteins in various lanes were approximately the same. An arrow indicates the band for the desired MBP-hPDE4B2. a, Cell lysate from the same positive clone of *E. coli* without induction by IPTG; b, the supernatant after dialysis of the dissolved precipitate from fractionation of the cell lysate with 60% saturation of ammonium sulfate; c, MBP-hPDE4B2 eluted with maltose from the amylase-resin column; d, Molecular weight marker as labeled; e, proteins eluted with the equilibrating buffer alone from the amylase-resin column.

dithioerythritol, and 1.0 mM *p*-methylsulfonyl fluoride), and treated with ultrasonic at 4 °C for 15 min in an ice-water bath. When the diluted cells had an absorbance below 0.5 at 600 nm, there were no detectable proteins in the pellet after ultrasonic treatment while MBP-hPDE4B2 with an expected molecular weight of about 109 kDalton was easily detected in the soluble lysate by sodium dodecyl polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1). When the diluted cells had an absorbance above 0.9 at 600 nm, less than 3% proteins was detected in the pellets, and their profiles were similar to those of the soluble lysate. Therefore, MBP-hPDE4B2 was soluble in *E. coli*, which was different from that in *E. coli* with a FLAG tag,[14] or in yeast with a His tag.[11]

The complete lysis of cells by ultrasonic treatment gave a large amount of cell lysate for handling (the culture medium in 700 ml gave about 320 mg protein in 120 ml lysate), which stimulated a trial purification of MBP-hPDE4B2 by ion-exchange chromatography (IEC). After induction with IPTG at 28 °C, a few proteins in the cell lysate passed out from a DEAE-Cellulose column (DE32, Amersham Pharmacia, NJ, USA), but no detectable proteins could be further eluted out by 2.0 M NaCl in an equilibrating buffer (10.0 mM Tris–HCl at pH 7.4, plus 10.0 mM $MgCl_2$ and 0.1 mM EDTA). When the proteins in the lysate were loaded on a CM-Cellulose column, MBP-hPDE4B2 and other proteins were found in the eluted solution without NaCl in the equilibrating buffer, but few proteins could be further eluted out from the column with 2.0 M NaCl in the equilibrating buffer. To facilitate the folding of proteins during syntheses, MBP-hPDE4B2 was induced by IPTG at 18 °C for 16 h. In this case, a few bound proteins on DEAE-Cellulose could be eluted with 2.0 M NaCl in the equilibrating buffer, which, however, contained no MBP-hPDE4B2. Therefore, IEC was ineffective to purify MBP-hPDE4B2.

In the cell lysate from *E. coli* after IPTG induction for 16 h, the proteins were fractionated with 60% ammonium sulfate to yield about 16% proteins in the precipitate, which was dissolved and dialyzed against a buffer (20.0 mM Tris–HCl buffer at pH 7.4, 10.0 mM $MgCl_2$, 0.20 mM *p*-aminobenzamidine, and 0.10 mM EDTA). After centrifugation at 4 °C, an aliquot of the supernatant containing 4.0 mg proteins was passed through an amylose-resin column (5.0 cm × 1.0 cm) pre-equilibrated with the equilibrating buffer (10.0 mM Tris–HCl at pH 7.4 plus 10.0 mM $MgCl_2$ and 0.1 mM EDTA). After the unbound proteins were eluted with this equilibrating buffer, 1.70 mg MBP-hPDE4B2 was eluted with 10.0 mM maltose in the equilibrating buffer (8% yield of proteins from cell lysate). Proteins from the cells transformed with the blank vector were fractionated with ammonium sulfate and eluted from an amylose-resin column to give endogenous PDEs.

In these two PDE preparations, proteins were quantified by the Bradford method using bovine serum albumin as the reference protein.[19] Hydrolysis of cAMP (Sigma-Aldrich, MO, USA) by PDEs was monitored by coupled end-point assay of phosphate with the malachite green (free base, Sigma-Aldrich), which was released from AMP by the action of calf intestinal alkaline phosphatase (Promega, Madison, WI, USA).[20] PDE reaction buffer contained 20.0 mM Tris–HCl buffer at pH 7.4, 10.0 mM $MgCl_2$ and 0.10 mM EDTA. One unit of PDE4 was the amount hydrolyzing one micromole cAMP per minute.

MBP-hPDE4B2 eluted with 10 mM maltose from the amylose-resin column showed about 35% homogeneity by SDS–PAGE, and its molecular weight was about 100 kD, close to that deduced from its coding sequence

(Fig. 2). Its highest specific activity reached 0.12 $U \cdot mg^{-1}$ protein at 60.0 μM cAMP, and the total activity was about 0.2 U from 4.0 mg proteins for affinity chromatography. Thus MBP-hPDE4B2 was about 0.01 $U \cdot mg^{-1}$ protein in cell lysate and about 5 $U \cdot l^{-1}$ in the culture medium. Based on the homogeneity, its specific activity was about 50% of that expressed in *E. coli* with a FLAG tag,[14] and about 35% of that expressed in yeast with a His tag.[11] The MBP tag accounted for 30% of the molecular weight of MBP-hPDE4B2, which might partially account for its lower specific activity than that with a FLAG tag in *E. coli*.[14] However, the productivity of MBP-hPDE4B2 was low, which might have been due to an absence of phosphorylation of hPDE4B2 synthesized in *E. coli*.[13,21–23]

