Steroid Receptor Coactivator-3 Is Required for Progesterone Receptor Trans-activation of Target Genes in Response to Gonadotropin-releasing Hormone Treatment of Pituitary Cells*

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Regulation of gonadotropin production involves interplay between steroids and neuropeptides, and we have examined the effects of gonadotropin-releasing hormones (GnRH I and GnRH II) on progesterone receptor (PR) activation in αT3-1 pituitary cells. Treatment with GnRHs activated a progesterone response element (PRE)-luciferase reporter gene, and this was blocked by protein kinase C and protein kinase A inhibitors but not by RU486. Treatment with GnRHs phosphorylated the PR at Ser294 and increased PR translocation to the nucleus within 1 h. Interactions between the PR and several coactivators were examined, and treatment with GnRHs specifically induced PR-steroid receptor coactivator-3 (SRC-3) interactions within 8 h. In chromatin immunoprecipitation assays, recruitment of PR and SRC-3 by the PREs of the luciferase reporter gene or the gonadotropin-α-subunit gene promoter was also increased by GnRHs within 8 h, while progesterone-induced recruitment of PR to the PREs occurred in association with much less SRC-3. A small interfering RNA knockdown of type I GnRH receptor levels reduced PR activation by GnRHs, while progesterone-dependent PR activation was unaffected. Moreover, small interfering RNA knockdown of SRC-3 abolished PRE-luciferase trans-activation by the PR in response to GnRHs. Collectively, these data indicate that PR activation by GnRHs in αT3-1 cells is type I GnRH receptor-mediated and that trans-activation of PR-responsive genes requires SRC-3 in this context.

The hypothalamic-pituitary-gonadal axis is regulated by a complex interplay between neuropeptides and steroid hormones. Ovarian steroids such as estradiol (E2) and progesterone (P4) exert positive and negative feedback controls at both hypothalamic and pituitary levels (1). The effects of P4 are mediated primarily through its binding to the progesterone receptor (PR), which belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors (2–4). Although the PR is encoded by a single gene, it has two distinct isoforms (PR-A and PR-B), generated by alternative promoter usage (5). In general, PR-B is transcriptionally active on most progesterone-responsive promoters, whereas PR-A acts in a more context-restricted manner and can antagonize the effects of PR-B (6). After binding to a progesterone response element (PRE), the receptors modulate target gene transcription by recruiting components of the transcriptional machinery directly or indirectly by interactions with coregulatory proteins (7), including members of the steroid receptor coactivator (SRC) family: SRC-1, SRC-2 (GRIP1/TIF2), and SRC-3 (ACTR/pCIP/RAC3/AIB1/TRAM1) (8). These coactivators serve as adaptors that potentiate the transcriptional activity of different steroid receptors through conserved NR box motifs (9), while their transcriptional activation domains mediate interactions with histone-modifying enzymes (10–12). Biochemical and protein-protein interaction studies suggest that SRCs function as components of large multiprotein complexes that integrate inputs from multiple signaling pathways (13).

The activation of PR and other nuclear hormone receptors was initially considered to be entirely steroid-dependent (14). However, non-steroidal agents including dopamine, growth factors, and protein kinase A (PKA) activators also stimulate steroid receptor activities in a ligand-independent manner (15, 16). While nuclear hormone receptor phosphorylation has been implicated in these ligand-independent events, they are not fully understood and may be effected at several levels (17). Nevertheless, steroid receptors are phosphorylated on multiple sites in response to hormone or kinase-signaling pathways (17, 18, 19).
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18), and the PR is phosphorylated, primarily on serine residues (19).

Hypothalamic release of gonadotropin-releasing hormone (GnRH I) plays a pivotal role in pituitary gonadotropin secretion (20, 21). In pituitary gonadotrope cells, binding of GnRH I to its G protein-coupled receptor (GnRH I) initiates the protein kinase C (PKC)/mitogen-activated protein kinase signaling pathway to stimulate transcription of the gonadotropin α-subunit gene (22). A second form of GnRH (GnRH II) exists (23), which acts on reproductive tissues (24) and promotes human chorionic gonadotropin secretion by cytotrophoblastic cells (25). However, its mechanisms of action and physiological significance are poorly understood.

