Regulation of Cytosolic Prostaglandin E Synthase

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Biosynthesis of prostaglandin E_2 (PGE_2), the most common prostanoid with potent and various biological activities, is regulated by three sequential steps of cyclooxygenase (COX) pathway. We reported the molecular identification of cytosolic prostaglandin E synthase (cPGES), a terminal enzyme of the COX-mediated PGE_2 biosynthetic pathway. Of interest, it is identical to the co-chaperone p23 that binds to heat shock protein 90 (Hsp90). Incubation of recombinant cPGES/p23 and Hsp90 resulted in a remarkable increase in PGES activity *in vitro*. Furthermore, A23187-induced PGE_2 generation in 3Y1 cells was suppressed by Hsp90 inhibitors, which destabilized the cPGES/p23-Hsp90 complex, and reduced cPGES/p23 activity and PGE_2 production to basal levels. Next, we found that cPGES/p23 underwent serine phosphorylation, which was accelerated transiently after cell activation. In activated cells, cPGES/p23 phosphorylation occurred in parallel with increased cPGES/p23 enzymic activity and PGE_2 production from exogenous and endogenous arachidonic acid, and these processes were facilitated by Hsp90 that formed a tertiary complex with cPGES/p23 and protein kinase CK2. Treatment of cells with inhibitors of CK2 and Hsp90 and with a dominant-negative CK2 attenuated the formation of the cPGES/p23-CK2-Hsp90 complex and attendant cPGES/p23 phosphorylation and activation. Mutations of either of two predicted CK2 phosphorylation sites on cPGES/p23 (Ser^{113} and Ser^{118}) abrogated its phosphorylation and activation both *in vitro* and *in vivo*. These results provide the first evidence that the cellular function of this eicosanoid-biosynthetic enzyme is under the control of a molecular chaperone and its client protein kinase.

Key words —— prostaglandin E synthase, molecular chaperone, phosphorylation, prostaglandin E_2, protein kinase CK2, heat shock protein 90

INTRODUCTION

Prostaglandin E_2 (PGE_2) is the most common prostanoid, being produced by a variety of tissues and cells, and has a broad range of biological activity. Three kinds of enzymes, phospholipase A_2 (PLA_2), cyclooxygenase (COX), and terminal PGE synthase (PGES) are involved in biosynthesis of PGE_2 and each of the three enzymatic steps involves multiple enzymes and isozymes that can act in different phases of cell activation.[1–5] Arachidonic acid released from membrane phospholipids by cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) is then supplied to either of the two COX isozymes, COX-1 or COX-

2. COX-1, a constitutive COX isozyme, contributes mainly to immediate PG generation, whereas COX-2, an inducible enzyme, mediates delayed PG generation. The COX metabolite PGH_2 is then isomerized to PGE_2 by the terminal PGES enzymes.

Cytosolic PGES (cPGES) is a 23 kDa glutathione (GSH)-requiring enzyme expressed constitutively in a wide variety of cells.[6] This enzyme is selectively coupled with COX-1 to mediate immediate PGE_2 production. Of particular note, cPGES is identical to the co-chaperone p23, which is associated with heat shock protein 90 (Hsp90).[7] In contrast, the GSH-dependent, membrane-bound PGES (mPGES)-1 is markedly induced by proinflammatory stimuli, down-regulated by glucocorticoids, and functionally coupled with COX-2.[8,9] mPGES-2 has a glutaredoxin- or thioredoxin-like domain and is activated by several thiol reagents.[10] This enzyme is constitutively expressed in various

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cells and can be coupled with both COX-1 and COX-2.[11]

Here, we briefly review our recent studies on the regulatory mechanism of cPGES/p23 in rat fibroblastic 3Y1 cells. Association of cPGES/p23 with Hsp90 was increased in cells stimulated with Ca^{2+} mobilizer, accompanied by concomitant increases in cPGES/p23 enzymic activity and PGE_2 production.[12] cPGES/p23 also underwent phosphorylation by protein kinase CK2, leading to increase in cPGES/p23 enzymic activity and association of cPGES/p23 with Hsp90.[13] Taken together, our results suggest that the cellular PGE_2-biosynthetic function is under the control of a molecular chaperone and its client protein kinase.

