Transcriptional Regulation of Prostaglandin Synthase 2 Gene Expression by Platelet-derived Growth Factor and Serum*

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Weilin Xie and Harvey R. Herschman‡

From the Departments of Biological Chemistry and Molecular and Medical Pharmacology, and the Molecular Biology Institute, UCLA School of Medicine, Los Angeles, California 90095

Prostaglandin synthase 2 (PGS2) is an immediate-early gene induced in a variety of cellular contexts. We investigate here the transcriptional activation of the murine PGS2 gene in NIH 3T3 cells, in response to the mitogens platelet-derived growth factor (PDGF) or serum. Site-directed mutagenesis experiments demonstrate that a consensus cyclic AMP response element (CRE) in the murine PGS2 promoter is essential for optimal PGS2 gene expression in response to PDGF or to serum. Overexpression of c-Jun potentiates PDGF- or serum-induced luciferase expression from a reporter construct containing the first 371 nucleotides of the PGS2 promoter. In contrast, overexpression of other transcription factors binding to the CRE element of the PGS2 gene inhibits induction by PDGF or serum. Moreover, positioning the c-Jun activation domain next to the minimal PGS2 promoter via a GAL4 DNA binding site rather than the CRE is sufficient to permit serum or PDGF stimulation of luciferase expression from this modified reporter construct. PDGF or serum treatment both activate c-Jun N-terminal kinase (JNK), the mitogen-activated protein kinase responsible for phosphorylation and activation of c-Jun. Cotransfection of plasmids expressing dominant-negative Ras, Rac1, MEKK-1, or JNK along with the [PGS2]luciferase reporter prevents induction by PDGF or serum, demonstrating that serum and PDGF induction of the PGS2 gene in NIH 3T3 cells requires activation of a Ras/Rac1/MEKK-1/JNK kinase/JNK signal transduction leading to phosphorylation of c-Jun. Additional cotransfection experiments with plasmids expressing dominant-negative Raf1 and ERK demonstrate that induction of PGS2 gene expression by PDGF and serum also requires activation of a Raf1/mitogen-activated protein kinase kinase (MAPKK)/ERK signal transduction pathway.

Expression of mitogen-induced primary response/immediate-early genes is rapidly induced at the transcriptional level by growth factors. Induction of primary response/immediate-early genes does not require protein synthesis; activation of pre-existing transcription factors as a result of the signal generated by occupancy of the growth factor receptor is sufficient for transcriptional activation of these genes (1). Serum, platelet-derived growth factor (PDGF),1 and other mitogens stimulate in fibroblasts the expression of many immediate-early genes, including transcription factors, secreted proteins, protein kinases, protein phosphatases, and an inducible form of prostaglandin synthase (1, 2).

Prostaglandins play key roles in many biological processes, including cell division, blood pressure regulation, immune responses, ovulation, bone development, wound healing, and water balance. Altered prostanol production is associated with a variety of illnesses, including bone resorption, cardiovascular disease, acute and chronic inflammation, atherosclerosis, and colon cancer (3, 4). Prostaglandin synthase (PGS), also known as cyclooxygenase, is the key enzyme in the conversion of free arachidonic acid to PGH₂, the common precursor to all prostaglandins, prostanoylns, and thromboxanes (3, 4).

Prostaglandin synthase 2 (PGS2) was initially cloned from fibroblasts either (i) treated with mitogens (5, 6) or (ii) expressing the viral v-src oncogene (7). Following its initial identification, PGS2 has been shown to be inducible by distinct stimuli in a variety of cells (8). The ligand-induced synthesis of PGS2 is required for mitogen-stimulated prostaglandin synthesis in fibroblasts (9, 10) and endotoxin-stimulated prostaglandin synthesis in macrophages (9). However, the molecular mechanisms by which PGS2 gene expression is elevated in various cells in response to various inducers has only recently begun to be elucidated.

