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Novel roles for GATA transcription factors in the regulation of steroidogenesis☆

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Abstract

Steroidogenesis is a tightly regulated process that is dependent on pituitary hormones. In steroidogenic tissues, hormonal stimulation triggers activation of an intracellular signalling pathway that typically involves cAMP production, activation of PKA, and phosphorylation of target transcription factors. In the classic cAMP signalling pathway, phosphorylation of CREB (cAMP response element (CRE)-binding protein) and its subsequent binding to cAMP-response elements (CREs) in the regulatory regions of target genes play a key role in mediating cAMP responsiveness. However, the cAMP responsive regions of several genes expressed in steroidogenic tissues do not contain consensus CREs indicating that other transcription factors are also involved. We have been studying the role played by the GATA family of transcription factors. GATA factors are expressed in a variety of tissues including the adrenals and gonads. Since the regulatory regions of several steroidogenic genes contain GATA elements, we have proposed that GATA factors, particularly GATA-4 and GATA-6, might represent novel downstream effectors of hormonal signalling in steroidogenic tissues. In vitro experiments have revealed that GATA-4 is indeed phosphorylated in steroidogenic cells and that phosphorylation levels are rapidly induced by cAMP. GATA-4 phosphorylation is mediated by PKA. Phosphorylation increases GATA-4 DNA-binding activity and enhances its transcriptional properties on multiple steroidogenic promoters. We now define a new molecular mechanism whereby phospho-GATA factors contribute to increased transcription of steroidogenic genes in response to hormonal stimulation.

Keywords: GATA-4; cAMP; Phosphorylation; Protein kinase; Trophic hormones

1. The GATA family of transcription factors

1.1. The six vertebrate GATA factors

GATA regulatory motifs were originally identified a little more than a decade ago in studies of erythroid-specific gene expression [1,2]. A novel transcription factor that specifically bound to GATA *cis*-elements was cloned from erythroid cells and named GATA-1 [3]. GATA-1 was shown to contain a DNA-binding domain that consisted of two similar zinc fingers with the distinctive form C-X_2-C-(X_{17})-C-X_2-C [2,3]. Since the cloning of the prototypic GATA-1 factor, five additional vertebrate factors (GATA-2 to GATA-6), having similar DNA-binding domains, have been identified [2,4].

The six vertebrate GATA factors can be divided into two subgroups based on similarities in primary amino acid sequence and spatiotemporal expression patterns: GATA-1/2/3 and GATA-4/5/6. The *GATA-1/2/3* genes are predominantly expressed in hematopoietic cell lineages [1], whereas the *GATA-4/5/6* genes are predominantly expressed in the heart, gut, gonads, and adrenals [5]. Complementary in vitro and in vivo approaches have established that these factors play essential roles in cell differentiation, organogenesis, and cell-specific gene expression in such diverse processes as hematopoiesis [2,4,6], adipogenesis [7], heart tube formation [5,8], female genitourinary tract development [9], and sex determination and differentiation [10,11]. The in vivo relevance of GATA factor function in humans is now supported by the identification of conditions, such as dyserythropoietic anemia and thrombocytopenia, human hypoparathyroidism, sensorineural deafness, renal anomaly (HDR) syndrome, and morbid obesity, that are associated with mutations in the coding regions of certain GATA genes [12,13], or mutations in consensus GATA motifs present in the promoter regions of specific target genes [14].

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1.2. Functional specificity of GATA factors