The classical “type I” GnRH receptor mediates the actions of GnRH in the pituitary but is present in many other cell types throughout the body. Although a second “type II” GnRH receptor has also been identified in some primates (26, 27), the gene encoding this receptor is inactivated or deleted from the genome of other mammals, including humans and mice (28).

Cross-talk between the PR and GnRH I has been implicated in a GnRH I self-priming mechanism in the pituitary (29, 30), which is defined as an enhanced LH secretion by pituitary gonadotropes in response to a second stimulation by GnRH I (31). This response appears to depend upon the capacity of estrogens to induce PR expression in gonadotropes (31), but it is completely absent in PR knock-out mice (32). It has therefore been suggested that activation of type I GnRH in gonadotropes, which ultimately activates the PR in a ligand-independent manner (33). However, the mechanisms responsible for GnRH I self-priming and the ligand-independent activation of the PR by GnRHs in gonadotropes are still unclear. We have therefore explored the effects of GnRH I and GnRH II on ligand-dependent and ligand-independent activation of the PR in αT3-1 mouse pituitary cells, in which GnRH I and II act via a common type I GnRH receptor.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The gonadotrope-derived clonal αT3-1 cell line was provided by Dr. P. L. Mellon (Department of Reproductive Medicine, University of California, San Diego, CA). The αT3-1 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Burlington, Canada) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air. The cells were passaged when they reached about 90% confluence using EDTA solution (0.05% trypsin, 0.5 mM EDTA).

Plasmids and siRNAs—A PRE-luciferase reporter plasmid containing two copies of a consensus PRE upstream of the thymidine kinase promoter, was provided by Dr. D. P. McDonnell (Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC). Plasmid DNAs for transfection studies were prepared using Qiagen plasmid maxi kits following the manufacturer’s suggested procedure. The concentration and integrity of DNA were determined by measuring absorbance at 260 nm and agarose gel electrophoresis, respectively. The siRNA (5'-TGACGGTTGCATTGTGACACCCATCTTCAAGGAGAAGTGCAATGCAGC-3') for type I GnRH receptor was produced using pSuper.gfp/neu vector. Two siRNAs for SRC-3 (siSRC-3(a) (5'-UUACUCUGCUUUCUUUGGCC) and siSRC-3(b) (30)) were obtained from Qiagen (Chatsworth, CA).

PRE-luciferase Reporter Gene Assays—Transient transfections were performed using FuGENE 6.0 (Roche Diagnostics, Quebec, Canada) following the manufacturer’s procedure. To correct for the transfection efficiencies of various luciferase constructs, the Rous sarcoma virus (RSV)-lacZ plasmid was co-transfected into cells. Briefly, 4 × 105 αT3-1 cells were seeded into six-well tissue culture plates 1 day before transfection in 2 ml phenol red-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% charcoal-dextran-treated fetal bovine serum (HyClone Laboratories, Inc.), which was used as standard culture medium in all experiments unless indicated. One microgram of the PRE-luciferase reporter plasmid and 0.5 μg of RSV-lacZ were dissolved in 100 μl of standard culture medium containing 3 μl of FuGENE 6.0 without serum. The DNA mixture was incubated for 45 min at room temperature and then applied to the cells. Incubation of the cells with transfection medium continued for 24 h at 37 °C in 5% CO2 and a further 48 h in culture medium with F12 (2 × 10-10 M) prior to treatments with GnRHs (I or II) or P4. The cellular lysates were collected with 150 μl of reporter lysis buffer and assayed for luciferase activity, and β-galactosidase activity to normalize transfection efficiencies, with commercially available reagents (Promega Corp., Nepean, Canada). Promoter activities were calculated as the luciferase activity/β-galactosidase activity.

