Transcription Factor GATA-4 Is Activated by Phosphorylation of Serine 261 via the cAMP/Protein Kinase A Signaling Pathway in Gonadal Cells*

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Gonadal gene expression is regulated by pituitary hormones acting through the cAMP/protein kinase A (PKA) signal transduction pathway. The downstream molecular effectors of these signals, however, have yet to be fully understood. We have recently shown that cAMP stimulation of gonadal cells leads to phosphorylation of the transcription factor GATA-4, a key regulator of gonadal gene expression, thus suggesting that this factor might be a novel target for the cAMP/PKA signaling pathway. We now show that the rapid phosphorylation of GATA-4 induced by cAMP in vivo can be blocked by a PKA-specific inhibitor but not by mitogen-activated protein kinase inhibitors, indicating that GATA-4 is predominantly phosphorylated by PKA in response to cAMP in gonadal cells. In addition, using in vitro kinase assays, we show that PKA phosphorylation of GATA-4 occurs predominantly on an evolutionarily conserved serine residue located at position 261. Phosphorylation of GATA-4 Ser261 by PKA enhances its transcriptional activity on different gonadal promoters, an effect that was markedly reduced with a S261A mutant. Moreover, the S261A mutant blunted cAMP-induced promoter activity in gonadal cells. Finally, PKA-dependent phosphorylation of GATA-4 also led to enhanced recruitment of the CREB-binding protein coactivator. This recruitment and transcriptional cooperation were dramatically impaired with the S261A mutant. Thus, our results identify GATA-4 as a novel downstream effector of cAMP/PKA signaling in gonadal cells, where phosphorylation of Ser261 and recruitment of CREB-binding protein likely represent a key mechanism for conveying the cAMP responsiveness of gonadal genes that lack classical cAMP regulatory elements.

Gene expression in the gonads is under the influence of the pituitary trophic hormones luteinizing hormone and follicle-stimulating hormone. Binding of these hormones to their G-coupled receptors activates adenylate cyclase leading to increased cAMP production. In the classical cAMP signaling pathway, cAMP associates with the regulatory subunits of the protein kinase A (PKA) holoenzyme, allowing dissociation of its catalytic subunits, which then translocate to the nucleus and phosphorylate target proteins (1, 2). The best studied target of PKA is the transcription factor cAMP-responsive element-binding protein (CREB), which binds as a dimer to the 8-bp palindromic sequence CRE found in the regulatory region of some cAMP-regulated genes. Phosphorylation of CREB Ser133 by PKA allows recruitment of the coactivator CREB-binding protein (CBP), which contacts the transcriptional machinery leading to increased gene transcription (3). In the gonads, however, several cAMP-regulated genes, such as steroidogenic acute regulatory protein (Star), steroid 17α-hydroxylase (Cyp17), P450scCYP11A1, inhibin α (Inha), and aromatase (Cyp19), lack consensus CRE elements. Therefore, transcription factors in addition to CREB must be acting as downstream effectors of cAMP signaling in gonadal cells.

There are several factors that have been shown to be activated by cAMP in gonadal cells. These include stimulatory protein 1 (Sp1), upstream stimulatory factor, estrogen receptor α/β, activating protein 1 (AP-1), CCAAT/enhancer-binding protein (C/EBP) β, and Nur77/NR4A1 (4–8). Although these transcription factors have been shown to contribute to the hormonal regulation of gonadal genes, most are ubiquitously expressed and respond to several stimuli other than cAMP. Moreover, not all cAMP-responsive gonadal promoters lacking CRE elements have binding sites for these factors. In the gonads, the orphan nuclear receptor steroidogenic factor 1 (SF-1/NR5A1) has been proposed to be a candidate factor downstream of cAMP/PKA. In support of this hypothesis, SF-1-binding sites are found in several hormonally regulated genes. Indeed, a role for SF-1 in the cAMP-dependent stimulation of several gonadal genes has been described (9). Although SF-1 can be phosphorylated by PKA in vitro (10), SF-1 is constitutively phosphorylated in vivo by the mitogen-activated protein kinase (MAPK) pathway, and its phosphorylation levels are not affected by cAMP treatment (11). Unfortunately, the absence of adrenals and gonads in SF-1 knockout mice (12–14) precludes a definitive answer to the role of this factor in these tissues. However, in other steroidogenic tissues normally expressing SF-1, such as the placenta and skin, expression of the cAMP-regulated genes Cyp11A1 and Cyp17 was normal in SF-1−/−.