We previously demonstrated that the cyclic AMP response element (CRE) of the PGS2 promoter is required for v-src-induced expression of a PGS2 promoter-luciferase reporter (11). We originally anticipated that either CREB or another member of the ATF family of transcription factors would mediate v-src induction of PGS2 gene expression via this CRE. However, when expression vectors encoding wild-type CREB, ATF-2, or ATF-3 were expressed with the PGS2 reporter, induction by co-expressed v-src was blocked, rather than enhanced (12). The c-Jun transcription factor, a member of the bZIP transcription factor family, forms heterodimers with ATF transcription factors that bind to CRE sequences (13, 14). Moreover, c-Jun-containing AP-1 complexes can also bind to CRE sequences (15, 16). We found that endogenous c-Jun can bind to the CRE of the PGS2 promoter, as demonstrated by antibody supershifts in electrophoretic mobility shift analyses (12). These experiments and additional co-transfection experiments suggested that c-Jun plays a major role in the transcriptional activation of the PGS2 gene by pp60src, the product of the v-src oncogene, via the CRE of the PGS2 promoter (12). Serum and PDGF are strong inducers of PGS2 gene expression in

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‡ To whom correspondence should be addressed: Rm. 341, Molecular Biology Institute, UCLA, Los Angeles, CA 90095. Tel.: 310-825-8735; Fax: 310-825-1447; E-mail: harvey@LBES.medsch.ucla.edu.

1 The abbreviations used are: PDGF, platelet-derived growth factor; ATP, activating transcription factor; JNK, c-Jun N-terminal kinase; JNKK, c-Jun N-terminal kinase kinase; ERK, extracellular signal-regulated kinase; CRE, cyclic AMP response element; PGS, prostaglandin synthase; DN, dominant-negative; CREB, cyclic AMP response element binding protein; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; TNF, tumor necrosis factor.

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murine fibroblasts (5, 17). In this report, we investigate the cis-acting elements of the PGS2 gene regulatory region, the transcription factors, and the signal transduction pathways necessary for PGS2 induction by PDGF and serum.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—We previously referred to PGS2 as 12-O-tetradecanoylphorbol-13-acetate-induced Sequence 10 or TIS10 (5). Consequently, our promoter constructs historically have the TIS10 designation (11, 17). The luciferase reporter constructs pTIS10-371LUC and pTIS10-49LUC, containing the first 371 or 40 nucleotides 5′ of the PGS2 transcription start site, were described previously (11, 17). The overlapping CRE and E-Box sites of the PGS2 5′ regulatory region are located between nucleotides −56 to −45 (17). Thus the pTIS10-371LUC reporter contains these CRE and E-Box cis-acting sites of the PGS2 promoter. In contrast, the pTIS10-371LUC construct has the CRE and E-Box sites deleted. Bridge polymerase chain reaction (18) was used to create pTIS10-373CRE*+LUC, a reporter plasmid in which the CRE located 56 nucleotides 5′ from the start site of transcription is converted from CGTCA to atTCA, and to create pTIS10-371-EBox*LUC, a reporter plasmid in which the overlapping E-Box located 33 nucleotides 5′ from the start site of transcription is converted from CAGCCG to CACGct. pTIS10-373CRE*+LUC and pTIS10-371-EBox*LUC reporter constructs were transiently transfected into NIH 3T3 cells in 60-mm culture dishes. A second set of cells was transfected with pTIS10-371-EBox*LUC, a similar reporter plasmid in which the E-Box was mutated. A final set of cells was transfected with pTIS10-373CRE*LUC, a reporter plasmid in which the CRE was mutated.

The transfected cells were treated with either 20% fetal serum or 10 ng/ml PDGF. Three plates of NIH 3T3 cells were used for each transfection condition. Cells were harvested 6 h after treatment with mitogens and assayed for luciferase activity and total protein. Data are expressed as averages ± standard deviations.

**RESULTS**

**The CRE Site at Nucleotide −56 of the PGS2 Promoter Is Essential for Optimal PDGF and Serum Induction.** We previously referred to PGS2 as TIS10 (4). Our promoter constructs thus historically have the TIS10 designation (8). The PGS2 luciferase reporter pTIS10-373LUC (4 µg), containing nucleotides −371 to +70 of the murine PGS2 promoter fused to the luciferase reporter gene, was transfected into NIH 3T3 cells in 60-mm culture dishes. A second set of cells was transfected with pTIS10-373-EBox*LUC, a similar reporter plasmid in which the E-Box was mutated. A final set of cells was transfected with pTIS10-373CRE*LUC, a reporter plasmid in which the CRE was mutated.

