Role of Upstream Stimulatory Factor Phosphorylation in the Regulation of the Prostaglandin G/H Synthase-2 Promoter in Granulosa Cells*

Khampoune Sayasith, Jacques G. Lussier, and Jean Sirois†

From the Centre de Recherche en Reproduction Animale and the Département de Biomédecine Vétérinaire, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec J2S 7C6, Canada

To investigate the role of USF phosphorylation in the regulation of the PGHS-2 promoter in granulosa cells, promoter activity assays were performed in primary cultures of bovine granulosa cells transfected with the chimeric PGHS-2 promoter/luciferase (LUC) construct −149/−2PGHS-2LUC. Transfections were done in the absence or presence of forskolin; the protein kinase A (PKA) inhibitor H-89; or an expression vector encoding USF1, USF2, the catalytic subunit of PKA (cPKA), or a PKA inhibitor protein (PKI). Electrophoretic mobility shift assays were performed to study USF/DNA interactions using granulosa cell nuclear extracts and a 32P-labeled proximal PGHS-2 promoter fragment containing the E-box element. The results showed that forskolin stimulation and cPKA overexpression caused a marked and significant increase in USF-dependent DNA binding and PGHS-2 promoter activities (p < 0.05). In contrast, both activities were decreased by H-89 treatment or PKI overexpression. Reverse transcription-PCR analyses revealed that these treatments had similar effects on endogenous PGHS-2 mRNA levels in granulosa cells. Cotransfection studies with a USF2 mutant lacking N-terminal activation domains (U2A1–220) repressed forskolin-, cPKA-, and USF-dependent PGHS-2 promoter activities. Electrophoretic mobility shift assays showed that U2A1–220 was able to compete with full-length USF proteins and to saturate the E-box element. Immunoprecipitation/Western blot analyses revealed an increase in the levels of phosphorylated USF1 and USF2 after forskolin treatment, whereas chromatin immunoprecipitation assays showed that binding of USF proteins to the endogenous PGHS-2 promoter was stimulated by forskolin. Site-directed mutagenesis of a consensus PKA phosphorylation site within USF proteins abolished their transactivating capacity. Collectively, these results characterize the role of USF phosphorylation in PGHS-2 expression and identify the phosphorylation-dependent increase in USF binding to the E-box as a putative molecular basis for the increase in PGHS-2 promoter transactivation in granulosa cells.

Prostaglandins are important regulators of a number of biological processes, including ovulation, which involves a complex series of biochemical events culminating in the rupture of the preovulatory follicle (1–3). Evidence for a relationship between prostaglandins and ovulation emerged in the 1970s, when studies in a number of species revealed a marked increase in the levels of follicular prostaglandins just prior to ovulation and the ability of indomethacin to block ovulation (reviewed in Refs. 1 and 4). Subsequent investigations in rats showed that this preovulatory rise in follicular prostaglandin synthesis results from gonadotropin-dependent, cell type-specific induction of prostaglandin G/H synthase (PGHS).1 The first rate-limiting enzyme in prostaglandin biosynthesis from arachidonic acid, in granulosa cells (5–7). Purification and N-terminal sequencing of the PGHS enzyme induced in rat granulosa cells helped characterize the presence and physiological significance of a then distinct PGHS isoform, now known as PGHS-2 (also referred to as cyclooxygenase-2) (8). The selective induction of PGHS-2 in rat preovulatory follicles proved to be a conserved mechanism by which the synthesis of prostaglandins necessary for ovulation is regulated in different species (9–15). Ultimately, genetic studies underscored the essential role of the enzyme in ovulation, as mice deficient in PGHS-2 have an anovulatory phenotype that can be reversed with exogenous prostaglandin E2 (16–18). Interestingly, the genetic background of the PGHS-2-null mice was shown to markedly influence the anovulatory/ovulatory phenotype (19).

The gonadotropin-dependent induction of PGHS-2 observed in preovulatory follicles in vivo has been recapitulated in vitro, as numerous agonists acting primarily through the protein kinase A (PKA) pathway were shown to increase expression of the PGHS-2 transcript and protein in primary cultures of granulosa cells (7, 9, 11, 20, 21). Incubation with the transcriptional inhibitor α-amanitin abolishes the agonist-dependent increase in PGHS-2 expression, clearly indicating that the phenomenon is dependent on transcription (20). The cloning of the rat and bovine PGHS-2 promoters and their characterization using homologous granulosa cell cultures revealed in both species that the proximal 150–200 bp located upstream of the transcriptional start site are sufficient to confer basal and forskolin/gonadotropin-inducible promoter activities (21, 22). Although a number of consensus cis-acting elements were identified within this proximal region, site-directed mutagenesis identified the E-box element as the predominant element involved in the regulation of PGHS-2 promoter activities in preovulatory granulosa cells (21, 23).