All vertebrate GATA proteins contain a conserved DNA-binding domain composed of two multifunctional zinc fingers. The C-terminal zinc finger is required for site-specific recognition and DNA-binding to the core WGATAR motif, whereas the N-terminal zinc finger contributes to the specificity and stability of the DNA-binding [15–17]. Since members of the GATA family share a highly homologous DNA-binding domain, they all exhibit similar DNA-binding properties [18,19], and consequently, have been reported to be functionally interchangeable in some in vitro assays [20,21]. This clearly contrasts, however, with their non-redundant functions in vivo [9,20,22–30]. The functional specificity of GATA factors is achieved, at least in part, via protein–protein interactions with other cell-restricted factors [5,8]. Indeed, GATA-1, -2, and -3 have been shown to interact with several cell-restricted or ubiquitously expressed factors such as RBTN2, NF-E2, EKLF, AP-1, SCL/Tal1, Pit1, PU.1, and Sp1 to control the activity of erythroid-, lymphoid-, and pituitary-specific promoters and enhancers [31–38]. Similarly, GATA-4 has been reported to interact with the homeoprotein Nkx2.5 and the myocyte enhancer factor MEF2 to direct cardiac-specific gene expression [39–41], and with C/EBPβ and the orphan nuclear receptor SF-1 to synergistically activate several gonadal promoters [11,42–45]. In addition to the above-mentioned GATA-interacting factors, a new family of large multitype zinc finger proteins termed Friend of GATA (FOG-1 and FOG-2) have been identified through their ability to interact with the N-terminal zinc fingers of GATA factors [46–50]. Like GATA-1, FOG-1 is highly expressed in developing hematopoietic cells. Similarly, FOG-2 is co-expressed with GATA-4 in the heart, brain, and gonads. Mouse knockout studies have revealed that FOG proteins, like their GATA counterparts, have crucial developmental functions in vivo. Thus, the lack of FOG-1 leads to a block in erythroid and megakaryocytic differentiation [51], while genetic ablation of FOG-2 lead to defects in heart morphogenesis and coronary vascular development [52–54], as well as impaired gonad development [10]. While FOG proteins do not appear to directly bind to DNA, they act as either enhancers or repressors of GATA transcriptional activity depending on the cell and promoter context being studied [46–50,55–57].

2. Expression and role of GATA factors in steroidogenic tissues

Besides the hematopoetic and cardiac systems, GATA factors are also prominently expressed in a variety of other tissues such as the gut, pituitary, brain, adrenals and gonads, where they control many important physiological processes through the regulation of numerous downstream target genes [32,43,58–66]. Since the adrenals and gonads represent the main steroid-producing organs, this suggests that GATA factors likely play an essential role in steroidogenesis. As summarized in Table 1, steroidogenic tissues express two GATA factors: GATA-4 and GATA-6. In the human and mouse adrenals, both GATA-4 and GATA-6 are highly expressed at the fetal stage. In the postnatal adrenal, however, GATA-4 expression is down-regulated whereas GATA-6 expression remains high [67,68]. In the gonads, both GATA-4 and GATA-6 are expressed in the ovarian steroidogenic compartment throughout development [69,70], whereas in the testis, GATA-4 is the predominant GATA factor of both fetal and postnatal Leydig cells [59,71–73]. In addition to GATA-4, Leydig cells and certain Leydig cell-derived cell lines also express the FOG-1 and FOG-2 proteins [57], suggesting a role for these cofactor proteins in Leydig cell function. Indeed, in the mouse, *FOG-2* gene inactivation or a targeted mutation of the GATA-4 protein that prevents its interaction with FOG-2 leads to a block in the expression of several steroidogenic markers including P450scc, 3βHSD, and P450c17 [10]. Although at present, it remains unclear whether the lack of steroidogenic markers in these animals is the result of an intrinsic defect in Leydig cell gene expression or due to a block in fetal Leydig cell development.

Table 1
Expression of GATA transcription factors in steroidogenic tissues

Gonads	Adrenals
GATA-4	
Testis	Fetal adrenal cortex [67,68]
Fetal and postnatal Leydig cells [59,71–73]	
Several Leydig cell lines	
MA-10 [44,57,75,76]	
mLTC-1 [57]	
I-10 [88]	
Ovary	
Fetal and postnatal granulosa cells [69,70]	
Adult theca cells [69,70]	
GATA-6	
Testis	Fetal and postnatal adrenal cortex [67,68]
Not in steroidogenic lineage	Several adrenal cell lines
	NCI-H295R [68]
Ovary	
Granulosa cells [69,70]	
Theca cells [69,70]	

Consistent with a role for GATA factors in the regulation of steroidogenesis, the promoters of several steroidogenic genes contain one or more consensus GATA regulatory motifs (Table 2). Using in vitro transactivation experiments, we and others have confirmed that some of these steroidogenic promoters are indeed targets for GATA factors. Those studied to date include the promoters for steroidogenic factor 1 (SF-1/NR5A1) [43], 17β-hydroxysteroid dehydrogenase type 1 [74], P450 aromatase [43], and steroidogenic acute regulatory protein (StAR) [43,44,75–77]. In addition to their