The transfected cells were treated with either 20% fetal serum or 10 ng/ml PDGF. Three plates of NIH 3T3 cells were used for each transfection condition. Cells were harvested 6 h after treatment with mitogens and assayed for luciferase activity and total protein. Data are expressed as averages ± standard deviations.

**Fig. 1. The CRE site of the PGS2 regulatory region is essential for optimal PDGF and serum induction.** We previously referred to PGS2 as TIS10 (4). Our promoter constructs thus historically have the TIS10 designation (8). The PGS2 luciferase reporter pTIS10-373LUC (4 µg), containing nucleotides −371 to +70 of the murine PGS2 promoter fused to the luciferase reporter gene, was transfected into NIH 3T3 cells in 60-mm culture dishes. A second set of cells was transfected with pTIS10-373-EBox*LUC, a similar reporter plasmid in which the E-Box was mutated. A final set of cells was transfected with pTIS10-373CRE*LUC, a reporter plasmid in which the CRE was mutated.

The transfected cells were treated with either 20% fetal serum or 10 ng/ml PDGF. Three plates of NIH 3T3 cells were used for each transfection condition. Cells were harvested 6 h after treatment with mitogens and assayed for luciferase activity and total protein. Data are expressed as averages ± standard deviations.

**The CRE Site at Nucleotide −56 of the PGS2 Promoter Is Essential for Optimal PDGF and Serum Induction in NIH 3T3 Cells.** The 371 nucleotides of the PGS2 promoter 5′ to the start site of transcription are sufficient for PGS2 gene induction by serum and PDGF (17). Because there are a limited number of cis-acting transcriptional regulatory elements within these 371 nucleotides (17), we utilized site-directed mutagenesis to generate luciferase reporter genes with specific mutations in potential regulatory sites. Transient transfection into NIH 3T3 cells was performed with each of these PGS2 reporters, and the ability of serum or PDGF to induce luciferase expression was examined.

**Mutation of the CRE element at nucleotides −56 to −51 of the PGS2 gene from CGTCA to atTCA reduced luciferase expression in serum- or PDGF-treated cells by about 70% when compared with the wild-type PGS2 regulatory region (Fig. 1).** In contrast, mutation of the overlapping E-Box at nucleotides −53 to −48 of the PGS2 gene from CACGTG to CACGct had no effect on PDGF- or serum-stimulated luciferase activity. Although PDGF has been reported to induce gene expression through an Sp-1 element, mutation of the consensus Sp-1 site located at nucleotides −239 to −234 in the 5′ regulatory region of the PGS2 gene (17) also had no effect on PDGF- or serum-induced luciferase activity (data not shown).

**Immunocomplex Assay for c-Jun N-terminal Kinase.** The JNK immunocomplex kinase assay was performed as described previously (12). Briefly, 6 µg of plasmid encoding Flag-epitope tagged JNK1 was transfected into NIH 3T3 cells in 100-mm dishes, using the calcium phosphate method (12). The cells were kept in 0.5% newborn calf serum for 24 h after transfection. Cells were stimulated with 20% serum (10 ng/ml) 30 min later, and the addition of the cell lysate to the assay is performed as described previously (11, 12). Triplelicate dishes were used for all transfections, and all experiments were repeated at least twice.
PGS2 Induction by PDGF and Serum

Overexpression of c-Jun augments PGS2 induction by serum and PDGF. A. NIH 3T3 cells were transfected with the c-Jun expression vector (2 μg) or the corresponding empty vector, along with the pTIS10.LUC luciferase expression vector (4 μg). Three plates were used for each transfection condition. Where indicated, the transfected cells were treated with either PDGF or serum. Control cells remained untreated. Cells were harvested 6 h after treatment with mitogens and assayed for luciferase activity and total protein. Three plates were used for each transfection condition. Data are expressed as averages ± standard deviations. B, the pGal4TIS10.LUC (4 μg) reporter construct containing five Gal4 DNA binding sites was cotransfected into NIH 3T3 cells, along with 2 μg of expression vectors encoding GAL4-DB, the Gal4 DNA binding domain; GAL4-Jun, the fusion protein of GAL4-DB and c-Jun; or GAL4-Jun 63/73, the fusion protein of GAL4DB and c-Jun in which serines 63 and 73 have been mutated to leucine. The luciferase reporter gene containing only the first 40 nucleotides of the PGS2 promoter, TIS10.LUC, expresses only minimal luciferase activity (11, 12). Because this plasmid does not contain the overlapping CRE and E-Box sites of the PGS2 promoter, it was not responsive to PDGF or serum stimulation (data not shown). If five Gal4 binding sites are added to this promoter, this [gal4-PGS2] reporter construct similarly expresses only minimal luciferase activity (12). Co-expression of the Gal4 DNA binding domain (GAL4-DB) does not enhance luciferase expression from the [gal4-PGS2] chimeric reporter vector, as shown in the first lane of the figure. Cells were treated with serum or PDGF as indicated in the figure. Cells were harvested 6 h after treatment with mitogens and assayed for luciferase activity and total protein. Data are expressed as averages ± standard deviations. Three plates were used for each transfection condition.