REGULATION BY Hsp90

Incubation of bacterial expression system-derived recombinant cPGES/p23 with Hsp90 *in vitro* in the presence of ATP and Mg^{2+} resulted in not only the formation of cPGES/p23-Hsp90 complex, but also a remarkable increase in cPGES activity (Fig. 1). This result is consistent with the fact that both ATP and Mg^{2+} are necessary for the formation of the cPGES/p23-Hsp90 complex.[14] Next, we examined interaction between cPGES/p23 and Hsp90 in rat fibroblastic 3Y1 cells, in which cPGES/p23 is the predominant PGES enzyme. Stimulation of 3Y1 cells with Ca^{2+}-ionophore A23187 elicited immediate PGE_2 syn-

thesis. The increase of PGE_2 synthesis was preceded by that of cPGES/p23 enzymic activity in cell lysates, which occurred within 1 min and peaked at 15 min. Immunoprecipitation of the cell lysates with anti-cPGES/p23 antibody revealed that more Hsp90 was co-precipitated with cPGES/p23 from A23187-treated cells than from untreated cells, indicating that there was an increased association between cPGES/p23 and Hsp90 following cell activation. To assess whether cPGES/p23 activation occurs even by physiological stimulus, 3Y1 cells were treated with bradykinin. Stimulation of cells with bradykinin resulted in increased PGE_2 synthesis and PGES activity in cell lysates, which were accompanied by increased association between cPGES/p23 and Hsp90.

Treatment of the cells with the Hsp90 inhibitors, geldanamycin and novobiocin, each of which binds to the ATP-binding site on Hsp90 and inhibits its chaperone function,[15, 16] reduced this stimulus-increased association between cPGES/p23 and Hsp90 to a basal level. The dissociation of the cPGES/p23-Hsp90 complex by the Hsp90 inhibitors was accompanied by suppression of bradykinin-induced PGE_2 synthesis and cPGES/p23 activation. Thus, it appears that stimulus-induced formation of the cPGES/p23-Hsp90 complex is correlated with the activity of cPGES/p23 to synthesize PGE_2 in cells.
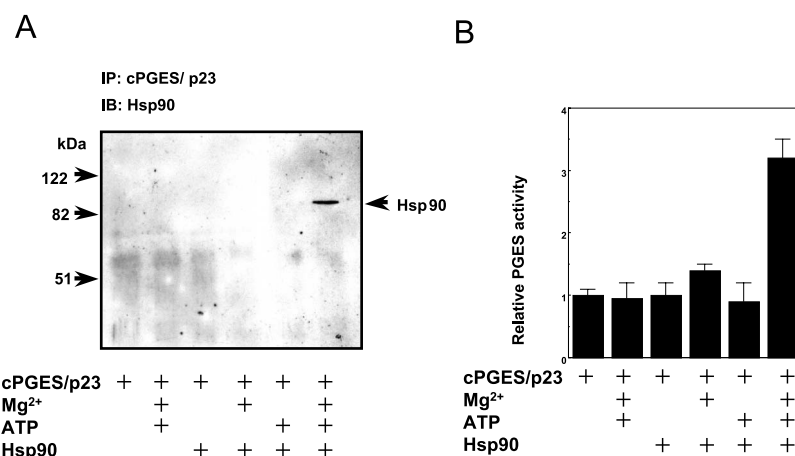

Fig. 1. *In Vitro* Association of cPGES/p23 and Hsp90 Increases cPGES/p23 Enzymic Activity.

    E. coli-derived recombinant cPGES/p23 and bovine Hsp90 were incubated for 5 min at 24°C in the presence or absence of 5 mM ATP and/or 5 mM $MgCl_2$. (A) The reaction mixtures were immunoprecipitated (IP) with anti-cPGES/p23 antibody, and the precipitates were subjected to SDS-PAGE/immunoblotting (IB) with anti-Hsp90 antibody. (B) The reaction mixtures were taken for PGES enzyme assay. Values represent fold increases in PGES activity relative to the activity of cPGES/p23 alone.