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PKA-mediated Activation of GATA-4 and Recruitment of CBP

Fig. 1. GATA-4 is phosphorylated by PKA in vivo. A, schematic representation of the experimental protocol used to analyze the in vivo phosphorylation status of GATA-4 in cAMP-stimulated MA-10 Leydig cells. Time (t) is in hours. B, MA-10 cells were incubated with [32P]orthophosphate for 2 h and then stimulated with 0.5 mM db-cAMP for an additional 2 h in the presence or absence of the signaling pathway inhibitors H89 (PKA pathway), SB203580 (p38 MAPK pathway), or PD90059 (ERK1/2 pathway). The phospho-GATA-4 protein was purified and detected as described under "Experimental Procedures." The phosphorylation levels of GATA-4 were quantified by densitometry. Total GATA-4 protein was revealed by immunoblotting (lower panel).

mice (13, 15, 16). Therefore, SF-1 might not be the predominant downstream effector of cAMP signaling in gonadal cells.

In addition to SF-1 regulatory elements, many cAMP-regulated gonadal promoters also contain GATA motifs for the binding of members of the GATA family of transcription factors. There are six vertebrate GATA factors (named GATA-1 to GATA-6). Four of these are expressed in the mammalian gonads: GATA-1, GATA-2, GATA-4, and GATA-6 (17–24). In particular, GATA-4 is strongly expressed from the onset of gonadal development and is later found in multiple cell lineages including testicular Sertoli and Leydig cells and granulosa cells of the ovary (17). Because the function of these cells is under hormonal control, GATA-4 may represent a key effector of hormonal signaling in gonadal cells. In agreement with this, GATA-4 has been shown to regulate several CAMP-dependent gonadal promoters including StAR, inhibin α, and aromatase PII (25). In addition, we have recently shown that cAMP stimulation of gonadal cells leads to phosphorylation of GATA-4 (26). Although PKA was a likely candidate, the kinase(s) activated by cAMP in gonadal cells and responsible for directly phosphorylating GATA-4 remained unknown.

We now report that GATA-4 is indeed a key effector of cAMP signaling in gonadal cells. Using MA-10 Leydig cells, we show that GATA-4 is phosphorylated in response to cAMP stimulation predominantly through PKA and not the MAPK signal transduction pathway. CAMP-induced phosphorylation occurs on Ser261, an evolutionarily conserved residue of the GATA-4 protein. Additionally, we provide a novel mechanism whereby activation of GATA-4 by PKA regulates multiple target genes via an enhanced recruitment of the CBP transcriptional coactivator.

EXPERIMENTAL PROCEDURES

Plasmids—Luciferase reporters for the murine ~902-bp StAR, ~218-bp aromatase PII, and ~679-bp inhibit α promoters have been described previously (25). The ~447-bp steroid 17α-hydroxylase promoter was amplified by PCR from rat genomic DNA (forward primer, 5'-CAGCTTGTGAGTGCACCTATG-3'; reverse primer, 5'-CCAGGTGGGTGGCCTATGAGC-3'); the mutated sequence contains a species-conserved PKA consensus phosphorylation site (indicated in bold type) located within the zinc finger region. The GATA-4 protein was amplified using 1 μg of different GST-GATA-4 fusion proteins as described under "Experimental Procedures." In the total protein gel (lower panel), the asterisks highlight the positions of the various GST-GATA-4 fusion proteins. The multiple bands represent different proteolytic fragments for each fusion protein. N-term, N-terminal; C-term, C-terminal; Zn, zinc finger.

Production of GST-GATA-4 Fusion Proteins—GST-GATA-4 fusion proteins were obtained by cloning various regions of rat GATA-4 in frame with GST using the pGEX2T vector (Amersham Biosciences). Fusion proteins were obtained by cloning various regions of rat GATA-4 in frame with GST using the pGEX2T vector (Amersham Biosciences).

Cell Culture and Transfections—CV-1 and L fibroblast cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. The MA-10 mouse Leydig tumor cells were grown in Waymouth's medium containing 15% horse serum (30). CV-1 cells were transfected in 24-well plates using the calcium phosphate precipitation method (25). MA-10 cells were seeded in 12-well plates and transfected using the Lipofectamine reagent (Invitrogen). In brief, 1.5 μg of STAR-luciferase reporter and 500 ng of either an empty expression vector or expression vectors for full-length GATA-4 or GATA-4 S261A were transfected with 5 μl of Lipofectamine reagent/well in serum-free OptiMEM medium (Invitrogen). The cells were harvested 24 h after transfection.
and antibiotic-free media. *Renilla* (pGL-TK) luciferase (Promega, Madison, WI) was used as an internal control. Complete medium was added 6 h after transfection. The next morning, the cells were treated with either vehicle (H₂O) or 0.5 mM dibutyryl cAMP (db-cAMP; Sigma-Aldrich) for 6 h and then harvested and analyzed using the Dual-Luciferase® reporter assay system from Promega. The data reported represent the averages of at least three experiments, each done in duplicate.

