Induction and Activation of Stat 5 in the Ovaries of Pseudopregnant Rats*

SUSAN J. RUFF, SUSAN LEERS-SUCHETA, MICHAEL H. MELNER, AND STANLEY COHEN

Departments of Biochemistry (S.J.R., S.C.), Obstetrics and Gynecology (S.L.-S., M.H.M.), and Cell Biology (S.L.-S., M.H.M.), Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

ABSTRACT

PRL acts in the ovary to promote the maintenance and function of the corpus luteum. However, the cellular signals that induce these responses have not been clearly defined. In the present report we demonstrate that Stat 5, previously identified as a transcription factor activated by PRL in the mammary gland, is also activated by PRL in the ovaries of pseudopregnant rats. Intraperitoneal injection of PRL into pseudopregnant rats results in the tyrosine phosphorylation and nuclear translocation of Stat 5. This activated Stat 5 possesses DNA-binding activity for a sequence containing the PRL-inducible element. In addition, we report that luteinization of the PMSG-primed ovary by the administration of hCG is accompanied by an induction of Stat 5 protein. (Endocrinology 137: 4095–4099, 1996)

During early pregnancy and pseudopregnancy, PRL secreted by the pituitary is important for the maintenance of luteal functions (6). During midpregnancy, placental lactogens become critical for continued luteal function (7–9). In infraprimate species, including the rat, these lactogenic hormones regulate the synthesis of progesterone and peptide hormones by the corpus luteum (4–5). If implantation and placentation have not occurred, placental lactogens will be absent, resulting in luteolysis or regression of the corpus luteum. The mechanisms by which PRL or placental lactogens are able to direct the sustenance of the corpus luteum have not been clearly defined.

The Stat family of proteins (signal transducers and activators of transcription) have been characterized as latent cytoplasmic transcription factors that become activated through phosphorylation of tyrosine residues and are translocated to the nucleus in response to a variety of extracellular signaling proteins (cytokines, growth factors, and hormones) (10, 11). In mammary glands, PRL stimulates the synthesis of casein by activating a DNA-binding activity specific for a response element upstream of the β-casein promoter (the PRL-inducible element (PIE)) (12). This DNA binding activity was purified from sheep mammary glands and identified as a 92-kDa tyrosine-phosphorylated protein that was named mammary gland factor (13). The complementary DNA encoding this protein was sequenced and found to contain sequence homologies with known members of the Stat family of transcription factors and was, therefore, renamed Stat 5 (14).

In the present report we demonstrate that PRL is capable of inducing the tyrosine phosphorylation, nuclear translocation, and DNA-binding activity of Stat 5 in the ovaries of pseudopregnant rats. In addition, the expression of Stat 5 protein is increased in the ovaries of pseudopregnant rats during ovulation and luteinization.

Materials and Methods

Twenty-six-day-old Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). HCG (CR127) and PRL (oPRL-18) were gifts from the National Hormone and Pituitary Program and the NIDDK. Immobilon-P membranes were obtained from Millipore (Bedford, MA). Antibody to phosphotyrosine (RC20H), monoclonal anti-Stat 3, and anti-Stat 5 were obtained from Transduction Laboratories (Lexington, KY). The polyclonal antibody to Stat 5 (sc-835 X) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat antirabbit IgG labeled with horseradish peroxidase was obtained from Cappel Laboratories (Warrington, PA). Enhanced chemiluminescence reagent was purchased from Amersham (Arlington Heights, IL). Prestained mol wt standards were obtained from Life Technologies (Gaithersburg, MD). Avidin-agarose was purchased from Pierce Chemical Co. (Rockford, IL). All oligonucleotides were obtained from Oligo's Etc. (Wilsonville, OR). Poly(dI-dC)-poly(dI-dC) was obtained from Pharmacia Biotech (Piscataway, NJ). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Induction of pseudopregnancy

Immature female rats were primed by a single sc injection of 20 IU PMSG, a glycoprotein hormone that possesses primarily FSH activity and partial LH activity. After 48 h, these PMSG-primed rats were treated with a single sc injection of 10 IU hCG, a hormone that exhibits LH activity. This previously described treatment regimen stimulates the development of multiple follicles, ovulation, and the formation of multiple pseudopregnant corpora lutea (21). Further treatments of these pseudopregnant animals were made 96 h after the injection of hCG. These animals are referred to throughout the text as day 5 pseudopregnant rats.

