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Expression and purification of human mPGES-1 in *E. coli* and identification of inhibitory compounds from a drug-library

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Human microsomal prostaglandin E synthase-1 (mPGES-1) is a membrane associated protein that catalyzes the conversion of prostaglandin H_2 (PGH_2) into prostaglandin E_2 (PGE_2). In this study, the expression of human mPGES-1 in *E. coli* was significantly enhanced by modifying the utility of specific codons and the recombinant mPGES-1 was efficiently purified to homogeneity. The K_m and V_{max} of the purified enzyme were determined and the trimeric state characterized by chemical cross-linking with glutaraldehyde. The purified mPGES-1 was used for the screening of a chemical library of bioactive or drug compounds to identify novel inhibitors, and oxacillin and dyphylline were identified as moderately inhibiting mPGES-1 with IC_{50} values of 100 and 200 μM, respectively. As these compounds competitively inhibited the catalysis of PGH_2, their binding sites appeared to be located near the PGH_2 binding pocket. [BMB reports 2008; 41(11): 808-813]

INTRODUCTION

Synthesized from membrane-derived arachidonic acid via the reactions of cyclooxygenases and prostanoid synthases, prostaglandins have multiple roles in human physiological processes (1, 2). Arachidonic acid is converted by cyclooxygenase-1 (COX-1) or cycloxygenase-2 (COX-2) to PGH_2 and then metabolized by specific prostanoid synthases to PGE_2, PGI_2, PGD_2, PGF_2, or thromboxanes. In particular, prostaglandin E syntheases (PGESs) convert PGH_2 to PGE_2, which regulates multiple physiological processes including inflammation (3-5), reproduction (6), or tumorigenesis (7, 8). Proinflammatory stimuli induce the production of PGE_2 in macrophages and other tissues (3, 4, 9-12) and elevated levels of PGE_2 have been shown to mediate fever (13) and pain (14).

Three kinds of human PGE synthases have been identified: a cytosolic prostaglandin E_2 synthase (cPGES), which consists of 160 amino acids (15), and two microsomal membrane- associated prostaglandin E_2 synthases (mPGES-1 and mPGES-2) with 152 (9) and 373 amino acid residues (16), respectively. Two of these synthases, cPGES and mPGES-2, are constitutively expressed and promote immediate PGE_2 production via constitutive COX-1 as part of cellular homeostatic maintenance (15). In contrast, mPGES-1 and COX-2 expression can be induced by proinflammatory stimuli in various tissues (3, 4, 10). Traditional nonsteroid anti-inflammatory drugs (NSAID) or specific COX-2 inhibitors lower the level of PGE_2 and inhibit the synthesis of PGI_2, thromboxane A, or PGD_2 (17). Hence, the selective inhibition of mPGES-1 would generate anti-inflammatory effects without unwanted side effects involving homeostasis. Gene knockout experiments with mice lacking mPGES-1 showed impaired inflammatory and pain responses (18), implying that mPGES-1 may be a promising drug target against chronic inflammatory diseases such as rheumatism (19).

Recently, a few compounds capable of inhibiting the catalytic activity of mPGES-1 have been reported. A stable PGH_2 analog (20) and NS-398 (21) were shown to inhibit mPGES-1 with a marginal potency (IC_{50} = 10-20 μM). Structure-activity relationship (SAR) studies using MK-886, an inhibitor of 5-lipooxygenase activating protein (FLAP), led to the generation of highly potent mPGES-1 inhibitors having an indole carboxylic acid structure (22), but they displayed low potency and selectivity in cell-based experiments, indicating that further improvement and experimentation is required (22). High throughput screening of these inhibitors allowed the identification of novel mPGES-1 inhibitory compounds, and optimization of one selected compound identified a highly potent phenanthrene imidazole derivative (MF63) with low IC_{50} value (μM range) and high bioavailability (23).

Although the development of novel inhibitors of mPGES-1 has received great attention, the variety of inhibitors with different chemical backbone structures and mPGES-1 structure information remain limited although the structure of mPGES-1 has been modeled and the substrate binding sites characterized (24). A higher resolution structure of mPGES-1 is required to facilitate the design of novel inhibitors and the optimization of previously identified inhibitors. In this report, the expression of recombinant mPGES-1 in *E. coli* has been optimized, the expressed protein purified, and its oligomeric state characterized. Additionally, a commercial library consisting of bioactive and drug compounds was screened for inhibitory compounds with new structural scaffolds.