Immunoprecipitation and Western Blot Analysis—The cells were washed twice with ice-cold phosphate-buffered saline and solubilized in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40) at 4 °C for 30 min. Endogenous PRs were immunoprecipitated from transfected cell extracts with PR antibody (10 μg/ml) for 1 h at 4 °C, followed by incubation with protein A-magnetic beads (BioLabs, Inc., Ipswich, MA) for 1 h at 4 °C. The beads were washed three times with lysis buffer. The PR-bound proteins were released by incubating the beads in SDS-PAGE sample buffer containing mercaptoethanol (5 min, 95 °C) and were subjected to electrophoresis on a 10% SDS-PAGE gel. The separated proteins were transferred electrophoretically onto Hybond-C (Amer sham Biosciences, Morgan, Ontario, Canada). The resulting Western blots were blocked with Tris buffered saline (20 mM Tris-Cl, pH 7.4, 500 mM NaCl, 0.1% Tween 20) containing 5% (w/v) nonfat milk for 2 h before addition of antibodies. Antibodies were obtained from Upstate, Lake Placid, NY (SRC-1, catalogue number 05-522; GRIP-1, catalogue number 06-986; SRC-3, catalogue number 05-490), Neomarker, Fremont, CA (Ser298, catalogue number sc-13124; type I GnRH, catalogue number MS-1139), or Santa Cruz Biotechnology, Inc., Santa Cruz, CA (pCAB, catalogue number sc-13124; gonadotropin α, catalogue number sc-18224). The anti-PR (phospho-Ser400) antibody was provided Dr. C. A. Lange (Department of Medicine, University of Minnesota). Incubation with primary and secondary antibodies and washing of blots were performed in Tris-buffered saline with 0.1% Tween 20. The ECL (Amer sham Bio-
sciences, Bucks, UK) was used for detection, and signals were visualized by exposure to Kodak X-Omat film.

**Immunocytochemistry**—Monolayer-cultured αT3-1 cells were grown in standard culture medium without serum for 16 h and subsequently incubated with serum or hormones (see figure legends for details). After stimulation, the cells were washed in phosphate-buffered saline and fixed in 4% paraformaldehyde for 15 min. Endogenous peroxidase was blocked with 0.35% H$_2$O$_2$ for 10 min, and fixed cells were incubated with anti-PR antibody. For a negative control, the first antibody was replaced with the same concentration of rabbit IgG. Detection of the primary antibody was performed with an ABC peroxidase staining kit (DakoCytomation, Corp., Carpinteria, CA).

**Chromatin Immunoprecipitation (ChIP) Assay**—Unless otherwise stated, all reagents, buffers, and supplies were included in a ChIP-IT$^\text{TM}$ kit (Active Motif, Inc., Carlsbad, CA). Briefly, the αT3-1 cells were cross-linked with 1% formaldehyde for 10 min at room temperature. After washing and treatment with glycine Stop-Fix solution, the cells were re-suspended in lysis buffer and incubated for 30 min on ice. The cells were homogenized, and nuclei were re-suspended in shearing buffer and subjected to optimized ultrasonication conditions to yield 100–400-bp DNA fragments. The chromatin was pre-cleared with protein G beads and incubated (overnight at 4°C) with 1 µg of the following antibodies: negative control mouse IgG, anti-PR, and anti-SRC-3. Protein G beads were then added to the antibody/chromatin incubation mixtures and incubated for 1.5 h at 4°C. After extensive washings, immunoprecipitated DNA was removed from the beads in an elution buffer. To reverse cross-links and remove RNA, 5 M NaCl and RNase was added to the samples and incubated for 4 h at 65°C. The samples were then treated with proteinase K for 2 h at 42°C, and the DNA was purified using gel exclusion columns. The purified DNA was subjected to PCR amplification (1 cycle of 94°C for 3 min; 40 cycles of 94°C for 20 s; 64°C for 30 s and 72°C for 30 s) of the PRE-luciferase promoter using specific forward (5’-AGAACCTTTGCTTGGTTCG-3’) and reverse (5’-AATAG-CAGACACTCTATGCCC-3’) primers and the PRE within the proximal promoter of the gonadotropin α-subunit gene (MGI: 88390) using forward (5’-ATGGAATATATAGAACACA-3’) and reverse (5’-CATTATTTCAACGAGAACCAG-3’) primers. As an input control, 10% of each chromatin preparation was used. The PCR products were resolved by electrophoresis in a 2.5% acrylamide gel and visualized after ethidium bromide staining.

