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Differential Roles for Signal Transducers and Activators of Transcription 5a and 5b in PRL Stimulation of ERα and ERβ Transcription

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PRL has been shown to stimulate mRNA expression of both ERα and ERβ in the rat corpus luteum and decidua of pregnancy. To investigate whether PRL may stimulate ER expression at the level of transcription and which transcription factors may mediate this stimulation, we have cloned the 5′-flanking regions of both rat ER genes. A constitutively active PRL receptor (PRL-R_{CA}) stimulated both ERα and ERβ promoter activity, indicating that PRL is acting to stimulate ER transcription. Putative signal transducer and activator of transcription (Stat)5 response elements were identified at −189 in the ERα promoter and at −330 in the ERβ promoter. Mutation of these response elements or overexpression of dominant negative Stat5 prevented stimulation of ERα and ERβ promoter activity, indicating that PRL regulation of ER expression requires both intact Stat5 binding sites as well as functional Stat5. Interestingly, either Stat5a or Stat5b could stimulate ERα transcription while stimulation of ERβ occurred only in the presence of Stat5b. Through mutational analysis, a single nucleotide difference between the ERα and ERβ Stat5 response elements was shown to be responsible for the lack of Stat5a-mediated stimulation of ERβ. These findings indicate that PRL stimulation of ER expression occurs at the level of transcription and that PRL regulation of ERα can be mediated by either Stat5a or Stat5b, while regulation of ERβ appears to be mediated only by Stat5b. (*Molecular Endocrinology* 15: 2172–2181, 2001)

IN THE PREGNANT rat, E2 is a potent tropic hormone, which stimulates both progesterone biosynthesis and luteal cell hypertrophy (1). However, the stimulatory effect of E2 depends upon previous exposure of the corpus luteum to PRL or PRL-related hormones from placental origin (2). This prerequisite was shown to be due to PRL stimulation of E2 binding activity and mRNA levels for both ERα and ERβ (2, 3). In addition to the corpus luteum, PRL has been shown to stimulate E2 binding activity or mRNA levels in the rat decidua (4), mammary gland (5), and liver (6). The mechanism of PRL action on ER expression, however, is not known.

Although PRL has been shown to activate multiple signaling pathways, including MAPK (7–11), PKCδ (12), c-src (13–16), and PI3K (17–20), the major and most comprehensively studied pathway activated by PRL is the janus kinase 2/signal transducer and activator of transcription 5 (Jak2/Stat5) pathway. PKCδ may be involved in PRL regulation of relaxin expression in the rat corpus luteum (12) whereas PI3K and/or MAPK may regulate PIM-1 expression in Nb2 cells (11). However, the mechanisms of by which PRL regulates gene expression through these pathways are not fully understood. In contrast, PRL has clearly been shown to regulate gene transcription through the Jak2/Stat5 pathway. This pathway has been implicated in the regulation of numerous genes by PRL, including milk proteins in the mammary gland (21), α2-macroglobulin in the corpus luteum (22), sodium-dependent bile acid cotransporter in rat liver (23), the CIS gene promoter in COS cells (24), the 3β-hydroxysteroid dehydrogenase gene promoter in HeLa cells (25), the PRL receptor (PRL-R) gene in insulin-producing INS-1 cells (26), and the aP2 promoter in NIH-3T3 cells (27).

In the general Jak/Stat signaling paradigm, the PRL-R dimerizes upon ligand binding (28). This causes activation of the tyrosine kinase, Jak2, which undergoes autophosphorylation and subsequently phosphorylates the receptor on tyrosine residues (28, 29). The phosphorylated tyrosines on the receptors and Jak2 become docking sites for the SH2 domains of

Abbreviations: CA-5a, CA-5b, Constituitively activated Stat5a and Stat5b; DN-5a, dominant negative Stat5a; β-gal, β-galactosidase; GAS, γ-interferon-activating sequence; Jak2, janus kinase 2; Mut-5b, mutant Stat5b; PRL-R, PRL receptor; PRL-R_{CA}, constitutively active PRL-R; Stat5, signal transducer and activator of transcription 5; WCE, whole-cell extracts.

Stat proteins (30, 31). Jak2 can thus phosphorylate and activate the recruited Stat proteins. The phosphotyrosine residues on the Stat proteins can serve as docking sites for the SH2 domain of another Stat protein so that Stats can either homo- or heterodimerize and translocate to the nucleus (32). By binding to cognate response elements located upstream of their responsive genes, Stat proteins can interact with basal transcriptional machinery and thereby regulate transcription (33).

Two forms of Stat5, Stat5a and Stat5b, were shown to transduce PRL signaling (33, 34). Although encoded by different genes, they are approximately 95% homologous at the protein level. These proteins contain a single conserved tyrosine residue in the C terminus (Y694 in Stat5a and Y699 in Stat5b), which becomes phosphorylated by Jak2 in response to PRL and is necessary for regulation of gene transcription (33, 34). Both Stat5a and Stat5b recognize the same DNA binding site, or GAS site (γ-interferon-activating sequence; TTCNNNGAA), and can mediate PRL-induced transcription (34). Stat5a and Stat5b have been knocked out, either independently or together, and several key differences between these two transcription factors were observed (35, 36). Without Stat5a, mammary gland maturation and function is impaired, while male patterns of liver function appear to be disrupted when Stat5b is absent (35–37). The reproductive phenotype in these mice is not clear. In the single Stat5a or Stat5b knockouts, no reproductive defects were observed, while the double knockout was infertile (36). However, in another Stat5b knockout, the ability to maintain pregnancy was reduced (35).

One of the major functions of PRL in luteal function is to stimulate ER expression and thereby maintain luteal responsiveness to E2 (2). To examine whether this stimulation occurs at the level of transcription, a 2-kb genomic fragment of the rat ERβ promoter region was isolated and sequenced. The rat ERα promoter was also cloned. Sequence analysis has revealed that both contain putative Stat5 response elements. Both promoters were found to be stimulated by PRL and to require intact Stat5 binding sites and functional Stat5. However, PRL stimulation of ERβ could be mediated by Stat5b only. The lack of ERβ responsiveness to Stat5a was found to be due to a single nucleotide difference in the ERβ Stat5 response element.