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Received 8 July 2008, Accepted 31 July 2008

Keywords: Codon usage, Inhibitor, mPGES-1, Overexpression, Screening

RESULTS AND DISCUSSION

Optimization of mPGES-1 expression in *E. coli*

The expression of membrane associated proteins in heterologous hosts has had limited success, as in the case of COX-1 (25) or cytochrome P450 (26). In many cases, overexpression of membrane proteins was found to decrease growth rates or induce cell-death in the expression hosts. Expression of recombinant human mPGES-1 in insect cells or *E. coli* has been reported, expressed in the latter at a level of 0.2-1 mg per L of culture (27) in the membrane fraction; and in a baculovirus system, the expression levels were similar to *E. coli* expression systems (28).

There are several factors that affect the level of recombinant protein expression in *E. coli* other than the strength of the promoters. The frequency of codons that are rarely used in the target sequence inserted into *E. coli* is one of the critical factors that determine the expression level. Several rare codons in the coding sequence of human mPGES-1 are suppressed in the Rosetta strain, but it has three CGG codons that are not suppressed by the Rosetta strain and are rarely used in *E. coli*. To avoid the potential retardation of mPGES-1 translation, all three CGG codons were changed to CGC codons, highly recognized by the tRNAs in *E. coli*, creating a mutant mPGES-1 with three silent mutations. When the wild-type and mutant sequences were expressed in various *E. coli* strains, the Rosetta (DE3) strain had a 5-7-fold greater expression relative to the BL21(DE3) strain. In addition, the codon-frequency mutant displayed an approximate 2-3-fold increase in expression relative to the wild-type (Fig. 1A, B). These results indicated that the presence of rare codons in human mPGES-1 hindered high-level expression in *E. coli*, and the introduction of *E. coli*- friendly codons and the use of *E. coli* strains supplemented with tRNAs for rare codons effectively increased the expression level of these proteins.

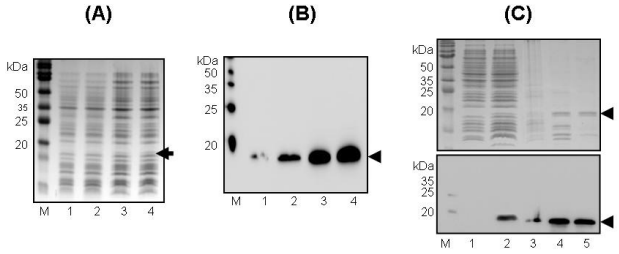

Fig. 1. Overexpression and purification of mPGES-1. (A) Expression of weight and codon frequency of mutant mPGES-1 in *E. coli* as determined by 18% SDS-PAGE. (B) Western blot using anti-His-tag antibodies: lane M, molecular weight markers; lane 1, crude extract of BL21(DE3) expressing wt-mPGES-1; lane 2, crude extract of Rosetta (DE3) expressing wt-mPGES-1; lane 3, crude extract of BL21(DE3) expressing codon frequency mutant mPGES-1; and lane 4, crude extract of Rosetta (DE3) expressing codon frequency mutant mPGES-1, ~20 μg of proteins loaded per lane. (C) Purification of mPGES-1. Fractions from each purification step analyzed by 18% SDS-PAGE (upper panel) and western blot (lower panel): lane M, molecular weight markers; lane 1, crude extract of Rosetta (DE3) cells; lane 2, crude extract (about 20 μg of protein) Rosetta (DE3) expressing codon frequency mutant mPGES-1; lane 3, membrane fraction from cell lysate; lane 4, the purified mPGES-1 after Ni-NTA column (about 0.5 μg); lane 5, the purified mPGES-1 (about 0.5 μg) after Q-sepharose column. Arrows indicate the expressed mPGES-1.

Purification and characterization of mPGES-1

The majority of the expressed mPGES-1 was recovered in the membrane fraction, completely dissolved in 4% Triton X-100 solution, and successfully purified to homogeneity using Ni-NTA and ion exchange columns (Fig. 1C). A purified 18.8 kDa band, detected with anti-His tag antibody, was observed after ion exchange chromatography with a SP-column and matched the calculated size (19 kDa) of the recombinant His-tag labeled mPGES-1. The final yield of the purified protein was 0.5 mg per L culture.

The enzymatic properties of the purified mPGES-1 were characterized by measurement of the reaction rate of the purified mPGES-1 at different concentrations of PGH_2 using a competition assay with PGE_2-labeled alkaline phosphatase to PGE_2-specific antibody (Fig. 2). The K_m and V_{max} values, calculated from Lineweaver-Burk plot as 2.4 ± 0.25 μM and 3.5 ± 0.08 $nmol.s^{-1}$, respectively, were in the same range as the mPGES-1 obtained from insect cells (28).