Preparation of ovarian extracts for isolation of nuclei and immunoprecipitation

Solutions of PRL (1 mg/ml) in Dulbecco's PBS or PBS alone were injected ip at a dose of 5 μl/g BW into day 5 pseudopregnant rats. Rats
were then killed in a CO₂ chamber at the indicated times. The ovaries were removed; stripped of adhering fat, connective tissue, and oviducts; and immediately frozen in liquid nitrogen. Each ovary from the PRL-injected animals weighed approximately 0.1 g. Nuclei were isolated from 0.2 g frozen ovaries, purified by centrifugation through a 2.2-M sucrose cushion, and extracted with 50 µl 0.2 M NaCl, as previously described (22). Portions (25 µl; 25 µg protein) of the nuclear extracts were resolved by SDS-PAGE (7%), transferred to Immobilon, and immunoblotted with the RC20H antiphosphotyrosine antibody fragment. Protein binding was detected with enhanced chemiluminescence reagent (ECL). The blot was stripped, blotted with a 1:1000 dilution of monoclonal anti-Stat 5 for 1 h, followed by a 1:2000 dilution of goat antimouse IgG labeled with horseradish peroxidase for 1 h. Antibody binding was detected with ECL.

For the immunoprecipitations, all manipulations were performed at 4°C. A 10% (wt/vol) ovarian lysate was prepared in buffer A (10 mM Tris, pH 8.3; 0.1 mM NaCl; 1% Triton X-100; 1 mM EDTA; 1 mM sodium orthovanadate; 50 mM sodium molybdate; and 0.5 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged for 30 min at 100,000 × g to remove insoluble debris. The soluble lysate (500 µl; 10 mg protein) was immunoprecipitated with the polyclonal antiserum to Stat 5 (2 µg) overnight, followed by the addition of 50 µl protein A-Sepharose (50% slurry) for 1 h. The precipitated sample was washed three times in buffer A, and the bound proteins were eluted by boiling in 100 µl 2× SDS sample buffer (22). The samples were resolved on SDS-PAGE (7%). Western blot analysis, after transfer to Immobilon, was carried out as described above.

Affinity purification and identification of PIE-binding proteins

Solutions of PBS or PRL (1 mg/ml) were injected ip into day 5 pseudopregnant rats at a dose of 5 µl/g BW, and the ovaries were removed and immediately frozen in liquid nitrogen. A 20% (wt/vol) homogenate was made in buffer B (20 mM HEPES, pH 7.9; 0.1 M sodium chloride; 1 mM sodium orthovanadate; 10 mM sodium fluoride; and 50 µM sodium molybdate) and centrifuged at 100,000 × g for 1 h. The resulting supernatant (900 µl; 11 mg protein) was supplemented with glycerol to 10%, with dithiothreitol to 1 mM, and with 10 µg poly(dI-dC)-poly(dI-dC) in a final volume of 1 ml and incubated at 4°C for 30 min. Biotinylated PIE oligonucleotide (2 µg) was added, and the mixture was incubated for 12 h, followed by the addition of 1 µg poly(dI-dC)-poly(dI-dC) to 1 mM and incubated at 4°C for 30 min. The resulting supernatant (900 µl; 11 mg protein) was supplemented with glycerol to 10%, with dithiothreitol to 1 mM, and with 10 µg poly(dI-dC)-poly(dI-dC) in a final volume of 1 ml and incubated at 4°C for 30 min. Biotinylated PIE oligonucleotide (2 µg) was added, and the mixture was incubated for 12 h, followed by the addition of 1 µg poly(dI-dC)-poly(dI-dC) to 1 mM and incubated at 4°C for 30 min. The resulting supernatant was centrifuged, and the sedimented material was washed three times with buffer B. The bound proteins were eluted by boiling in 2× Laemmli buffer (100 µl) for 5 min, resolved by SDS-PAGE (7%), transferred to Immobilon, and immunoblotted with the monoclonal antibody to Stat 5, and detected as described above. The blot was stripped and reprobed with a 1:1000 dilution of the monoclonal antibody to Stat 3 for 1 h, followed by secondary antibody incubation and ECL detection, as described above.

