Protein Kinase A-Dependent Synergism between GATA Factors and the Nuclear Receptor, Liver Receptor Homolog-1, Regulates Human Aromatase (CYP19) PII Promoter Activity in Breast Cancer Cells

Marie France Bouchard, Hiroaki Taniguchi, and Robert S. Viger

Ontogeny-Reproduction Research Unit (M.F.B., H.T., R.S.V.), Centre Hospitalier Universitaire de Québec-Centre Hospitalier de l’Université Laval Research Centre, Ste-Foy, Québec, Canada G1V 4G2; Department of Obstetrics and Gynecology (R.S.V.), Centre de Recherche en Biologie de la Reproduction, Faculty of Medicine, Université Laval, Québec, Canada G1K 7P4

Cancers, including that of the breast, are the result of multiple contributing factors including aberrant gene expression. Indeed, the CYP19 gene encoding P450 aromatase, the key enzyme for estrogen biosynthesis, is up-regulated in breast tumors predominantly via the cAMP-responsive gonad-type PII promoter, ultimately leading to increased intratumoral estrogen production and tumor growth. Thus, identifying the molecular factors involved in aromatase PII promoter regulation is essential for our understanding and treatment of the disease. Because we have previously shown activity of the murine aromatase PII promoter to be markedly up-regulated by GATA factors with respect to the gonads, we hypothesized that GATA factors are also key determinants of human PII promoter-driven aromatase transcription in breast tumors. We now show that GATA3 and GATA4 are indeed expressed in several breast cancer cell lines. Consistent with the cAMP dependence of the PII promoter, activation elicited by GATA3 or GATA4 alone and the striking synergism between GATA3 or GATA4 and the nuclear receptor liver receptor homolog (LRH)-1 was intimately linked to forskolin treatment or overexpression of protein kinase A (PKA) catalytic subunit. PKA-mediated phosphorylation increases the interaction between GATA3 and LRH-1 and the requirement for PKA in aromatase PII promoter stimulation involves at least three specific amino acid residues: GATA3 Ser308, GATA4 Ser261, and LRH-1 Ser469. Finally, we show that the human LRH-1 promoter is itself a target for GATA factors. Thus, taken together, our results suggest that GATA factors likely contribute to aberrant aromatase expression in breast tumors through two distinct, yet complementary mechanisms. (Endocrinology 146: 4905–4916, 2005)

Breast cancer is one of the most common forms of cancer affecting women and over the past two decades, the rate of breast cancer incidence has been increasing for both premenopausal and postmenopausal women (1). Like all cancers, the development of breast cancer is accompanied by several molecular changes such as overexpression of transcription factors, steroid hormones, and growth factors and their related receptors that ultimately disrupt the normal balance between cell differentiation and proliferation. Breast cancers can be divided into two groups based on their ability to respond to antihormonal therapy. More than half of these are hormone-dependent carcinomas in which estrogens play a predominant role in tumor growth (2). The principal source of estradiol (E2) biosynthesis in premenopausal women is the ovary. After menopause, extragonadal sites of estrogen synthesis such as the bones, liver, muscles, brain, skin, and adipose tissue, including the breast (3), become the primary source of estrogen production. In malignant breast tissue, E2 concentrations are 10–50 times higher than plasma levels (4). These abnormally high E2 levels are due to local production by the tumor itself rather than an enhancement of uptake from the plasma (5). Thus, identifying the factors involved in E2 synthesis in breast tumors is an essential part of our understanding of the disease.

The rate-limiting step in E2 biosynthesis is catalyzed by the enzyme P450 aromatase. Although the enzyme is present in normal breast, aromatase activity is present at much higher levels in individuals with breast carcinomas (6–8). In breast tumors, aromatase expression and activity is highest in the adipose stromal cell compartment (composed of a pool of undifferentiated fibroblasts) rather than in mature adipocytes (9); lower levels are also present in epithelial cells (10–12). Moreover, xenograft and transgenic mice studies have revealed that high aromatase activity is necessary and sufficient to induce intratumoral E2 production in the absence of circulating ovarian estrogens (5, 13). The human aromatase gene (CYP19) is a complex gene composed of nine translated exons (exons II–X) and several different untranslated first exons that result from alternative promoter usage (a diagrammatic representation of the CYP19 gene is shown in Fig. 1A). Aromatase gene expression is therefore driven in a tissue-specific manner through the use of multiple promoters that are differentially regulated via a host of different
cytokines, hormones, transcription factors, and signaling pathways (14). In normal breast and other adipose tissues, CYP19 expression is mainly under the control of the glucocorticoid-stimulated promoter I.4 (15, 16). Surprisingly, increased CYP19 expression in adipose tissue from women with breast cancer is not due to an increase in promoter I.4-driven expression but rather to an important activation of promoters I.3 (another adipose-type promoter), I.7 (endothelial-type), and PII (gonad-type) (6, 12, 17–20). Thus, CYP19 promoter switching in breast tumors is a hallmark of the disease.

Prostaglandin E2 (PGE2) is an important secretory product of breast cancer epithelial and fibroblast cells as well as macrophages infiltrating the tumor site (21). Overexpression of cyclooxygenase 2 in the mammary gland of transgenic mice induces tumorigenesis; an effect that is dependent on a functional prostaglandin EP2 receptor suggesting that PGE2 is the major eicosanoid involved (22). In breast tumors, PGE2/EP receptor activation raises intracellular cAMP levels and stimulates CYP19 expression via promoters I.3 and PII (23–27). Further support for the involvement of PGE2 is the recent demonstration that cyclooxygenase inhibitors suppress CYP19 expression and activity in breast cancer cells (28).