When this [gal4-PGS2] reporter is cotransfected with the vector expressing a fusion protein of the Gal4 DNA binding domain and the c-Jun activation domain (amino acids 5–200; GAL4-Jun), luciferase activity is elevated as a result of the unstimulated basal activity of the overexpressed GAL4-c-Jun protein (Fig. 2B). When cells containing the [gal4-PGS2] reporter and the GAL4-c-Jun expression vector are exposed to serum or to PDGF, the mitogen treatments significantly enhance luciferase expression. We conclude that the c-Jun activation domain, if properly positioned “upstream” of the minimal PGS2 promoter, can drive either PDGF- or serum-induced gene expression. In contrast, GAL4-CREB protein is unable to mediate serum- or PDGF-stimulated luciferase expression from the [gal4-PGS2] reporter (data not shown).

Phosphorylation of serine residues Ser63 and Ser73 is necessary for transcriptional activation of c-Jun at AP-1 sites (24, 25). To determine whether phosphorylation at these two serines is required for PDGF- or serum-induced, c-Jun-mediated PGS2 induction, we used an expression vector encoding a fusion protein, GAL4-Jun 63/73, in which these serines are replaced by leucines. The GAL4-Jun 63/73 fusion protein is unable to mediate either basal expression, PDGF activation, or serum activation of the [gal4-PGS2] reporter (Fig. 2B). We conclude that phosphorylation of the c-Jun activation domain at serines 63 and 73 is required for PDGF- or serum-induced, c-Jun-mediated transcriptional activation from the PGS2 regulatory region.

Diagram of luciferase activity after transfection with different vectors and treatments. The x-axis represents different treatments (C-JUN, PDGF, and SERUM), and the y-axis represents luciferase units/μg protein. The diagram shows that c-Jun enhances luciferase expression from the PGS2 promoter in response to serum or PDGF (Fig. 2A). These data are consistent with the suggestion that c-Jun mediates serum and PDGF induction of PGS2 gene expression.

We previously demonstrated that wild-type CREB does not mediate v-src induction of PGS2 gene expression (12). In contrast to the results with c-Jun, when wild-type CREB was coexpressed with the TIS10.LUC PGS2 reporter gene, induction by serum or PDGF was partially blocked, rather than enhanced (data not shown). The inability of transfected wild-type CREB to enhance PDGF or serum-stimulated luciferase expression and the ability of c-Jun to enhance this induction suggest that like v-src induction, serum and PDGF induction is mediated by activation of c-Jun and not a member of the ATF transcription factor family.

Transcriptional Activity from the Minimal PGS2 Promoter by the c-Jun Activation Domain Can Be Enhanced by Serum or PDGF—We wanted to determine whether the activation domain of the c-Jun transcription factor, when positioned at the location of the CRE site of the PGS2 promoter, can be transcriptionally activated by serum or PDGF treatment regardless of the DNA binding site. We used a minimal PGS2 promoter (from nucleotides −40 to +3) to prepare a luciferase reporter construct in which the CRE sequence in the PGS2 luciferase reporter is replaced with five yeast Gal4 transcription factor DNA binding sites from the gal4 gene (12). This reporter construct was transfected into NIH 3T3 cells, along with plasmids that express fusion proteins between the Gal4 DNA binding domain and the activation domains of several ATF or bZIP transcription factors. Basal, serum-stimulated, and PDGF-stimulated luciferase activities were then analyzed. The CRE and E-Box elements of the PGS2 promoter are absent from this reporter construct. Consequently, activation domains can be analyzed for signal transduction-stimulated transcriptional activity when placed immediately upstream of the basal PGS2 promoter, without interference from endogenous transcription factors.

