Molecular Identification of Cytosolic Prostaglandin E2 Synthase That Is Functionally Coupled with Cyclooxygenase-1 in Immediate Prostaglandin E2 Biosynthesis*

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The abbreviations used are: PG, prostaglandin; cPGES, cytosolic prostaglandin E2 synthase; COX, cyclooxygenase; GLUT, glucose transporter; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; C180 cells, Chinese hamster ovary cells; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PLA2, phospholipase A2; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PLA2, phospholipase A2.

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Here we report the molecular identification of cytosolic glutathione (GSH)-dependent prostaglandin (PG) E2 synthase (cPGES), a terminal enzyme of the cyclooxygenase (COX)-1-mediated PG biosynthetic pathway. GSH-dependent PG activity in the cytosol of rat brains, but not of other tissues, increased 3-fold after lipopolysaccharide (LPS) challenge. Peptide microsequencing of purified enzyme revealed that it was identical to p23, which is reportedly the weakly bound component of the steroid hormone receptor/hsp90 complex. Recombinant p23 expressed in Escherichia coli and 293 cells exhibited all the features of PG activity detected in rat brain cytosol. A tyrosine residue near the N terminus (Tyr9), which is known to be critical for the activity of cytosolic GSH S-transferases, was essential for PGES activity. The expression of cPGES/p23 was constitutive and was unaltered by proinflammatory stimuli in various cells and tissues, except that it was increased significantly in rat brain after LPS treatment. cPGES/p23 was functionally linked with COX-1 in marked preference to COX-2 to produce PG_E2 from exogenous and endogenous arachidonic acid, the latter being supplied by cytosolic phospholipase A2, in the immediate response. Thus, functional coupling between COX-1 and cPGES/p23 may contribute to production of the PG_E2 that plays a role in maintenance of tissue homeostasis.

 Biosynthesis of prostaglandin (PG) E2, the most common prostanooid with potent bioactivities, is regulated by three sequential steps of the cyclooxygenase (COX) pathway. Phospholipase A2 (cPLA2) initiates this pathway by releasing arachidonic acid (AA) from membrane glycerophospholipids. Of more than 10 members of the PLA2 family characterized to date, cysotic PLA2 (cPLA2) and several secretory PLA2s are involved in supplying AA to either of the two COX isoforms, COX-1 and COX-2, depending upon the phases of cell activation (1–3). The constitutive COX-1 is mainly utilized in immediate PG_E2 biosynthesis, which occurs within several minutes after stimulation with Ca2+-mobilizers, whereas the inducible COX-2 mediates the delayed PG_E2 biosynthesis, which lasts for several hours following proinflammatory stimuli. Although COX-1 and COX-2 have been reported to exhibit subtle differences in AA requirements in that COX-2 is favored over COX-1 at low AA concentrations (3–5) and subcellular localizations (6), their functional segregation in the PG_E2 biosynthetic response cannot be fully explained only by these aspects. The activity of PGES, which catalyzes conversion of COX-derived PGH2 to PG_E2, has been detected in both cytosolic and microsomal fractions of various cells, and in most, if not all, cases it requires glutathione (GSH) for optimal activity (7–9). Although several groups have attempted to purify this critical enzyme to near homogeneity for the last 20 years (7–9), such trials have been unsuccessful. The PGES enzyme purified from human brain cytosol was identified as a GSH S-transferase (GST), which converts PGH2 to PG_E2, PGD2, and PGF2α, nonspecifically (9). GSH-independent PGES with a molecular mass of 31 kDa was recently purified from bovine heart (10). Interestingly, PGES activity has been shown to be strongly induced by proinflammatory stimuli in macrophages (11, 12). More recently, microsomal GST-like 1 (MGST1-L1), a member of the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily, has been shown to exhibit significant PGES activity (13, 14).