In classic steroidogenic tissues such as the gonads, the regulation of the aromatase PII promoter has been intensely studied.
studied. Transgenic experiments have revealed that 278 bp of the proximal human aromatase PII promoter are sufficient for proper spatiotemporal and hormonal regulation in the ovary (29). Within this region are found binding sites for cAMP-responsive element binding protein and members of the NR5A family of nuclear receptors, steroidogenic factor-1 (NR5A1/SF-1/Ad4BP) and liver receptor homolog-1 (NR5A2/LRH-1/FTF) (30, 31). Although SF-1 is not detected in breast tumors, LRH-1 is strongly expressed and has been shown to be able to bind to and activate the human aromatase PII promoter in 3T3-L1 preadipocytes, especially in the presence of forskolin stimulation (32). However, LRH-1 and cAMP-responsive element binding protein are also expressed in many other tissues in which aromatase is not, suggesting that specificity of aromatase expression in breast tumors and other steroidogenic tissues requires the participation of other transcription factors. Indeed, members of the CCAAT/enhancer binding protein (C/EBP) and GATA families of transcription factors have also been shown to be able to bind and activate the aromatase PII promoter (33–35).

There are six vertebrate GATA factors that can be divided into two groups based on sequence homology and hormone distribution: GATAs 1/2/3 and GATAs 4/5/6. Mouse knockout models have revealed that these proteins are critical regulators of cell fate specification, cell differentiation, organogenesis, and tissue-specific gene expression in many tissues (36–39). In addition to their critical role in regulating key developmental processes, GATA factors have recently been implicated as potential regulators of cell fate specification, cell differentiation, organogenesis, and tissue-specific gene expression in many tissues (36–39). In addition to their critical role in regulating key developmental processes, GATA factors have recently been implicated as potential regulators of cell fate specification, cell differentiation, organogenesis, and tissue-specific gene expression in many tissues (36–39). In addition to their critical role in regulating key developmental processes, GATA factors have recently been implicated as potential regulators of cell fate specification, cell differentiation, organogenesis, and tissue-specific gene expression in many tissues (36–39).

In the present study, we used the MCF-7 breast cancer cell line to demonstrate that the human aromatase PII promoter is turned on in breast cancer, and there is a strong possibility that GATA factors contribute to CYP19 function in breast cancer cells.

In the present study, we used the MCF-7 breast cancer cell line to demonstrate that the human aromatase PII promoter is turned on in breast cancer, and there is a strong possibility that GATA factors contribute to CYP19 function in breast cancer cells.

Materials and Methods

Plasmids

The −430-bp human aromatase PII (−430 to +40) and −460-bp human LRH-1 (−460 to +50) promoters were amplified by PCR from human genomic DNA and cloned into the BamHI/KpnI sites of a modified pXP1 luciferase reporter vector (47). The primer pairs used were 5′-ACGGATCCGCTCTTACGTGACAACCTCTAC-3′ and 5′-ACGGTAC- CACAGAATCCCCTAAAAGGTTC-3′ for the human aromatase PII promoter and 5′-ACGGATCCCCATAGCTGGGAATGTCACTT G-3′ and 5′-ACGGTACACCGAAGCTTTTCTGCTGAGTTCTC-3′ for the human LRH-1 promoter. The identity of both promoters was confirmed by sequencing. The minimal (−80 bp) human aromatase PII promoter construct was obtained by PCR using the −430-bp construct as template and the following primer pair: 5′-ACGGAATCTTGGTTGA CTTGTAACC-3′ and 5′-ACGGTACACAGAATCCCCTAAAAGGTTC C-3′. The GATA3 expression plasmid was made by cloning the full-length GATA3 cDNA (kindly provided by Doug Engel, University of Michigan, Ann Arbor, MI) into the Xhol/BamH1 sites of a previously described pcDNA3 expression vector modified to provide its own ATG (48). Expression plasmids for GATA4, GATA4 S261A, the GATA dominant-negative competitor (GATA DN), and protein kinase A (PKA) catalytic subunit-α have been previously reported (34, 48–50). The GATA3 5308A and LRH-1 5469A point mutations were obtained by site-directed mutagenesis of the corresponding wild-type GATA3 and LRH-1 cDNAs using a QuickChange XL mutagenesis kit (Stratagene, La Jolla, CA) and the following oligonucleotide pairs: GATA3 5308A (5′-CAAGCCCCGACGGTGCAGCGAGAAGGACGAC-3′); 5′-CCCTCTGCTTCTTTGCTGCGGACGGTTGGGTTCG-3′); LRH-1 5469A (5′-CGACTACCGAATCCCCGATCGATTAGACGTCGATCC-3′); 5′-GAGATTTCCTAAGCTGTCATGAG-3′. The human LRH-1 expression plasmid was generously provided by Luc Bélanger (Laval University, Québec, Canada).

Cell culture and transfections

All cell lines used in this study are available from American Type Culture Collection (Manassas, VA). BT-20 and MCF-7 cells were grown in Eagle’s MEM supplemented with 10% fetal bovine serum (FBS; Invitrogen Canada, Burlington, Canada), ZR-75–1 cells in RPMI 1640 containing 10% FBS, and T-47D cells in DMEM containing 10% FBS, according to the recommendations provided by American Type Culture Collection. HeLa cells were grown in DMEM/F12 containing 10% FBS. All transfections were performed in 24-well plates using the calcium phosphate precipitation method (51). MCF-7 and T-47D cells were plated at an initial density of 6.0 × 10^6 cells/well and transfected 24 h later. Culture medium was changed 12–16 h after transfection, and the cells were finally harvested the following day by lysing the cells in 50 μl lysis buffer [100 mM Tris-HCl (pH 7.9), 0.5% Igepal (Sigma-Aldrich Canada, Oakville, Canada), and 5 mM dithiothreitol]. An aliquot of the lysate was then assayed for luciferase activity using an AutoLumatPlus LB 953 luminometer (Berthold Technologies USA, Oak Ridge, TN) and luciferase (BD Canada, Oakville, Canada) as substrate. The phRL-TK Renilla luciferase vector was used as internal control (Promega, Madison, WI). In all experiments, the total amount of DNA was kept constant at 2 μg/well using Sp64 (Promega) as carrier DNA. Data reported represent the average of at least three experiments, each done in duplicate.