**Nuclear Extracts and Western Blots**—Nuclear extracts were prepared by the procedure described by Schreiber et al. (31). Recombinant GATA proteins were obtained by transfecting L cells with expression vectors for the different GATA-4 proteins. In Western analyses, 10-μg aliquots of nuclear extract were separated by SDS-PAGE and transferred to Hybond polyvinylidene difluoride membrane (Amersham Biosciences). Immunodetection of the native GATA-4 protein was achieved using a GATA-4 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). Hemagglutinin-tagged GATA-4 proteins were detected using an anti-hemagglutinin antibody (BD Biosciences Canada, Mississauga, Canada), and a VECTASTAIN®-ABC-Amp™ Western blot detection kit (Vector Laboratories, Burlington, CA). Daulux™ (Vector Laboratories) was used as chemiluminescent substrate.

**Analysis of in Vivo GATA-4 Phosphorylation**—MA-10 cells were seeded in 6-well plates using complete media. The next day, the cells were washed and maintained in phosphate-free Dulbecco’s modified Eagle’s medium for 30 min. ³²P]Orthophosphate (0.5 mCi/ml) was added, and the cells were incubated for an additional 2 h. The cells were then lysed and treated with either vehicle or different signaling pathway inhibitors (50 μM PD90059 (ERK1/2 pathway), 10 μM SB203580 (p38 MAPK pathway), and 10 μM H89 (PKA pathway) from Calbiochem, San Diego, CA) for 30 min. The cells were finally stimulated for 2 h by adding vehicle (H₂O) or 0.5 mM db-CAMP. The 2-h cAMP treatment period is within the range of times that others have reported for the induction of GATA phosphorylation in various systems using either *in vivo* phosphorylation or increased GATA binding as functional endpoints (32–35). After stimulation, the cells were washed and lysed in 300 μl of radioimmunoprecipitation buffer (10 mM sodium phosphate, pH 7.2, 1% Igepal (Sigma-Aldrich), 0.1% SDS, 1% deoxycholate, 2 mM EDTA, 150 mM NaCl, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml of the protease inhibitors apotinin, leupeptin, and pepstatin (Sigma-Aldrich Canada)). Radiolabeled GATA-4 was purified by immunoprecipitation with 2 μl of GATA-4 antiserum (Santa Cruz) for 2 h at 4 °C. A 20-μl aliquot of protein G-Sepharose (Amersham Biosciences) was then added, and incubation was continued for an additional 2 h at 4 °C. Immunoprecipitated complexes were washed five times in radioluciferase reaction buffer, resuspended in 1× Laemmli loading buffer, heated at 95 °C for 5 min, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Labeled GATA-4 protein was visualized by autoradiography.

**In Vitro Phosphorylation Assay**—In vitro kinase assays were performed using 1 μg of the different GST-GATA-4 proteins and 10 units of PKA catalytic subunit for 30 min at 30 °C (New England Biolabs, Mississauga, Canada) in a total volume of 40 μl containing 1 μl (10 μCi) of [γ-³²P]ATP (3000 Ci/mmol) in the presence of 50 μM of cold ATP. The reactions were terminated by adding 1× Laemmli loading buffer and heating the mixture for 5 min at 95 °C. After SDS-PAGE, the labeled proteins were transferred to polyvinylidene difluoride membrane and visualized by autoradiography. The total protein was visualized by Coomassie staining of the gel.

**Pull-down Assay**—In *vitro* pull-down assays were performed as previously described (26). A portion of the CBP protein (amino acids 1200–1900) was ³⁵S-labeled using the TNT transcription/translation kit from Promega. The GST-G4-ABC fusion protein (500 μg) was added, and the cells were incubated for an additional 2 h at 4 °C. After SDS-PAGE, the labeled GST-G4-ABC fusion protein was purified by immunoprecipitation with 2 μl of GST-G4-ABC antiserum (Santa Cruz) for 2 h at 4 °C. A 20-μl aliquot of protein G-Sepharose (Amersham Biosciences) was then added, and the antibody was incubated for an additional 2 h at 4 °C. Immunoprecipitated GST-G4-ABC fusion protein was washed five times in radioluciferase reaction buffer, resuspended in 1× Laemmli loading buffer, heated at 95 °C for 5 min, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Labeled GST-G4-ABC fusion protein was visualized by autoradiography.