In this study, we report the molecular identification of cytosolic PGES (cPGES), a GSH-requiring enzyme that is expressed ubiquitously in a wide variety of cells and tissues. Importantly, this enzyme is capable of converting COX-1-, but not COX-2-, derived PGH2 to PG_E2 efficiently. Our present results, together with identification of the inducible membrane-associated PGES that is preferentially coupled with COX-2 as described in the accompanying paper (15), revealed that segregated utilization of the biosynthetic enzymes in different phases of PG production also occurs at the step of terminal synthases.

EXPERIMENTAL PROCEDURES

Animals—Wistar rats (7 weeks old, male) were purchased from Japan Bio-Supply Center (Tokyo, Japan). Rabbits (New Zealand White, 1-kg body weight, female) were from Saitama Experimental Animal Supply (Saitama, Japan).

Cells—Human embryonic kidney 293 cells were obtained from Japanese Cancer Resources Bank. Rat fibroblastic 3Y1 cells were donated by Dr. Y. Uehara (National Institute of Infectious Disease, Tokyo, Japan). Human cervix epithelial HeLa cells, human stomach MKN45 cells, human glial U251 cells, human fibroblastic W138 cells, human
neuroblastoma GOTO cells, Chinese hamster ovary (CHO) cells, mouse osteoblastic MC3T3-E1 cells, and mouse fibroblastic L929 cells were obtained from the RIKEN Cell Bank. GOTO, HEK293, CHO, MKN45 and L929 were cultured in RPMI 1640 medium (Nissui Pharmaceutical) containing 10% fetal calf serum (FCS; Bioserum), 1% ITS, 2.5% and 1Y1 in complete adjuvant. Serum titers were checked by the enzyme-linked immunosorbent assay (ELISA) method. Briefly, 1 μg of each plasmid was mixed with 4 μl of LipofectAMINE and 6 μl of Plus reagent in 200 μl of Opti-MEM, left for 15 min, and then added to cells that had attained 60% confluence in six-well plates (Iwaki Glass) in 1 ml of Opti-MEM. After incubation for 4 h, 2 ml of fresh culture medium was added. After 18 h, the medium was replaced with 2 ml of fresh medium, and the culture was continued for 3 days. In order to establish stable transfectants, cells transfected with each cDNA were cloned by limiting dilution in 24- or 48-well plates in 1 and 0.5 ml of culture medium, respectively. The supernatants were subjected to RNA blotting and immunoblotting, as described below.

Cell Activation—All procedures were described in our previous reports (5-7). Briefly, 293S cells were seeded on the bottom of 24- or 48-well plates in 1 and 0.5 ml of culture medium, respectively. After culture for 4 days, the cells were washed once with culture medium and then incubated with 250 μl (24-well plate) or 100 μl (48-well plate) of various concentrations of AA or 10 μM A23187 in medium containing 1% FCS for 30 min or 1 ng/ml IL-1β in medium containing 10% FCS for 4 h. The supernatants were subjected to the PGE2 enzyme immunoassay. Activation of other cell lines was carried out in a similar way.

Preparation of Antibody against cPGES/p23—The (His)6-tagged cPGES/p23 (500 μg) in 500 μl of phosphate-buffered saline was mixed with an equal volume of Freund’s incomplete adjuvant and injected into rabbits. Immunization was repeated every 3 weeks with the same amount of the antigen mixed with an equal volume of Freund’s incomplete adjuvant. Serum titers were checked by the enzyme-linked immunosorbent assay (see below), followed by Western blotting (see below) using the purified recombinant (His)6-tagged cPGES/p23 and the lysate of HeLa cells.

In the enzyme-linked immunosorbent assay, 1 μg/ml recombinant (His)6-tagged cPGES/p23 was coated on Immulon 2 plates (Dynatech Laboratories) (50 μl/well) overnight at 4 °C. Subsequent procedures were performed at room temperature. After washing with 10 mM Tris-HCl (pH 7.4) containing 0.05% Tween 20 and 150 mM NaCl (TBS-T), the plates were incubated for 1 h with 5% skim milk in PBS. After 6 washes with TBS-T, serial dilutions of rabbit antisera were added to the plates (50 μl/well) and incubated for 1 h. After 6 washes with TBS-T, the plates were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (50 μl/well) at a 1:1,000 dilution in TBS-T. The plates were incubated with o-phenylenediamine. After termination of the reaction by adding 4 N H2SO4, absorbance at 490 nm was measured.