REGULATION BY PHOSPHORYLATION

There are several potential phosphorylation sites in the primary amino acid sequence of human cPGES/p23[17, 18] [Fig. 2(A)]. To clarify whether or not cPGES/p23 undergoes phosphorylation in cells, 3Y1 cells were pre-cultured with [^{32}P]orthophosphate, and cPGES/p23 protein was immunoprecipitated from the cell lysates of these cells, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography. There was a significant incorporation of radioactivity into cPGES/p23 protein even under basal culture conditions, and this phosphorylation was significantly increased in cells stimulated for 1 min with A23187. Phospho-amino acid analysis revealed that this phosphorylation occurred predominantly on serine, but not threonine or tyrosine, residues with or without A23187 treatment. Protein phosphatase treatment of cell lysate decreased cPGES/p23 activity by >90%, suggesting that phosphorylation of cPGES/p23 is crucial for its enzymic function. Judging from the effects of several protein kinase inhibitors on conversion of exogenous arachidonic acid to PGE$_2$ in 3Y1 cells, protein kinase CK2 may be involved in phosphorylation and activation of cPGES/p23, and protein kinase C (PKC) and Ca^{2+}/calmodulin kinase II (CaMKII) are not. To confirm this possibility, we examined using an *in vitro* kinase assay under conditions where recombinant cPGES/p23 was incubated with recombinant CK2 in the presence of Mg^{2+} and [^{32}P]ATP. A marked incorporation of ^{32}P into cPGES/p23 protein was observed, indicating that cPGES/p23 is directly phosphorylated by CK2 *in vitro* [Fig. 2(B)]. Furthermore, the enzymatic activity of cPGES/p23 was significantly increased following incubation with CK2. When Hsp90 was added to this *in vitro* kinase assay mixture, there were further increases in ^{32}P incorporation into cPGES/p23 protein and in cPGES/p23 enzymatic activity [Fig. 2(B)]. To clarify whether cPGES/p23 directly associates with CK2 or not, a mixture of the two was immunoprecipitated with anti-cPGES/p23 antibody and then immunoblotted with anti-CK2 antibody. CK2 was co-precipitated with cPGES/p23, and vice versa. Hsp90 was added to this mixture, it was co-precipitated with cPGES/p23 and CK2 by anti-cPGES/p23 antibody, and more CK2 was associated with cPGES/p23. These results suggest that the formation of the ternary complex containing cPGES/p23, CK2 and Hsp90 facilitates phosphorylation of cPGES/p23 by CK2.

Next, we examined whether the formation of cPGES/p23-CK2-Hsp90 complex with its at-
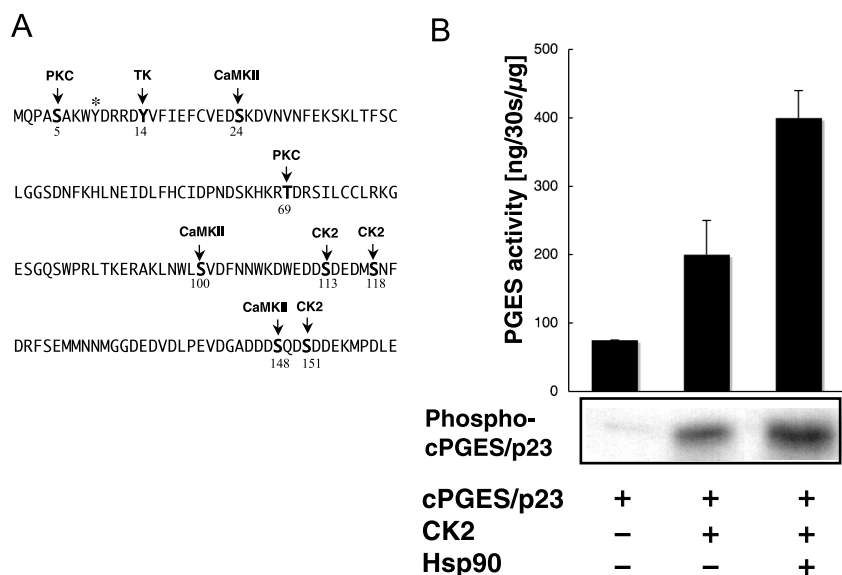

Fig. 2. *In Vitro* Phosphorylation and Activation of cPGES/p23 by CK2 and Augmentation by Hsp90.

(A) Potential phosphorylation sites on human cPGES/p23 predicted from its primary amino acid sequence. The asterisk indicates a tyrosine residue required for enzyme catalysis.[6] TK; tyrosine kinase, PKC; protein kinase C, CaMKII; Ca^{2+}/calmodulin-dependent protein kinase II. (B) *E. coli*-derived recombinant cPGES/p23 was incubated for 30 min at 30°C with recombinant CK2 with or without Hsp90 in the presence of Mg^{2+} and [^{32}P]ATP. After brief boiling, samples were applied to SDS-PAGE followed by autoradiography (lower panel). Aliquots of samples were taken for PGES enzyme assay (upper panel).