RESULTS

Our laboratory has previously shown, using semiquantitative RT-PCR, that PRL stimulates both ERα and ERβ mRNA levels in corpora lutea of pregnant rats and in primary cultures of luteinized granulosa cells (3). Since this method does not provide information as to the differential levels of expression between two genes, we have used quantitative real-time RT-PCR. Known amounts of rat ERα and ERβ cDNA were used to generate standard curves for analysis of mRNA levels in experimental samples amplified in parallel reactions (Fig. 1A). In corpora lutea on d 7 of preg-

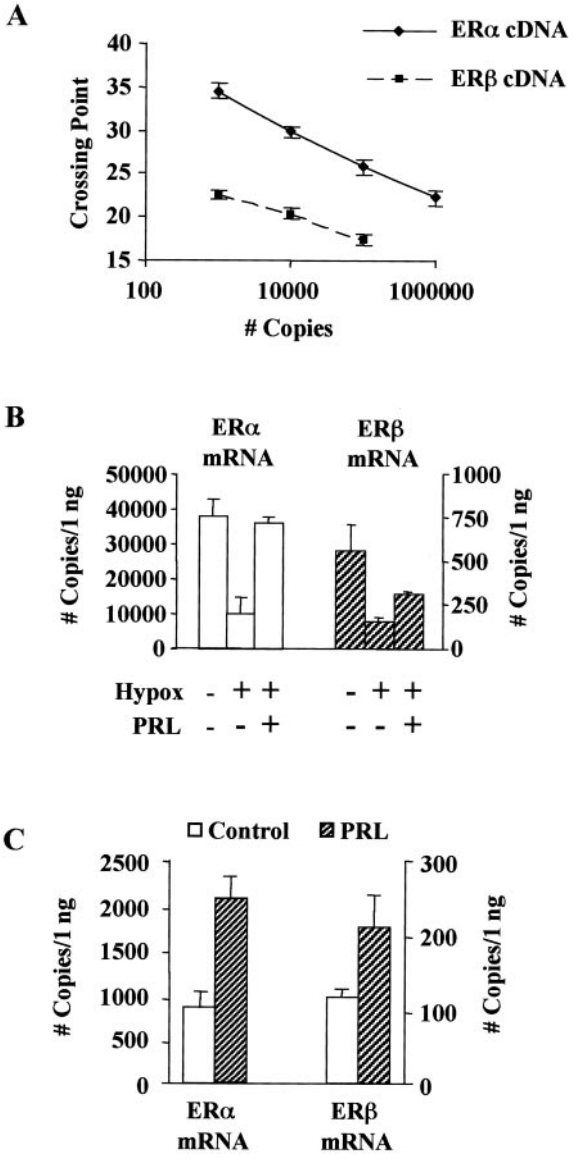

Fig. 1. Standard Curves for Real-Time Quantitative PCR

A, Known amounts of ERα and ERβ cDNA, ranging from 10^3 to 10^7 copies/μl and 10^3 to 10^6 copies/μl, respectively, were amplified as described in *Materials and Methods*. The crossing point represents the number of cycles required to reach a chosen level of fluorescence, at which all standards and samples were in the linear range of amplification. The crossing point was then plotted against the number of copies of cDNA/μl, and the linear regression equation through the data points was used to determine the number of copies of ERα or ERβ in reverse transcribed RNA samples. B, Real-time quantitative PCR was carried out for ERα and ERβ using mRNA from corpora lutea of pregnant rats, hypophysectomized rats, and hypophysectomized rats treated with PRL. C, Real-time quantitative PCR was carried out for ERα and ERβ using mRNA from primary luteinized granulosa cells that had been treated with 1 μg/ml PRL for 12 h.

nancy, approximately 45,000 copies of ERα and 550 copies of ERβ were detected in samples corresponding to 1 ng of RNA (Fig. 1B). In rats, hypophysectomized on d 3 of pregnancy, ERα and ERβ levels were reduced to approximately 30% of the control levels. Sustained treatment with PRL induced a 3.5-fold induction of ERα expression and a 2-fold induction of ERβ expression. In contrast to corpora lutea, in which there was approximately 70 times more ERα than ERβ, luteinized granulosa cells cultured for 72 h expressed only 8 times more ERα than ERβ, with approximately 850 copies of ERα and 120 copies of ERβ per ng of starting RNA (Fig. 1C). After a 12-h treatment with PRL, a 2.4-fold increase in ERα and a 1.8-fold increase in ERβ expression was observed. These findings confirm that PRL can stimulate both ERα and ERβ mRNA expression and further demonstrate a much higher level of ERα than ERβ in corpora lutea of pregnancy and in primary luteinized granulosa cells.

To investigate whether PRL can regulate ER expression at the level of transcription, the ERα and ERβ 5′-flanking regions were cloned, and promoter-reporter constructs were prepared as described in *Materials and Methods*. A putative Stat5 response element (5′-TTCTAGGAA-3′), which represents a perfect consensus Stat5 binding site, was located at −180 bp in the ERα promoter region. In addition, a putative Stat5 response element (3′-TTCTGGTAA-5′) with one nucleotide difference (*underlined*) from the consensus sequence was located at −330 bp in the ERβ promoter region (Fig. 2). CHO cells were transfected with either the ERα or ERβ promoter-luciferase reporter constructs (ERα-luc, ERβ-luc) together with an expression vector for a constitutively active PRL-R (PRL-R_{CA}) (38). Control cells were transfected with an expression vector for PRL-R_L, which, in the absence of any exogenous PRL treatment, served as a control. The active PRL-R has previously been shown to signal to the β-casein promoter in much the same way as PRL acting through the long form of its receptor but in the absence of exogenous PRL treatment (38). The presence of PRL-R_{CA} caused a marked phosphorylation of both Stat5a and Stat5b (Fig. 3A) and induced a 10-fold stimulation of ERα-luc activity (Fig. 3B, *left*) and a 5-fold stimulation of ERβ-luc activity (Fig. 3B, *right*), indicating that PRL can induce phosphorylation of Stat5a and Stat5b as well as regulate both ERα and ERβ expression at the level of transcription.