Table 2
Potential target genes for GATA factors in steroidogenic tissues

Gene	References
Steroidogenic acute regulatory protein (*StAR*)	[43,44,75,76]
Aromatase PII promoter (*Cyp19*)	[43]
17α-Hydroxylase (*Cyp17*)	up
P450 side-chain cleavage (*Cyp11A1*)	up
Luteinizing hormone receptor (*LH-R*)	uc
21-Hydroxylase (*Cyp21*)	uc
11β-Hydroxylase (*Cyp11B1*)	up
Adrenocorticotropin hormone receptor (*ACTH-R*)	uc
17β-Hydroxysteroid dehydrogenase type 1 (*HSD17B1*)	[74]
3β-Hydroxysteroid dehydrogenase	up

uc: uncharacterized. Consensus GATA elements found within the proximal 5′ regulatory elements available from the GenBank database. up: unpublished data from our laboratory.

ability to directly stimulate transcription of target steroidogenic promoters, we have also shown GATA factors to regulate target promoters via a synergistic interaction with the orphan nuclear receptor SF-1. SF-1 was originally identified as an important regulator of the genes encoding the P450 steroid hydroxylases [78]. Subsequent studies revealed that SF-1 was in fact a critical regulator of gene expression in the adrenal gland and at all levels of the reproductive axis [78]. While SF-1 is clearly essential for the development and function of steroidogenic tissues, SF-1 alone cannot account for the tissue-specific expression of steroidogenic enzyme encoding genes. Therefore, the participation of other transcription factors is required to establish this specificity. Since GATA factors are co-expressed with SF-1 in steroidogenic tissues, they represent natural transcriptional partners for SF-1. In agreement with this hypothesis, we have shown that SF-1 can directly interact with GATA factors [42], resulting in a synergistic activation of multiple SF-1-dependent promoters, the most notable being the murine aromatase PII promoter [43]. Taken together, these data point to GATA factors being critical regulators of steroidogenic gene expression and function, a title that was until now solely held by SF-1.

3. GATA-4: a novel downstream effector of the cAMP/PKA signalling pathway in steroidogenic cells

Adrenal and gonadal steroidogenesis is tightly regulated by the pituitary trophic hormones ACTH, LH, and FSH. Hormone-induced steroidogenesis is predominantly mediated through the cAMP-dependent intracellular signalling pathway leading to activation of protein kinase A (PKA). PKA then translocates to the nucleus and phosphorylates target proteins [79,80]. The best studied target of cAMP/PKA signalling is the transcription factor CREB (cAMP response element (CRE)-binding protein) which binds as a dimer to the 8 bp palindromic sequence CRE found in the regulatory region of some cAMP-regulated genes [81]. In steroidogenic tissues, however, several cAMP-regulated genes, such as steroidogenic acute regulatory protein, steroid 17α-hydroxylase (*Cyp17*), P450scc (*Cyp11A1*), and aromatase (*Cyp19*), lack consensus CRE elements. Therefore, transcription factors besides CREB must be acting as downstream effectors of cAMP signalling in steroidogenic cells. Since expression of several steroidogenic genes is stimulated by cAMP, it would therefore be reasonable for a single transcription factor to act as a common effector of hormonal signalling. As previously pointed out, the promoters of several steroidogenic enzyme encoding genes contain binding sites for both SF-1 and GATA factors. A role for SF-1 in the cAMP-dependent stimulation of several steroidogenic genes has already been proposed [78]. Although SF-1 can be phosphorylated by PKA in vitro [82], SF-1 is constitutively phosphorylated in vivo by the mitogen-activated protein kinase (MAPK) pathway and its phosphorylation levels are not apparently affected by cAMP treatment [83]. Thus, CREB and SF-1 might not be the only effectors of cAMP signalling in steroidogenic cells.