**Data Analysis**—Data are presented as the mean ± S.D. and were analyzed by analysis of variance followed by Tukey’s multiple comparison test. $p < 0.05$ was considered statistically significant.

**RESULTS**

**Transactivation of PR by GnRH I and GnRH II in αT3-1 Cells**—The ability of GnRHs to activate PR-mediated transcription in αT3-1 cells was studied in the absence or presence of P4. In our initial experiments, αT3-1 cells were transfected with the PRE-luciferase reporter plasmid and then treated with either GnRH I or II ($10^{-7}$ M) alone or with P4 ($10^{-7}$ M) alone. Under these conditions, P4 increased the transcriptional activity of PR in a time-dependent manner with maximal activation at 24 h, while GnRH I and II showed maximal effects on PR activation at 8 h (Fig. 1A). When these effects of GnRH I and II were studied in the presence of $10^{-7}$ M P4, this resulted in a synergistic increase in PR trans-activation of the reporter plasmid after an 8 h treatment (Fig. 1B).

These initial experiments led us to suspect that the temporal difference in stimulation of PR by GnRHs and P4 could be attributed to PR acting through ligand-independent and ligand-dependent pathways, respectively. To explore this, cells were cotreated with PKA (H89), or PKC inhibitors (staurosorpin and GF109203X), a PR antagonist (RU486), or a GnRH I receptor antagonist (Antide). This showed that cotreatments with staurosorpin, GF109203X, H89, and Antide completely blocked the trans-activation of the PR that was mediated by GnRHs, while RU486 did not (Fig. 2). By contrast, activation of the PR by P4 was blocked completely by RU486 under the same conditions (data not shown).

**Treatment with GnRHs Affects PR Phosphorylation and Subcellular Distribution**—The majority of PR phosphorylation sites contain a Ser-Pro consensus sequence for proline-directed kinases (34). Since PKC and PKA inhibitors reduced the transcriptional activity of the PR, we investigated whether the PR is phosphorylated by GnRHs or P4. Ser$^{295}$ and Ser$^{300}$ of the human PR are conserved in the murine PR and are hyperphos-
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FIGURE 3. Regulation of PR phosphorylation at Ser294 by GnRHs. The αT3-1 cells expressing endogenous PR-A and PR-B isoforms were treated with 10⁻⁷ m GnRH I, GnRH II, or P4 for 1–8 h. Equal amounts of cell lysates (100 µg) were electrophoresed on SDS-7% PAGE gels, transferred to nitrocellulose, and Western blotted using antibodies specific for either PR-A and PR-B (upper panel), phospho-Ser294 PR (middle panel), or actin as a control (lower panel). Control (C) represents untreated cells at time 0.

phorylated in response to ligand and mitogens (18, 34). Moreover, Ser400 phosphorylation mediates ligand-independent transactivation of the CDK-2 gene by the human PR (17).

To investigate the regulation of PR phosphorylation in αT3-1 cells, antibodies that recognize both isoforms of PR (PR-A and PR-B) as well as antibodies against phosphorylated-PR at Ser294 or Ser400 were used in Western blotting experiments (Fig. 3). This demonstrated that both PR-A and PR-B isoforms are present in αT3-1 cells, but we were unable to detect PR phosphorylated at Ser400 in these cells. Low levels of Ser294-phosphorylated PR-B were detected, while phosphorylation of PR-A at this site was essentially undetectable. Although phosphorylation of mouse PR-B at Ser294 in αT3-1 cells tended to increase at 1–4 h following treatment with GnRH I or GnRH II (Fig. 3), the increases are not significant (p < 0.08). By contrast, there was no increase in PR-B phosphorylation at this site after P4 treatment within this time frame.