PGS2 Induction by PDGF and Serum
Recent studies have demonstrated that JNK is the primary MAP kinase responsible for ligand-stimulated c-Jun phosphorylation in vivo (26–28). JNKs can be phosphorylated and activated in response to TNF-α (29), environmental stress (29), UV light (27), and the oncogene v-src (12). If serum or PDGF stimulate PGS2 expression via a c-Jun-dependent pathway in NIH 3T3 cells, then we would expect these ligands to also stimulate JNK activity. To test this hypothesis, we transfected NIH 3T3 cells with Flag epitope-tagged JNK1 and treated cells with either PDGF or serum. Flag-JNK1 was then immunoprecipitated with an anti-Flag monoclonal antibody. Immunocomplex kinase assays were then performed using GST-c-Jun recombinant protein as substrate (12, 22). Both PDGF and serum treatment activate JNK enzyme activity in NIH 3T3 cells (Fig. 3), as expected if c-Jun plays a major role in transcriptional activation of the PGS2 gene in response to these agents.

The Ras/Rac1/MEKK-1/JNK/JNK Signal Transduction Pathway Mediates Both Serum- and PDGF-induced PGS2 Gene Expression—Recent experiments from several laboratories have demonstrated that ligand-stimulated phosphorylation of JNK is mediated by activation of Ras and Rac, two small GTP-binding proteins (30, 31). Ras activation, in many contexts, leads to activation of MEKK-1, the first protein kinase in the MEKK-1/JNK/JNK kinase cascade that phosphorylates and activates c-Jun (32, 33). We next wanted to determine whether the Ras/Rac1/MEKK1/JNK pathway plays a role in the serum- and PDGF-stimulated induction of PGS2 gene expression. We used a group of dominant-negative mutants that interfere with the Ras/Rac1/MEKK1/JNK pathway to investigate this question.

Expression of kinase negative, dominant-negative JNK1, which interferes with the phosphorylation of c-Jun, can attenuate luciferase expression from the TIS10 -371-LUC reporter gene in response to PDGF or serum (Fig. 4A). Expression of a kinase-defective, dominant-negative MEKK-1 mutant also efficiently inhibited the PDGF and serum stimulation of PGS2 gene expression (Fig. 4B). These data suggest that the MEKK-1/JNK/JNK pathway plays a role in serum- or PDGF-induced expression of the PGS2 gene in NIH 3T3 cells.

Both serum and PDGF stimulation of 3T3 cells cause activation of Ras; in response to these treatments Ras exchanges GDP for GTP and Ras-GTP activates several protein kinase cascades leading to transcriptional activation and altered gene expression (32, 33). If Ras plays a role in serum- or PDGF-stimulated PGS2 gene expression, a DN-Ras protein should block induction. This is the case; expression of DN-Ras can, in a dose-dependent manner, substantially suppress serum-stimulated luciferase expression from the TIS10 -371-LUC luciferase reporter and block all PDGF-stimulated luciferase activity (Fig. 4C).

The intermediate mediators from Ras to MEKK-1 are not well understood. Recently several groups showed that Rac1 and CDC42H, members of the Rho family of GTPases, are involved in transducing the signals from Ras to MEKK-1, leading to activation of JNK (reviewed by Vojtek and Cooper (34)). Dominant-negative Rac1 potently attenuates both PDGF- and serum-stimulated luciferase expression from the TIS10 -371-LUC reporter gene (Fig. 4D), in agreement with the proposed role of Rac1 in this pathway. In contrast, an activating Rac1 mutation (RacV12) does not interfere with serum- or PDGF-stimulated luciferase expression.

These data establish one pathway required for induction of PGS2 gene expression in response to PDGF or serum. Both serum- and PDGF-stimulated PGS2 gene expression require Ras/Rac1 activation, leading to activation of the MEKK1/JNK/JNK kinase cascade. Phosphorylation of c-Jun by JNK results in transcriptional activation of the PGS2 gene, mediated by the CRE located at nucleotides (-56 to -52) of the PGS2 regulatory region.