As previously mentioned, many cAMP-regulated steroidogenic promoters contain GATA regulatory motifs for the binding of members of the GATA family of transcription factors. Thus, GATA factors might also be targets of the hormone-induced cAMP/PKA signalling pathway in steroidogenic cells. Consistent with this hypothesis, we have recently reported that GATA-4 is indeed a phosphoprotein in MA-10 Leydig cells [44]. We investigated the in vivo phosphorylation status of GATA-4 in response to cAMP stimulation using a phospho-(Ser/Thr) PKA substrate antibody that specifically recognizes serine or threonine residues that have been phosphorylated by PKA. As shown in Fig. 1A, endogenous GATA-4 protein is constitutively phosphorylated in unstimulated MA-10 cells (vehicle lane). Following cAMP stimulation, however, GATA-4 phosphorylation levels are significantly increased by about five-fold (cAMP lane). This could be specifically attributed to increased phosphorylation since total GATA-4 protein levels remained unchanged after the same treatment (Fig. 1B). In vitro kinase assays further confirmed that GATA-4 can be directly phosphorylated by PKA (Fig. 1C). Analysis of the GATA-4 primary amino acid sequence reveals the presence of one putative PKA consensus phosphorylation site that matches the high-affinity sequence RRXS. This serine residue is located at amino acid position 261 (Ser261) between the two zinc fingers of the DNA-binding domain, a region perfectly conserved between the rat, mouse, human, bovine, rabbit, frog, fish, and chick GATA-4 proteins. Therefore, this serine residue likely constitutes the phosphoacceptor site on the GATA-4 protein that is targeted by PKA in response to cAMP stimulation in steroidogenic cells. Studies regarding the relevance of Ser261 in PKA-mediated phosphorylation of GATA-4 are currently underway.

If cAMP/PKA-mediated phosphorylation of GATA-4 plays a role in hormone-induced steroidogenic gene expression, then phosphorylation of GATA-4 might enhance its DNA-binding and/or transactivation properties on target

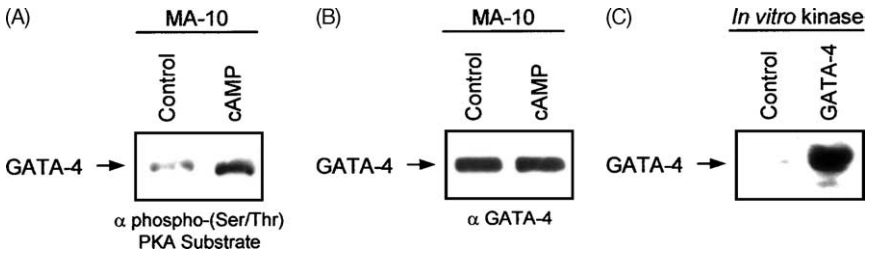

Fig. 1. Phosphorylation of GATA-4 via the cAMP/PKA pathway. (A) GATA-4 is phosphorylated in response to cAMP in MA-10 cells. GATA-4 protein present in nuclear extracts prepared from either unstimulated (vehicle) or dibutyryl (db) cAMP-stimulated MA-10 cells was purified by immunoprecipitation using an antibody against GATA-4. Immunoprecipitates were then subjected to SDS-PAGE, transferred to PVDF membrane, and revealed with an anti-phospho-(Ser/Thr) PKA substrate antibody. (B) Total GATA-4 protein from unstimulated (vehicle) or db-cAMP-treated MA-10 cells was detected using a commercially available GATA-4 antiserum. (C) Unprogrammed rabbit reticulocytes (control lane) or in vitro translated GATA-4 protein (GATA-4 lane) were purified by immunoprecipitation with a GATA-4-specific antiserum and the immunoprecipitates were then used in an in vitro PKA phosphorylation assay in the presence of [γ^{32}P]ATP. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and visualized by autoradiography. Figure adapted from [44] with permission from The Endocrine Society®.