1 The abbreviations used are: PGHS, prostaglandin G/H synthase; PKA, protein kinase A; USF, upstream stimulatory factor; EMSA, electrophoretic mobility shift assay; β-Br-CAMP, β-bromo-CAMP; εPKA, catalytic subunit of PKA; PKI, PKA inhibitor protein; CaMK, Ca2⁺/calmodulin-dependent protein kinase; wt, wild-type; LUC, luciferase; PMA, phorbol 12-myristate 13-acetate; ChIP, chromatin immunoprecipitation; hCG, human chorionic gonadotropin.
Upstream stimulatory factor (USF) 1 and USF2 are ubiquitous proteins characterized by highly conserved C-terminal basic helix-loop-helix and leucine zipper domains responsible for dimerization and DNA binding activities (24, 25). These transactivating factors are known to bind to the E-box promoter element and to regulate the transcription of several genes (26–32). Immunoblotting and electrophoretic mobility shift assays (EMSAs) provided evidence for the presence of USF proteins in granulosa cell nuclear extracts and for their ability to interact with the E-box element present in the PGHS-2 promoter (21, 23). More direct evidence for a role of USF proteins in PGHS-2 promoter transactivation came from the demonstration that overexpression of full-length USF proteins markedly increases and overexpression of a dominant-negative USF2 mutant represses PGHS-2 promoter activities in a E-box-dependent manner in granulosa cells (33). However, the absence of changes in the endogenous levels of full-length USF proteins during the ovulatory process in rat and bovine granulosa cells (21, 23) raises questions as to the precise mechanism controlling USF-dependent PGHS-2 activation. Considering that USF phosphorylation has been implicated in modulating the DNA binding and transcriptional activities of other genes (34–36), the specific objective of the present study was to determine the potential role of USF phosphorylation in the regulation of the PGHS-2 promoter in preovulatory granulosa cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**[α-32P]dCTP was purchased from PerkinElmer Life Sciences (Woodbridge, Ontario, Canada). Lipofectamine Plus reagent, TRizol total RNA isolation reagent, a 1-kb DNA ladder, synthetic oligonucleotides, and culture medium were obtained from Invitrogen (Burlington, Ontario). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). The 5′-end labeling system, the Dual-Luciferase reporter assay system, and plasmids pGEM-T Easy and pRL-SV40 were obtained from Promega Corp. (Madison, WI). Hybrid-P polyanhydridole difluoride membranes, Rainbow molecular weight markers, the ECL Plus Western blotting system, horseradish peroxidase-linked donkey anti-rabbit secondary antibody, and restriction enzymes were obtained from Amersham Biosciences (Baie D’Urfé, Québec, Canada). Polyclonal antibodies against USF1 and USF2 and protein A/G-Sepharose beads were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-serine (Ser 259), anti-phospho-threonine (Thr 257), and anti-phospho-serine (Ser 262) antibodies were from Cell Signaling Technology (Sudbury, Canada). "Transfection and Reporter Activity Assays—Primary cultures of bovine granulosa cells, transient transfections, and reporter activity assays were performed as described previously (21, 33). Bovine granulosa cells in culture were transfected with the SV40 Renilla luciferase control vector (pRL-SV40) to perform internal normalization. Three hours after transfection, cells were incubated in fresh culture medium for 36 h in the absence (control) or presence of forskolin (10 μM), E-B-CAMP (1 mM), or phorbol 12-myristate 13-acetate (PMA; 100 nM). After the culture period, the cells were prepared, and firefly and Renilla luciferase activities were determined using the Dual-Luciferase assay system and a Lumat LB 9507 luminometer (Berthold Technologies).**

**Granulosa Cell Nuclear Extracts and EMSAs—**Confluent cultures of granulosa cells (100-mm dish) were transfected with the U1wt, U2wt, U1A1–129, or U2A1–220 expression construct (4 μg of construct/dish) followed by the PGHS-2 promoter reporter (627 bp) and Renilla-LUC (90 fmol/well) construct, with pGL3-control as a dual-luciferase negative control. After overnight incubation, the cells were transfected with the SV40 Renilla luciferase control vector (pRL-SV40) to perform internal normalization. Three hours after transfection, cells were incubated in fresh culture medium for 36 h in the absence (control) or presence of forskolin (10 μM). Twenty-four hours after incubation, the cells were harvested, and lysates were prepared as described previously (21, 22, 33). Protein concentration was determined by the method of Bradford (37) using the Bio-Rad protein assay. EMSAs were performed as described (21, 33). In some experiments, extracts were preincubated for 15 min with phosphatase type VII (0.5–1 U) prior to addition of other reagents to study the effect of protein dephosphorylation on binding activities.