RNA isolation, first-strand cDNA synthesis, and RT-PCR analysis

Total cellular RNA was prepared from BT-20, MCF-7, T-47D, and ZR-75–1 cells by the acid guanidinium thiocyanate-phenol-chloroform method (52). First-strand cDNA was prepared from each RNA sample using SuperScriptII reverse transcriptase (Invitrogen Canada). These cDNAs were then used in RT-PCRs to detect the presence of GATA3, GATA-4, and LRH-1 transcripts using the following primer sets: GATA3 (5′-AGGACGAGAAAGT GCCCTC-3′; 5′-AGGTCCCTCTGCCCTGCT-3′), GATA4 (5′-TTCTGAGATGTTTACGACCTC-3′; 5′-TAAAGGACTGGTCGTTCTT-3′), and LRH-1 (5′-TGGATAATCGTCTCAGGTATCCAGGCC-3′; 5′- AGTCTAGAAGCAGCTTCATTGGCATC-3′). The integrity of the different cDNAs was verified by amplifying tubulin as a control gene.

Nuclear extracts, Western blot, and DNA-binding assays

Nuclear extracts from the different breast cancer cell lines and recombinant GATA and LRH-1 proteins (wild-type and mutated constructs) were overexpressed in HeLa cells by the procedure described by Schreiber et al. (53). For the Western blot analyses, 10-μg aliquots of nuclear extract were separated by SDS-PAGE and transferred to Hybond polyvinylidene difluoride membrane (Amersham Biosciences, Baie-D’Urfé, Canada). Immunodetection of the GATA, GATA4, and LRH-1 proteins was achieved using commercial antisera (Santa Cruz Biotechnology, Santa Cruz, CA) and a VECTASTAIN ABC-Amp Western blot detection kit (Vector Laboratories Canada, Burlington, Canada). Duolux (Vector Laboratories Canada) was used as chemiluminescent substrate. EMSAs were performed using a 32P-labeled double-stranded oligonucleotide corresponding to the LRH-1 (sense oligo: 5′-GATCCCTTCATACGAGAGAAGGTTC-3′) or the LRH-1 (antisense oligo: 5′-GATCCCTTCATACGAGAGAAGGTTC-3′).
TCAGAAATGC-3'; antisense oligo: 5'-GATCTGCTATTCTGACCTGT-GTAGAG-3') and GATA (sense oligo: 5'-GATCCAAGGTCTCATCACG-ACAAA-3'; antisense oligo: 5'-GATTTGCTGTCGAACTTGG-3') elements of the proximal human aromatase PII promoter. Binding reactions were done using 10 μg of nuclear extract in 20 μl buffer [4 mM Tris-HCl (pH 7.9), 24 mM NaCl, 0.4 mM EDTA (pH 8.0), 0.4 mM dithiothreitol, 5 mM MgCl₂, 10% glycerol, and 1 μg polydeoxynucleosinic-deoxyribonucleic acid] for 1 h at 4°C. When using in vitro-translated GATA3 or LRH-1 protein, 2-μl aliquots of protein and 100 ng deoxyribonucleosinic-deoxyribonucleic acid were used. GATA- and LRH-1-containing complexes were subsequently analyzed by electrophoresis through a 4% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 200 V for 1.5 h at 4°C followed by autoradiography.

**Mammalian two-hybrid assay**

A protein-protein interaction assay between GATA3 and LRH-1 was studied using the CheckMate mammalian two-hybrid system (Promega). The C-terminal domain of LRH-1 (amino acids 250–495) was generated by PCR (oligo pair: 5'-ATGCGGCCGCGATGATTAAGCCCAAGCGAAGGC-3' and 5'-ATGCGGCCGCTTATGCTCTTTTGGCATGC-3') using the LRH-1 cDNA as template and the product was fused to the GAL4 DNA binding domain by cloning into the SalI/NotI sites of the pBIND vector. Similarly, the entire coding region of GATA3 was obtained by PCR (oligo pair: 5'-GGCTGCGACTATTTGAGAGGAGGACTACC-3' and 5'-ATGGGCGGCCAATACCCATGCGGATTGAC-3') using the GATA3 cDNA as template, and the product was fused with the VP16 activation domain by cloning into the SalI/NotI sites of the pACT vector. The different constructs, used alone or in combination, were transfected in HeLa cells (plated at an initial density of 2.0×10⁴ cells/well) along with the pG5lac reporter, which contains five copies of a GAL4 binding site driving expression of luciferase. Transfection conditions were as described above.