steroidogenic promoters. Although GATA-4 protein levels in MA-10 cells were not altered by cAMP treatment (Fig. 1B), electrophoretic mobility shift assays revealed that GATA-4-binding activity was significantly increased (Fig. 2A). Supershift experiments using a GATA-4-specific antiserum revealed that the increase in GATA-binding was solely attributable to GATA-4. To assess the effect of GATA-4 phosphorylation on its transcriptional activity, heterologous CV-1 cells were cotransfected with expression vectors for GATA-4 and PKA catalytic subunit α. As shown in Fig. 2B, co-transfection of PKA led to a marked enhancement of the ability of GATA-4 to transactivate a representative steroidogenic promoter, the murine StAR promoter. Similar experiments performed with PKC did not result in an enhancement of GATA-4 transcriptional activity (our unpublished observations), indicating that GATA-4 is not a target for PKC phosphorylation. In support of a role for phosphorylation in the enhancement of GATA-4 transcriptional activity, the protein phosphatase inhibitor okadaic acid (OA) enhances GATA-4-dependent transactivation (G4 + OA in Fig. 2B) almost to the same extent as overexpression of PKA catalytic subunit (G4 + PKA in Fig. 2B). GATA-4 transcriptional activity is maximal when PKA is overexpressed in the presence of OA (G4 + OA + PKA in Fig. 2B). In addition to the StAR promoter (Fig. 2), the PKA-mediated enhancement of GATA-4 transcriptional activity also observed a number of other target promoters including P450c17 and aromatase PII (our unpublished observations). Moreover, similar experiments performed with GATA-6 revealed that its transactivation potential can also be enhanced by PKA [44]. Taken together, these data support a role for GATA factors as downstream effectors of

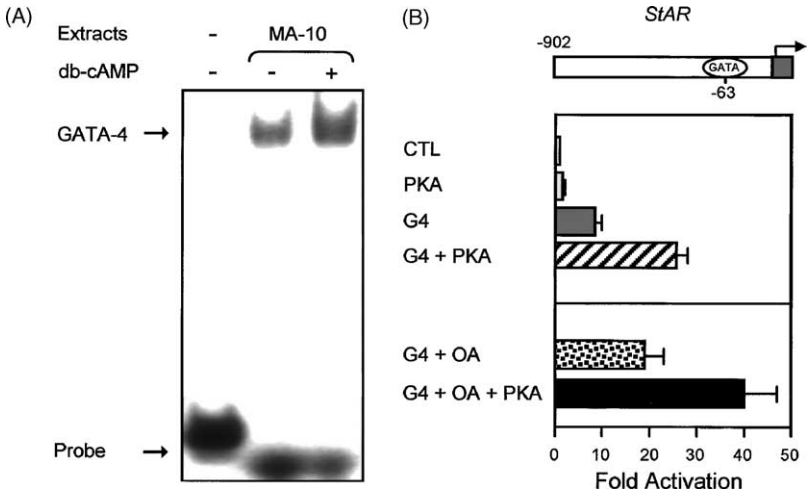

Fig. 2. Phosphorylation potentiates GATA-4 DNA-binding and transactivation properties. (A) cAMP stimulation increases GATA-binding activity in MA-10 cells. Nuclear extracts were isolated from unstimulated or db-cAMP-treated (0.5 mM) MA-10 cells. The electrophoretic mobility shift assay was performed using 5 μg of nuclear extract and a ^{32}P-labelled probe corresponding to the GATA element of the murine StAR promoter. (B) PKA-mediated phosphorylation enhances the GATA-4-dependent activation of the StAR promoter. CV-1 cell fibroblasts were transfected with the −902 bp StAR promoter along with either an empty expression vector (CTL) or expression vectors for PKA, GATA-4 (G4), GATA-4 and PKA (G4 + PKA), GATA-4 and the protein phosphatase inhibitor okadaic acid (G4 + OA), or GATA-4 in the presence of both PKA and okadaic acid (G4 + OA + PKA). All promoter activities are reported as fold activation over control (±S.E.M.).

hormone-induced steroidogenesis in both the gonads (where GATA-4 is expressed) and the adrenals (where GATA-6 is expressed).