RNA Blotting—Approximately equal amounts (~10 μg) of the total RNAs obtained from the transfected cells were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were

### Functional Coupling between COX-1 and cPGES

**Materials**—The goat anti-human COX-2 and rabbit anti-human cPLA2 antibodies were purchased from Santa Cruz. The rabbit anti-mouse COX-1 antibody was provided by Dr. W. L. Smith (Michigan State University, East Lansing, MI) and was affinity purified with human COX-2 and mouse COX-2 were described previously (2, 16). LipofectAMINE Plus, LipofectAMINE 2000, Opti-MEM, and TRIzol reagent were obtained from Life Technologies. Bacterial LPS (Salmonella minnesota Re 595), 1-chloro-2,4-dinitrobenzene (CDNB), indo-methacin, and gSH were purchased from Sigma. Ethacrynic acid, 1,2-dichloro-4-nitrobenzene, and p-nitrophenethyl bromide were from Wako. Freund’s complete and incomplete adjuvants were from Difco Laboratories. AA, PGH2, rabbit anti-human COX-1 antibody, and the enzyme immunoassay kits for PGE2 were from Cayman Chemical.

Oligonucleotide primers were from Amersham Pharmacia Biotech. Geneticin, hygromycin, and the mammalian expression vectors pCR3.1 and pCDNA3.1/hyg(+) were purchased from Calbiochem. Human and mouse interleukin (IL)-1β, mouse COX-1 antibody was provided by Dr. W. L. Smith (Michigan State University, East Lansing, MI). cDNA probes for human COX-1, COX-2, and pCDNA3.1/hyg(+) were purchased from Invitrogen. A23187 was purchased from Calbiochem. Human IL-1β and mouse COX-1 cDNA were obtained from the American Type Culture Collection (Rockville, MD). Human p23 cDNA (18) was subcloned into pET21c (Novagen) and transformed into E. coli BL21 (DE3) (Strategene). The cells were cultured until they reached the late lag phase, and 0.3 mM isopropyl-1-thio-β-D-galactopyranoside was added to induce protein production. After an overnight incubation, bacterial cell pellets were lysed in 20 mM Tris-HCl (pH 8) containing 0.5 mM NaCl, 10% glycerol, and 6 μM guanidine HCl by stirring for 50 min at room temperature. After centrifugation at 15,000 g for 30 min at 4 °C, the resulting supernatants were applied to a nickel-nitriilotriacetic acid-agarose column (Qiagen) preequilibrated with 100 mM NiSO4 at a flow rate of 10 ml/h. After washing, the bound protein was eluted with the same buffer containing 20–60 mM imidazole in a stepwise manner.

**Site-directed Mutagenesis**—To obtain cPGES/p23 Y9N mutant, mismatched primer PCR was carried out using ex Tag polymerase with cPGES/p23 cDNA as a template and the primers 5′-ATG CAG CCT GCT TCT GCA AAG TGG AAC GGC-3′ (the mutated base is underlined) and 5′-TTA CTC CAG ATC TGG CAT-3′. PCR conditions were 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 30 s, for 25 cycles. The fragment obtained was subcloned into pCR3.1 and sequenced.