**In vitro kinase and glutathione-S-transferase (GST) pull-down assays**

*In vitro* kinase assays using GST-GATA3 and GST-LRH-1 fusion proteins were done as previously described (48, 54). The GST-GATA3 fusion protein was obtained by transferring the C-terminal portion (amino acids 301–443) of GATA3 generated by PCR (oligo pair: 5'-ATGCGGCCGCGATGATTAAGCCCAAGCGAAGGC-3' and 5'-GGCTGCGACTATTTGAGAGGAGGACTACC-3') using the GATA3 cDNA into the NotI/SalI sites of the pGEX-2T cloning vector (Amersham Biosciences). A similar approach was used to transfer the C-terminal domain of LRH-1 (amino acids 250–495) by PCR (oligo pair: 5'-ATGGGCGGCCAATACCCATGCGGATTGAC-3' and 5'-GGCTGCGACTATTTGAGAGGAGGACTACC-3') using the full-length LRH-1 cDNA as template. Fusion proteins were produced using the Escherichia coli strain BL21 and purified using glutathione resin (Amersham Biosciences). GST pull-down assays were carried out as previously outlined (48). The 35S-labeled GATA3 and LRH-1 proteins were obtained using the TnT in vitro transcription/translation kit (Promega).

**Results**

**GATA factors and LRH-1 are expressed in multiple breast cancer cell lines but by themselves are poor activators of human aromatase PII promoter activity**

Owing to its strong expression in both breast adipose and breast cancer tissues and its ability to activate the aromatase PII promoter, the LRH-1 transcription factor was identified as an important regulator of estrogen synthesis in the breast (27, 32). However, because LRH-1 is readily detected in normal breast tissue (32), it is unlikely that LRH-1 alone accounts for the up-regulation of aromatase PII promoter activity in breast cancer. As shown in Fig. 1A, the proximal aromatase PII promoter contains, in addition to LRH-1, species-conserved binding elements for members of the GATA family of transcription factors. In the human sequence, these corre-spond to nucleotide positions −180 bp (distal GATA element) and −170 bp (proximal GATA element). To begin to address the role of GATA factors in aromatase PII promoter activity in breast cancer cells, we screened a number of breast cancer cell lines (BT-20, MCF-7, T-47D, ZR-75–1) for GATA expression by RT-PCR (Fig. 1B). Although transcripts for GATA1, GATA2, GATA5, and GATA6 could not be detected (data not shown), the GATA3 and GATA4 genes were significantly expressed (Fig. 1B). LRH-1 transcripts were also readily detected in all four cell lines tested. Western blot analysis confirmed that the cell lines also express GATA proteins (Fig. 1C) that are capable of binding to the proximal GATA element of the human aromatase PII promoter (Fig. 1D). The GATA4 protein was present only at appreciable levels in BT-20 cells and is likely the reason for the two bands (both GATA3 and GATA4) observed in the EMSA when using extracts from these cells (Fig. 1D, first panel). The four breast cancer cell lines also expressed LRH-1 protein (Fig. 1C). The two LRH-1 protein bands detected were consistent with those reported for other cell lines and tissues (55). To assess the GATA dependence of the aromatase PII promoter, MCF-7 cells were cotransfected with a −430-bp human aromatase PII promoter-luciferase reporter (containing the consensus GATA and LRH-1 regulatory elements) along with expression vectors for GATA3, GATA4, or LRH-1 (Fig. 1E). Surprisingly, these factors by themselves were very poor activators of the human aromatase PII promoter in MCF-7 cells. Similar results were obtained with the other breast cancer cell lines (data not shown).

**The GATA- and LRH-1-mediated transactivation of the human aromatase PII promoter and potent synergism between the factors in breast cancer cells is PKA dependent**

In steroidogenic tissues, we and others have demonstrated that GATA factors act in synergy with the nuclear receptor SF-1 to activate multiple promoters (34, 56, 57). This includes the murine aromatase PII promoter (34). Because the SF-1 regulatory element is conserved in the human aromatase PII promoter and can be bound by LRH-1 (32), we hypothesized that a similar cooperation between GATA factors and LRH-1 might be responsible for the up-regulation of CYP19 transcription in breast tumors. However, as shown in Fig. 2A, cotransfection of either GATA3 or GATA4 and LRH-1 in MCF-7 cells produced little more in the way of aromatase PII promoter activation over the individual factors. It was therefore clear that an additional key component was missing. The fact that the aromatase PII promoter is a classic cAMP-responsive promoter regulated by the PKA signaling pathway was a strong hint that the elusive factor might be PKA itself. To test this hypothesis, MCF-7 cells were transfected with the −430-bp aromatase PII promoter-luciferase reporter along with expression vectors for GATA3, GATA4, and LRH-1 and then stimulated with either 10 μM forskolin (Fig. 2B) or cotransfected with an expression vector coding for a constitutively active PKA catalytic subunit (Fig. 2C). In both cases, overexpression of PKA or forskolin stimulation (which stimulates adenylate cyclase ultimately leading to endogenous PKA activation) not only enhanced the transcriptional properties of the individual factors on the human aromatase PII
GATA Regulation of the CYP19 Promoter

Fig. 2. PKA stimulates the transactivational properties of GATA3, GATA4, and LRH-1 and synergism between the factors on the human aromatase PII promoter. A, In the absence of forskolin treatment or PKA overexpression, GATA3 and GATA4 only weakly synergize with LRH-1 on the human aromatase PII promoter. The −430-bp human aromatase PII promoter was cotransfected in MCF-7 breast cancer cells with either an empty expression vector (control) or expression vectors for GATA3 (50 ng), GATA4 (50 ng), LRH-1 (100 ng), or a combination of GATA3 or GATA4 and LRH-1 as indicated. *, Significantly different from control, GATA3, GATA4, and LRH-1 groups (\( P < 0.05 \), Kruskal-Wallis ANOVA followed by Mann-Whitney \( U \) tests). B, Forskolin treatment markedly enhances GATA/LRH-1 synergism and the transcriptional activities of the individual factors on the −430-bp human aromatase promoter. MCF-7 cells were transfected as described in A but treated for 4 h before harvesting the cells with either 10 \( \mu \)M forskolin or dimethylsulfoxide as control (no treatment). C, A similar experiment was carried out using overexpression of PKA catalytic subunit-\( \alpha \) (25 ng) instead of forskolin treatment. For all transfection experiments, data are reported as fold activation over the empty (control) expression vector (±SEM). For B and C, groups with similar letters are not significantly different from one another (\( P > 0.05 \), Kruskal-Wallis ANOVA followed by Mann-Whitney \( U \) tests).