Since phosphorylation has been reported to influence the cellular distribution of the PR (35), the subcellular localization of PR was examined after treatment with GnRHs over a period of 24 h (Fig. 4). When αT3-1 cells were cultured for 16 h in serum-free medium, the PR was predominantly cytoplasmic (Fig. 4A), while immunoreactive PR is located predominantly in the nucleus of αT3-1 cells cultured in the presence of serum (Fig. 4B). Importantly, when the cells in serum-free medium were treated with 10⁻⁷ m GnRH I or GnRH II, the PR accumulated in the nucleus within 1 h (Fig. 4, C and D), and this persisted up to 24 h (data not shown).

Treatment with GnRHs Promotes PR Interactions with SRC-3 and Their Corecruitment to PREs of Target Genes—To examine whether the PR associates with specific coactivators in αT3-1 cells after treatment with GnRHs, we immunoprecipitated cell lysates with anti-PR antibody and then immunoblotted the released proteins with antibodies to various coactivators. As shown in Fig. 5A, there was no increase in the co-immunoprecipitation of pCAF, SRC-1, or SRC-2 with the PR after cells were stimulated with GnRH I or GnRH II. By contrast, both GnRHs increased interaction of the PR with SRC-3, and this again was most apparent at 4–8 h after treatment with GnRH I.

Since P4 binding to the PR promotes its interaction with SRC-3 (11), we further compared a P4-dependent and ligand-independent (i.e. GnRH-mediated) recruitment of SRC-3 by PR in αT3-1 cells after 8 h of treatment. Under these conditions, none of the hormones influence the total level of either PR-A or PR-B. Although the amount of SRC-3 that immunoprecipitates with PR after GnRH I treatment increased to about the same extent as that observed after P4 treatment, there was a more modest increase in the ligand-independent interactions between SRC-3 and the PR after GnRH II treatment (Fig. 5B).

Since SRC-3 possesses histone acetyltransferase activity, which effects chromatin remodeling and transcription (36), we explored the possibility that GnRH I or II treatments influence PR-mediated assembly of SRC-3 at PREs within target genes by ChIP assays. For this purpose, we first used the same synthetic PRE containing reporter gene construct that was introduced by transfection efficiency, and PRE-reporter gene activities were expressed in terms of luciferase activity/β-galactosidase activity. Control cells were not treated with GnRHs. Bars represent S.D.
which was much greater than the recruitment of SRC-3 to this site after an 8-h treatment with P4. Interestingly, although treatment with either GnRHS caused a similar level of PR recruitment to this PRE, the increase in SRC-3 recruitment to this site after GnRH II treatment was not as effective as after GnRH I treatment, but it was still greater than observed after P4 treatment (Fig. 6A).

To determine whether GnRHS can regulate an endogenous target gene (21) in this way, we used ChIP assays to evaluate the recruitment of the PR and SRC-3 to the PRE within the gonadotropin α-subunit gene promoter in αT3-1 cells (Fig. 6B). In this experiment, we observed a very rapid (within 1 h) recruitment of PR to this PRE after treatment with GnRH I or GnRH II, and this was more pronounced and longer lasting after GnRH I treatment. However, both GnRHS appear to recruit more PR to this site than after treatment with P4. Interestingly only GnRH I treatment resulted in a clear and consistent increase in recruitment of SRC-3 to the gonadotropin α-subunit gene PRE (Fig. 6B).

To confirm that GnRH treatments influence gonadotropin α-subunit gene expression under these conditions, we performed a Western blot to measure gonadotropin α-subunit levels in the cells. This showed that GnRH I and GnRH II both increase the cellular gonadotropin α-subunit content within the same time frame, i.e. 8 h after treatment (Fig. 6C), while P4 does not (data not shown). These results are in line with the changes in recruitment of PR and SRC-3 to the PRE within the gonadotropin α-subunit gene promoter after treatment with GnRHS and P4 (Fig. 6B) and our observations that GnRH I consistently enhances transactivation of target genes by PR to a greater extent than GnRH II.