Zinc chloride at 50.0 μM had no effects on endogenous PDEs, whereas it inhibited MBP-hPDE4B2 by 90%, an effect consistent with that on human PDE4 expressed in *E. coli* or in insect cells.[14] Moreover, Michaelis-Menten constant (K_m) of endogenous PDEs from *E. coli* was 28 ± 1 μM ($n = 3$), while K_m of MBP-hPDE4B2 was 10 ± 2 μM ($n = 3$), consistent with that of human PDE4 with a FLAG tag produced in *E. coli*.[14] Furthermore, the inhibition by rolipram (ICN 159810, stock solution at 10.0 mM in dimethylsulfone) on these two preparations of PDEs was much different. The half-inhibition concentration of rolipram was determined by regression analysis of the linear part of a plot of inhibition percentages to logarithmic concentrations of rolipram, from which the dissociation constant was derived.[20,24] As to endogenous PDEs, rolipram showed an IC_{50} above 200 μM with a dissociation constant above 60 μM. As to MBP-hPDE4B2, rolipram showed an IC_{50} of 52 ± 2 nM at 60.0 μM cAMP and a dissociation constant of 10 ± 2 nM ($n = 3$), consistent with that on human PDE4 having the high-affinity binding site.[12,14,25] Besides, at 30.0 μM cAMP, rolipram had the dissociation constant of 10 ± 1 nM ($n = 2$), confirming that rolipram was its competitive inhibitor.[24] The recombinant expression of human PDE4 in yeast with a His tag gave both soluble and insoluble hPDE4s of different kinetic properties.[11] Therefore, after one-step affinity chromatography with amylose-resin, the MBP-hPDE4B2 preparation showed kinetic homogeneity and was suitable for screening of its inhibitors.

In conclusion, MBP-hPDEB2 was successfully expressed as a soluble active protein in *E. coli*, and the MBP tag could also be used to produce fused proteins of other PDE isozymes for screening of their selective inhibitors, respectively.

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References

1) Conti M and Jin SL, *Prog. Nucl. Acid Res. Mol. Biol.*, **63**, 1–38 (1999).
2) Wong WSF and Koh DSK, *Biochem. Pharmacol.*, **59**, 1323–1335 (2000).
3) Tasken K and Stokka AJ, *Biochem. Soc. Trans.*, **34**, 476–479 (2006).
4) Conti M, Richter W, Mehats C, Livera G, Park JY, and Jin C, *J. Biol. Chem.*, **278**, 5493–5496 (2003).
5) Banner KH and Trevethick MA, *Trends Pharmacol. Sci.*, **25**, 430–436 (2004).
6) Houslay MD, Schafer P, and Zhang KY, *Drug Discov. Today*, **10**, 1503–1519 (2005).
7) Lugnier C, *Pharmacol. Ther.*, **109**, 366–398 (2006).
8) Adcock IM, Caramori G, and Chung KF, *Lancet*, **372**, 1073–1087 (2008).
9) Lipworth BJ, *Lancet*, **365**, 167–175 (2005).
10) Kroegel C and Foerster M, *Exp. Opin. Investig. Drugs*, **16**, 109–124 (2007).
11) Wilson M, Sullivan M, Brownn N, and Hously MD, *Biochem. J.*, **304**, 407–415 (1994).
12) Wang P, Myers JG, Wu P, Cheewatrakoolpong B, Egan RW, and Billah MM, *Biochem. Biophys. Res. Commun.*, **234**, 320–324 (1997).
13) Rocque WJ, Holmes WD, Patel IR, Dougherty RW, Ittoop O, Overton L, Hoffman CR, Wisely GB, Willard DH, and Luther MA, *Protein Expr. Purif.*, **9**, 191–202 (1997).
14) Lario PI, Bobechko B, Bateman K, Kelly J, Vrielink A, and Huang Z, *Arch. Biochem. Biophys.*, **394**, 54–60 (2001).
15) Terpe K, *Appl. Microbiol. Biotechnol.*, **60**, 523–533 (2003).
16) Feher A, Boross P, Sperka T, Oroszlan S, and Tozser J, *Protein Expr. Purif.*, **35**, 62–68 (2004).
17) Kohl T, Schmidt C, Wiemann S, Poustka A, and Korf U, *Proteome Sci.*, **6**, 4 (2008).
18) Li C, Tian M, Yuan Y, and Zhou Q, *Assay Drug Dev. Technol.*, **6**, 803–810 (2008).
19) Bradford MM, *Anal. Biochem.*, **72**, 248–254 (1976).
20) Zhu S, Gan Z, Li Z, Liu Y, Yang X, Deng P, Xie Y, Yu M, Liao H, Zhao Y, Zhao L, and Liao F, *Anal. Chim. Acta*, **636**, 105–110 (2009).
21) Sette C and Conti M, *J. Biol. Chem.*, **271**, 16526–16534 (1996).
22) MacKenzie SJ, Baillie GS, McPhee I, MacKenzie C, Seamons R, McSorley T, Millen J, Beard MB, van Heeke G, and Houslay MD, *Br. J. Pharmacol.*, **136**, 421–433 (2002).
23) Houslay MD and Adams DR, *Biochem. J.*, **370**, 1–18 (2003).
24) Cheng Y and Prusoff WH, *Biochem. Pharmacol.*, **22**, 3099–3108 (1973).
25) Rocque WJ, Tian G, Wiseman JS, Holmes WD, Zajac-Thompson I, Willard DH, Patel IR, Wisely GB, Clay WC, Kadwell SH, Hoffman CR, and Luther MA, *Biochem.*, **36**, 14250–14261 (1997).