RESULTS

**GATA-4 Is Phosphorylated through the cAMP/PKA Pathway**

Recent results obtained in our laboratory using an antibody against a phosphoserine residue indicated that the GATA-4 transcription factor was phosphorylated in response to cAMP stimulation of MA-10 cells (26). Although our results suggested that PKA might be responsible for GATA-4 phosphorylation, the exact signaling pathway and kinase involved had not been identified. To further analyze the phosphorylation status of GATA-4 and identify the signaling pathway involved, ³²P]orthophosphate metabolic labeling studies were performed in quiescent and db-cAMP-stimulated MA-10 Leydig cells; the experimental protocol is depicted in Fig. 1A. As shown in Fig. 1B, GATA-4 is constitutively phosphorylated in these cells in the absence of any stimulation. GATA-4 phosphorylation levels, however, were significantly increased (more than 3.5-fold) following db-cAMP treatment (Fig. 1B, top panel). This increase occurred without a change in total GATA-4 protein levels (Fig. 1B, lower panel), which is consistent with previous studies showing that hormonal stimulation of MA-10 cells has no effect on GATA-4 mRNA and protein levels (7, 26, 36, 37). Because GATA-4 has recently been shown to be phosphorylated at amino acid Ser₁⁰⁶ through the p38 and ERK1/2 MAPK pathways in cardiac cells (33, 35) and because cAMP is also known to activate the MAPK pathways (6), we used different inhibitors to identify which signaling pathway is responsible for GATA-4 phosphorylation in gonadal cells. As shown in Fig. 1B, cAMP-induced GATA-4 phosphorylation was completely abrogated by H89, a PKA inhibitor, but not by SB203580 or PD90059, which are inhibitors of the p38 and ERK1/2 MAPK pathways, respectively. The inhibitory effect of H89 was not simply due to a blockade of basal GATA-4 phosphorylation because H89 treatment in the absence of cAMP only resulted in a slight decrease in phosphorylation levels (Fig. 1B, right panel). Thus, PKA appears to be the predomi-
nant kinase responsible for cAMP-induced GATA-4 phosphorylation in MA-10 cells.

To map the relevant phosphoacceptor site, we next examined the GATA-4 primary amino acid sequence for potential PKA phosphorylation sites. One putative PKA consensus phosphorylation site that matches the high affinity RRXS motif is present at amino acid position 261 (Ser261) between the two zinc fingers of the DNA-binding domain (Fig. 2A). This region is perfectly conserved between the rat, mouse, human, bovine, rabbit, frog, fish, and chick GATA-4 proteins (Fig. 2A). To characterize the ability of this site to undergo PKA-mediated phosphorylation, in vitro kinase assays were performed using various GST-GATA-4 fusion proteins (Fig. 2B). PKA efficiently phosphorylated a GATA-4 C-terminal fusion protein (GST-G4 255–440) containing the consensus PKA motif, whereas N-terminal constructs lacking this region (GST-G4 1–200 and GST-G4 1–260) could not be phosphorylated by PKA. Therefore, the PKA target site(s) on GATA-4 are located between amino acids 261 and 440. Consistent with this, mutation of Ser261 (GST-G4 255–440 S261A) or a further N-terminal deletion that removes Ser261 (GST-G4 302–440) severely impaired PKA-mediated phosphorylation (Fig. 2B, lower panel). As revealed by total protein staining (Fig. 2B, lower panel), the absence of phosphorylation observed with GST fusion proteins lacking Ser261 was not due to low amounts of protein. The multiple bands likely represent different proteolytic fragments of the various fusion proteins. Thus, these results suggest that Ser261 is a predominant phosphoacceptor site of the GATA-4 protein targeted by the cAMP/PKA signaling cascade in MA-10 Leydig cells. The weak phosphorylation observed with the GST-G4 255–440 S261A and GST-G4 302–440 constructs, however, suggests that there might also be low affinity phosphorylation sites in the C-terminal portion of the GATA-4 protein.