**Chromatin Immunoprecipitation (ChIP)—**ChIP assays were performed as described previously (29) with minor modifications. Briefly, untransfected granulosa cells or granulosa cells transfected with the U2wt or U2A1–220 expression construct were cultured for 36 h in the absence (control) or presence of forskolin (10 μM). After the end of the culture period, protein/DNA cross-linking was performed by incubating cells with minimal essential medium containing 1% formaldehyde for 20 min at 37°C. Cells were harvested in phosphate-buffered saline containing 1% SDS, 10 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, protease inhibitors (15 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were sonicated (five cycles, 10 s/cycle) using a Branson Model 450 Sonifier to obtain DNA fragments of 0.5–1 kb, and debris were removed by low speed centrifugation. For each chromatin extract supernatant, one-third was kept as DNA input (positive control for DNA); one-third was incubated with anti-USF2 antibody (Santa Cruz Biotechnology, Inc.); and the remaining third was incubated with normal rabbit serum (negative control). After overnight incubation at 4°C, lysates were incubated with protein A/G-Sepharose beads for 2 h and subsequently washed five times with phosphate-buffered saline by repeated centrifugation/resuspension cycles. Beads were incubated at 65°C for 4 h and treated with proteinase K for 2 h at 50°C to reverse protein/DNA cross-links. DNA was recovered by phenol/chloroform extraction and ethanol precipitation, and PCR was performed using sense (5′-TCCTG CCCC A ACCCG G GGTCT GGCCCC AAATG TT-3′) and antisense (5′- CTCCTG ACGTTC C ATGGC GATG AGG-3′) primers specific for the bovine PGHS-2 promoter. The expected PCR product (180 bp), which contains the USF-binding E-box cis-element, was analyzed by 2% agarose gel electrophoresis.

**RNA Extraction and Reverse Transcription-PCR Analysis—**Total RNA was extracted from granulosa cells cultured under various experimental conditions using TRIzol reagent. The One-Step RT-PCR system was used to characterize expression of bovine USF1, USF2, PGHS-2, and glyceraldehyde-3-phosphate dehydrogenase mRNAs. Reactions were performed as described previously (33) with sense and antisense primers specific for USF1 (5′-CGTTC AGCAAG TGATG AGG-3′ and 5′-CTCTG GAGTCA AATCT CACCT-3′), generating a 627-bp...
USF Phosphorylation and PGHS-2 Promoter Regulation

RESULTS

PKA Pathway-dependent Control of USF-regulated PGHS-2 Promoter Activities in Granulosa Cells—To characterize the role of the PKA pathway in basal and USF-regulated PGHS-2 promoter activities, granulosa cells were cotransfected with the promoter construct −149/−2PGHS-2.LUC and the vector expressing USF1 or USF2, and cells were cultured in the absence or presence of a PKA activator (forskolin or 8-Br-cAMP) and inhibitor (H-89) and a protein kinase C activator (PMA). The results show that forskolin and 8-Br-cAMP significantly increased basal PGHS-2 promoter activity (3.3 ± 0.4 (control) versus 6.2 ± 0.5 (forskolin) and 5.5 ± 0.4 (8-Br-cAMP); p < 0.05), whereas the phorbol ester PMA had no effect on cells not overexpressing USP proteins (Fig. 1A). Cotransfections with the USF1 or USF2 expression vector resulted in an increase in basal promoter activity, which was further stimulated by forskolin and 8-Br-cAMP, but not by PMA (Fig. 1A). Incubation of cells with the PKA inhibitor H-89 blocked forskolin-stimulated PGHS-2 promoter activity in untransfected granulosa cells or in those transfected with the USF1 and USP2 expression vectors (Fig. 1B). Interestingly, H-89 also reduced USF1- and USF2-regulated PGHS-2 promoter activities in cells not stimulated with forskolin (7.5 ± 0.8 versus 3.8 ± 0.4 (USF1) and 13.0 ± 2.7 versus 3.4 ± 0.1 (USF2); p < 0.05) (Fig. 1B), suggesting the presence of some basal level of PKA activation in control cells. Thus, these results suggest that PKA activation is a potent regulator of USF-dependent PGHS-2 promoter activity in granulosa cells.

To study directly the role of PKA in USF-regulated PGHS-2 promoter activity, cPKA was overexpressed in cells cotransfected with −149/−2PGHS-2.LUC and U1wt or U2wt. The results show that cPKA overexpression caused a marked increase in USF1- and USF2-regulated PGHS-2 promoter activities, with 5.4- and 4.0-fold increases, respectively, compared with cells not transfected with cPKA (p < 0.05) (Fig. 2A). In contrast, cotransfections with vectors expressing CaMKII and CaMKIV, two kinases known to phosphorylate some transcription factors and to up-regulate gene transcription (41), had little or no effect on promoter activity. To investigate the role of the E-box in the cPKA-stimulated, USF-regulated PGHS-2 promoter, cotransfections were performed with −149/−2PGHS-2.Ebox.LUC, which contains a mutated E-box element in the context of the −149/−2 PGHS-2 promoter fragment. The results reveal that mutation of the E-box element abolished USF1- and USF2-stimulated PGHS-2 promoter activities or severely repressed cPKA-stimulated activities (p < 0.05) (Fig. 2B). The PKA inhibitor H-89 decreased cPKA- and USF-stimulated PGHS-2 promoter activities in a dose-dependent manner, with the highest amount of H-89 (10 µM) causing an 88–94% reduction in promoter activities (p < 0.05) (Fig. 2C).