To examine whether PRL-R_{CA} activation of Stat5 was required for its stimulatory effect on ERα and ERβ promoter activity, CHO cells were transfected with expression vectors for a dominant negative Stat5a (DN-5a) or a mutant Stat5b (Mut-5b). The DN-5a contains a C-terminal deletion in the transactivation domain and is transcriptionally inactive (39). The Mut-5b contains a four-amino acid substitution in the DNA binding domain, which prevents it from entering the nucleus, binding DNA, and therefore regulating tran-

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-2023  AAAAAAATTT ATTATTTATT TTTATGTGTA TGGTGTTTTG CTGGCATGTG TGTAAGTAGA TGTACCATAT
-1953  ATCGCCGTGC ATGGAGGCTG AGAAGACAGT ATTGGTTCCC CTGGGCATGG AGTCAACGAG GACTGGAGTT
-1883  ACAGGCAATT GTGAGCCACC ATGTGGGTGC TGGAACTGAG CCCAGACCTT ATGGAAACCC TTTCGCTTTC
-1813  CTTGGGTTCT GTGGACTAAA CTCAGGTCTG TCAGCTAGCT TGGCTGGTCC ATTTATTGGT TGAGCCATCC
-1743  TGGCCTCCCT CCAGATGAAT CTTGTTAACA AGTACCATTA AATGAACTGA TTGTTTAAGA CCTCTACTTC
-1673  AACTTTGCTA CCCAAGGCAG AAGGAGGAAG GACTGAGTGA TACAGGACAC ACAGTCTCTC GGGAAAGGGT
-1603  ACAGGGATGT GCAGATTGGG GAGTACCAAT CTCACCCTTT CCTTTCCTCA CCGGAATGGT CTCAGTAGGT
-1533  AGCCCCTCTC CTGTCCACTC TACCTTTCCT AACCCTCAAG GATGACATTT TACCTTATAA ATGGGGAAGA
-1463  GTACAGGACT CTTAGCTTAA CTGAAACGTG CACATAAGAC GATGGGGGCA GTGAGGTTTG GACGCTTTGT
-1393  ATAGTTTTAA AGTAGGTTCT TTTCACATGT GTAGTCTGTG GCGCTACCTG TGGGACTCAT AAACCCAGGC
-1323  CTCAGGAAGG TCCCCAAAGG CATTTTGGTT ACACTGAAAT GTTGAGGTTG GCGTTGTACA AGTTTCCCTC
-1253  CAAATACCCC TTGGTTTTCT TCTATTACAT TTCCATCCAT CAAGAAACAC ATTCCGGGCC TGAAGAGACA
-1183  GCTCCGCTGT TAAGATAGCA AGCTGCTCTT GCATAGAAGC TGAGTTCTGC CCCAGCACCC ATGCCCAGGA
-1113  GGGAATCCCA CACCTATATT CTCCGTGGGC ATCTGCACGC GTGCCCTCAA GCCCACGTGT GCATAATTTA
-1043  AAAGATAAAA AGTAGATCTT TGGGGCTGGA GAGATGGCTC GAGAGGTTAA GAGCACTGGC TGCTCTTCCA
-973   GAGATTCTGA GTTCAAATCC CAGCAACCAC ATGGTGGCTT CGAACCATCT GTAATGGGAT CCGGTGCCCT
-903   CTTCTGGTGT GCTCGAAGAC AGCTATAGTG TACTCATTAA ATAAATCTTT AAAAAAAAAA AGTAGATCTT
-833   TGAAACCCTT CATAAACGCC CCATGATGTG TGCGGATGGC CTGACCACTA ATTAGGTACA CGAGTGACAC
-763   CAGGTCGGCC TACAGGTCAT TTGTTTTCCT TTTAACTTTT GCCACCACAG ACTGCCTTAA AGCCAATTGT
-693   TTCTTAAAAA AAAACAAAAA AACAAAAAAA CAAAAAAACA AAAAACTAAG GCGTGATCCC TGCCTTTGTA
-623   GAGTAGGGTC ACTTCAGGTT AACGCTGTGG AGCTGGGAGG ATCCGAGCTG CCATCAGGGG GCGCCAGAGA
-553   CCCTTTGGGA CAAGGAAGAG AGGAGCCCTG TAAAAGTGTA GGCAGATTTT TTCAGGTGAA TTCCCTCGTT
-483   TTTTTTTTTT TCTCTCCACT TCCATCGCCA GCCGCTGGTA GACACGCATT CAAACCTCCG CCTCCAGCCC
-413   TTGCCTTTCT GGGGCTCAAG CTAACACTCC CTGGAAAGGG ATCTGATTTC ATTACTCCGT TATTACTGCT
                    STAT5  RE
-343   TATTTCGGTG CTATTACCAG AACCCGGGGC CTGGCCCATG CTTCTTAAGG AGAGTTGTCA GACCTAAGCA
-273   TTTAATGACA CGCGCGTGGG TCAAAGTATG GAAACAAGAT GAAGTGTGGT CCCTGGACGC ACGCAGTCAA
-203   GTCCAGAATT CCTGGGGATG GTCAGAAATT CCCATTCCCG GGCTCCATCT GGTTCTCCAT CGTCAGTCTC
-133   TTGGAAGGGG GGTGTCCCTA GTGGATGACT GTGAAGAGGC TGGAGGTCGA CATCCAGTGG ATCCGGTTGC
-63    GCGGCGCGGG AGTGCCTGAA ATCTCCGGCT CCGGGTTTGT CACGTGGACT TAGCTACCCT CCCAGACTCT
+8     TTTCTAGGTC TTTAAAAGAC GCACTAACAT CCGTTAGTC                          +1
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Fig. 2. Rat ERβ Gene Promoter Region

The rat ERβ 5′-flanking region was cloned as described in *Materials and Methods*. This regulatory region of the ERβ gene spans from −2,023 bp to +46 bp and contains a putative Stat5 response element at −330 bp (*underlined* and *labeled*). The second gene-specific primer used for cloning the ERβ promoter is *underlined*.