Since GATA factors have been shown to interact with a variety of different factors [5], PKA-mediated phosphorylation of GATA-4 could potentially modulate its ability to transcriptionally cooperate with other transcription factors in steroidogenic cells. Indeed, we have recently shown this to be the case for the synergistic cooperation between GATA-4 and C/EBPβ on the murine StAR promoter [44]. Both factors have been shown to be required for full StAR promoter activity in steroidogenic cells [75,76]. By themselves, GATA-4 and C/EBPβ are respectively strong and weak activators of StAR promoter activity [44]. We then surmised that the two factors might synergistically activate the StAR promoter. In spite of a direct physical interaction between GATA-4 and C/EBPβ, no transcriptional synergism was observed on the StAR promoter [44]. However, in the presence of PKA, synergism could readily be observed [44]. Interestingly, PKA-dependent synergism between GATA-4 and C/EBPβ on the StAR promoter was also observed with GATA-6, as well as between GATA-4 and two other C/EBP proteins, C/EBPα and C/EBPδ [44]. The fact that synergy was not limited to specific members of the GATA and C/EBP family of factors indicates that this mechanism might be responsible for the regulation of *StAR* gene expression in steroidogenic tissues where GATA and C/EBP factors are co-expressed. In addition to the StAR promoter, PKA also enhances synergism between GATA-4 and SF-1, especially on the aromatase promoter. Therefore, in addition to directly modulating its DNA-binding and transcriptional activities, PKA-mediated phosphorylation of GATA-4 also enhances its ability to transcriptionally cooperate with at least two other transcription factors (C/EBPβ and SF-1) on different steroidogenic promoters.

4. Perspectives

Although recent studies have focused on the role of GATA-4 in basal and cAMP-induced StAR transcription [44,75,76], it is likely that expression of other steroidogenic genes will also be regulated by GATA factors. Consistent with this, GATA factors are found in all major