As a complementary approach to study the role of the PKA pathway in the USF-dependent regulation of the PGHS-2 promoter, the effect of overexpression of PKI (42) was tested in granulosa cells transfected with USP expression vectors. The results reveal a dose-dependent reduction in PGHS-2 promoter activities with increasing amounts of PKI transfected, with the highest amount of PKI (25 pmol) resulting in 57–79% and 75–80% inhibition of basal and forskolin-dependent promoter activities, respectively, in USF1- or USF2-overexpressing cells (p < 0.05) (Fig. 3, A and B).

To determine whether the effects of PKA activators and inhibitors on PGHS-2 promoter activity relate to changes in mRNA levels, cells were cultured for 24 h in the absence or presence of forskolin, cPKA, H-89, or PKI, and reverse transcription-PCR analyses were performed to study changes in the levels of the PGHS-2 transcript. The results show that the levels of PGHS-2 mRNA were low in control cultures, but markedly increased after stimulation with forskolin or trans-

---

**FIG. 1.** PKA activator- and USF-dependent regulation of the PGHS-2 promoter in granulosa cells. A, bovine granulosa cells were cotransfected with the promoterless plasmid pGL3-Basic (Basic) or −149/−2PGHS-2.LUC (−149/−2PGHS) in the absence or presence of forskolin (10 µM), PMA (100 nM), 8-Br-cAMP (1 mM), or the PKA inhibitor H-89 (10 µM). B, cotransfections were performed with −149/−2PGHS-2.LUC and the U1wt or U2wt expression vector. After transfection, cells were cultured for 36 h in the absence (Control) or presence of forskolin (FSK; 10 µM), 8-Br-cAMP (1 mM), or PMA (100 nM). C, results reveal that mutation of the E-box element abolished USF1- and USF2-stimulated PGHS-2 promoter activity, whereas the phorbol ester PMA had no effect in cells not overexpressing USP proteins (Fig. 1A). Cotransfections with the USF1 or USF2 expression vector resulted in an increase in basal promoter activity, which was further stimulated by forskolin and 8-Br-cAMP, but not by PMA (Fig. 1A). Incubation of cells with the PKA inhibitor H-89 blocked forskolin-stimulated PGHS-2 promoter activity in untransfected granulosa cells or in those transfected with the USF1 and USF2 expression vectors (Fig. 1B). Interestingly, H-89 also reduced USF1- and USF2-regulated PGHS-2 promoter activities in cells not stimulated with forskolin (7.5 ± 0.8 versus 3.8 ± 0.4 (USF1) and 13.0 ± 2.7 versus 3.4 ± 0.1 (USF2); p < 0.05) (Fig. 1B), suggesting the presence of some basal level of PKA activation in control cells. Thus, these results suggest that PKA activation is a potent regulator of USF-dependent PGHS-2 promoter activity in granulosa cells.
U1wt or U2wt in the absence (−) or presence of the vector expressing cPKA, CaMKII, or CaMKIV as described under “Experimental Procedures.” All cultures were cotransfected with the SV40 Renilla luciferase vector (pRL-SV40) as an internal control to normalize experimental reporter activity. B, cotransfections were performed with −149/−2PGHS-2 ΔE-box.LUC (−149/−2PGHS-3E-box) and the U1wt or U2wt expression vector in the absence (−) or presence of the vector expressing cPKA. C, cotransfections were performed with −149/−2PGHS-2.LUC and cPKA in the absence (Control) or presence of U1wt or U2wt. After transfection, cells were incubated for 36 h in the absence or presence of graded doses (0–10 μM) of H-89. Results are presented as relative luciferase activity (firefly/Renilla; mean ± S.E. of triplicate cultures from three experiments).

FIG. 2. Effect of cPKA overexpression on USF-regulated PGHS-2 promoter activity in granulosa cells. A, bovine granulosa cells were cotransfected with −149/−2PGHS-2-LUC (−149/−2PGHS) and U1wt or U2wt in the absence (−) or presence of the vector expressing cPKA, CaMKII, or CaMKIV as described under “Experimental Procedures.” All cultures were cotransfected with the SV40 Renilla luciferase vector (pRL-SV40) as an internal control to normalize experimental reporter activity. B, cotransfections were performed with −149/−2PGHS-2 ΔE-box.LUC (−149/−2PGHS-3E-box) and the U1wt or U2wt expression vector in the absence (−) or presence of the vector expressing cPKA. C, cotransfections were performed with −149/−2PGHS-2.LUC and cPKA in the absence (Control) or presence of U1wt or U2wt. After transfection, cells were incubated for 36 h in the absence or presence of graded doses (0–10 μM) of H-89. Results are presented as relative luciferase activity (firefly/Renilla; mean ± S.E. of triplicate cultures from three experiments).

Effect of U2Δ1–220 on PKA- and USF-dependent PGHS-2 Promoter and Protein/DNA Binding Activities—To further investigate the molecular basis for the regulation of the PGHS-2 promoter by USF proteins following PKA activation, promoter activity and protein/DNA binding assays were performed in the presence of the U2Δ1–220 expression construct, which encodes an N-terminally truncated form of USF2 lacking essential transactivation domains but containing dimerization and DNA-binding domains (34). The results show that simultaneous cotransfections with U2Δ1–220 and −149/−2PGHS-2.LUC had no effect on basal and forskolin-inducible PGHS-2 promoter activities, but caused a marked reduction in cPKA-mediated activities (p < 0.05) (Fig. 4A). In contrast, U2Δ1–220 blocked the basal, forskolin-, and cPKA-dependent PGHS-2 promoter activities in cultures transfected with the U1wt and U2wt expression constructs (p < 0.05) (Fig. 4A).