tendant phosphorylation is crucial for the PGE$_2$-biosynthetic function of cPGES/p23 *in vivo*. Stimulation of 3Y1 cells with bradykinin resulted in immediate PGE$_2$ synthesis, which increased linearly during the initial 3 min and then reached a plateau, correlating with transient phosphorylation of cPGES/p23 which peaked at 1 min and then decreased to the basal level by 5–10 min [Fig. 3(A)]. In parallel with cPGES/p23 phosphorylation, cPGES/p23 enzymic activity in cell lysates was increased markedly at 1 min and declined at 10 min [Fig. 3(B), lower panel]. Association of cPGES/p23 with CK2 was also markedly elevated at 1 min and then returned to the basal level. Furthermore, association of cPGES/p23 with Hsp90 was transient, peaking at 1 min and returning to the basal level by 10 min [Fig. 3(B), upper panel]. Moreover, the CK2 inhibitor 5,6-dichloro-1-*β*-D-ribofuranosylbenzimidazole (DRB) or the Hsp90 inhibitor geldanamycin markedly inhibited bradykinin-induced PGE$_2$ synthesis, cPGES/p23 activation, and association of cPGES/p23 with CK2 and Hsp90 [Fig. 3(C), (D)]. These results suggest

that Hsp90 acts as scaffold protein that brings cPGES/p23 and CK2 in proximity, thereby allowing their efficient functional interaction under physiological conditions. To verify the contribution of CK2 to cPGES/p23 activation, we took advantage of DN-CK2, which harbors a mutation (D156A) in the kinase domain and thereby blocks the cellular functions of CK2.[19] Overexpression of DN-CK2 in 3Y1 cells resulted in decrease of phosphorylation of cPGES/p23 and association of cPGES/p23 with Hsp90 relative to those in mock-transfected cells. Accordingly, cPGES/p23 enzymic activity in the lysates of DN-CK2-transfected cells was approximately half that of mock-transfected cells. Moreover, PGE$_2$ synthesis by DN-CK2-transfected cells from exogenous arachidonic acid or in response to A23187 was also reduced by half as compared with that of mock-transfectant. These results indicate that complex formation of cPGES/p23 with CK2 and Hsp90, and concomitant phosphorylation by CK2 are essential for the activation of cPGES/p23 in cells.

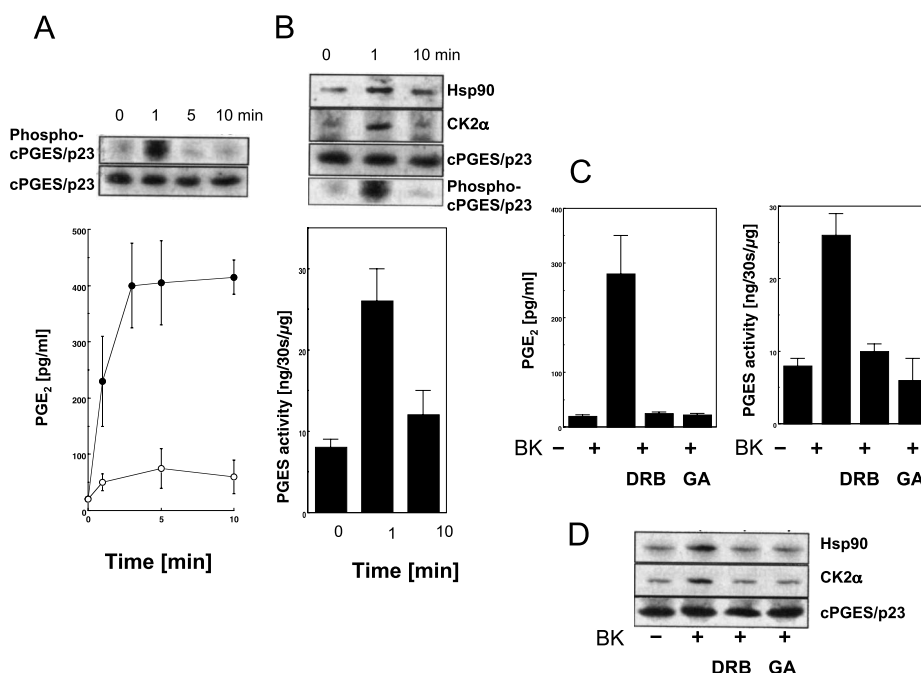To address which residues of cPGES/p23 are

Fig. 3. Bradykinin (BK)-induced Phosphorylation and Activation of cPGES/p23 in 3Y1 Cells.