The Ras/Raf1/Mapk/K/Erk Signal Transduction Pathway Also Participates in Both Serum- and PDGF-induced PGS2 Gene Expression—In addition to the MEKK1/JNK/JNK kinase cascade, Ras-GTP activates several additional kinase-mediated signal transduction pathways. Each pathway leads to phosphorylation and activation of distinct subsets of transcription factors (32–35). As described above, the immediate downstream effector of Ras leading to activation of JNK enzyme activity and phosphorylation of c-Jun is the MAP kinase kinase kinase, Raf1. Raf1 then activates distinct protein kinase cascade, RAF1/MEKK1/ERK, that leads to phosphorylation of transcription factors such as TCF/Elk-1 and C-MYC (32–35). To determine whether the Raf1 kinase cascade is also involved in serum- or PDGF-stimulated PGS2 gene expression, we examined the effect of dominant-negative mutants of the components of the Raf/MEKK1/ERK pathway on luciferase induction from the TIS10 -371-LUC reporter gene.

Expression of kinase-defective, dominant-negative ERK1 or ERK2 proteins blocks both serum- and PDGF-stimulated luciferase induction from the TIS10 -371-LUC reporter gene (Fig. 5A). ERK1 and ERK2 are the distal kinases in this Ras-activated kinase cascade. Raf1 is the proximal kinase in this signal transduction pathway. We also performed transfection analyses using a dominant-negative Raf1 expression plasmid. DN-Raf1, like DN-ERK1 and DN-ERK2, also attenuates induction from the PGS2 promoter in response to serum or PDGF (Fig. 5B). These data suggest that activation of the Raf1/MEKK1/ERK pathway(s) are also required for induction of PGS2 gene expression by serum or PDGF in NIH 3T3 cells.

DISCUSSION

Cis-acting Elements of the PGS2 Regulatory Region That Mediate PGS2 Gene Expression—We have previously identified the CRE located at nucleotides (-56/-52) 5′ from the start site of transcription of the murine PGS2 gene as the key cis-acting element required for transcriptional activation of the PGS2 gene in response to expression of pp60src oncogene (12). In contrast, mutational and co-transfection experiments demonstrated that the overlapping E-Box located at nucleotides (-53/-48) plays little or no role in PGS2 induction by v-src. In this current study we show that the CRE of the PGS2 promoter also plays a major regulatory role in PDGF and serum induction of PGS2 gene expression and that the E-Box has little or no role. The corresponding CRE of the human PGS2 gene also plays a key role in PGS2 expression in human monocytes (36).

Sirois and Richards (37) reported that a CAAT/NFILβ/C/
EBP) site located 140 nucleotides 5' of the transcriptional start site of the rat PGS2 gene plays a major regulatory role in the induction of PGS2 message in rat ovarian granulosa cells in response to pituitary hormones or forskolin stimulation. However, those studies used a hybrid PGS2 promoter in which nucleotides (2195/2110) were fused to a minimally active PGS2 promoter from nucleotides (252/232). In a more recent study, Morris and Richards (38) report that, using a region of the rat PGS2 promoter extending from nucleotide 2195 to nucleotide 132, mutation of the NF-IL6(C/EBP) site has no effect on induction of reporter gene activity by luteinizing hormone and gonadotrophin-releasing hormone in cultured rat ovarian granulosa cells. Morris and Richards (38) report that mutational analysis has identified the critical cis-acting element for luteinizing hormone or gonadotrophin-releasing hormone induction of PGS2 promoter activity in rat ovarian granulosa cells as the E-Box, CACGTC, which shares identical sequence and location with the E-Box of the murine PGS2 promoter. The rat PGS2 promoter does not contain the CGTCA CRE present at nucleotide 256 in the murine and human promoters (39); instead this sequence is AGTCA. It will be of great interest to compare the expression of the rat and murine PGS2 genes in ovarian granulosa cells and in fibroblasts, in response to inducers such as luteinizing hormone, gonadotro-
phosphorylation, v-src, and PDGF, and determine the roles of the CRE and E-Box sequences in these cell-type specific responses in different species.