The ability of U2Δ1–220 to compete with full-length USF1 and USF2 for binding the PGHS-2 promoter was tested in vitro by EMSAs using nuclear extracts prepared from granulosa cells transfected with the truncated or wild-type USF expression vectors and the 32P-labeled −149/−2 PGHS-2 promoter fragment. A major protein-DNA complex was formed with extracts prepared from USF2-transfected (Fig. 4B, band a, lane 1) and U2Δ1–220-transfected (band b, lane 9) cells. As expected, the complex containing N-terminally truncated USF2 (Fig. 4B, band b) migrated faster than the complex containing full-length USF2 (band a). Competition assays clearly revealed the ability of increasing amounts of the U2Δ1–220 extract to displace full-length USF2 on the −149/−2 PGHS-2 promoter fragment (Fig. 4B, lanes 2–8). Moreover, U2Δ1–220 was able to displace full-length USF1, although not as efficiently as USF2 (Fig. 4C). EMSA performed with an extract prepared from mock-transfected granulosa cells confirmed the presence of a complex known to contain endogenous USF proteins (Fig. 4D, band d, lane 2) as shown previously (21, 33).

To demonstrate the capacity of full-length USF2 and N-terminally truncated USF2 to interact with the endogenous
**USF Phosphorylation and PGHS-2 Promoter Regulation**

**PGHS-2 promoter in vivo**, untransfected granulosa cells and those transfected with the U2wt or U2Δ1–220 expression vectors were cultured in the absence or presence of forskolin, and protein/DNA interactions were studied by ChIP analyses. The results show that endogenous USF2, U2wt, and U2Δ1–220 effectively bound the PGHS-2 promoter in vivo (Fig. 5, lanes 3, 9, and 15). Interestingly, binding of full-length USF2 and truncated USF2 to the endogenous PGHS-2 promoter appeared to be increased after forskolin treatment (Fig. 5, lanes 6 versus lane 3 for endogenous USF2, lane 12 versus lane 9 for U2wt, and lane 18 versus lane 15 for U2Δ1–220). In contrast, negative controls employing normal rabbit serum instead of anti-USF2 antibody for ChIP revealed very low or no binding activity, as expected (Fig. 5, lanes 2, 5, 8, 11, 14, and 17).

**Phosphorylation Status of USF Proteins and Its Effect on Protein/DNA Binding Activities**—To further investigate whether changes in PKA-dependent phosphorylation alter the ability of USF proteins to bind to the PGHS-2 promoter, EMSAs were performed with the −149/−2 PGHS-2 promoter fragment and nuclear extracts obtained from USF-transfected granulosa cells cultured in the absence or presence of forskolin or cotransfected with the cPKA or PKI expression vector. Alternatively, the effect of protein dephosphorylation on USF/DNA binding activity was studied by EMSAs with cell extracts treated with phosphatase. The results show that the intensity of the major protein-DNA complex formed with the USF2 cell extract increased after forskolin treatment or cPKA overexpression (Fig. 6A, band a, lane 4 versus lanes 5 and 6, respectively), but markedly decreased after PKI overexpression (band a, lane 4 versus lane 7). Likewise, the complex formed with extracts from USF2-transfected cells was reduced after phosphatase treatment in control (Fig. 6A, lane 4 versus lane 1), forskolin-treated (lane 5 versus lane 2), and cPKA-treated (lane 6 versus lane 3) cells. Comparable results were obtained when identical treatments were performed with cells transfected with full-length USF1, albeit some of the effects were more modest (Fig. 6B). Similar studies performed with N-terminally truncated USF2 construct U2Δ1–220 showed that the intensity of the protein-DNA complex increased after forskolin and cPKA transfection (Fig. 6C, band c, lane 1 versus lanes 2 and 3, respectively), but decreased after PKI transfection (band c, lane 1 versus lane 4, respectively). Phosphatase treatment reduced the formation of the U2Δ1–220 protein-DNA complex in control (Fig. 6C, lane 1 versus lane 5), forskolin-treated (lane 2 versus lane 6), and cPKA-treated (lane 3 versus lane 7) cells. Similar results were observed with the N-terminally truncated USF1 construct U1Δ1–129 (Fig. 6D).