Expression of cPGES/p23 in 293 Cells—The cDNA flanking the entire open reading frame of human cPGES/p23 was subcloned into the mammalian expression vector pCDNA3.1/hyg(+) and transfected into 293 cells stably expressing human COX-1 or COX-2, which we had previously established (2), using LipofectAMINE Plus according to the manufacturer’s instruction. Briefly, 1 μg of each plasmid was mixed with 4 μl of LipofectAMINE and 6 μl of Plus reagent in 200 μl of Opti-MEM, left for 15 min, and then added to cells that had attained 60% confluence in six-well plates (Iwaki Glass) in 1 ml of Opti-MEM. After incubation for 4 h, 2 ml of fresh culture medium was added. After 18 h, the medium was replaced with 2 ml of fresh medium, and the culture was continued for 3 days. In order to establish stable transfectants, cells transfected with each cDNA were cloned by limiting dilution in 96-well plates (Iwaki Glass) in culture medium supplemented with 50 μg/ml hygromycin. After 3–4 weeks of culture, single colonies were picked up and expanded. Expression of cPGES/p23 and each COX was assessed by RNA blotting and immunoblotting, as described below.

**Cell Activation**—All procedures were described in our previous reports (5-7). Briefly, 293S cells were seeded on the bottom of 24- or 48-well plates in 1 and 0.5 ml of culture medium, respectively. After culture for 4 days, the cells were washed once with culture medium and then incubated with 250 μl (24-well plate) or 100 μl (48-well plate) of various concentrations of AA or 10 μM A23187 in medium containing 1% FCS for 30 min or 1 ng/ml IL-1β in medium containing 10% FCS for 4 h. The supernatants were subjected to the PGE2 enzyme immunoassay. Activation of other cell lines was carried out in a similar way.

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In the enzyme-linked immunosorbent assay, 1 μg/ml recombinant (His)6-tagged cPGES/p23 was coated on Immulon 2 plates (Dynatech Laboratories) (50 μl/well) overnight at 4 °C. Subsequent procedures were performed at room temperature. After washing with 10 mM Tris-HCl (pH 7.4) containing 0.05% Tween 20 and 150 mM NaCl (TBS-T), the plates were incubated for 1 h with 5% skim milk in PBS. After 6 washes with TBS-T, serial dilutions of rabbit antisera were added to the plates (50 μl/well) and incubated for 1 h. After 6 washes with TBS-T, the plates were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (50 μl/well) at a 1:1,000 dilution in TBS-T. After washing, the plates were incubated with o-phenylenediamine. After termination of the reaction by adding 4 N H2SO4, absorbance at 490 nm was measured.

**RNA Blotting**—Approximately equal amounts (~10 μg) of the total RNAs obtained from the transfected cells were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were
then probed with the respective cDNA probes that had been labeled with [α-32P]dCTP (Amersham Pharmacia Biotech) by random priming (Takara Biomedicals). All hybridizations were carried out as described previously (19).

SDS-PAGE/Immunoblotting—Cell lysates (10⁵ cells/ml) or culture supernatants were subjected to SDS-PAGE using 15% (w/v) gels for cPGES/p23 and 10% gels for COXs under reducing conditions. The separated proteins were electrophoreted onto nitrocellulose membranes (Schleicher & Schuell) using a semidry blotter (MilliBlot-SDE system; Millipore). The membranes were probed with the respective antibodies and visualized using the ECL Western blot system (PerkinElmer Life Sciences), as described previously (19).

Immunofluorescent Confocal Microscopic Analysis—Cells were seeded onto cover glasses (Matsunami Glass) at 1 × 10⁵ cells/ml and cultured for 1 day. After removing the supernatants, the cells were fixed with 10% (v/v) formalin in PBS for 30 min at 4 °C. The cells were then treated 0.2% (v/v) Triton X-100 for 2 min, washed six times, and incubated for 1 h with 3% (w/v) BSA in PBS (PBS-BSA). After three washes, the cells were incubated with rabbit anti-cPGES/p23 antibody (1:200 dilution) in PBS-BSA for 2 h, washed three times, and then incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:100 dilution) in PBS-BSA for 1 h. After six washes, the coverslips were mounted on glass slides using Perma Fluor (Japan Tanner) and examined using a Fluoview laser fluorescence microscope (Olympus).