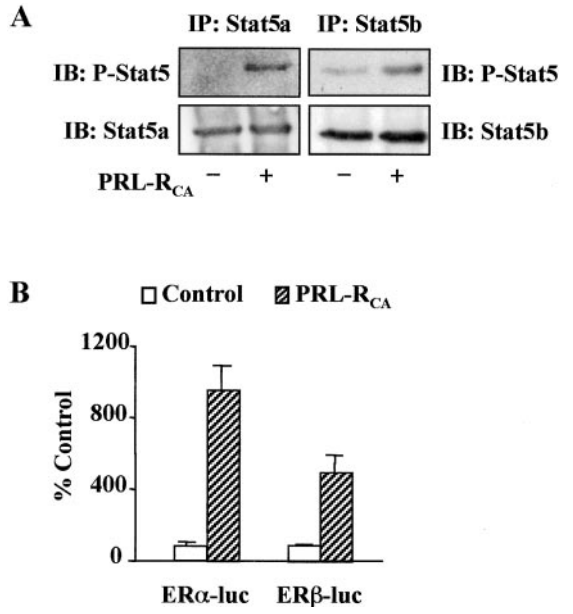


Fig. 3. Effect of PRL-R$_{CA}$ on Stat5 Phosphorylation and ERα and ERβ Promoter Activity in CHO Cells

A, CHO cells were cultured in six-well plates and transfected with 2 μg/well expression vectors for PRL-R$_{CA}$ or PRL-R$_L$ (control). Forty-eight hours after the start of transfection, WCE were prepared and Stat5a and Stat5b were immunoprecipitated using specific antibodies to each form of Stat5. Western blotting was performed first using an antibody, which recognizes the tyrosine-phosphorylated form of both Stat5a and Stat5b (Y694 and Y699, respectively). The blots were stripped and reprobed using the same antibodies for immunoprecipitation. B, Cells were transfected as described in panel A with the addition of 0.5 μg/well ERα-luc or ERβ-luc. Luciferase activity was measured in each well and normalized to the total protein level within that well. The experiment was repeated three times with triplicate wells for each group. The data represent the combined mean ± SEM for all three experiments.

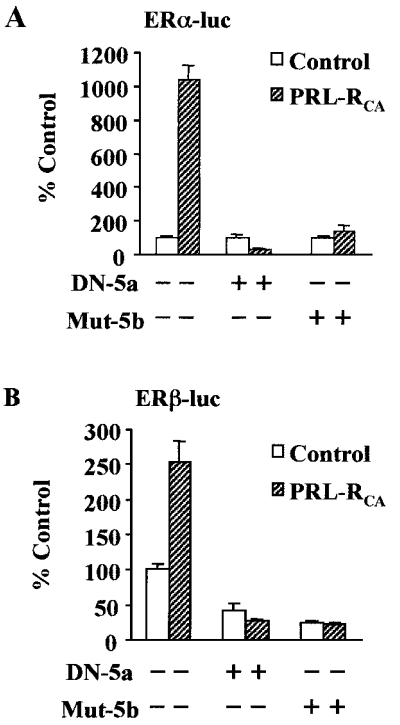


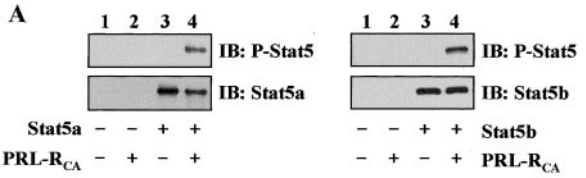
Fig. 4. Effect of Dominant Negative Stat5 on PRL-R$_{CA}$ Stimulation of ERα and ERβ Promoter Activity in CHO Cells

CHO cells were cultured and transfected as described for Fig. 3. In addition, each well was transfected with 1 μg/well DN-Stat5a or Mut-Stat5b. Luciferase activity was measured in each well and normalized to the total protein level within that well. The experiment was repeated three times with triplicate wells for each group. The data represent the combined mean ± SEM for all three experiments.

scription (40, 41). Both of these Stat5 expression vectors prevented PRL-R$_{CA}$ stimulation of ERα and ERβ promoter-driven luciferase activity (Fig. 4), indicating that PRL-R$_{CA}$ regulation of both ERα and ERβ transcription requires a functional Stat5.

The ability of PRL-R$_{CA}$ to regulate ERα-luc and ERβ-luc was also studied in COS cells. In contrast to CHO cells, COS cells express very low levels of endogenous Stat5a and Stat5b, which could not be detected by Western analysis (Fig. 5A, lanes 1 and 2). When COS cells were transfected with expression vectors for either Stat5a or Stat5b, high levels of Stat5 expression were detected and both became phosphorylated in response to PRL-R$_{CA}$ (Fig. 5A, lanes 3 and 4). We next examined the capacity of each Stat5 to transactivate the ERα and ERβ promoters. As shown in Fig. 5B, ERα promoter activity was stimulated 2-fold by PRL-R$_{CA}$ in the presence of either Stat5a or Stat5b. In contrast, ERβ promoter activity was stimulated by PRL-R$_{CA}$ only in the presence of Stat5b (Fig. 5B). In the presence of Stat5a, PRL-R$_{CA}$ had little or no effect on ERβ-driven reporter activity. These findings provide additional evidence that PRL can regulate ER expression at the level of transcription. In addition, it appears that ERα is responsive to either Stat5a or Stat5b while ERβ may be responsive only to Stat5b.

Because these studies in both CHO and COS cells consist of a reconstructed PRL signaling pathway, we questioned whether PRL utilizes the same pathway to regulate expression of the endogenous ERα and ERβ genes. To address this question, primary luteinized granulosa cells were used. PRL is known to induce phosphorylation of Stat5 in these cells; however, it is not known whether this is due to Stat5a or Stat5b activation (22, 42). Primary cells were cultured for 72 h and then treated with PRL for 5 min. After immunoprecipitation and Western blotting, it was found that primary luteinized granulosa cells express both Stat5a and Stat5b and both become highly phosphorylated in response to PRL (Fig. 6A). To examine the possibility that Stat5a and Stat5b can differentially affect stimulation of endogenous ERα and ERβ mRNA, primary luteinized granulosa cells were transfected with expression vectors for constitutively active Stat5a or Stat5b (CA-5a or CA-5b). These constitutively active Stat5s were generated by random mutagenesis and



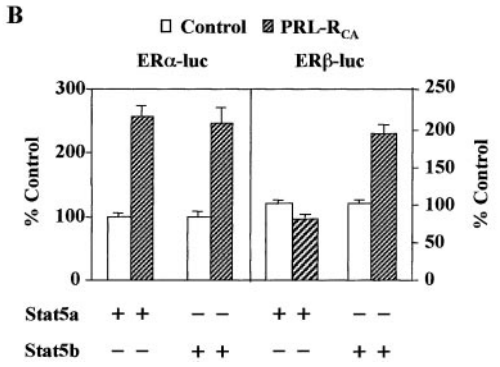
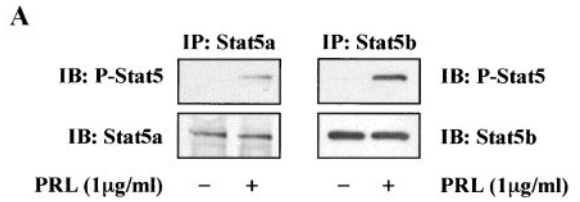