Mutational analysis of the human PGS2 gene demonstrated that both the CRE and the NF-IL6(C/EBP) cis-acting elements play functional roles in PGS2 induction by a combination of endotoxin and phorbol ester in bovine arterial endothelial cells (39). Mutual analysis of the murine PGS2 regulatory region also identified the corresponding NF-IL6(C/EBP) element as a mediator of TNFα-induced PGS2 gene expression in the murine osteogenic MC3T3-E1 cell line (40). TNFα induction of PGS2 gene expression in MC3T3-E1 cells is also mediated by an NFκB site located 400 nucleotides from the start site of transcription (40). Thus, to date, only the CRE, E-Box, NF-IL6(C/EBP), and NFκB elements have been implicated in ligand-induced expression of the PGS2 gene. A number of other potential cis-acting regulatory sequences, including binding sites for AP2, Sp1, MEF-2, and GATA transcription factors, have been identified in the regulatory regions of PGS2 genes. However, none of these has yet been demonstrated to play a role in ligand-induced modulation of PGS2 gene expression.

**Transcription Factors That Mediate Induction of PGS2 Gene Expression**—The CRE of the PGS2 gene can be recognized in electrophoretic gel shift mobility experiments by a variety of proteins, including CREB and c-Jun (12). Using similar analyses, the NFκB element of the PGS2 gene can be recognized by NFκB p50 and p65 molecules and the NF-IL6(C/EBP) element of the PGS2 gene can be recognized by NF-IL6(C/EBP)β (40). The E-Box of both the murine and rat PGS2 promoters can be bound by multiple proteins from both fibroblast (12, 14) and granulosa cell (38) nuclear extracts. Antibody supershift analyses identified one of these E-Box binding proteins as upstream stimulatory factor (38).

Gel shift experiments identify candidate transcription factors that may mediate gene expression. However, binding of a transcription factor to a presumptive regulatory DNA sequence from a gene in question does not provide sufficient evidence to conclude that a particular transcription factor is either necessary or sufficient for regulation of gene expression in vivo. In fact, such experiments can be misleading. Thus, although both recombinant and endogenous CREB bind strongly to the murine PGS2 CRE in gel shift experiments (11, 12, 38), co-transfection experiments demonstrate that CREB does not mediate CRE-dependent PGS2 gene expression in response to v-src expression (12), serum treatment, or PDGF exposure in NIH 3T3 fibroblasts.

Experiments utilizing co-transfection of PGS2 reporter constructs with a variety of transcription factors that recognize the PGS2 CRE element are consistent with the suggestion that c-Jun mediates v-src (12), PDGF, and serum induction (Fig. 2) of PGS2 gene expression. Co-transfection of C/EBPδ, which binds to NF-IL6(C/EBP) sequences, increases PGS2 promoter activity from a PGS2 reporter construct in endotoxin/phorbol ester-stimulated bovine aortic endothelial cells (39), suggesting that C/EBPδ may play a role in PGS2 induction. Again, enhancement of gene expression by over-expression of a transcription factor is consistent with but does not conclusively demonstrate that the same transcription factor mediates expression of the gene in question in a physiological induction response. Co-transfection experiments with PGS2 reporter vectors and expression vectors either for other transcription factors recognizing the PGS2 NF-IL6(C/EBP) element, transcription factors that bind to the NFκB element, or transcription factors that bind to the E-Box have not been reported.

We demonstrated that the c-Jun transactivation domain, when placed next to the PGS2 minimal promoter by fusion to the DNA binding domain of the yeast GAL4 transcription factor and binding of this chimeric protein to Gal4 DNA-binding sites, can drive v-src (12), serum- and PDGF-induced (Fig. 2) gene expression. Induction by these agents is eliminated when the sites of JNK phosphorylation are mutational altered in the c-Jun transactivation domain of the GAL4-Jun chimeric transcription factor. These results demonstrate that phosphorylation of the c-Jun transactivation domain plays a critical role in PDGF and serum-induced transcription from the PGS2 gene, just as it does in v-src-induced expression of the PGS2 gene.

c-Jun is a member of the bZIP transcription factor superfamily and can form heterodimers with members of the Jun, Fos, and ATF subfamilies of the bZIP family (13–16). We do not, as yet, know whether the c-Jun homodimer or a heterodimer between c-Jun and another member(s) of the bZIP family is required for serum- or PDGF-stimulated PGS2 gene expression in fibroblasts.