Transfection of Antisense cPGES/p23 cDNA into 3Y1 Cells—Approximately 4 μg of cPGES/p23 cDNA subcloned into pCIG3.1 in an inverse direction were incubated with 5 μl of LipofectAMINE 2000 reagent in 200 μl of Opti-MEM for 15 min at room temperature and then added to cells that had attained 60–80% confluence in six-well plates and been supplemented with 800 μl of Opti-MEM. After incubation for 6 h at 37 °C, 1 ml of DMEM supplemented with 2% FCS was added, and the culture was continued for another day. Then the cells were trypsinized, seeded onto 24-well plates, and cultured for 2 days. After washing once with DMEM, the cells were stimulated for 30 min with 10 μl A23187 in DMEM or for 1 h with 1 mg/ml mouse IL-1β and mouse TNFα in DMEM containing 2% FCS. The supernatants were taken for PGE2 enzyme immunoassay, and the cells were subjected to cPGES enzyme assay and immunoblotting.

RESULTS

Detection of PGES Activity in the Cytosol of Rat Tissues—In an effort to identify PGES isoforms, we measured PGES activity, which converts PGH2 to PGE2, in the cytosol of various rat tissues before and after injection of LPS. All tissues examined contained significant PGES activity that was not affected by LPS, except that the activity in brain increased up to 3-fold 48 h after LPS challenge (Fig. 1A). This activity was stimulated markedly by GSH and was inhibited by CDNB, a substrate for several GST enzymes (20) (Fig. 1B). As shown in Fig. 1C, the activity obtained from LPS-treated rat brains was significantly higher than that obtained from untreated animals. When the fractions containing PGES activity were then applied to Superdex 200 gel filtration, there were three major peaks that exhibited significant PGES activity, among which only the activity eluted in fractions corresponding to a molecular mass of ~50 kDa (around fraction 78) showed several fold higher activity than that in replicate fractions purified from rats not treated with LPS (Fig. 2C). On SDS-PAGE, this activity comigrated with a major protein with an apparent molecular mass of 26 kDa, which was detected more faintly in the untreated group. The specific activity of the peak fraction purified from LPS-treated rat brains after gel filtration was estimated to be approximately 5 μmol/ min/mg of protein. This activity showed dependence on GSH and was inhibited by CDNB (data not shown). There was no detectable GST activity toward several cytosolic GST substrates, such as CDNB, 1,2-dichloro-4-nitrobenzene, p-nitrophenethyl bromide, and ethacrynic acid (data not shown). On the other hand, the other two higher molecular weight PGES peaks, which were eluted in fractions 53 and 69 in both LPS-treated and -untreated groups (Fig. 2C), were fairly insensitive to CDNB (data not shown). These results suggest that there are several proteins that exhibit PGES activity with different enzymatic properties in the cytosol.

Molecular Identification of Cytosolic PGES—Peptide mapping of the 26-kDa protein revealed that the partial amino acid sequences (MDPASAKWDYRRDDYVFIEFC, KSKLCCFSCGL, and IDLFLHCDIPN) were identical to those of the corresponding portions (1–20, 33–42, and 53–62, respectively) of human p23, a cytosolic protein that is the weakly bound component of the steroid hormone receptor/hsp90 complex with a putative chaperone function (18, 21). We therefore isolated the full-length human p23 cDNA from HeLa cells and expressed it in E. coli as a C-terminally (His)₆-tagged protein. The recombinant protein purified by nickel-chelating column had significant PGES activity in the presence of GSH and was inhibited by CDNB (Fig. 3A), whereas formation of other PGs was negligible (data not shown). The Kᵢ and Vmax values of the recombinant protein for PGH₂ were estimated to be 14 μM and 190 nmol/ min/mg of protein, respectively, in our in vitro assay system. GST activity was undetected when CDNB, 1,2-dichloro-4-nitrobenzene, p-nitrophenethyl bromide, and ethacrynic acid were used as substrates (data not shown). Furthermore, PGES activity in lysate of p23-transfected HEK293 cells was stimulated markedly by GSH as compared with mock-transfected cells, and was inhibited by CDNB (Fig. 3B). Thus, we conclude that p23 indeed possesses PGES activity, and therefore designate it cPGES/p23 (c stands for cytosolic) hereafter.