PKA-mediated phosphorylation activates GATA-4—If cAMP/PKA phosphorylation of GATA-4 plays a role in hor-

Fig. 4. GATA-4 Ser261 is required for the PKA-dependent enhancement of GATA-4 transcriptional activity. A, the ability of the full-length wild-type (FL) and mutant (FL S261A) GATA-4 proteins to activate the −902-bp murine StAR promoter (white bars) and respond to PKA stimulation (black bars) was assessed by cotransfection experiments in CV-1 cells. S261A represents a point mutation in the GATA-4 protein that prevents phosphorylation by PKA. B, Western blot (WB) detection of L cell nuclear extracts (10 μg) overexpressing hemagglutinin-tagged GATA-4 proteins used in A. αHA, anti-hemagglutinin. C, stimulation of GATA-4 transcriptional activity by the MAPK pathway requires the GATA-4 N-terminal domain. CV-1 cells were cotransfected with the −902-bp murine StAR promoter along with an empty expression vector (CTL) or expression vectors for full-length GATA-4 (FL) or an N-terminal deletant (ΔN2), in the absence (−) or presence of expression vectors for PKA, wild-type MEK1 (MEK1 WT), or a constitutively active form of the kinase (MEK1 CA). D, Western blot detection of L cell nuclear extracts overexpressing the full-length (FL) or ΔN2 GATA-4 proteins alone or GATA-4 FL in combination with the different kinases.
PKA-mediated activation of GATA-4 and recruitment of CBP

PKA-dependent Activation of GATA-4 Requires Ser^{261}—To establish the importance of Ser^{261} for basal and PKA-stimulated GATA-4 transcriptional activities, the full-length wild-type (FL) or mutated (FL-S261A) GATA-4 proteins were used in cotransfection experiments (Fig. 4A). Consistent with its role in PKA-mediated phosphorylation (Fig. 2), mutation of GATA-4 Ser^{261} (FL-S261A) markedly reduced its activation by PKA without affecting basal transactivation (Fig. 4A). This decrease was not due to differences in expression, nuclear localization, or stability of the two different GATA-4 constructs because both the wild-type and S261A GATA-4 proteins were expressed at similar levels (Fig. 4B). The residual enhancement of GATA-4-S261A by PKA is likely attributable to low affinity phosphorylation site(s) located in the C-terminal region of the GATA-4 protein (Fig. 2B).

As previously mentioned, the GATA-4 protein contains another phosphorylation site at Ser^{165}, which is a target of the p38 and ERK1/2 MAPK in cardiac cells (33, 35). Therefore, it remained possible that the PKA enhancement of GATA-4 transcriptional activity could be indirectly mediated through the MAPK pathway. Although a constitutively active form of MAPK/ERK kinase 1 (MEK1 CA) could enhance full-length GATA-4 transcriptional activity (Fig. 4C), this enhancement was lost when the N-terminal domain of GATA-4 containing the MAPK target Ser^{165} was removed (ΔN_{2}). In contrast, PKA could still enhance the activity of this same construct. This differential effect was not due to differences in the amount of GATA-4 protein present because both the full-length (FL) and ΔN_{2} GATA-4 constructs were expressed at similar levels (Fig. 4D). Moreover, the enhancing effects of PKA and MEK1 were not due to increases in GATA-4 expression because the total GATA-4 protein levels were not altered by either kinase (Fig. 4D). Therefore, GATA-4 appears to contain at least two independent phosphorylation sites of which Ser^{261} is indispensable for the maximal transcriptional enhancement by PKA.

Because the GATA-4 Ser^{261} mutation blunted PKA-mediated transcriptional enhancement in heterologous cells, we next examined the effect of this mutation on cAMP-induced StAR promoter activity in MA-10 cells (Fig. 5). Stimulation of MA-10 cells with db-cAMP activated the StAR promoter about 6-fold; cAMP induction was not affected by exogenously expressed GATA-4 (Fig. 5, compare the gray and stippled bars). This induction, however, was blunted by overexpression of the non-phosphorylatable GATA-4 S261A mutant (Fig. 5, solid bar), which competes with endogenous GATA-4 protein for DNA binding. Thus, these results suggest that phosphorylation of GATA-4 Ser^{261} is essential to obtain maximal activation of GATA- and cAMP-dependent gonadal promoters in response to hormonal stimulation.