Fig. 5. Effect of PRL-R_{CA} on Stat5 Phosphorylation and ERα and ERβ Promoter Activity in COS Cells

A, WCE were prepared from COS cells transfected with 1 μg/well Stat5a (*left panels*) or 1 μg/well Stat5b (*right panels*) and 2 μg/well PRL-R_{CA} or PRL-R_L (control). Western blotting was performed as described for Fig. 3. B, Luciferase activity was measured in COS cells that had been transfected with either 0.5 μg/well ERα-luc (*left*) or ERβ-luc (*right*), 0.5 μg/well β-gal, 1 μg/well Stat5a or Stat5b, and 2 μg/well PRL-R_{CA} or PRL-R_L (control). Luciferase activity was normalized to the β-gal activity within that well. The experiment was repeated seven times with triplicate wells for each group. The data represent the combined mean ± SEM for all seven experiments.



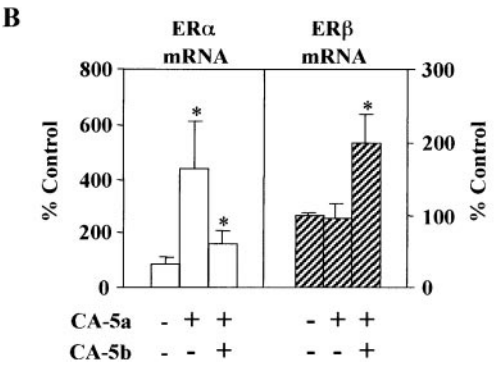


Fig. 6. Stat5 Phosphorylation and Regulation of ERα and ERβ mRNA in Primary Luteinized Granulosa Cells

A, Primary luteinized granulosa cells were cultured for 72 h and then treated with PRL (1 μg/ml) for 5 min. Immunoprecipitation and Western blotting were performed as described for CHO cells. B, Primary luteinized granulosa cells were transfected with expression vectors for CA-5a or CA-5b (1 μg/well). Thirty-six hours after the start of transfection, total RNA was isolated and quantitative real-time RT-PCR was carried out for ERα and ERβ as described in *Materials and Methods* and Fig. 1. The data for the CA-5a and CA-5b groups were normalized to the level of ERα or ERβ mRNA expression in the control group and presented here as the mean ± SEM for six different transfected wells for each group. *, $P < 0.05$ compared with empty vector control as determined by ANOVA followed by Dunnett's multiple range test.

found to contain two mutations, one in the DNA binding domain (H299R) and one in the transactivation domain (S711F). They have been shown to be constitutively phosphorylated, located in the nucleus, and capable of binding DNA and regulating gene transcription in the absence of any cytokine stimulation (43). CA-5a induced a 4-fold stimulation of endogenous ERα mRNA while CA-5b stimulated ERα mRNA expression 2-fold (Fig. 6B). In contrast, ERβ expression was stimulated 2-fold only in the presence of CA-5b. These results confirm our findings that Stat5b, and not Stat5a, can mediate regulation of ERβ while stimulation of ERα can be mediated by either Stat5a or Stat5b.

Because both ER promoters contain putative Stat5 response elements, we next examined whether these were essential for regulation by PRL. Mutations were made to each promoter so that the Stat5 response elements were no longer capable of binding Stat5. Stimulation of both ERα and ERβ promoter-driven luciferase activity by PRL-R_{CA} was completely prevented by mutation to the Stat5 response elements (Fig. 7), indicating that PRL stimulation of ER expression requires intact Stat5 DNA binding sites. Because the ERα and ERβ promoter Stat5 response elements contain a 1-bp difference (GAA for ERα and TAA for ERβ), we next investigated whether this single nucleotide could explain the differential responsiveness of ERα and ERβ to Stat5a. A single nucleotide mutation was made to the ERα promoter (ERα-M_1) so that it resembled the ERβ response element (GAA to TAA), and the corresponding mutation was made to ERβ (ERβ-M_1) so that it resembled the ERα response element (TAA to GAA). The mutated ERα promoter (ERα-M_1) containing the ERβ Stat5 response element was still stimulated by PRL-R_{CA} in the presence of Stat5a or Stat5b, although the degree of stimulation was markedly reduced (Fig. 8A). Of great interest was our finding that a single nucleotide mutation to the Stat5 response element of the ERβ promoter (ERβ-M_1) rendered ERβ now highly responsive to Stat5a (Fig. 8B). In addition, stimulation of the mutated ERβ promoter was significantly increased in the presence of Stat5b

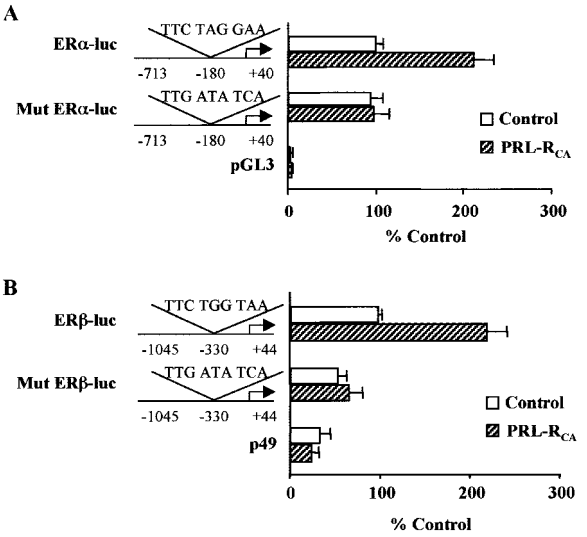


Fig. 7. Role of Putative Stat5 Response Elements in PRL-R_{CA} Stimulation of ERα and ERβ Promoter Activity

COS cells were transfected with 0.5 μg/well wild-type, mutated, or promoter-less reporter constructs for ERα-luc (A) or ERβ-luc (B). In addition, each well was transfected with 0.5 μg β-gal, 1 μg Stat5b, and 2 μg PRL-R_{CA} or PRL-R_L (control). Forty-eight hours after the start of transfection, luciferase activity was measured in each well and normalized β-gal activity within that well. The experiment was repeated four times with triplicate wells for each group. The data represent the combined mean ± SEM for all four experiments.