Characterization of oligomeric state of mPGES-1

The apparent size of mPGES-1-Triton X-100 complex was previously measured as 215 kDa in hydrodynamic studies and determined to be a trimer in the protein-detergent complex (27). Thus, to chemically cross-link the purified His-tag labeled mPGES-1 and determine its oligomeric state, the enzyme was incubated with glutaraldehyde and analyzed by SDS-PAGE, yielding protein bands at 38 and 57 kDa detected by anti-His tag antibody and corresponding to dimeric and trimeric forms of mPGES-1 (Fig. 3A). When lysozyme, a monomeric protein, was treated with the same concentration of glutaraldehyde, only the monomer band was detected (Fig. 3B), indicating that the high-molecular weight bands observed after cross-linking mPGES-1 represent true oligomers rather

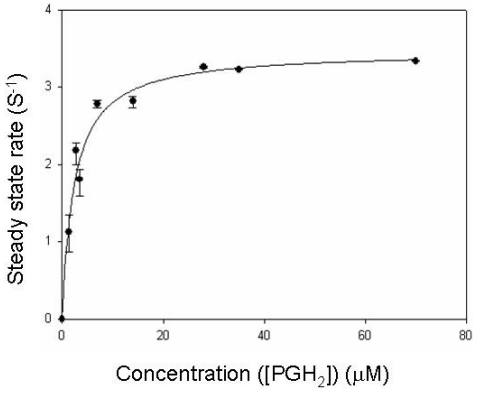

Fig. 2. Enzymatic properties of the purified recombinant mPGES-1. The activity of the mPGES-1 measured in various PGH_2 concen-trations.

than the random cross-linking of monomers. Also worthy of note, the intensity of the 57 kDa band was higher than the 38 kDa band, indicating that the recombinant mPGES-1 produced here were mainly trimers, which was consistent with the hydrodynamic behavior of mPGES-1 (27).

Identification and characterization of novel mPGES-1 inhibitors

The initial screening of a chemical library of 1,040 bioactive or drug compounds to identify novel mPGES-1 inhibitors yielded oxacillin and dyphylline as having inhibitory activity. Further analysis showed that these two compounds inhibited mPGES-1 within the concentration range of 100-200 μM, and IC_{50} values of oxacillin (Fig. 4A) and dyphylline (Fig. 4B) were calculated as 0.11 mM and 0.23 mM, respectively. The inhibition mechanisms of dyphylline and oxacillin were examined by measuring K_m and V_{max} values from Eadie-Hofstee plots of mPGES-1 activity in the presence of these compounds. The K_m values of mPGES-1 in the presence of dyphylline or oxacillin increased, whereas the V_{max} values were unchanged in their Eadie-Hofstee plots (Fig. 4C and 4D indicating that these compounds were competitive inhibitors and suggested that both oxacillin and dyphylline were bound close to the PGH_2 binding pocket of mPGES-1. Oxacillin is an antibiotic that contains a β-lactam ring structure and dyphylline is bronchodilator agent that contains a methylxanthine structure, neither of which structural moieties have been previously reported as inhibitors of mPGES-1. Optimization of oxacillin or dyphylline, however, is required for developing highly potent mPGES-1 inhibitors.

In summary, an optimized expression of human mPGES-1 in *E. coli* was achieved and the purified enzyme shown to be primarily in the trimeric state and to possess enzymatic characteristics consistent with previously reported parameters. The mPGES-1 product was then used to screen for inhibitors with novel structures from a commercial chemical library of bioactive compounds, identifying two compounds, oxacillin and dyphylline, for the first time as competitive inhibitors of mPGES-1 Although their IC_{50} values were relatively higher than known inhibitors, the novel inhibitory activity of oxacillin and dyphylline against mPGES-1 may provide valuable insight for the design of more, potent mPGES-1 inhibitors.

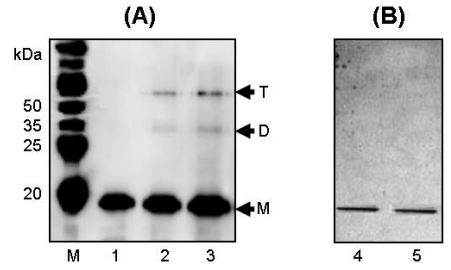

Fig. 3. Chemical cross-linking of mPGES-1. (A) Partially purified mPGES-1 (10 μg) cross-linked with: lane 1, nothing; lane 2, 0.05%; or lane 3, 0.1% glutaraldehyde, and mPGES-1 detected by western blotting with antibodies after SDS-PAGE. The positions of the monomer (M), dimer (D) and trimer (T) indicated by the arrows. (B) Lysozyme was incubated with: lane 4, nothing or lane 5, 0.05% glutaraldehyde and analyzed by SDS-PAGE.