Cooperation between GATA-4 and CBP on Gonadal Promoters Is PKA-dependent—The CBP transcriptional coactivator is an important regulator of gene expression through its ability to interact and cooperate with several transcription factors such as CREB in response to cAMP/PKA signaling (3). Because p300/CBP has also been reported to physically interact in vitro with the DBD of GATA-4 (38), we tested whether GATA-4 could cooperate with CBP on a series of gonadal promoters and whether this cooperation required protein phosphorylation by PKA (Fig. 6). No significant cooperation between GATA-4 and CBP was observed in the absence of PKA on the StAR, 17α-hydroxylase, aromatase, and inhibin α promoters (Fig. 6A, open bars). In the presence of PKA, however, strong synergy was observed on all natural promoters tested (Fig. 6A, solid bars). In addition, the PKA-dependent cooperation between GATA-4/CBP was also observed on a synthetic reporter (Fig. 6B, left panel) consisting of a single copy of the consensus GATA element from the proximal StAR promoter fused to the unresponsive minimal Müllerian inhibiting substance promoter (Fig. 6B, right panel). Once again, this effect was independent of changes in total GATA-4 protein levels (Fig. 6C). Thus, GATA-4 binding to DNA is necessary and sufficient for the PKA-dependent GATA-4/CBP transcriptional cooperation.

Next, the domain of GATA-4 required for the PKA-dependent synergy with CBP was mapped (Fig. 7). The GATA-4 protein contains two independent activation domains (ADs) that flank its DBD. Deletion of the GATA-4 C-terminal AD (ΔC_{1}) did not impair synergy. However, deletion of both GATA-4 ADs (ΔN_{1}C_{1}) leaving only the DBD, severely blunted cooperation with CBP. These results suggest that maximal cooperation with CBP requires at least one functional AD and an intact GATA-4 DBD, the latter being involved in DNA binding and direct physical interaction with p300/CBP (38). In agreement with these requirements, a heterologous AD from the viral protein VP16 fused to the GATA-4 DBD (VP16-ΔN_{1}C_{1}) could restore PKA-dependent cooperation with CBP (Fig. 7). Because PKA-mediated phosphorylation of Ser^{261} is important to activate the GATA-4 transcription factor, we next tested whether phosphorylation of GATA-4 Ser^{261} is also required for transcriptional synergism with CBP. As shown in Fig. 7, mutation of GATA-4 Ser^{261} into alanine markedly impaired transcriptional synergism with CBP (50-fold activation compared with 125-fold for wild-type GATA-4). Thus, phosphorylation of GATA-4 Ser^{261} is likely an important mechanism for the re-

![Fig. 5](https://example.com/fig5.png)

**FIG. 5.** The S261A mutant impairs cAMP-induced StAR promoter activity in MA-10 cells. The ~902-bp murine StAR promoter was transfected in MA-10 cells along with an empty expression vector (CTL) or expression vectors for wild-type GATA-4 (G4) or the S261A mutant (G4 S261A). Transfected cells were stimulated with vehicle (−) or 0.5 mM db-cAMP (+) for 6 h prior to harvesting. All results are reported as fold activation over the respective controls (±S.E.).
PKA-mediated Activation of GATA-4 and Recruitment of CBP

**Recruitment of CBP and transcriptional cooperation with GATA-4 is PKA-dependent.** A, CV-1 cells were cotransfected with the indicated promoter constructs along with an empty expression vector (CTL) or expression vectors for GATA-4 (G4), CBP, or a combination of GATA-4 and CBP (G4+CBP) and with (+) or without PKA (−). B, similar experiments were performed using a synthetic reporter consisting of one copy of the GATA element from the StAR promoter fused to the unresponsive minimal Mullerian inhibiting substance promoter. All transfection results are reported as fold activation over control (±S.E.). C, Western blot (WB) detection of L cell nuclear extracts overexpressing GATA-4 or GATA-4 in combination with PKA, CBP, or both PKA and CBP.

**PKA-mediated Phosphorylation of GATA-4 Ser261 Enhances the Physical Interaction with CBP.**—The fact that transcriptional cooperation between GATA-4 and CBP requires PKA-mediated phosphorylation of GATA-4 Ser261 suggests that protein phosphorylation by PKA might also enhance the interaction between the two proteins. To test this possibility, in vitro interaction experiments were performed using GST-GATA-4 fusion proteins and a fragment of the CBP protein (amino acids 1200–1900) previously shown to interact with GATA-4 (38). As previously reported by Dai and Markham (38), GATA-4 and CBP do interact in the absence of PKA phosphorylation (Fig. 8, lane 2). Consistent with the transcriptional cooperation data (Fig. 7), PKA-mediated phosphorylation of the wild-type GATA-4 fusion protein (GST-G4 255–440 WT) enhanced the interaction with CBP (Fig. 8, lane 3). In contrast, no increase was observed with the GATA-4 S261A mutant that cannot be phosphorylated by PKA (Fig. 8, lane 5).