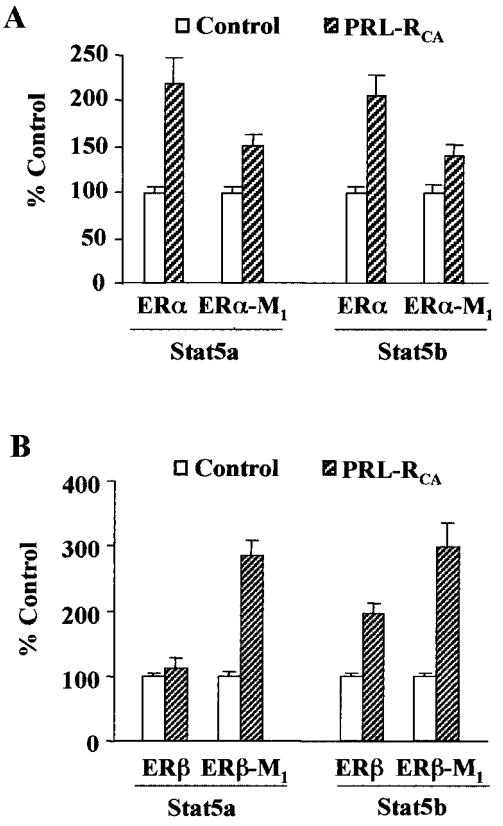


Fig. 8. Comparison of ERα and ERβ Consensus and Nonconsensus Stat5 Response Elements in Stat5-Mediated Regulation by PRL-R_{CA}

COS cells were transfected with 0.5 μg/well ERα-luc, ERα M1-luc, ERβ-luc, or ERβ M1-luc, 0.5 μg β-gal, 1 μg Stat5a or Stat5b, and 2 μg PRL-R_{CA} or PRL-R_L (control). ERα constructs are shown in panel A and ERβ constructs are shown in panel B. Forty-eight hours after the start of transfection, luciferase activity was measured in each well and normalized β-gal activity within that well. The experiment was repeated six times with triplicate wells for each group. The data represent the combined mean ± SEM for all six experiments.

as well (Fig. 8B). Based on these results, it appears that the nonconsensus Stat5 binding site in the ERβ promoter both prevents its responsiveness to Stat5a and limits its responsiveness to Stat5b.

DISCUSSION

In the rat corpus luteum of pregnancy, PRL is known to stimulate expression of both ERα and ERβ mRNA, leading to a functionally significant increase in the number of E2 binding sites (3, 44). Results from our current studies indicate that PRL causes this increase in ER expression at the level of transcription, which is mediated by the transcription factor Stat5. Using real-time quantitative RT-PCR, we provide evidence that ERα mRNA expression is far more abundant in both corpora lutea and luteinized granulosa cells than is ERβ. This is expected since previous work from Dr. Park-Sarge's group (45) has shown that the LH surge specifically down-regulates ERβ expression but has no effect on ERα levels. Also, Dr. Joanne Richards' laboratory (46) found low expression of ERβ mRNA and no ERβ DNA binding activity in luteinized granulosa cells. In addition, although the ERβ knockout mouse is subfertile, it is capable of supporting a full-term pregnancy, suggesting that luteal function is not compromised in the absence of ERβ and that ERα is sufficient to sustain pregnancy (47). Nevertheless, PRL is capable of stimulating expression of both ERα and ERβ at the level of transcription. Although ERβ may not be crucial for luteal function, the ability of PRL to stimulate ERβ may be important in other PRL target tissues. In addition, multiple hormones and cytokines are capable of activating Stat5 and may therefore be capable of regulating ER expression through a similar pathway.

Perhaps the most intriguing finding from these studies was that ERα and ERβ appear to be regulated differently by Stat5a and Stat5b. In COS and primary luteinized granulosa cells, the ERα promoter and endogenous gene were responsive to both Stat5a and Stat5b. In contrast, in each of the models used, the findings support the conclusion that ERβ is far more responsive to Stat5b than to Stat5a. In CHO cells, dominant negative expression vectors for both Stat5a and Stat5b were capable of preventing stimulation of

both ERα and ERβ. This finding suggests then that the DN-5a may prevent stimulation of ERβ by forming heterodimers with endogenous Stat5b. Alternatively, overexpression of these Stats may be capable of preventing the endogenous Stat5 from being activated through competition for Jak2 substrate binding sites. This lack of stimulation by Stat5a on ERβ expression, however, may not be an issue in the corpus luteum since the major Stat5 expressed is Stat5b (data not shown). Whether PRL can or cannot regulate ERβ transcription in other tissues, such as the mammary gland, where Stat5a plays a major role in PRL signaling, remains to be investigated. The differential regulation of ERα and ERβ by Stat5a and Stat5b may be one mechanism that contributes to the tissue-specific pattern of ER expression.

The lack of ERβ stimulation by Stat5a was attributed to a single nucleotide in the Stat5 response element, which both prevented ERβ responsiveness to Stat5a and limited its responsiveness to Stat5b. When this single nucleotide was introduced into the ERα response element, responsiveness to both Stat5a and Stat5b was significantly reduced. These results are somewhat expected since a nonconsensus binding site should be less effective at driving gene expression than would be a consensus site. Also, when this single nucleotide was mutated in ERβ, so that it resembled the consensus ERα Stat5 response element, ERβ transcription in the presence of either Stat5a and Stat5b was markedly increased. These findings could explain, in part, why ERβ expression is so much lower in the corpus luteum than is ERα. However, these results do not explain the complete lack of ERβ responsiveness to Stat5a. If this nucleotide were the sole explanation, then it would be expected that ERα would be unresponsive to Stat5a once it too had the same nucleotide as ERβ. This suggests that within the ERα promoter some additional regulatory region may be capable of enhancing Stat5a action. It is possible that the differential action of Stat5a and Stat5b on these two promoters may be explained by the ability of Stat5 to form stable tetramers through protein-protein interactions involving a tryptophan residue, which is conserved in all Stats and a lysine residue in the Stat5 N-terminal domain (48). Interestingly, only Stat5a tetramers and not Stat5b tetramers were shown to bind the multiple GAS sites in the CIS gene promoter (24). In this same promoter, Stat5b preferentially bound as a dimer. The study of Stat5a dimer and tetramer DNA binding sites revealed that Stat5a tetramers could bind to a wider range of nonconsensus sites, which a Stat5a dimer could not bind. A spacing of 6 bp between tandem GAS sites was the preferred distance for Stat5a tetramer binding (49). Also, it appears that two full GAS sites are not completely necessary for Stat5a tetramer binding since one of the sites could be replaced with a GAS half-site (24). Although we have not found additional GAS sites or half-sites in either promoter, it is possible that some nonconsensus site in the ERα promoter could be essential for its responsiveness to Stat5a while the ERβ promoter may not be capable of supporting Stat5a tetramer binding.