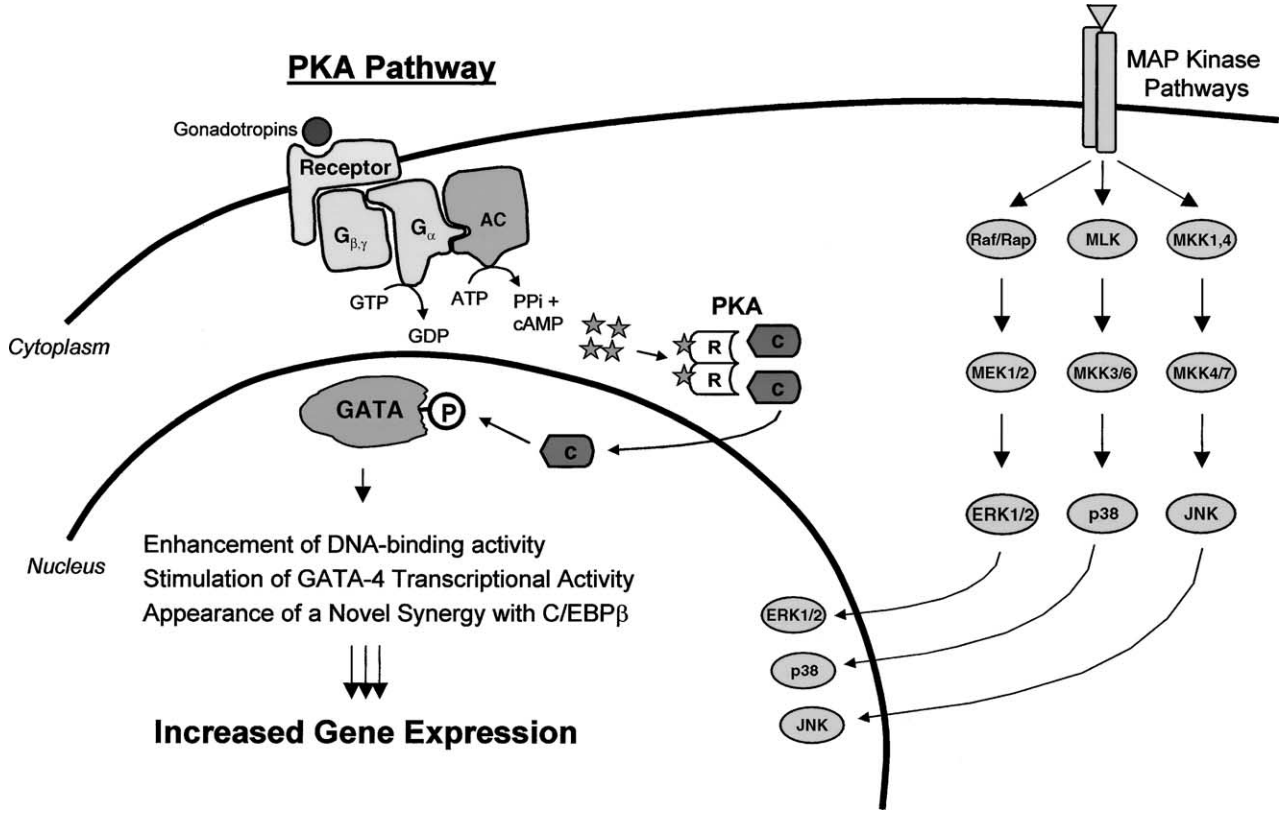

Fig. 3. Modulation of steroidogenic gene expression through phosphorylation of GATA factors. Two pathways are known to induce GATA-4 phosphorylation: the cAMP/PKA pathway (left panel) and the MAP kinase pathway (right panel). In the cAMP/PKA pathway, the binding of pituitary trophic hormones (H) to their respective G-protein coupled receptors triggers the activation of adenylate cyclase (AC) which increases intracellular cAMP production (depicted by stars) from ATP. cAMP then binds the regulatory (R) subunit of protein kinase A (PKA) allowing dissociation of the PKA catalytic (C) subunit and its translocation to the nucleus where it can phosphorylate GATA factors (GATA-4 and/or GATA-6). GATA-4 is also directly phosphorylated by Erk1/2 and p38 upon activation of the MAP kinase pathway triggered by mitogen and stress signals. GATA phosphorylation mediated by either or both pathways contributes to increased expression of GATA-dependent target genes through multiple mechanisms as depicted.

steroidogenic tissues (Table 1) and the promoters of several steroidogenic genes contain consensus GATA regulatory elements (Table 2). In both the gonads and the adrenals, phosphorylation of GATA-4 and/or GATA-6 is likely to be the major consequence of hormonal signalling through the cAMP/PKA pathway. However, recent data on the cAMP regulation of GATA-6 in both granulosa–luteal and adrenals cells [68,70], suggest that cAMP might also directly stimulate *GATA-6* gene expression. In contrast, GATA-4 expression levels in steroidogenic cells appear to be unaffected by cAMP stimulation [44,76].

As previously mentioned, modulation of the intrinsic transcriptional properties of GATA-4 and its ability to cooperate with other transcription factors are two mechanisms (which are not mutually exclusive) whereby steroidogenic gene expression might be regulated by the PKA-mediated phosphorylation of GATA factors. Another potential mechanism involves protein–protein interactions with co-activators. Indeed, both GATA-4 and GATA-6 have been shown to interact with p300/CBP in the absence of any post-translational modification [84,89]. PKA-mediated phosphorylation of GATA factors could conceivably enhance their ability to recruit CBP to target steroidogenic promoters and thereby enhance gene transcription. This mechanism would be reminiscent of the classic interaction between CBP and phospho-CREB in response to cAMP signalling. Importantly, this novel mechanism of GATA phosphorylation and recruitment of CBP would provide new insights into our understanding of the hormone-dependent regulation of steroidogenic genes that lack classic cAMP regulatory elements (CREs).

Besides the cAMP/PKA pathway, the MAPK signalling pathway is also emerging as an important regulator of gonadal and adrenal steroidogenesis [85]. Interestingly, GATA-4 has recently been reported to be a direct target of the MAPK ERK1/2 in the heart. In this tissue, MAPK-mediated phosphorylation of GATA-4 occurs on Ser105 which is a consensus phosphorylation site for MAPK [86,87]. Although this amino acid is not a target for PKA phosphorylation, it might still be an important target for MAPK in steroidogenic cells via stimuli that do not necessarily implicate cAMP. Therefore, divergent signalling pathways and kinases might converge on GATA-4 and/or GATA-6 to regulate steroidogenic gene expression and function (Fig. 3). Through this mechanism, GATA factors could represent the cornerstone of a large transcriptional complex (involving other transcription factors such as SF-1, C/EBPβ, and the co-activator CBP/p300) that is required for the activation of different sets of genes in response to hormone, mitogen, and stress signals in steroidogenic tissues.

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