**DISCUSSION**

In gonadal cells, it is well established that most gonadotropin-regulated events, such as increased steroidogenic gene expression in Leydig cells, are mediated through the cAMP/PKA signaling pathway (39). In several tissues, including the gonads, the classic downstream effector of cAMP/PKA signaling is the phospho-CREB/CBP complex, which specifically binds to CRE regulatory elements leading to stimulation of target promoters (1, 2). Several cAMP-regulated gonadal genes, however, lack consensus CRE elements, which suggests that transcription factors other than CREB are responsible for conveying the cAMP responsiveness of those genes. Based on its expression in multiple cell types of the testis and ovary and on the fact that it can activate several gonadal promoters (40), GATA-4, much like CREB, is likely a common regulatory factor that coordinates the cAMP responsiveness of multiple gonadal genes. Indeed, our present data strongly support a role for GATA-4 as a novel downstream effector of hormonal signaling in gonadal cells.

**GATA-4: a Novel Mediator of cAMP Signaling in Gonadal Cells**—Orthophosphate metabolic labeling of MA-10 cells confirmed that endogenous GATA-4 protein is directly phosphorylated by PKA in response to cAMP stimulation (Fig. 1). The consensus PKA phosphorylation site that we have identified (GATA-4 Ser261) is perfectly conserved across several species. This strong conservation suggests a critical role for this site that has been preserved during evolution. In addition to GATA-4, all other vertebrate GATA proteins as well as GATA factors from Drosophila (dGATA-A) and sea urchin (SpGATAc)
GATA-4 Ser261.

and CBP requires phosphorylation of GATA-4 (ΔN,C1) consisting of the VP16 activation domain fused to the GATA-4 zinc finger region was used to show that transcriptional cooperation with CBP is transferable. The results are shown as fold activation over control (± S.E.).

Fig. 7. PKA-dependent transcriptional cooperation between GATA-4 and CBP requires phosphorylation of GATA-4 Ser261. CV-1 cells were cotransfected with the 902-bp murine Star promoter along with 50 ng of expression vector encoding full-length GATA-4 (FL) or different mutated GATA-4 proteins and expression vectors for PKA and/or CBP. A GATA-4 fusion protein (VP16-ΔN,C1) consisting of the VP16 activation domain fused to the GATA-4 zinc finger region was used to show that transcriptional cooperation with CBP is transferable. The results are shown as fold activation over control (± S.E.).

Several GATA factors have already been implicated as downstream effectors of signaling pathways in other systems. For example, phosphorylation of GATA-3 has been shown to be involved in the regulation of interleukin-5 gene expression in T helper 2 cells (32). In hematopoietic cells, interleukin-3-dependent phosphorylation of GATA-2 stimulates proliferation of hematopoietic progenitors (43), and phosphorylation of GATA-1 regulates erythroid maturation of murine erythroleukemia cells (44). GATA-4 is also phosphorylated in response to hypertrophic stimuli in the heart (33–35, 45–48). Although some of those cases involved cAMP production, GATA phosphorylation was not directly mediated by PKA but rather involved activation of the MAP kinases p38 and ERK1/2. This can be achieved by cross-talk between the cAMP and MAPK pathways, which involves a new class of cAMP-binding proteins termed Epac (exchange protein activated by cAMP) that bind to and activate Ras-related small GTPases, thus providing entry into the ERK1/2 or p38 MAPK pathways (49, 50). In the heart, MAPK-mediated phosphorylation of GATA-4 occurs on Ser105, which is a consensus phosphorylation site for MAPK (33, 35). Although GATA-4 could be potentially phosphorylated by MAPK on Ser105 by cAMP stimulation of MA-10 Leydig cells, cAMP-induced phosphorylation was not blocked by MAPK inhibitors (Fig. 1). This suggests that cross-talk between cAMP and the MAPK pathway is not responsible for cAMP-induced phosphorylation of GATA-4 in these cells. Rather, GATA-4 is predominantly phosphorylated on Ser261, which lies within a consensus motif (RXS) for PKA phosphorylation. To our knowledge, this is the first demonstration of cell signaling that involves direct phosphorylation of a GATA factor by PKA. The fact that GATA-4 is preferentially phosphorylated by PKA on Ser261 in MA-10 cells does not formally exclude a role for Ser105 phosphorylation. In fact, gonadal cells do respond to certain stimuli that activate the cAMP pathway but without the involvement of PKA (6). Therefore, divergent signaling pathways and kinases might converge on GATA-4 to regulate gonadal gene expression and function. Interestingly, such a mechanism has recently been described for the yeast GATA factor Gln3, which acts as a final effector of two key nutrient and sensing pathways via differential phosphorylation by two different kinases (51).