**Signal Transduction Pathways That Mediate Induction of PGS2 Gene Expression**—There are a large number of distinct ligands and physiological stimuli that can induce PGS2 gene expression, in some cases in very cell-specific fashions (8). Consequently, there are likely to exist varied and in some cases cell-specific signal transduction pathways leading to transcription factor activation that results in elevated transcription from the PGS2 gene. For example, both serotonin (41) and endothelin (42) can induce PGS2 gene expression in mesangial cells. Although induction of PGS2 expression by both ligands appears to be sensitive to inhibitors of tyrosine kinases, down-regulation of protein kinase C prevents subsequent PGS2 induction by serotonin (41) but not PGS2 induction by endothelin (42). In contrast, PGS2 induction in human skin fibroblasts by interleukin-1β is not blocked by tyrosine kinase inhibitors (43). Moreover, ceramide treatment elevates PGS2 levels in murine fibroblasts, suggesting that lipid second messengers may also mediate PGS2 gene expression (44). We conclude that a variety of signaling pathways are likely to couple various ligand-receptor interactions with the modification/activation of alternative transcription factors necessary to elicit ligand-stimulated elevation of transcription from the PGS2 gene.

Induction of PGS2 gene expression in NIH 3T3 cells in response to expression of the v-src oncogene (12), to serum stimulation, or to PDGF stimulation requires activation of two Ras-dependent signaling pathways. Ras activates the MEKK-1/JNK/JNK kinase cascade, leading to phosphorylation of c-Jun and stimulated transcription from the CRE of the PGS2 gene. For the PDGF-induced response we can now identify the pathway of PGS2 induction, from (i) PDGF receptor occupancy to (ii) activation of the Ras/Rac1/MEKK-1/JNK/JNK signal transduction pathway, leading to (iii) phosphorylation of the c-Jun transcription factor, which causes (iv) transcriptional activation of the PGS2 gene via the CRE cis-acting regulatory element. A similar sequence from receptor occupancy, through signal transduction, to activation of a specific transcription factor and stimulation of transcription from a defined cis-acting element has not been described for any other ligand-induced PGS2 induction pathway.

Ras activation also stimulates activation of the Raf/ MAPKK/ERK pathway(s). The Elk1 component of the TCF/ Elk1 transcription factor complex can be phosphorylated and activated by both JNK and ERK MAP kinases (45). Phosphorylated TCF/Elk1 can activate gene expression through the serum response element present in the 5’ regulatory region of many immediate early/promise response genes. These data establish the serum response element as a site of integration of these two Ras-mediated signal transduction pathways. How-
ever, the PGS2 gene does not contain a serum response element. c-Jun is phosphorylated by JNK but not by the ERK MAP kinases. Ras/Raf1/MAPKK-activated ERK may phosphorylate an as yet unidentified transcription factor that also participates in the v-src-induced (12), serum-induced, and PDGF-induced (Fig. 5) expression of PGS2 message. The transcription factor activated by this second Ras-mediated pathway may be a heterodimer partner of c-Jun active at the PGS2 gene CRE element. The signal transduction pathways leading to activation of the transcription factors mediating induction of the PGS2 gene via the NFκB element (40), via the NF-IL6(C/ EBP) element (39), or via the E-Box (38) have not yet been reported.

TNFα activates JNK and leads to c-Jun phosphorylation in mesangial cells (29). Surprisingly, Yamamoto et al. (40) report that they “could not demonstrate the involvement of the CRE motif in the TNFα-dependent cyclooxygenase-2 induction in MC3T3-E1 cells.” It will be of great interest to determine whether (i) TNFα activates JNK-dependent c-Jun phosphorylation in MC3T3-E1 cells and (ii) whether a c-Jun/CRE-mediated pathway of PGS2 gene expression can be demonstrated in MC3T3-E1 cells, in response to serum, PDGF, or v-src expression. These and similar experiments will help to determine the degree of cell and ligand specificity that exists in the use of alternative transcription factors, signal transduction pathways, and cis-acting PGS2 promoter elements, for ligand-induced expression from the PGS2 gene.

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