Results

Detection of PIE-binding complexes in nuclear extracts from the ovaries of PRL-treated rats

The ovaries of rats primed with PMSG or day 5 pseudopregnant rats contain synchronously proliferating granulosa and luteal cells, respectively. Treatments of both of these cell types with PRL has been shown to enhance progesterone production (23). In the mammary gland, PRL has been shown to induce a DNA-binding activity specific for the PIE (13). To determine whether PIE-binding activity could be induced by PRL in the ovary, rats primed with PMSG or day 5 pseudopregnant rats were treated with PRL or PBS for 15, 30, or 60 min. Nuclear extracts from the ovaries of PBS- and PRL-treated rats were analyzed for the ability to form specific PIE-binding complexes in a gel shift assay. Nuclear extracts from the ovaries of PMSG-primed rats, whether treated or not treated with PRL, contained no detectable PIE-binding activity (data not shown). However, the administration of PRL to day 5 pseudopregnant rats resulted in the appearance of one PIE-binding protein complex in nuclear extracts (labeled A in Fig. 1), whereas nuclear extracts from the ovaries of PBS-injected pseudopregnant animals had no detectable PIE-binding activity. This PIE-binding activity was maximal 30 min after the injection of PRL (Fig. 1). From this experiment we conclude that PRL induces a DNA-binding activity specific for PIE in the ovaries of day 5 pseudopregnant rats.
Detection of tyrosine-phosphorylated proteins in nuclear extracts from the ovaries of PRL-treated rats

It had been previously reported that in mammary glands, Stat 5 is tyrosine phosphorylated and capable of binding to PIE in response to PRL (13, 14). In liver, both Stat 1 and Stat 5 are tyrosine phosphorylated and capable of complexing with PIE in response to epidermal growth factor (16). We, therefore, analyzed nuclear extracts prepared from the ovaries of control and PRL-treated pseudopregnant rats for the presence of phosphotyrosine-containing proteins. Two major tyrosine-phosphorylated bands (92 and 90 kDa) could be seen after PRL injection (Fig. 2, left panel). The concentrations of these nuclear proteins were highest at 30 min. Other minor tyrosine-phosphorylated proteins (120 and 50–55 kDa) were occasionally detected in nuclear extracts; however, changes in the intensities of these bands did not consistently correlate with PRL treatment. No consistent change in protein phosphotyrosine content could be detected in nuclear extracts after PBS injection (Fig. 2, left panel).

To determine whether the PRL-inducible 90- to 92-kDa tyrosine-phosphorylated proteins seen in the day 5 pseudopregnant rat ovarian nuclear extracts were related to Stat 5, the antiphosphotyrosine immunoblot was reprobed with a monoclonal anti-Stat 5 antibody. This procedure revealed the presence of Stat 5 in the nuclear extracts from PRL-injected animals, but not in nuclear extracts from PBS-injected animals (Fig. 2, right panel). The data suggest that Stat 5 is tyrosine phosphorylated and translocated to the nucleus of day 5 pseudopregnant rat ovarian cells after the administration of PRL. It should be noted that the peak of PIE-binding activity in nuclear extracts from PRL-treated animals correlated in time with the appearance of tyrosine-phosphorylated Stat 5 in these extracts. Whether the appearance of the 90- to 92-kDa doublet in immunoblots of nuclear extracts is due to changes in the phosphorylation state of Stat 5 and/or alternate isoforms of Stat 5 is not known (15).