To characterize the phosphorylation status of USF proteins in granulosa cells, nuclear protein extracts were prepared from untransfected cells and USF-overexpressing cells before and after stimulation with forskolin in vitro or hCG in vivo. Immunoprecipitation was performed with specific anti-USF antibodies, and immunoprecipitated proteins were analyzed by Western blotting with anti-phosphoserine and anti-phosphothreonine antibodies. The results reveal that the levels of phosphorylated USF1 were...
very low in unstimulated cells transfected with the USF1 expression vector, but increased after forskolin treatment (Fig. 7A, lane 2 versus lane 3). The levels of USF2 phosphorylated at serine and threonine residues were moderate in control cells transfected with the USF2 construct, but appeared to increase after forskolin treatment (Fig. 7A, lane 4 versus lane 5). When identical experiments were performed with cells overexpressing N-terminally truncated USF proteins, the levels of phosphorylated U1Δ1–129 and U2Δ1–220 were markedly increased after forskolin treatment (Fig. 7B, lane 2 versus lane 3 for U1Δ1–129 and lane 4 versus lane 5 for U2Δ1–220). Likewise, experiments performed with untransfected granulosa cells revealed that the levels of endogenous phosphorylated USF1 and USF2 rose after forskolin stimulation (Fig. 7C, lane 2 versus lane 5 for USF1 and lane 3 versus lane 6 for USF2).

Interestingly, a similar pattern was observed in nuclear extracts of granulosa cells obtained from preovulatory follicles isolated before and after hCG treatment, with the results showing an increase in phosphorylated USF1 and USF2 18 h after hCG treatment (Fig. 7D, lane 2 versus lane 4 for USF1 and lane 3 versus lane 5 for USF2). In all studies, immunoprecipitation with normal rabbit serum failed to reveal an immunoreactive signal, as expected (Fig. 7, A, B, and D, lanes 1; and C, lanes 1 and 4).

Effect of USF Phosphorylation Site Mutants on PGHS-2 Promoter Activity—Inspection of USF1 and USF2 amino acid sequences with the phosphorylation site prediction server NetPhos 2.0 (available at www.cbs.dtu.dk) revealed several putative phosphorylatable residues; however, only one serine residue in USF1 (Ser257) and one in USF2 (Ser259) were identified as putative consensus PKA phosphorylation sites. To determine their potential involvement in USF action on PGHS-2 promoter activity, PCR-based site-directed mutagenesis was used to substitute the phosphorylatable serine with a non-phosphorylatable alanine, and single point mutants were tested in promoter activity assays. The results show that the substitution of Ser262 in USF1 (U1S262A) severely impaired basal (5.6 ± 0.5 (U1wt) versus 1.7 ± 0.3 (U1S262A)) and forskolin-dependent (25.4 ± 2.6 (U1wt) versus 8.8 ± 0.8 (U1S262A)) PGHS-2 promoter activities (p < 0.05) (Fig. 8).

Similarly, the substitution of Ser259 with Ala in USF2 (U2S259A) markedly decreased basal (17.8 ± 2.7 (U2wt) versus 2.1 ± 0.3 (U2S259A)) and forskolin-dependent (39.5 ± 2.4 (U2wt) versus 9.8 ± 1.6 (U2S259A)) PGHS-2 promoter activities (p < 0.05) (Fig. 8). The levels of PGHS-2 promoter activities observed in the presence of U1S257A and U2S259A were not significantly different from those in cells not transfected with the USF constructs, suggesting that this single point mutation rendered the mutant constructs nonfunctional (Fig. 8). In contrast, substitutions of Ser269 in USF1 (U1S269A) and of Ser275 in USF2 (U2S269A and U2S275A, respectively) had no marked effect and led to PGHS-2 promoter activities very similar to those with wild-type USF constructs (Fig. 8).

DISCUSSION

Ovulation has been compared with an acute self-controlled inflammatory reaction, as both processes share similar phenomena such as hyperemia, edema, leukocyte extravasation, and tissue damage and repair (4, 43). The biosynthesis of prostaglandins, which are known mediators of inflammation, has been shown to be essential for the process of follicular rupture, with the induction of PGHS-2 in granulosa cells ap-
The biological activity of transcription factors is often regulated by post-translational modifications, with protein phosphorylation being one of the most common post-translational modifications in eukaryotic cells. Phosphorylation has been described for various transcription factors or coactivators, but additional studies will be required to address this issue.

The putative consequence of PKA-dependent USF phosphorylation is the apparent increase in USF binding affinity. Further evidence for a link between PKA-mediated USF phosphorylation and PGHS-2 gene expression has not been established. The present study has provided complementary evidence for a link between PKA-mediated USF phosphorylation and PGHS-2 promoter transactivation in granulosa cells.

The diagram illustrates immunoprecipitation/Western blot analyses of phosphorylated USF proteins in granulosa cells in vitro and in vivo. A, nuclear extracts were prepared from granulosa cells transfected with U1wt or U2wt and cultured in the absence (−) or presence (+) of forskolin (FSK). Extracts (10 µg of protein/reaction) were then subjected to immunoprecipitation and Western blot analyses as described for “Experimental Procedures.” B, nuclear extracts (10 µg of protein/reaction) were prepared from granulosa cells transfected with the U1wt expression vector and cultured in the absence (−) or presence (+) of forskolin. C, nuclear extracts were prepared from transfected granulosa cells cultured for 36 h with forskolin (10 µM) and subjected to immunoprecipitation and Western blot analyses as described for A. D, extracts of nuclear proteins were prepared from granulosa cells of preovulatory follicles isolated 0 and 18 h after an ovulatory dose of hCG and subjected to immunoprecipitation and Western blot analyses as described for A.