Type I GnRH Receptor and SRC-3 Are Required for GnRH-mediated PR Activation—Since mouse αT3-1 cells only possess the type I GnRH receptor, we used siRNA to decrease its expression in order to determine if it mediates the ligand-independent transactivation of the PR by GnRH I or GnRH II in these cells (Fig. 7). A Western blot demonstrated that the siRNA treatment very effectively decreased type I GnRH receptor levels prior to the introduction of the PRE-luciferase reporter gene (Fig. 7). When these cells were then treated with GnRH I or GnRH II, the PRE-reporter gene was reduced substantially over that observed in cells that contain normal levels of the type I GnRH receptor (Fig. 7). In this context, it should also be noted that the siRNA-induced loss of type I GnRH receptor had no influence on the ligand (P4)-dependent transactivation of the PRE-reporter gene (Fig. 7). These data confirm
that the type I GnRH receptor mediates the ligand-independent activation of the PRE-reporter gene by GnRH I and GnRH II.

We also used siRNAs to explore whether SRC-3 is essential for the GnRH-induced trans-activation of the PRE-luciferase reporter gene. Transfection of αT3-1 cells with two siRNAs resulted in substantial decreases in the cellular content of SRC-3, as shown by Western blotting, with a greater decrease being observed with the siSRC-3(b) treatment (Fig. 8A). The results of this experiment are particularly important because they demonstrate that loss of SRC-3 has a much greater impact on the rapid (within 8 h), ligand-independent effects of the GnRHS on PRE-luciferase reporter gene activation, as compared with the ligand (P4)-dependent transactivation of the PR within this same time frame. In fact treatment with siSRC-3(b) completely blocked the ligand-independent transactivation of the PRE-luciferase reporter by both GnRHS acting either alone (Fig. 8A) or in synergy with P4 (Fig. 8B).

DISCUSSION

The main function of GnRH I in the pituitary is to promote gonadotropin secretion (20, 21). In female rats, sequential treatments of GnRH I enhance substantially the production of gonadotrophins (33), and this self-priming effect is thought to involve the PR because it is absent in PR knock-out mice (32). Moreover, this effect has been reported to be due to the ligand-independent activation of the PR by GnRH I in primary pituitary cell cultures (33). We confirmed this latter observation by using an established mouse pituitary cell line (αT3-1 cells) and have also shown that GnRH II promotes the ligand-independent activation of the PR in these cells. Although both GnRHS function rapidly in this context, i.e. within 8 h, GnRH I consistently evoked a more robust response than GnRH II. However, our data indicate that the ligand-independent activation of the PR by both GnRHS is mediated via the type I GnRHR and involves the PKA and PKC pathways.

Numerous studies have indicated that the ligand-independent activation of nuclear hormone receptors, including the PR, involve an alteration in the phosphorylation of the receptors themselves (17, 18, 37) or their various coregulatory proteins...
Treatment with GnRHs, and this again was most evident after GnRH I treatment. Thus, PR phosphorylation and its translocation to the nucleus appear to occur prior to its increased association with SRC-3.

To explore the relevance of GnRH-induced interactions between PR and SRC-3 in relation to the ligand-independent activation of PR responsive genes, we performed ChIP assays to examine the loading of PR and SRC-3 onto PREs within the promoters of a transiently transfected reporter gene, as well as the most relevant endogenous GnRH-responsive gene in the pituitary (i.e. the gonadotropin α-subunit gene). In these assays, a rapid and robust recruitment of both PR and SRC-3 to the multiple PREs within the PRE-luciferase reporter gene was observed after GnRH I treatment. Although GnRH II increased recruitment of PR to this PRE, it occurred in concert with much less SRC-3 than that observed after GnRH I treatment. However, in both cases, treatments with GnRHs elicited a more robust response than that observed after the ligand-dependent recruitment of PR to the PRE. In the context of the PRE within the gonadotropin α-subunit gene promoter, it appears that GnRH I and GnRH II treatments cause a very rapid (within 1 h) loading of the PR onto the PRE, which in the case of GnRH I treatment clearly occurs prior to the recruitment of SRC-3. However, again treatments with either GnRHs caused a much more robust recruitment of PR to the endogenous PRE within 8 h of treatment, as compared with P4 treatment. The relevance of these effects was confirmed by showing that GnRH treatments increased the expression of the gonadotropin α-subunit gene within the same time frame.