To confirm that the Stat 5 protein was tyrosine phosphorylated after the injection of PRL, total cell extracts from the ovaries of PBS- and PRL-injected animals were immunoprecipitated with the antibody specific for Stat 5. The precipitated proteins were resolved by SDS-PAGE and then analyzed by immunoblotting with an antibody to phosphotyrosine and an antibody to Stat 5. The anti-Stat 5 immunoprecipitate contained a tyrosine-phosphorylated protein of approximately 92 kDa only when prepared from the ovaries of PRL-treated animals and not from those of control animals (Fig. 3, left panel). Probing the blot with the monoclonal antibody to Stat 5 revealed that Stat 5 was present in immunoprecipitates prepared from ovaries of both control and PRL-treated animals (Fig. 3, right panel). We interpret the detection of multiple Stat 5 bands after treatment with PRL to reflect different phosphorylation states or isoforms of this protein. From these experiments we conclude that Stat 5 is tyrosine phosphorylated in pseudopregnant rat ovaries after the administration of PRL.

Affinity purification of PIE-binding proteins

To demonstrate that Stat 5 was responsible for the PIE-binding activity detected in the ovary, the PIE-binding proteins were affinity purified. Cytosolic extracts from the ovaries of day 5 pseudopregnant rats treated with either PBS or PRL for 30 min were incubated with biotinylated PIE and avidin-agarose. The adsorbed proteins were eluted, separated by SDS-PAGE, and analyzed by immunoblotting with monoclonal antibodies to phosphotyrosine and Stat 5. Two major tyrosine-phosphorylated bands of 92 and 90 kDa were present among the proteins adsorbed by biotinylated PIE in the cytosol from PRL-treated animals (Fig. 4a). No tyrosine-
INDUCTION AND ACTIVATION OF STAT 5 IN OVARIES

Endo. 1996
Vol 137. No 10

A

<table>
<thead>
<tr>
<th>PIE-biotin</th>
<th>Adsorbent</th>
<th>Blot Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PY</td>
<td>+</td>
<td>Stat 5</td>
</tr>
<tr>
<td>- PRL</td>
<td>-</td>
<td>- PRL</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>PIE</th>
<th>Probe Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>- PRL</td>
</tr>
<tr>
<td>+ PRL</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 4. Identification of PIE-binding proteins. a, Affinity purification. Ovaries from PBS- or PRL-treated rats were homogenized, and cytosolic extracts were adsorbed with PIE-biotin-avidin-agarose as described in Materials and Methods. The adsorbed proteins were resolved by SDS-PAGE and assayed by immunoblot with antibodies to phosphotyrosine and Stat 5. b, Supershift analysis. Nuclear extracts prepared from PBS- or PRL-treated day 5 pseudopregnant rats were assayed for their ability to bind PIE as described in Fig. 1. Antibodies specific for Stat 5 were included in the gel shift reaction where indicated. The positions of the PIE complex (A) and supershift are indicated.

phosphorylated proteins were detected on the biotinylated PIE-avidin-agarose beads after incubation with ovarian extracts from PBS-injected rats. Reprobing of the blot with a monoclonal antibody specific for Stat 5 revealed that Stat 5 was adsorbed to PIE only from extracts of PRL-treated animals (Fig. 4a). The detection of multiple Stat 5 bands among the biotinylated PIE-binding proteins again may reflect different phosphorylation states or isoforms of this protein. From this experiment we conclude that Stat 5 is tyrosine phosphorylated and capable of binding PIE in response to PRL in cytosolic extracts of the ovaries of day 5 pseudopregnant rats.

To directly determine whether the PRL-induced PIE-binding complexes present in nuclear extracts contained Stat 5, we performed supershift analyses with an antibody specific for this protein (Fig. 4b). Little PIE-specific binding was detectable in nuclear extracts from the ovaries of PBS-treated day 5 pseudopregnant rats. Nuclear extracts from the ovaries of PRL-treated day 5 pseudopregnant rats contained one specific PIE-binding complex (labeled A in Fig. 4b). An antibody specific for Stat 5 supershifted this PRL-inducible PIE-A complex. From this experiment we conclude that Stat 5 is a component of the PIE-A binding complex.