MATERIALS AND METHODS

Materials

Escherichia coli strain DH5α was used for the amplification of

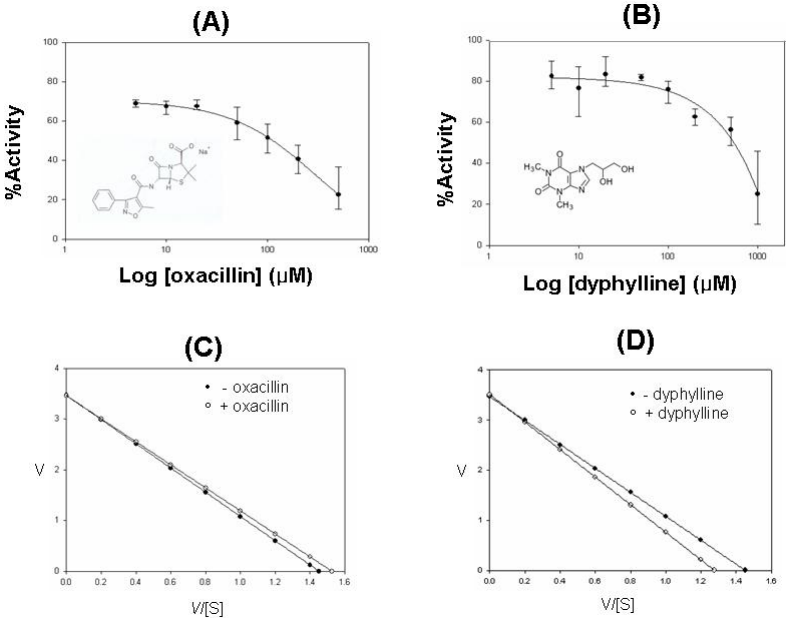

Fig. 4. Concentration dependent inhibitory activities of oxacillin (A) and dyphylline (B). The Eadie- Hofstee plot for the inhibition by oxacillin (C) and dyphylline (D).

the expression vector and *E. coli* Rosetta (DE3) (Novagen, USA) used for the expression of mPGES-1. Luria Broth medium and agar (Merck, Germany) was used for the *E. coli* growth media, all restriction enzymes used for DNA digestion obtained from New England Biolabs (USA), and isopropyl-β-D-thiogalactopyranoside (IPTG) obtained from Bio Basic, Inc., Canada. The mouse anti-His-tag antibody, horseradish peroxidase (HRP) labeled anti-mouse antibody, stable peroxidase substrate buffer, and polyvinylidene fluoride (PVDF) membrane for western blotting were purchased from Santa Cruz, USA, GE Healthcare, Pierce, USA, and Amersham Biosciences, Sweden, respectively. Prostaglandin H_2 (PGH_2), oxacillin, and dyphylline were obtained from Sigma, USA and anti-PGE_2 mouse antibody, alkaline phosphatase conjugated with PGE_2, and 96-well EIA plates coated with a goat anti-Mouse antibody, used for the assay of mPGES-1, obtained from Assay Designs, USA. The chemical library for screening was obtained from MicroSource, USA.

Construction of expression vector of mPGES-1

The plasmid DNA harboring the full-length cDNA of human mPGES-1 was obtained from Professor Hoon Cho at Chosun University. The coding region mPGES-1 (gi:38505195) from the plasmid DNA was amplified by polymerase chain reaction (PCR) using primer 1 (5′-ATGCCTGCCCACAGCCTG-3′) and primer 2 (5′-TCACAGGTGGCGGGCCGC-3′) (Cosmo Genetech, Korea) and the amplified DNA fragment inserted into the TA vector. The 0.5 kb *Nde* I/*Eco*R I fragment of the recombinant TA vector was ligated into the *Nde* I and *Eco*R I restriction site of pET-28a vector (Novagen, USA) to generate pPGES and the plasmid designed to produce full-length mPGES-1 with His_6 tag at the N-terminus. Three CGG codons of mPGES-1, encoding the 40, 73, and 122nd arginines of mPGES-1, were changed to CGC codons by site-directed mutagenesis, introduced sequentially using a quick-change kit (Stratagene, USA), to generate pPGES-mut in which all CGG codons were changed to CGC, which occurs in high frequency in *E. coli*. The coding sequence of mPGES-1 and mutations in pPGES and pPGES-mut, respectively, were confirmed by DNA sequencing (Cosmo Genetech, Korea).