Although the homology between cPGES/p23 and other known cytosolic GSTs (including hematopoietic PGD₂ synthase (Ref. 22)) is low (~20%), near the N terminus cPGES/p23 has a tyrosine residue (Tyr⁰) that is conserved in several other cytosolic GSTs as well as hematopoietic PGD₂ synthase (Fig. 4A). The tyrosine residue in this position serves as a GSH acceptor, thereby being essential for catalytic activity (20, 22). As shown
in Fig. 4B, the cPGES/p23 mutant in which Tyr⁹ was replaced by Asn exhibited virtually no PGES activity when transfected into HEK293 cells.

Expression of cPGES/p23 in Various Cells and Tissues—RNA blot analysis showed that cPGES/p23 was most abundantly expressed in the testis, and was also expressed in various tissues of the rat (Fig. 5A). In most tissues, expression was unchanged following LPS treatment. Exceptionally, cPGES/p23 mRNA expression in brain was increased approximately 3-fold after treatment with LPS (Fig. 5A), a result consistent with increased PGES activity in brain cytosol fraction (Fig. 1A). Confocal microscopic analysis using an anti-cPGES antibody revealed that cPGES is located in the cytosol of these cells (Fig. 6).

Functional Coupling between cPGES/p23 and COXs—To assess whether cPGES/p23 plays a role in PGE₂ production by live cells, human cPGES/p23 cDNA/pCR3.1 or the control vector was measured in the presence or absence of 1 mM GSH and 1 mM CDNB.
stable transfectants (Fig. 7A). Whereas cells expressing COX-1 alone produced significantly more PGE₂ than control cells, particularly when a high concentration (10 μM) of AA was added, as reported previously (2), cotransfection of COX-1 and cPGES/p23 led to a marked increase in PGE₂ that was detectable at lower AA concentrations (Fig. 7B, left). Approximately 10-fold increase in PGE₂ formation by COX-1-cPGES/p23 co-transfectants relative to that by COX-1 single transfectants at all AA doses tested (Fig. 7B, left) appeared to correlate with the expression levels of overexpressed versus endogenous cPGES/p23 (Fig. 7A). On the other hand, PGE₂ generation by COX-2-expressing cells was increased minimally even after cPGES cotransfection (Fig. 7B, right).

To assess the metabolism of endogenous AA, these transfectants were stimulated for 30 min with A23187 (immediate response) or for 4 h with IL-1 (delayed response). Although A23187-induced PGE₂ generation was not increased significantly in cells coexpressing COX-1 and cPGES/p23, further introduction of cPLA₂, which caused the burst release of AA (1), led to a dramatic increase in the production of PGE₂ (Fig. 7C, left). A23187-induced PGE₂ generation by cPLA₂-COX-1 cotransfectants was about one tenth that by cPLA₂-COX-1-cPGES/p23 cotransfectants (Fig. 7C, left), suggesting that the former occurs through the pathway involving overexpressed cPLA₂-COX-1 and endogenous cPGES/p23. In contrast, PGE₂ generation by A23187-stimulated COX-2-expressing cells was increased only modestly when cPLA₂ and cPGES/p23 were coexpressed (Fig. 7C, left). Moreover, cPGES/p23 did not promote delayed PGE₂ biosynthesis induced by IL-1 even when combined with cPLA₂ and either of the two COX isozymes (Fig. 7C, right). Collectively, these results suggest that cPGES/p23 mediates COX-1-dependent immediate PGE₂ synthesis.