The transcriptional enhancement elicited by PKA or forskolin treatment (Fig. 2) suggests that phosphorylation of GATA factors and/or LRH-1 plays a key role in aromatase PII promoter activation in breast cancer cells. We previously demonstrated that the GATA4 transcription factor is indeed a target for PKA-mediated phosphorylation (48, 54). Using commercially available PKA catalytic subunit along with GST-GATA3 and GST-LRH-1 fusion proteins in in vitro ki-
for PKA phosphorylation (48). Because coexpression of PKA is essential for the dramatic synergism between GATA3 and LRH-1 on the human aromatase PII promoter (Fig. 2), PKA-mediated phosphorylation of GATA3 and/or LRH-1 likely alters the functional properties of one or both factors. We first studied DNA-binding (Fig. 5B). Pretreatment of in vitro-translated GATA3 or LRH-1 with PKA catalytic subunit and ATP, however, did not alter the DNA-binding properties of either factor (compare Fig. 5B, lanes 2 and 5 with lanes 3 and 6). Moreover, the stimulatory effects of PKA in transfection assays (Fig. 2) was not due to increases in GATA and/or LRH-1 expression because total protein levels for either protein, as assessed by Western blot, were not altered by the kinase (data not shown).

Because posttranslational modifications of transcription factors are known to affect the recruitment of transcriptional partners and/or cofactors, we next used GST pull-down assays to examine whether the direct physical interaction between GATA3 and LRH-1 could be enhanced by PKA-mediated phosphorylation (Fig. 5C). The effect of phosphorylation on protein-protein interaction was studied in both orientations: phosphorylation of GATA3 and interaction with untreated 35S-labeled LRH-1 (lanes 1–3) and conversely, phosphorylation of LRH-1 and interaction with untreated 35S-labeled GATA3 (lanes 4–6). In both cases, PKA-mediated phosphorylation markedly enhanced the interaction between the proteins (compare Fig. 5C, lanes 3 and 6 with lanes 2 and 5), suggesting that one of the roles of PKA in human aromatase PII promoter regulation in breast cancer cells is the enhancement of interactions between transcription factors acting directly at the level of the PII promoter.

We recently demonstrated that GATA4 is phosphorylated in a PKA-dependent manner at position Ser261 in gonadal cells, resulting in enhanced GATA4-dependent transactiva-
proteins that were phosphorylated (EMSA was performed using B, PKA does not affect GATA3 or LRH-1 DNA binding properties. Blue stain of the total protein used in each phosphorylation reaction. Upper panel, whereas the lower panel (input protein) is a Coomassie phosphorylated fusion proteins (phospho protein) are indicated in the proteins as described in A. Interaction between the two proteins. A, performed using 2 μg of either GST-GATA3 or GST-LRH-1 fusions proteins as described in Materials and Methods. The 32P-labeled phosphorylated fusion proteins (phospho protein) are indicated in the upper panel, whereas the lower panel (input protein) is a Coomassie blue stain of the total protein used in each phosphorylation reaction. B, PKA does not affect GATA3 or LRH-1 DNA binding properties. EMSA was performed using in vitro-translated GATA3 or LRH-1 proteins that were phosphorylated (+PKA) or not (−PKA) via an in vitro phosphorylation reaction described above but with unlabeled ATP. C, PKA-mediated phosphorylation enhances the physical interaction between GATA3 and LRH-1. The GST fusions proteins described in A were used in pull-down assays with either in vitro-translated 35S-labeled full-length LRH-1 or GATA3 proteins. To study the effect of PKA on the GATA3-LRH-1 protein-protein interaction, both proteins were phosphorylated using PKA catalytic subunit as described in B.

Fig. 5. PKA phosphorylates GATA3 and LRH-1 and enhances the interaction between the two proteins. A, In vitro kinase assays were performed using 2 μg of either GST-GATA3 or GST-LRH-1 fusions proteins as described in Materials and Methods. The 32P-labeled phosphorylated fusion proteins (phospho protein) are indicated in the upper panel, whereas the lower panel (input protein) is a Coomassie blue stain of the total protein used in each phosphorylation reaction. B, PKA does not affect GATA3 or LRH-1 DNA binding properties. EMSA was performed using in vitro-translated GATA3 or LRH-1 proteins that were phosphorylated (+PKA) or not (−PKA) via an in vitro phosphorylation reaction described above but with unlabeled ATP. C, PKA-mediated phosphorylation enhances the physical interaction between GATA3 and LRH-1. The GST fusions proteins described in A were used in pull-down assays with either in vitro-translated 35S-labeled full-length LRH-1 or GATA3 proteins. To study the effect of PKA on the GATA3-LRH-1 protein-protein interaction, both proteins were phosphorylated using PKA catalytic subunit as described in B.