In conclusion, it is clear that PRL regulation of ER expression is at the level of transcription and that Stat5 mediates this regulation. Furthermore, the Stat5 response elements within the ERα and ERβ are necessary for this stimulation. Our data also demonstrate that ERα and ERβ are differentially responsive to Stat5a and Stat5b and that a single nucleotide in the ERβ promoter can explain its lack of responsiveness to Stat5a. And finally, this increase in ER transcription represents a functional stimulation of ER expression since only a modest increase of E2 binding sites by PRL in the corpus luteum is sufficient to render the corpus luteum responsive to E2.

MATERIALS AND METHODS

Materials

PMSG, human CG, DMEM/F12 (1:1), DMEM, horseradish peroxidase conjugated secondary antibodies, and all other reagent grade chemicals were obtained from Sigma (St. Louis, MO). OptiMem, LipofectAMINE, α-MEM medium, and Trizol were purchased from Life Technologies, Inc. (Gaithersburg, MD). The Advantage RT-for-PCR kit and the chemiluminescence β-gal substrate were from CLONTECH Laboratories, Inc. (Palo Alto, CA). FBS was from HyClone Laboratories, Inc. (Logan, UT). DNA Master SYBR Green I was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Trypsin-EDTA, antibiotics, and antimycotics were from Mediatech (Herndon, VA). Antibodies to Stat5a, Stat5b, and phosphorylated Stat5a/5b were from Upstate Biotechnology, Inc. (Lake Placid, NY). Protein A/G agarose beads and the enhanced chemiluminescence detection reagents were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The luciferase assay substrate and reporter lysis buffer were purchased from Promega Corp. (Madison, WI).

Animal Models

Pregnant and immature female Sprague Dawley rats were obtained from Sasco Animal Labs (Madison, WI) and housed under controlled conditions of light and temperature with free access to standard rat chow and water. All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

To determine the effect of PRL on ER expression, mRNA was obtained from corpora lutea from pregnant rats that had either been left intact, hypophysectomized, or hypophysectomized and treated with PRL as previously published (3). To obtain primary luteinized granulosa cells, follicular development was induced in immature rats (24–26 d of age) by injection of 15 IU PMSG ip. An ovulatory dose of human CG (10 IU, ip) was given 48 h later. Luteinized granulosa cells were harvested and cultured as previously described (42). Transfection of primary cells was carried out in OptiMem using LipofectAMINE according to the manufacturer's directions.

Real-Time, Quantitative RT-PCR

RNA from tissue and cell cultures was isolated using Trizol according to the manufacturer's instructions. Reverse tran-

scription was carried out using reagents from the Advantage RT-for-PCR kit according to the manufacturer's instructions. One microgram of total RNA was used for the reverse transcription reaction, and the product was diluted to a final volume of 100 μl by adding diethyl pyrocarbonate-treated H_2O. To generate standard curves for quantitative PCR, rat ERα and ERβ cDNA, which was kindly provided by Dr. Maruyama (50), were diluted to concentrations ranging from 10^3 to 10^7 copies/μl. Five-microliter aliquots of standards or diluted reverse transcription products were combined with 2 μl 10× DNA Master SYBR Green I, 1.6 μl $MgCl_2$ (3 mM final concentration), and specific primers for rat ERα or ERβ (0.5 μM final concentration). The primers used have been previously published (3). Reactions were carried out in glass capillary tubes in a total volume of 20 μl. The DNA Master SYBR Green I mix contains *Taq* DNA polymerase, reaction buffer, deoxynucleotide triphosphate, 10 mM $MgCl_2$, and SYBR Green I dye, which is a specific fluorescence dye for double-stranded DNA. PCR reactions were performed in the Roche Lightcycler instrument and the accompanying software was used for data analysis (Roche Molecular Biochemicals, Mannheim, Germany). After a 2-min denaturation, PCR cycles were carried out as follows: 0 sec at 95 C, 10 sec at the annealing temperature, and 15 sec at 72 C. For ERα, 40 cycles at an annealing temperature of 63 C were used; for ERβ, 35 cycles at an annealing temperature of 69 C were used. At the end of each cycle, the amount of double-stranded DNA was monitored by measuring the level of SYBR Green I fluorescence. After the completion of all cycles, a level of fluorescence was selected at which all of the standards and samples were within the linear range of amplification. The crossing point, or the number of cycles necessary for each sample or standard to obtain the selected level of fluorescence, was calculated using the Roche Lightcycler software. Based on these crossing points, a standard curve was generated, and the number of ERα or ERβ copies was calculated for each sample. The data presented represent the number of copies of ERα or ERβ in 1 ng of total RNA.

Cloning of the ERα and ERβ Promoters

The 5′-flanking region of the rat ERα gene was cloned using the rat PromoterFinder DNA Walking kit (CLONTECH Laboratories, Inc.). Five different genomic libraries were generated by digesting genomic DNA with 5 different restriction enzymes, namely *Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Ssp*I followed by ligation with a specifically designed PromoterFinder adapter. The ERα promoter region was amplified by nested PCR using the five different genomic libraries as templates and two sets of primers designed according to the published sequence of the ERα promoter (51). The first PCR reaction was carried out using the following primers: 5′-CCACTCATAAATCTCTT-GGTAACGGC-3′ and 5′-GAAGGAAGGAATGTGCTCGAAT-GATC-3′. A second PCR reaction was carried out using product from the first reaction and the following primers: 5′-CTGGGGTTGCAATTAGTC-ATTTAGGC-3′ and 5′-TCGC-GAATTCGAGTGGCGCGGTGTGTGATCAAG-3′. The second primer also included an attached *Eco*RI site for subsequent cloning. All five sources of genomic DNA yielded an amplified product of the expected size (880 bp). The PCR products were pooled, and the internal *Kpn*I site at −769 in the ERα promoter region and the added *Eco*RI site were used to subclone the PCR product into the pBluescript DNA vector (Stratagene, La Jolla, CA). Subcloning of the ERα promoter region into the pGL3-basic luciferase reporter vector (Promega Corp.) was carried out utilizing the *Kpn*I and *Bgl*II sites in the pGL3 vector and the *Kpn*I and *Bam*HI sites in the pBluescript vector. Both strands of the ERα promoter generated from different colonies were sequenced. Sequence analysis revealed two differences from the originally published sequence (G to C at −494, A to G at −346) (51).