To ensure that the preferential coupling between COX-1 and cPGES/p23 was not a peculiarity of the 293 transfectants, we next examined the functional coupling between the endogenous enzymes. When antisense cDNA for cPGES/p23 was transfected into rat fibroblastic 3Y1 cells, in which COX-1-dependent immediate and COX-2-dependent delayed PGE₂-biosynthetic responses occur in response to A23187 and IL-1/TNFα, respectively (23), the expression of cPGES/p23, but not COX-1, protein was decreased by half (Fig. 8A, inset), with a concomitant reduction of PGES activity in cell homogenates (Fig. 8B). Importantly, A23187-elicted immediate, but not IL-1/TNFα-induced delayed, PGE₂ generation was suppressed by half in cells transfected with the cPGES/p23 antisense cDNA (Fig. 8B).
DISCUSSION

In the present study, we have identified for the first time a cytosolic form of PGES, which is fully functional in mammalian cells. It is a GSH-requiring enzyme expressed in a wide variety of cells and tissues, and is identical to p23, a putative chaperone molecule that binds to the ATP-dependent conformation of hsp90 and stabilizes preformed steroid hormone receptor/hsp90 heterocomplexes (18, 21, 24). P23 was initially suggested to be required for refolding of the receptor to the steroid binding conformation (25). However, subsequent studies demonstrated that p23 is not essential for the folding change of the steroid hormone receptor (26) and that deletion of the p23 gene in yeast does not ablate glucocorticoid receptor action (27). It has recently been proposed that p23 might play some role(s) at later steps in intracellular receptor-mediated signal transduction, perhaps including receptor recycling and reversal of the response (28), and the assembly of active telomerase complex (29). However, the precise cellular functions of p23 have remained obscure.

Several lines of evidence suggest that p23 is a functional GSH-dependent PGES. First, recombinant expression of p23 in both bacteria and mammalian cells reproduced PGES activity indistinguishable from that of the enzyme purified from rat brain in terms of GSH requirement and CDNB sensitivity (Figs. 1–3). Unlike typical cytosolic GSTs (20), the GST activity of recombinant p23 toward several cytosolic GST substrates was negligible. These properties are similar to those of hematopoietic PGD2 synthase, another GSH-requiring terminal prostanoid synthase belonging to the class of GSTs (22). Second, as in the case of various cytosolic GSTs and hematopoietic PGD2 synthase (20, 22), a tyrosine residue near the N terminus of p23 is essential for enzyme activity (Fig. 4).
FIG. 8. Effect of cPGES/p23 antisense cDNA on PGE$_2$ generation in rat fibroblastic 3Y1 cells. A, the PGE$_2$ activity in the cytosol of 3Y1 cells 3 days after transfection with cPGES/p23 antisense cDNA/pCR3.1 or empty vector was assessed. The expression of cPGES/p23 and COX-1 proteins, as assessed by immunoblotting, is shown in the inset. B, the cells were stimulated for 30 min with 10 nM A23187 and for 12 h with 1 ng/ml mouse IL-1β plus 100 units/ml mouse TNFα to assess the immediate and delayed phases of PGE$_2$ generation, respectively. PGE$_2$ generation without stimuli was <1 ng/well in both phases (data not shown).

Moderate changes in expression of p23 mRNA and protein paralleled that in PGES activity in rat brain cytosol following LPS treatment (Figs. 1 and 5). Fourth, transfection of p23 into HEK293 cells dramatically increased the cells' capacity to produce PGE$_2$, particularly when combined with COX-1 (Fig. 7). Finally, reduction of p23 expression by antisense treatment led to a concomitant decrease in A23187-induced, COX-1-dependent PGE$_2$ generation in 3Y1 cells (Fig. 8). In contrast, p23 expression minimally affected COX-2-dependent PGE$_2$ generation (Figs. 7 and 8). Thus, cPGES/p23 is preferentially linked with COX-1, a constitutive COX isozyme, promoting the immediate PGE$_2$ biosynthetic response, and physiologically it may contribute to production of PGE$_2$ required for the maintenance of tissue homeostasis.