USF Phosphorylation and PGHS-2 Promoter Regulation

The diagram illustrates the effect of USF1 and USF2 phosphorylation site mutants on PGHS-2 promoter activities. A, bovine granulosa cells were co-transfected with the promoterless plasmid pGL3-Basic (Basic) or −149/−2PGHS-2.LUC (−149/−2PGHS) in the absence (−) or presence of the U2wt or U1wt expression construct; USF1 phosphorylation site mutant U1S257A or U1S262A; or USF2 phosphorylation site mutant U2S259A, U2S269A, or U2S275A as described for “Experimental Procedures.” B, results are presented as relative luciferase activity (firefly/Renilla; mean ± S.E. of triplicate cultures from four experiments). C, a list of the sense (S) and antisense (AS) mutagenic oligonucleotide primers used to generate USF1 (U1S257A and U1S262A) or USF2 phosphorylation site mutant U2S259A, U2S269A, or U2S275A as described for “Experimental Procedures.” Lowercase letters indicate nucleotide changes introduced into the USF sequence.
way in the regulation of the PGHS-2 promoter in bovine preovulatory granulosa cells is further underscored by the lack of effect of CaMKII and CaMKIV, two Ca\(^{2+}\)/calmodulin-dependent protein kinases known to activate the transcription of other genes (41, 53–56). Ultimately, the functional inactivation of USF proteins through a single point mutation within the USF1 (U1S257A) and USF2 (U2S259A) PKA phosphorylation sites provided further evidence for a relationship between PKA-mediated USF phosphorylation and PGHS-2 gene expression.

One remarkable feature regarding the control of PGHS-2 gene expression is the large variety of agonists known to stimulate its transcription, including numerous pro-inflammatory mediators, growth factors, oncogenes, and protein and steroid hormones (reviewed in Refs. 57 and 58). The 5′-flanking promoter region of the PGHS-2 gene contains a number of consensus cis-acting elements; of these, the cAMP response, NF-IL6 (CCAAT/enhancer-binding protein), and NF-xB elements have often been shown to directly influence the mitogenic/oncogenesis-dependent induction of PGHS-2 in various cell types (57–65). In contrast, the E-box element present within the proximal promoter region was not found to participate in PGHS-2 promoter activation (63, 65–67), except in few cases, such as in skin carcinoma cells, macrophages, and gastric epithelial cells (68–70). One notable exception is also the gonadotropin-dependent induction of PGHS-2 in rat and bovine granulosa cells, in which the E-box was shown to play a central role (21, 23, 33, 52). Indeed, a previous study showed that mutation of the E-box element results in a 91–92% reduction in basal and forskolin-dependent PGHS-2 promoter activities in bovine granulosa cells, whereas mutation of the bovine cAMP response element/activating transcription factor has no deleterious effect (21). The results from the present study confirm the key role of the E-box in granulosa cells because its mutation abolished USF-dependent PGHS-2 promoter activation. Moreover, the marked increase in PGHS-2 promoter activity caused by cPKA in USF-transfected cells was also severely repressed (>92%) when the E-box was mutated, clearly indicating the central function of the cis-element.

Overexpression of N-terminally truncated USF proteins served as an important approach to further characterize the potential role of protein phosphorylation in the regulation of the PGHS-2 promoter. These truncated proteins, U2Δ1–220 and U1Δ1–129 (33), lack N-terminal transactivation domains but contain the basic helix-loop-helix and leucine zipper domains responsible for dimerization and DNA binding activities (24, 25, 71). The results showing the ability of overexpressed U2Δ1–220 to block cPKA-regulated PGHS-2 promoter activities under basal conditions as well as forskolin- and cPKA-regulated PGHS-2 promoter activities in cells overexpressing USF1 or USF2 were of particular interest. These findings suggest that the phosphorylation events induced by cPKA and responsible for regulating the PGHS-2 promoter involve primarily USF proteins. It should also be noted that, under the present experimental conditions of simultaneous cotransfections of U2Δ1–220 and −149/−2PGHS-2.LUC, the inability of U2Δ1–220 to attenuate the forskolin-dependent activation of the PGHS-2 promoter in the absence of USF overexpression was expected, in keeping with a previous report (33). A rapid forskolin-dependent modification/activation of existing endogenous USF proteins prior to expression of sufficient USF mutant was proposed as the likely basis for this outcome. Indeed, experiments in which overexpression of U2Δ1–220 was allowed to proceed for 24 h prior to transfection of the promoter construct and stimulation with forskolin clearly revealed the ability of the mutant to block activation of the PGHS-2 promoter (33).