Mechanism of Phospho-GATA-4 Action in Gonadal Cells—In addition to stimulating GATA-4 transcriptional activity on several target promoters, phosphorylation of GATA-4 also modulates its ability to cooperate with certain transcription factors. For example, GATA-4 can synergize with SF-1 in the absence of PKA (27). GATA-4/SF-1 synergism was nonetheless enhanced by PKA (data not shown). PKA action is also essential for transcriptional synergism between GATA-4 and other transcription factors such as C/EBPβ (26) and CBP (this study). Thus, modulation of the intrinsic transcriptional properties of GATA-4 and its ability to cooperate with other transcription factors.

Fig. 8. PKA-mediated phosphorylation of GATA-4 Ser261 enhances recruitment of the CBP coactivator. In vitro pull-down assays were performed using 500 ng of bacterially produced phosphorylated (+PKA) or unphosphorylated (−PKA) GST-GATA-4 fusion proteins and an in vitro translated 35S-labeled fragment of the CBP protein (amino acids 1200–1900). After extensive washes, the bound proteins were separated on a 10% SDS-PAGE gel and visualized by autoradiography. Input corresponds to 10% of the total 35S-CBP used in each assay.

Control

|       | FL | S261A | ΔC1 | ΔN,C1 | VP16-ΔN,C1 |
|-------|----|-------|-----|-------|------------|
| PKA   | -  | -     | -   | -     | -          |
| PKA + CBP | -  | -     | -   | -     | -          |
| PKA + GST-G4 | -  | -     | -   | -     | -          |
| PKA + CBP + GST-G4 | -  | -     | -   | -     | -          |

|       | FL | S261A | ΔC1 | ΔN,C1 | VP16-ΔN,C1 |
|-------|----|-------|-----|-------|------------|
| PKA   | -  | -     | -   | -     | -          |
| PKA + CBP | -  | -     | -   | -     | -          |
| PKA + GST-G4 | -  | -     | -   | -     | -          |
| PKA + CBP + GST-G4 | -  | -     | -   | -     | -          |
PKA-mediated Activation of GATA-4 and Recruitment of CBP

factors are two mechanisms (which are not mutually exclusive) whereby gonadal gene expression might be regulated by the PKA-mediated phosphorylation of GATA-4. Another potential mechanism involves protein-protein interactions with coactivators. Although GATA-4 can interact with the coactivator CBP in the absence of any posttranslational modification, PKA-mediated phosphorylation of GATA-4 Ser(105) significantly enhanced this interaction (Fig. 8). Moreover, on target gonadal promoters, this enhanced phospho-GATA-4 Ser(105)/CBP interaction was critical for maximal GATA-4/CBP transcriptional synergism. Interestingly, these results are reminiscent of the interaction between CBP and phospho-CREB Ser(332). In the classical cAMP/KA pathway, cAMP elicits a 3- to 4-fold increase in CREB phosphorylation levels (52). This is very similar to our observed increase in GATA-4 phosphorylation levels after cAMP stimulation of MA-10 Leydig cells. Thus, it appears that phosphorylation of GATA-4, much like CREB, allows for a stronger interaction with CBP, ultimately leading to increased gene transcription. Importantly, this novel mechanism provides new insights into our understanding of the hormone-dependent regulation of genes that lack classical cAMP regulatory elements.

Because the GATA-4 protein contains two distinct phosphoacceptor sites (Ser(105) and Ser(261)), differential phosphorylation in response to various signals might constitute another important regulatory mechanism. Like GATA-4, CREB can also be phosphorylated on different serine residues by different kinases depending on the stimuli. For example, phosphorylation of CREB Ser(142) by the casein kinase II and Ca(2+)/calmodulin kinase II prevents CBP recruitment and target gene activation (3). This allows for signal discrimination through CREB leading to differential output. Although no data are currently available concerning coactivator recruitment upon phosphorylation of GATA-4 Ser(105) in response to a stress signaling pathway, it is still tempting to speculate that a similar mechanism could also exist for GATA-4. In this way, phosphorylation of a common transcriptional regulator by different kinases in response to stress or cAMP signals would translate into differential gene expression via modulation of coactivator recruitment. Through this mechanism, GATA-4 could represent the cornerstone of a large transcriptional complex (involving other transcription factors such as SF-1 and C/EBPβ, and the coactivator CBP/p300) that is required for the activation of different sets of genes in response to hormonal and stress signaling in the gonads and other endocrine tissues.

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