Induction of Stat 5 protein

Since both granulosa and luteal cells respond to PRL (23), it was surprising that PIE-binding activity could be detected in the ovaries of PMSG-primed rats only after treatment with hCG. One possibility for this observation is that insufficient amounts of Stat 5 protein are present in the ovaries of PMSG-primed animals before the administration of hCG, and that any activation in response to PRL was below the level of detection in our gel shift assay. To test this possibility, we analyzed total cell extracts from the ovaries of PMSG-primed and day 5 pseudopregnant rats for the presence of Stat 5 protein by direct immunoblot analyses. The concentration of Stat 5 protein was detectable, albeit low, in the ovaries of PMSG-primed rats. However, in the ovaries of day 5 pseudopregnant rats, we observed a substantial increase in the relative protein concentration of Stat 5. These results are illustrated in Fig. 5, with two animals in each group. As a control, the blot was reprobed with a monoclonal antibody specific for Stat 3. This control indicates that there is no apparent change in the relative protein concentration of Stat 3 after hCG administration. From this experiment we conclude that the expression of Stat 5 protein is induced after luteinization of the ovarian follicle.

Discussion

We demonstrated that the tyrosine phosphorylation of Stat 5 and its translocation to the nucleus is an event initiated in the ovaries of day 5 pseudopregnant rats after the administration of PRL. The genes that are regulated in the ovary in response to the activation of Stat 5 protein by PRL are not currently known. Even though Stat 5 DNA-binding activity was detected using a response element upstream of the \( \beta \)-casein promoter, we do not believe that \( \beta \)-casein gene expression is being activated in these ovaries. In view of the ability of PRL to maintain the corpus luteum, it is possible that PRL could act to inhibit the expression of genes involved in luteal regression and apoptosis.

It is interesting to consider the potential targets of Stat 5 that could be important for luteal function, such as genes in

Fig. 5. Induction of Stat 5 protein expression. Ovaries from PMSG-primed and day 5 pseudopregnant rats were extracted as described in Materials and Methods for direct Western blots, and aliquots (50 μl; 1 mg protein) were resolved by SDS-PAGE and analyzed by direct immunoblot with antibodies to Stat 5 and Stat 3.
the steroidogenic pathway, including P450 side-chain cleavage enzyme (CYP11A), and 3β-hydroxysteroid dehydrogenase as well as genes metabolizing steroids such as 20α-hydroxysteroid dehydrogenase (24). The promoter of the gonadal isomorph of human 3β-hydroxysteroid dehydrogenase (type II) contains a 9/9 match with the PIE (mammary gland factor) consensus recognition element at 118 to 119. The rat promoter of this gene has not yet been cloned or characterized. It is currently not known whether this element is functional, although previous data indicated that the expression of this gene in rat ovaries is regulated by PRL (17, 18). The human P450 side-chain cleavage enzyme promoter also contains two 9/9 putative Stat 5 recognition elements at −514 to −506 and at −2125 to −2116. The function of these putative recognition elements in PRL-induced transcriptional regulation of these genes is currently unknown.

The pseudopregnant model is ideal for determining the cellular signals occurring in the ovary in response to PRL. During the initial phase of pseudopregnancy, PRL is secreted by the pituitary and functions to promote maintenance of the corpus luteum. During the initial phase of pseudopregnancy, PRL is secreted in the late afternoon (19, 20). At times between these two surges, endogenous PRL concentrations are decreased by 10-fold. Our experiments were carried out between the diurnal PRL surges, and we were able to detect the effect of exogenous PRL.

Trophoblast tissue from uterine implantation of an embryo releases placental lactogens that bind to ovarian PRL receptors and promote maintenance of the corpus luteum. The injection of PRL should, therefore, mimic an implantation event. In view of our results, we conclude that the activation of Stat 5 may be one of the events initiated in the ovary after implantation of an embryo.

Luteal cells that develop in the ovaries of day 5 pseudopregnant rats have relatively more Stat 5 protein than the granulosa precursor cells. If Stat 5 is altering the transcription of the same genes in both cell types, then the increased amount of Stat 5 in corpora luteal cells may function to increase their transcriptional rates. Precedence for this idea comes from the observation that multiple response elements in tandem result in more transcription factor binding, thus increasing the chances that polymerase is recruited to the intended promoter. As maintenance of the corpus luteum is dependent on detecting and responding to a placental lactogen, the increase in Stat 5 transcription factor might ensure detection of the PRL signal. If this hypothesis is true, then an animal deficient in Stat 5 might be incapable of maintaining pregnancy.

References