The human LRH-1 promoter is a target for GATA factors in breast cancer cells

Because the mouse LRH-1 promoter has been shown to be activated by multiple GATA factors in liver cell lines (59), we hypothesized that expression of the LRH-1 factor, which cooperates with GATA factors on the aromatase PII promoter, could itself be a target for GATA factors in breast cancer cells. The human LRH-1 promoter, much like its mouse counterpart, contains at least three consensus GATA binding motifs in its proximal promoter region (Fig. 7). To begin to study the GATA-dependent regulation of this promoter, we cotransfected MCF-7 cells with a −460-bp human LRH-1 promoter-luciferase reporter along with expression plasmids for GATA3 or GATA4 in the presence or absence of PKA catalytic subunit. As shown in Fig. 7, GATA3 and especially GATA4 could activate the human LRH-1 promoter in MCF-7 cells. Similar results were also obtained with the other breast cancer cell lines, and the level of activation observed was similar, whether GATA3 and GATA4 were transfected alone or in combination (data not shown). As described for the human aromatase PII promoter, the transcriptional activation mediated by both GATA3 and GATA4 on the human LRH-1 promoter was markedly enhanced by coexpression of PKA catalytic subunit. The difference in transcriptional response elicited by GATA3 vs. GATA4 likely reflects a differential requirement of each protein for transcriptional cofactors rather than differences in DNA-binding because both proteins possess similar DNA-binding properties (60, 61).
FIG. 6. PKA-dependent enhancement of GATA3, GATA4, and LRH-1 transcriptional activities and GATA/LRH-1 synergism on the human aromatase PII promoter involve specific phosphoacceptor sites. A, Amino acid sequence alignments reveal the presence of species-conserved PKA phosphorylation sites in the mouse GATA3 (Ser308), rat GATA4 (Ser261), and human LRH-1 (Ser469) proteins. Zn, Zinc finger; LBD, ligand binding domain; DBD, DNA-binding domain. B, To test whether these serine residues were important for PKA responsiveness, the individual serines were mutated into alanines by site-directed mutagenesis. The ability of these mutant proteins (GATA3 S308A, GATA4 S261A, LRH-1 S469A) to respond to PKA and transcriptionally cooperate on the human aromatase PII promoter were then assessed by cotransfection experiments in MCF-7 cells. MCF-7 cells were cotransfected with the 430-bp human aromatase PII promoter, PKA catalytic subunit-α (25 ng), and expression vectors for the wild-type or mutated GATA3 (50 ng), GATA4 (50 ng), or LRH-1 (100 ng) proteins used alone or in combination as indicated. Data are reported as fold activation over the empty expression vector (± SEM). Luc, Luciferase. *, Significantly different from the respective wild-type (black bar) counterparts (P ≤ 0.05, Mann-Whitney U tests). C, The wild-type and mutated GATA and LRH-1 proteins are expressed at similar levels. Western blot (WB) detection of 10 μg of nuclear extract was obtained by overexpression of the recombinant proteins in HeLa cells.
regulated on adipocyte differentiation (45). Consistent with a role in adipogenesis, overexpression of both factors leads to a suppression of adipocyte differentiation due in part to suppression of peroxisome proliferator-activated receptor-\(\gamma\) transcription via an interaction between GATA and C/EBP family members directly at the level of the peroxisome proliferator-activated receptor-\(\gamma\) promoter (64). Thus, one potential role of GATA factors in breast tumors is to keep preadipocytes (which is a major source of aromatase activity) in an undifferentiated state. Because we previously demonstrated that the murine aromatase PII promoter is activated by GATA factors in a gonadal context (34), we now propose a second, yet equally important, role for GATA factors in the up-regulation of CYP19 expression in breast cancer cells. In this study, we have focused predominantly on GATA3 because it is the major GATA factor shown to be up-regulated in breast tumors (40, 41, 46) and expressed in breast cancer cell lines (Fig. 1) (33, 41, 46). Because all members of the GATA factor family recognize the same consensus binding motif in target promoters (60, 61), all vertebrate GATA proteins, including GATA2 (a marker of preadipocytes), should have the same capacity to activate the human aromatase PII promoter as we have described for GATA3 and GATA4.

The activation of the aromatase PII promoter in breast tumors is likely the result of aberrant expression of transcriptional activators acting directly on activation elements within the PII promoter. Indeed, previous studies with the LRH-1 and C/EBP\(\beta\) transcription factors have been shown to follow this general mechanism, and our current data with GATA factors is no exception. A previous study by Jin et al. (33), however, proposed that GATA factors (and in particular GATA4) modulate aromatase PII promoter activity in breast cancer cells by derepressing a silencer element, which they termed S2. The GATA binding motifs described in their S2 silencer region are the same as those described in this study (Fig. 1A). The difference is that their promoter studies were done using an artificial thymidine kinase reporter, whereas ours were done using the native human aromatase PII promoter. Although the mechanisms proposed are different, it is important to point out that the end result, up-regulation of CYP19 transcription, is the same.

The switch in aromatase promoter usage to the cAMP-dependent gonad type PII promoter in breast tumors suggests that transcriptional regulatory mechanisms present in the gonads are also active in breast cancer cells. In heterologous cells, the murine aromatase PII promoter is strongly activated by GATA factors, alone and in a synergistic cooperation with SF-1 (34). We were therefore surprised to find that in MCF-7 cells, GATA3 or GATA4, used alone or in combination with LRH-1 (the homolog of SF-1 present in breast tumors), did not transactivate the human aromatase PII promoter (Figs. 1E and 2A). The aromatase PII promoter, however, has been shown to be induced in response to tumor-derived factors such as PGE\(_2\) via cAMP and subsequent activation of PKA (25, 27). Therefore, we surmised that aromatase promoter PII activation involving GATA factors might be dependent on PKA. The fact that GATA factors are phosphorylated in different tissues by different signaling pathways (65–72), including the cAMP/PKA signaling pathway in steroidogenic cells of the gonads (48), strongly sup-