In these ChIP assays, substantially more SRC-3 appeared to be recruited to PREs by GnRHs than that observed after P4 treatment, while the PR-SRC-3 interactions observed in co-immunoprecipitation assays showed a similar pattern after treatments with both GnRHs and P4. As suggested by recent studies (40, 41), multiple cellular signaling pathways phosphorylate SRC-3 and regulate the activities of steroid receptors. It is therefore possible that GnRHs increase phosphorylation of SRC-3, and this induces recruitment of SRC-3 to PREs in a ligand-independent manner.

To demonstrate that SRC-3 plays a pivotal role in the GnRH-induced ligand-independent activation of the PR in αT3-1 cells, we substantially reduced their SRC-3 levels using an siRNA approach. These studies clearly indicated that loss of SRC-3 from the cells essentially eliminates the ability of GnRH I and GnRH II to activate the PRE-luciferase reporter gene in the absence or presence of P4. In this context, it also appears that loss of SRC-3 effects the ligand-independent activation of the reporter gene by GnRHs more effectively than that observed after P4 treatment. This suggests a qualitative difference in the transcriptional complexes that assemble at the PREs in response to the ligand-dependent versus ligand-independent activation of the PR.

Taken together, our studies indicate that the self-priming of gonadotropin gene expression in pituitary cells by GnRH is mediated via the type I GnRHR. More importantly, we show that treatment of αT3-1 cells with GnRHs, and GnRH I in particular, results in rapid changes in PR phosphorylation and its translocation to the nucleus, where it interacts with PREs fol-
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followed by the recruitment of SRC-3. We also provide evidence that the interaction between the PR and SRC-3 is essential for the ligand-independent trans-activation of the PR in response to GnRH I and GnRH II treatment.

REFERENCES

1. Shupnik, M. (1996) Biol. Reprod. 54, 279–286
2. Sartorius, C. A., Melville, M. Y., Howland, A. R., Tung, L., Takimoto, G. S., and Horwitz, K. B. (1994) Mol. Endocrinol. 8, 1347–1360
3. McDonnell, D. P., Clement, D. L., Herrmann, T., Goldman, M. E., and Pike, J. W. (1995) Mol. Endocrinol. 9, 659–669
4. McDonnell, D. P., Shabazz, M. M., Vegeto, E., and Goldman, M. E. (1994) J. Steroid Biochem. Mol. Biol. 48, 425–432
5. Kastner, P., Krust, A., Turcotte, B., Stropp, U., Chambon, P., and Gronemeyer, H. (1998) Nat. Rev. Endocrinol. 4, 117–128
6. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Peeters, B., and Chambon, P. (1999) EMBO J. 18, 254–265
7. Wu, R.-C., Smith, C. L., and O’Malley, B. W. (2005) Mol. Cell. Biol. 25, 7738–7747
8. Powers, R. F., McFadden, G., and O’Malley, B. W. (1998) Endocrinology 139, 1911–1921
9. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) EMBO J. 16, 2053–2058
10. Wang, X., Carroll, D. L., and O’Malley, B. W. (1995) Mol. Cell. Biol. 15, 3275–3282
11. Qiu, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Endocrinology 144, 1303–1310
12. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
13. Schnick, M., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) EMBO J. 22, 777–789
14. Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) EMBO J. 16, 5093–5103
15. Qiu, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
16. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
17. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
18. Zhang, Y., Beck, C., Poletti, A., Edwards, D., and Weigel, N. (1995) Mol. Endocrinol. 9, 1029–1040
19. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
20. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
21. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
22. Zhang, Y., Beck, C., Poletti, A., Edwards, D., and Weigel, N. (1995) Mol. Endocrinol. 9, 1029–1040
23. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
24. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
25. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
26. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
27. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
28. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
29. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
30. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
31. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
32. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
33. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
34. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
35. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
36. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
37. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
38. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
39. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
40. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095
41. Qi, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003) Mol. Endocrinol. 17, 628–642
42. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
43. Labriola, L., Salatino, M., Prieto, C. J., Privalovsky, M., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 4084–4095