(A) Time course of PGE$_2$ synthesis after incubation for the indicated periods with (●) or without (○) 10 μM BK (lower panel). ^{32}P-incorporation into cPGES/p23 (Phospho-cPGES/p23) at each time point was assessed immunoprecipitation and subsequent autoradiography, and equal precipitation of cPGES/p23 protein from each sample was verified by immunoblotting (upper panel). (B) Formation of the cPGES/p23-CK2-Hsp90 complex and cPGES/p23 phosphorylation after stimulation with BK for indicated periods were assessed by immunoprecipitation assay (upper panel). PGES activity in cell lysates at each time point was measured (lower panel). (C) Effect of 5,6-dichloro-1-*β*-D-ribofuranosylbenzimidazole (DRB) and geldanamycin (GA) on PGE$_2$ synthesis (left panel) and PGES activity in cell lysates (right panel). Cells were preincubated for 5 hr with inhibitors and then treated for 1 min with or without BK. (D) Effect of DRB and GA on the formation of the cPGES/p23-CK2-Hsp90 complex, as determined by immunoprecipitation and subsequent immunoblotting with the indicated antibodies.

phosphorylated by CK2 in cells, we constructed point mutants of cPGES/p23 in which each CK2-consensus Ser residue was replaced with Ala or Gly (S113A, S118A and S151G). These mutants and wild type (WT) of cPGES/p23 were transfected into 3Y1 cells, and their enzymic activities and PGE_2 synthesis were determined. cPGES/p23 enzymic activity was increased significantly in cells transfected with WT or S151G, whereas the activity in cells transfected with S113A or S118A was comparable with that in parental cells [Fig. 4(A)]. PGE_2 synthesis induced by bradykinin was markedly elevated when WT or S151G was transfected, whereas in the case of S113A or S118A it was not significantly altered [Fig. 4(B)]. Immunoprecipitation analysis of ^{32}P-pre-labeled cells revealed that phosphorylation of cPGES/p23 occurred in WT- or S151G-transfected cells, and not in S113A or S118A-transfected cells [Fig. 4(C)]. Judging from these results, we conclude that Ser^{113} and Ser^{118} are the predominant CK2 phosphorylation sites critical for cPGES/p23 activation in cells, and that phosphorylation of these two Ser residues promotes association of cPGES/p23 with Hsp90.

In summary, we propose that functional cPGES/p23 exists in cells as a multicomponent complex containing CK2 and Hsp90 as the minimal requirements. CK2 activated by upstream signals triggers dual phosphorylation of Ser^{113} and Ser^{118} on cPGES/p23, which in turn facilitates the recruitment of cPGES/p23 to the Hsp90 complex, leading to its full activation. In porcine endothelial cells, bradykinin induces phosphorylation of angiotensin-converting enzyme by CK2,[20] consistent with our findings that bradykinin elicits CK2-dependent cPGES/p23 activation, although the signaling from bradykinin to CK2 activation remains to be elucidated. Our results have provided the first evidence that the function of PGES is elegantly controlled by a particular protein kinase in cooperation with a molecular chaperone.

Fig. 4. Determination of CK2 Phosphorylation Sites Using cPGES/p23 Mutants.

(A) PGES activity in lysates of the transfectants. Values show relative enzymic activity, with that of parental cells regarded as 1. (B) PGE_2 synthesis by the transfectants and parental cells after treatment with bradykinin. (C) Phosphorylation of WT and mutant cPGES/p23 proteins. Cells pre-labeled with ^{32}P were lysed and were subjected to immunoprecipitation (IP) with anti-FLAG antibody. Individual samples were resolved by SDS-PAGE and were subjected to autoradiography or immunoblotting with anti-FLAG antibody.

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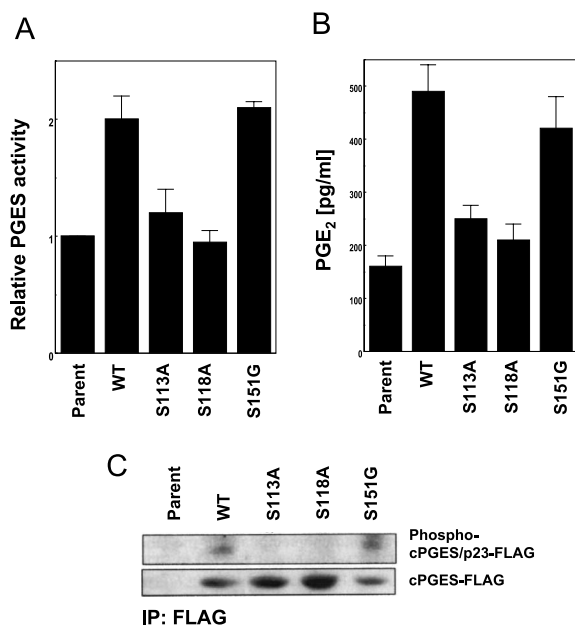
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