Since cPGES/p23 was eluted at >50 kDa on gel filtration (Fig. 2C), it may form a homodimer, as do cytosolic GSTs (20), and this has been recently verified by others (30). Alternatively, it may form a heterooligomer with other cellular components. The latter possibility is in line with the notion that p23 assembles in the hsp90-directed steroid hormone receptor (18, 21, 24–28) or telomerase (29) complex. Since the other PGS peaks detected on gel filtration (Fig. 2C) were CDNB-insensitive, it is likely that they reflect the presence of multiple forms of PGS other than CDNB-sensitive cPGES/p23 in cells.

That cPGES/p23 functions as a PGE$_2$-biosynthetic enzyme and as a putative chaperone of lipophilic steroid hormone receptors (18, 21, 24–28) implies its multifunctional role. This property is reminiscent of that of lipocalin-type PGD$_2$ synthase, which not only plays a role in production of PGD$_2$ in the central nervous system (31) but also binds to several lipophilic ligands, such as biliverdin, bilirubin, and thyroid hormones (32), and functions as a retinoid transporter (33). PGF synthase is a member of the aldo-keto reductase superfamily, the enzymes belonging to which exhibit reductase activities toward various carbonyl compounds in addition to PGH$_2$ (34, 35). Whether cPGES/p23 displays enzymatic activity toward lipophilic substances other than PGH$_2$ remains to be elucidated.

The $K_m$ value of the recombinant cPGES/p23 purified from E. coli was calculated to be 14 μM, which was comparable to the $K_m$ of other cytosolic terminal PG synthases, including lung-type (10 μM) and liver-type (25 μM) PGFSs (35) and hematopoietic PGD$_2$ synthase (500 μM) (36), and the $V_{max}$ value (190 nmol/min/mg) was also similar to that of PGFSs (200–400 nmol/min/mg) (35), although the in vitro assay conditions for each enzyme differ. It should be noted that the activity of bacterially expressed cPGES/p23 appeared to be approximately 1 order lower than that of the enzyme purified from rat brain or the recombinant enzyme expressed in HEK293 cells. This may be a reflection of the presence of certain interacting cofactor(s) in samples prepared from mammalian sources. It is also possible that in mammalian cells cPGES/p23 undergoes posttranslational modifications such as phosphorylation, which may lead to enzymatic activation. In support of this idea, cPGES/p23 has multiple putative phosphorylation sites (18). Alternatively, a weaker activity of the bacterially expressed enzyme may be caused by aberrant folding of the recombinant protein in bacteria, by the linkage of the artificial tag to the C terminus, or by the presence of substance(s), which interferes with measurement of the activity, in samples.

The reason why cPGES/p23 prefers COX-1 to COX-2 is currently unknown. cPGES/p23 is predominantly present in the cytosol (Fig. 6) distal from the perinuclear COXs (37, 38), and its cytosolic location was not altered by A23187 stimulation of cells (data not shown). Since COX-1, rather than COX-2, spreads into the cytosol along the endoplasmic reticular membrane (39, 40), albeit not in all cases (41), this subtle difference in subcellular localization of the two COXs might account, at least in part, for the COX-1 selectivity of cPGES/p23. Another possibility is the presence of certain cofactor(s) that may assist the functional coupling between COX-1 and cPGES/p23. Indeed, 5-lipoxygenase-activating protein acts as an essential cofactor that links cPLA$_2$ and 5-lipoxygenase by presenting AA released by the former to the latter in the leukotriene-biosynthetic pathway (42). As p23 reportedly assembles in a steroid hormone/hsp90 complex, which translocates from the cytosol into the nucleus in a ligand-dependent manner, steroid hormones may modulate the subcellular locations of cPGES/p23 and thereby alter its PGE$_2$-biosynthetic capacity in cells.
COX-2-dependent PGE2-biosynthetic pathway.

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