To isolate the regulatory region of the rat ERβ gene, we used the touchdown PCR amplification approach using the GenomeWalker kit (CLONTECH Laboratories, Inc.) according to the manufacturer's procedure. Two gene-specific primers were designed against the most 5′-end of sequences of the rat ERβ mRNA (gene-specific primer 1: 5′-AAGCTGCAAA-GATTACCCACGACTA-3′ and gene-specific primer 2: 5′-GACTAACGGATGTTAGTGCGTCTT-3′) (52). Thus, the expected gene-regulatory DNA would contain 46 bp of the 5′-end of the ERβ mRNA. The primary PCR amplification was carried out using the combined GenomeWalker libraries (1 μl) and the primer set of gene-specific primer 1 and adapter primer 1, under PCR conditions of 72 C for 4 min (7 cycles) and 67 C for 4 min (33 cycles). The secondary PCR amplification was carried out using 1 μl of the diluted primary PCR products (1:100) and the primer set of gene-specific primer 2 and adapter primer 2, under PCR conditions of 72 C for 4 min (5 cycles) and 67 C (22 cycles). This procedure yielded two prominent PCR fragments (~1 and ~2 kb) that were subsequently isolated, subcloned into PCR2.1 T/A overhang vector (CLONTECH Laboratories, Inc.), and sequenced using M13 forward and backward primers. Both contained the adapter 2 sequences at their 5′-end and the gene-specific primer 2 at their 3′-end. The inserts of these clones were isolated by restriction digestion using *Eco*RV/*Spe*I and subsequent fill-in reactions with Klenow, and inserted into the *Sma*I arms of the pUBT-luc vector (53). For these studies, the ~1 kb promoter region of the rat ERβ gene was used.

Mutations to ERα and ERβ Promoters

The first set of mutations made to the ERα and ERβ promoters consisted of six and five nucleotides, respectively, being changed to abolish the Stat5 binding sites. Oligonucleotide primers for these mutations were made as follows (mutated nucleotides *underlined*): ERα 5′-GCCAAGGGGGCTG-GAGTTTCTTGATATCATGCTGA-TTCTAGTGGTGCTACT-GCCG -3′ and ERβ 5′-ATTACTGCTTATTTCGGTGCTATGA-TATCAACCCGGGGCCTGGCCCATGC-3′. The second set of mutations made to the ERα and ERβ promoters consisted of a single nucleotide being changed. The consensus Stat5 site of the ERα promoter (TTCnnnGAA) was changed so that it resembled the Stat5 response element of the ERβ promoter (TTCnnnTAA). The nonconsensus ERβ Stat5 response element (TTCnnnTAA) was mutated so that it would resemble the ERα Stat5 response element (TTCnnnGAA). Oligonucleotide primers for these mutations were made as follows (mutated nucleotides *underlined*): ERα 5′-GGCTGGAGTT-TCTTCTAGTAAT-GCTGATTCTAGTGG-3′ and ERβ 5′-CGGTGCTATTCCCAGAACCCGG-GGCCTGG-3′. All mutations were made using the QuikChange Site-directed Mutagenesis kit according to the manufacturer's directions (Stratagene). The presence of the correct mutations was confirmed by DNA sequencing.

Culture and Transfection of CHO and COS Cells

CHO and COS cells were routinely cultured in α-MEM and DMEM/F12 (1:1), respectively. All media were supplemented with 10% FBS, 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml Amphotericin. Cultures were carried out at 37 C in a 5% CO_2, humidified atmosphere. For transient transfections, 100,000 cells were seeded per well in six-well plates and cultured for 24 h. Both CHO and COS cells were transfected using calcium phosphate DNA precipitation and were approximately 50% confluent at the start of transfection (54). In general, a total of 4–5 μg DNA were transfected per well, and the total amount of DNA was equalized with empty vector when necessary. Twenty-four hours after the start of transfection, media were changed to standard culture media supplemented with 1% FBS, and cells were cultured for an additional 24 h at 5% CO_2. The entire length of the experiments was standardized to 48 h from the start of transfection.

Reporter Assays

Luciferase and β-galactosidase (β-gal) activities were measured by first preparing cell lysates in 1× reporter lysis buffer. Luciferase activity driven by the ERα or ERβ promoter was measured by combining lysate with Firefly luciferase assay substrate and measuring luminescence for 10 sec on a Lumat LB 9507 Luminometer (EG&G Berthold, Oak Ridge, TN). As a control, cells transfected with the ERα or ERβ promoter were cotransfected with an expression vector for β-gal. β-gal Activity was measured in a separate aliquot of lysate by incubating with a luminescent β-gal substrate for 1 h at room temperature and then measuring luminescence for 5 sec. The luciferase activity was normalized to β-gal activity within the same well. In experiments done in CHO cells, luciferase activity was normalized to total protein levels in each well because of inconsistent β-gal expression.

Immunoprecipitation and Western Blotting

Whole cell extracts (WCE) from primary luteinized granulosa cells and cell lines were prepared by lysing cells in RIPA buffer (1× PBS, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 μM sodium orthovanadate, 10 μg/ml phenylmethylsulfonyl fluoride, and 30 μl/ml aprotinin. For immunoprecipitation, 500 μg of WCE were incubated with 4 μl anti-Stat5a or anti-Stat5b antibodies for 1 h at 4 C. Protein A/G agarose beads were added, and the mixture was incubated overnight at 4 C on a rocking platform. The beads were washed four times in PBS, resuspended in 2× electrophoresis buffer, and boiled for 5 min. For Western blots performed on WCE, protein was diluted in an equal volume of 2× electrophoresis buffer and boiled for 5 min. Twenty microliters of immunoprecipitated protein or 20 μg of WCE were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Western blotting was performed using protocols provided with the Stat5 antibodies.

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