![Fig. 7. GATA3 and GATA4 are strong activators of the human LRH-1 promoter in breast cancer cells. MCF-7 cells were cotransfected with a -460- to +50-bp human LRH-1 promoter construct and either an empty expression vector (−) or expression vectors for GATA3 (50 ng) or GATA4 (50 ng) in the absence or presence of an expression vector for PKA catalytic subunit-\(\alpha\) (25 ng). Results are shown as fold activation over the empty (control) expression vector (± SEM). Groups with similar letters are not significantly different from one another (\(P > 0.05\), Kruskal-Wallis ANOVA followed by Mann-Whitney \(U\) tests). Luc, Luciferase.](image-url)

**Discussion**

The majority of breast cancer cases present estrogen receptor-positive tumors that are dependent on local estrogen production for growth (62). Because elevated estrogen levels in breast tumors are due to abnormally high P450 aromatase (CYP19) activity, a better understanding of the molecular mechanisms that are responsible for aberrant CYP19 expression is key for the development of future therapeutic strategies. In breast cancer, up-regulation of CYP19 expression is the result of a switch from the adipose-specific promoter I.4 to the gonad-type, cAMP-regulated PII promoter. To date, the aromatase PII promoter has been studied by many laboratories and has been shown to be regulated by different signaling pathways and multiple transcription factors, most notably LRH-1 and C/EBP\(\beta\) (27, 32, 35). Although these studies have provided important insights into the transcriptional regulation of the CYP19 gene in breast cancer, the picture is far from complete. In the present study, we now identify members of the GATA family of transcription factors as new players in the regulation of aromatase PII promoter activity in breast cancer cells.

GATA factors are important regulators of cell fate specification, cell differentiation, and tissue-specific gene expression in many systems (36, 37, 39, 63). They are also markers of various cancers including those of the esophagus; stomach; adrenal; gonads; and, interestingly, the breast (40–44, 46). In white adipose tissue, expression of GATA2 and GATA3 is restricted to preadipocytes and markedly down-
ported this possibility. Indeed, we found that human aromatase PII promoter activation in breast cancer cells by GATA3 or GATA4, whether alone or in cooperation with LRH-1, was strictly dependent on forskolin treatment (Fig. 2B) or exogenously expressed PKA (Fig. 2C). Interestingly, LRH-1 transcriptional activity on the human aromatase PII promoter was also markedly up-regulated by either forskolin or PKA (Fig. 2, B and C). This increase was comparable with the increase in LRH-1 activity observed in LRH-1-transfected 3T3-L1 cells stimulated with PKA and phorbol 12-myristate 13-acetate (32). This suggests that LRH-1, much like GATA3 or GATA4, is a target for PKA in breast cancer cells. Our in vitro kinase assays (Fig. 5A), showed that LRH-1 is indeed a substrate for PKA-mediated phosphorylation.

We previously mapped a novel phosphorylation site (Ser261) in the GATA4 protein that is targeted by the PKA phosphorylation pathway in gonadal cells (48). We now show that this site also contributes to the PKA-mediated enhancement of GATA4 transcriptional activity on the human aromatase PII promoter in breast cancer cells (Fig. 6). To our knowledge, similar PKA phosphorylation sites have not yet been mapped in the GATA3 and LRH-1 proteins. By aligning the GATA3 and LRH-1 amino acid sequences across several different species, we identified potential PKA phosphorylation sites at Ser308 (corresponding to the mouse GATA3 protein) and Ser469 (corresponding to the human LRH-1 protein) that were also crucial for PKA-mediated enhancement of transcriptional activity of either factor on the human aromatase PII promoter in breast cancer cells. The Ser308 amino acid present between the two zinc fingers of GATA3 is in fact the equivalent of Ser261 in the GATA4 protein, whereas Ser469 present in the LRH-1 protein has not yet been described in the SF-1 protein. Interestingly, only the LRH-1 point mutant (LRH-1 S469A) decreased synergism between GATA factors and LRH-1. This suggests a mechanism whereby maximal aromatase PII promoter is achieved via phosphorylation of all factors involved but especially LRH-1.

Because PKA action was crucial for GATA/LRH-1-dependent activation of the human aromatase PII promoter, the next logical question to answer was: what is the role of PKA? Posttranslational modifications of transcriptional factors, including phosphorylation, are well known to affect the transcriptional properties of the target factors such as DNA-binding. In our case, however, the DNA-binding properties of GATA3 and LRH-1 were not altered by pretreatment with PKA (Fig. 5B). Phosphorylation of transcription factors, including members of the GATA and NR5A families of proteins, are also known to modulate the recruitment of other transcriptional partners or coactivators (48, 54, 73). We found that PKA significantly enhanced the interaction between GATA3 and LRH-1 (Fig. 5C), suggesting that one of the roles of PKA is to favor a stronger GATA-LRH-1 protein-protein interaction and resulting transcriptional synergism. Because the LRH-1 S469 phosphorylation mutant had a detrimental effect on GATA3/LRH-1 transcriptional synergism on the human aromatase PII promoter (Fig. 6B), we immediately tested whether this same mutation could prevent the enhanced GATA3/LRH-1 interaction elicited by PKA. This enhanced interaction, however, was maintained with the LRH-1 S469A mutant (data not shown). This suggests that although the LRH-1 Ser469 amino acid is required for max-

![Fig. 8. Proposed mechanism of action of GATA transcription factors in the control of aromatase gene expression in human breast cancer tumors. PGE₂ produced by the tumor activates PKA via the cAMP signaling pathway. PKA phosphorylates GATA3, GATA4, and LRH-1 in either tumorous epithelial or undifferentiated adipose stromal cells. cAMP-dependent aromatase PII promoter activity is then up-regulated through two different mechanisms: direct binding of GATA factors to the aromatase promoter and a synergistic interaction (=) between GATA and LRH-1. In this model, GATA factors have a dual role in driving breast tumor aromatase expression because LRH-1 promoter activity is itself markedly up-regulated by GATA factors. EP receptor, Prostaglandin E<sub>2</sub> receptor; G, G protein; AC, adenylate cyclase.](image-url)
imal GATA3/LRH-1 synergy on the human aromatase PII promoter, the PKA-dependent enhancement of a GATA3/LRH-1 interaction involves more than one or possibly other amino acids that are also targets for PKA. Another possibility is that phosphorylation of LRH-1 Ser469 may be required for the recruitment of other, as-yet-unidentified cofactors or transcriptional partners involved in human aromatase PII promoter activation.