Expression and purification of mPGES-1

The coding regions of human mPGES-1 in pPGES or pPGES-mut were expressed in various strains of *E. coli*, such as BL21 (DE3), Rosetta (DE3), and Rosetta (DE3)/pRARE, which contained the plasmids, grown at 37°C in LB medium containing 30 μg/ml kanamycin until the OD_{600} of the culture was 0.5-0.7. The temperature of the culture was then changed to the appropriate temperatures, IPTG added to 1 mM, and the cultures allowed to grow for different time intervals. The level of mPGES-1 expression was measured by western analysis using an anti-His tag antibody.

To obtain pure mPGES-1, Rosetta (DE3) cells harboring pPGES-mut were grown at 37°C in LB medium containing 30 μg/ml kanamycin and 34 μg/μl chloramphenicol until the OD_{600} of the culture reached 0.5-0.7. mPGES-1 expression was induced by addition of IPTG to 1 mM final concentration, further growth for 12 h at 18°C, and the cells harvested by centrifugation (5,000 g, 20 min) at 4°C . The cell pellet was suspended in 5 volumes of lysis buffer (15 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, and 1 mM GSH), lysed by ultrasonication, and removed by centrifugation (5,000 g, 10 min) at 4°C . The membrane fraction in the supernatant was precipitated by ultracentrifugation (100,000 g, 1 h) at 4°C, resuspended in 20 ml of solubilization buffer (15 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM PMSF, 1 mM GSH, and 4% Triton X-100) for 3 h on ice with stirring, and the insoluble material removed by ultracentrifugation (100,000 g, 30 min) at 4°C. The supernatant was next loaded onto a Ni-NTA chromatography column equilibrated with buffer A (15 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, and 0.2% Triton X-100), washed with buffer A and then washing buffer (50 mM imidazole in buffer A), and the bound protein eluted with elution buffer (200 mM imidazole in buffer A) and immediately loaded onto a mono Q-sepharose column.

Protein cross-linking

Chemical cross-linking of purified mPGES-1 was performed by incubating 10 μg of mPGES-1 with various concentrations of glutaraldehyde in 0.1 ml of buffer A for 10 min at room temperature, loading the samples onto 18% SDS-PAGE, and the proteins detected by western blotting with anti-His tag antibodies.

Western blot analysis

Proteins were resolved by SDS-PAGE (17.5% acrylamide) and transferred to PVDF membranes by applying 100 V for 2 h. After incubating the membranes with 5% skim milk PBST (PBS containing 0.1% Tween 20) for 1 h at room temperature, they were washed 4 times with 50 ml of PBST for 10 min, and incubated with a 1/500 dilution of anti His-tag antibody and a 1/2,000 dilution of horseradish peroxidase-linked anti mouse antibody in PBST for 2 h, followed by extensive washing with PBST. Immunodetection was performed using a Western Blot Chemiluminescence substrate reagent (Pierce, USA) per the instruction manual.

Assay

mPGES-1 activity was assessed, as described previously (29), with 50 ng of mPGES-1 in 100 μl of reaction buffer added to each well of a 96-well non-binding plate (Kartell, Italy) and incubated for 15 min at 20°C. The enzyme reaction was initiated by adding 20 μl of cold PGH_2 (final conc. 2.8 μM), incubated for 30 sec at room temperature, and terminated by adding 20 μl of $SnCl_2$ solution in 1 N HCl. After a 200-fold dilution with the Assay Buffer (Assay Designs Kit, USA), 100 μl of the diluted mixture was transferred to a 96-well EIA plate coated with a goat anti-Mouse antibody, 50 μl of anti-PGE_2 mouse antibody and 50 μl of the alkaline phosphatase conjugated to PGE_2 added to each well, the plates incubated for 2 h at room temper-

ature with moderate shaking, and finally washed with wash buffer (Assay Designs Kit). The color developing reaction was initiated by adding 200 µl of p-nitrophenyl phosphate (pNpp) substrate solution for 4 h at room temperature, terminated by adding 50 μL of stop solution, and the absorbance of the solution at 405 nm measured using a plate reader (DYNEX, USA). Inhibitory activity by test chemical compounds was measured by the incubation of 2 µl of a compound in dimethyl sulfoxide (DMSO) with mPGES-1 for 30 min prior to the addition of PGH_2. Protein concentrations were measured by the Lowry method (30) using bovine serum albumin as a standard.

Acknowledgments
This work was supported by grants from the 21C Frontier Research Program, Functional Proteomics Research, Ministry of Education, Science and Technology, Korea, and the Reaearch Program 2007 of Kookmin University in Korea

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