EMSAs indicated that U2Δ1–220 was able to compete with full-length USF proteins and saturate the E-box element and thus provided evidence for a plausible mechanism of action for this dominant-negative mutant. Interestingly, as observed for full-length proteins, the phosphorylation status of U2Δ1–220 and U1Δ1–129 seemed to affect their binding to the E-box. Indeed, forskolin treatment and cPKA overexpression increased and phosphatase treatment and PKI overexpression reduced the formation of truncated USF-DNA complexes in vitro. The forskolin-dependent phosphorylation of U2Δ1–220 and U1Δ1–129 was confirmed by immunoprecipitation/Western blot analyses, whereas ChIP assays clearly pointed to an increase in binding of U2Δ1–220 to the endogenous PGHS-2 promoter after forskolin treatment. The identification of functional PKA-dependent phosphorylation sites at Ser\(^{259}\) in full-length USF2 and at Ser\(^{257}\) in full-length USF1 is in keeping with a key role of the C terminus of USF proteins in PGHS-2 promoter binding and activation.

In summary, the present study has characterized for the first time a relationship between USF phosphorylation and PGHS-2 promoter activation in granulosa cells and has identified USF phosphorylation as a likely biochemical consequence of the activation of the PKA pathway by the luteinizing hormone preovulatory surge (72). The present investigation also established the apparent requirements for the control of PGHS-2 gene expression in granulosa cells, including an active cAMP/PKA signaling pathway, intact USF proteins, and a consensus E-box element. Although activation of the PKA pathway is an important trigger of this biochemical cascade, the potential downstream activation of other signaling pathways should also be considered (73). Last, a more comprehensive understanding of the molecular mechanisms involved in PGHS-2 gene expression in preovulatory granulosa cells will need to consider the potential involvement of transcriptional coactivators and chromatin epigenetic regulators.

Acknowledgments—We thank Dr. Richard Maurer for the generous gift of vectors expressing cPKA, PKI, CaMKII, and CaMKIV and Danielle Rannoux for technical assistance with the collection of ovaries.

REFERENCES

1. Armstrong, D. T. (1981) J. Reprod. Fertil. 62, 283–291
2. Murdoch, W. J., Hansen, T. R., and McPherson, L. A. (1993) Prostaglandins 46, 85–115
3. Sirois, J., Sayasith, K., Brown, K. A., Stock, A. E., Bouchard, N., and Dore, M. (2004) Hum. Reprod. Update. 10, 373–385
4. Espey, L. L., and Lipner, H. (1996) in Physiology of Reproduction (Knobil, E., and Neil, J. D., eds) Vol. 1, pp. 725–781, Raven Press, Ltd., New York
5. Hedin, L., Gaddy-Kurten, D., Kurten, R., DeWitt, D. L., Smith, W. L., and Richards, J. S. (1987) Endocrinology 121, 722–721
6. Hustig, R. L., Malik, A., and Clark, M. R. (1987) Mol. Cell. Endocrinol. 50, 237–246
7. Wong, W. Y., and Richards, J. S. (1991) Mol. Endocrinol. 5, 1269–1279
8. Sirois, J., and Richards, J. S. (1992) J. Biol. Chem. 267, 6382–6388
9. Sirois, J., Simmons, D. L., and Richards, J. S. (1992) J. Biol. Chem. 267, 11586–11592
10. Sirois, J. (1994) Endocrinology 135, 841–848
11. TsaI, S. J., Wiltbank, M. C., and Bodenstein, K. J. (1996) Endocrinology 137, 3348–3355
12. Sirois, J., and Dore, M. (1997) Endocrinology 138, 4427–4434
13. Joyce, J. M., Pendula, F. L., O’Brien, M., and Eppig, J. J. (2001) Endocrinology 142, 3187–3197
14. Duffy, D. M., and Stoffier, R. L. (2002) Hum. Reprod. 17, 2825–2831
15. Segi, E., Haraguchi, K., Sagimoto, Y., Tense, M., Tounekawa, H., Tamba, S., Tsuboi, K., Tanaka S., and Ichikawa, A. (2003) Biol. Reprod. 68, 804–811
16. Lim, H., Park, C., Das S., S. K., Dinach, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997) Endocrinology 197, 2825–2831
17. Davis, B. J., Lennard, D. E., Lee, C. A., Tiano, H. F., Morham, S. G., Wetsel, W. C., and Langenbach, R. (1999) Endocrinology 140, 2685–2695
18. Reese, J., Zhao, X., Ma, W. G., Brown, N., Maziau, T. J., and Dey, S. K. (2001) Endocrinology 142, 3198–3206
19. Wang, H., Ma, W. G., Tejada, L., Zhang, H., Morrow, J. D., Das, S. K., and Dey, S. K. (2004) J. Biol. Chem. 279, 10649–10658
20. Wong, W. Y., DeWitt, D. L., Smith, W. L., and Richards, J. S. (1989) Mol. Endocrinol. 3, 1714–1723
21. Liu, J., Antaya, M., Boerboom, D., Lussier, J. G., Silversides, D. W., and Sirois, J. (1999) J. Biol. Chem. 274, 35007–35015
22. Sirois, J., Levy, L. O., Simmons, D. L., and Richards, J. S. (1993) J. Biol. Chem. 268, 12199–12206
23. Morris, J. K., and Richards, J. S. (1996) J. Biol. Chem. 271, 16633–16643
24. Gregor, P. D., Sadawada, M., and Roeder, R. G. (1990) Genes Dev. 4, 1730–1740