The proximal LRH-1 gene promoter contains at least three consensus GATA binding motifs that are conserved between mouse and human. GATA factors are positive regulators of LRH-1 transcription in hepatic cells and perhaps human preadipocytes in which GATA expression coincides with LRH-1 expression (32, 45). We now show that the human LRH-1 promoter is also activated by GATA factors in breast cancer cells (Fig. 7). Interestingly, treatment of cultured human preadipocytes with forskolin or PGE2 has been reported to induce LRH-1 expression (27, 74). Because this induction was concomitant with the increase in CYP19 expression via promoter PII, LRH-1 gene induction has been proposed to occur through a mechanism whereby tumor-derived factors such as PGE2 (acting in part via the cAMP/PKA signaling pathway) turn on aromatase expression in breast tumors (27, 74). Our present data, showing that GATA-mediated activation of the human LRH-1 promoter is strongly enhanced by PKA (Fig. 7), would favor such a mechanism. However, because GATA factors are broadly expressed, it is unlikely that they alone would account for LRH-1 transcription in breast tumors and other adipose tissues. Indeed, LRH-1 promoter activity has been reported to be regulated by multiple transcription factors, at least in hepatocytes (59).

Taken together, our results identify GATA factors, acting in concert with LRH-1, as potentially important regulators of CYP19 expression in breast tumors. As presented in Fig. 8, we propose a model whereby they contribute to increased aromatase PII promoter-driven transcription through two complementary mechanisms: first, by direct binding of GATA factors (which are targets of the cAMP/PKA signaling pathway) to the aromatase PII promoter, and second, through a synergistic cooperation with LRH-1 whose promoter is also a target for regulation by PKA and GATA factors. Furthermore, the mechanism described here might also have implications for CYP19 expression in the gonads, such as granulosa cells of the ovary, in which LRH-1 and GATA factors (GATA4 and GATA6) are strongly expressed (75–78).

Acknowledgments

We thank Drs. Luc Belanger (Laval University, Quebec, Canada) and Doug Engel (University of Michigan, Ann Arbor, MI) for generously providing materials used in this study.

Received February 15, 2005. Accepted August 9, 2005.

Address all correspondence and requests for reprints to: Dr. Robert S. Viger, Ontogeny and Reproductive Research Unit, T1–49, Centre Hospitalier de l’Universite´ Research Centre, 2705 Laurier Boulevard, Ste-Foy, Quebec, Canada G1V 4G2. E-mail: robert.viger@crchul.ulaval.ca.

This work was supported by a research grant from the Cancer Research Society Inc. R.S.V. holds the Canada Research Chair in Reproduction and Sex Development.

References


10. Esteban JM, Warsi Z, Griendling KK, Hall P, Shively JE, Chen S 1996 Aromatase expression in breast cancer cells (Fig. 7). Interestingly, treatment of cultured human preadipocytes with forskolin or PGE2 has been reported to induce LRH-1 expression (27, 74). Because this induction was concomitant with the increase in CYP19 expression via promoter PII, LRH-1 gene induction has been proposed to occur through a mechanism whereby tumor-derived factors such as PGE2 (acting in part via the cAMP/PKA signaling pathway) turn on aromatase expression in breast tumors (27, 74). Our present data, showing that GATA-mediated activation of the human LRH-1 promoter is strongly enhanced by PKA (Fig. 7), would favor such a mechanism. However, because GATA factors are broadly expressed, it is unlikely that they alone would account for LRH-1 transcription in breast tumors and other adipose tissues. Indeed, LRH-1 promoter activity has been reported to be regulated by multiple transcription factors, at least in hepatocytes (59).

Taken together, our results identify GATA factors, acting in concert with LRH-1, as potentially important regulators of CYP19 expression in breast tumors. As presented in Fig. 8, we propose a model whereby they contribute to increased aromatase PII promoter-driven transcription through two complementary mechanisms: first, by direct binding of GATA factors (which are targets of the cAMP/PKA signaling pathway) to the aromatase PII promoter, and second, through a synergistic cooperation with LRH-1 whose promoter is also a target for regulation by PKA and GATA factors. Furthermore, the mechanism described here might also have implications for CYP19 expression in the gonads, such as granulosa cells of the ovary, in which LRH-1 and GATA factors (GATA4 and GATA6) are strongly expressed (75–78).

Acknowledgments

We thank Drs. Luc Belanger (Laval University, Quebec, Canada) and Doug Engel (University of Michigan, Ann Arbor, MI) for generously providing materials used in this study.

Received February 15, 2005. Accepted August 9, 2005.

Address all correspondence and requests for reprints to: Dr. Robert S. Viger, Ontogeny and Reproductive Research Unit, T1–49, Centre Hospitalier de l’Universite´ Research Centre, 2705 Laurier Boulevard, Ste-Foy, Quebec, Canada G1V 4G2. E-mail: robert.viger@crchul.ulaval.ca.

This work was supported by a research grant from the Cancer Research Society Inc. R.S.V. holds the Canada Research Chair in Reproduction and Sex Development.


40. Bouchard C