**Convergence of Progesterone with Growth Factor and Cytokine Signaling in Breast Cancer**

**PROGESTERONE RECEPTORS REGULATE SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION EXPRESSION AND ACTIVITY**

(Received for publication, June 1, 1998, and in revised form, August 21, 1998)

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STATS (signal transducers and activators of transcription) are latent transcription factors activated in the cytoplasm by diverse cell surface signaling molecules. Like progesterone receptors (PR), Stat5a and 5b are required for normal mammary gland growth and differentiation. These two proteins are up-regulated during pregnancy, a period dominated by high levels of progesterone. We now show that progesterin treatment of breast cancer cells regulates Stat5a and 5b, Stat3, and Stat1 protein levels in a PR-dependent manner. In addition, progesterin treatment induces translocation of Stat5 into the nucleus, possibly mediated by the association of PR and Stat5. Last, progesterone pretreatment enhances the phosphorylation of Stat5 on tyrosine 694 induced by epidermal growth factor. Functional data show that progesterin pretreatment of breast cancer cells enhances the ability of prolactin to stimulate the transcriptional activity of Stat5 on a β-casein promoter. Progesterone and epidermal growth factor synergize to control transcription from p21WAF1 and c-fos promoters. These data demonstrate the convergence of progesterone and growth factor/cytokine signaling pathways at multiple levels, and suggest a mechanism for coordination of PR and Stat5-mediated proliferative and differentiative events in the mammary gland.

Progesterone, acting through progesterone receptors (PR), is important in the control of breast cell proliferation and differentiation (1). Mice lacking PR exhibit incomplete mammary gland ductal branching and failure of lobulo-alveolar development (2). Interestingly, similar disruptions of mammary gland development and lactation are observed in mice upon deletion of several other genes including cyclin D1 (3), prolactin receptors (4), the activin/inhibin B gene (5) and most genes such as γ-casein. In addition, growth factors such as epidermal growth factor (EGF) can also stimulate Stat5, as well as Stat1 and Stat3, which then bind to STAT sites on numerous promoters (16). Phosphorylation of a tyrosine residue (tyrosine 694 in Stat5) and JAK2, induces phosphorylation of Stat5a and 5b, which then bind sites on the promoters of mammary-specific genes such as β-casein. In addition, growth factors such as EGF can also stimulate Stat5, as well as Stat1 and Stat3, which then bind to STAT sites on the promoters of growth regulatory genes such as p21WAF1 (13–15) and c-fos (16).

Despite conflicting in vivo and in vitro data regarding the proliferative versus growth inhibitory role of progesterone in the breast, the lack of mammary gland ductal branching and failure of lobulo-alveolar development observed in the PR-knockout mouse (2) demonstrates that PR must play a proliferative role during development. Interestingly, in vitro, progestins stimulate breast cancer cells to progress through one...
round of cell division accompanied by the induction of cyclin D1, p21 WAF1, EGF, EGFR, c-myc, and c-fos. This is followed by growth arrest at the G1/S phase of the second cycle (17–19). We have proposed that progesterone-arrested cells are poised to respond to secondary proliferative or differentiative signals ((18) and the accompanying paper (20)).

There is considerable evidence that progesterone and EGF have complementary effects on the mammary gland. Like progestins and PR, EGF and EGFR are required in the proliferative phase of mammary gland development (21, 22). Mice carrying a spontaneous mutation resulting in a critical amino acid substitution in the kinase domain of the EGFR have underdeveloped ductal trees and impaired lactation (23). Furthermore, progestin treatment: 1) up-regulates EGFR (17, 24, 25) and other type I growth factor receptors (20) in breast cancer cell lines; 2) enhances the ability of EGF to induce proliferation of breast cancer cells (18); and 3) potentiates EGF mediated signaling pathways (20).

Since STAT proteins are downstream effectors of ER (13–16) we postulated that cross-talk between progestrone and EGF occurs at the level of STATs. That steroid hormone and growth factor signaling pathways converge at STAT proteins is suggested by the recent report of a functional interaction between glucocorticoid receptors (GR) and STAT5 in which GR act as coactivators for Stat5-mediated induction of the β-casein promoter by glucocorticoids and prolactin (26). Conversely, Stat3 can act as a coactivator of GR-mediated transcription on a mouse mammary tumor virus promoter in the presence of interleukin-6 and dexamethasone (27).

We now report that progestin treatment of breast cancer cells up-regulates Stat5 and Stat3 protein levels in a PR-dependent manner. In addition, progestin treatment induces translocation of Stat5 into the nucleus, possibly mediated by the physical association of PR and Stat5 proteins. Functional data show that progestin treatment of breast cancer cells enhances the ability of prolactin to stimulate the transcriptional activity of Stat5 on a β-casein promoter, and synergizes with EGF to control transcription of the p21WAF1 and c-fos promoters. These data demonstrate the regulation of a key growth factor signaling molecule by progestrone and suggest a mechanism for coordination of PR and Stat5-mediated proliferative and/or differentiative events in the mammary gland.

MATERIALS AND METHODS

Cell Lines and Reagents—The wild type PR-positive T47Dco breast cancer cell line and its clonal derivatives T47D-Y, T47D-YA, and T47D-YB, have been described (28). Cells are routinely cultured in 75-cm² plastic flasks and incubated in 5% CO₂ at 37 °C in a humidified environment. The stock medium consists of Eagle’s minimum essential medium with Earle’s salts (MEM), containing 1-glutamine (292 μg/liter) buffered with sodium bicarbonate (2.2 g/liter), insulin (6 ng/ml), and 5% fetal bovine serum (Hyclone, Logan, UT) without antibiotics. For routine subculturing, cells are diluted 1:20 into new flasks once per week, supplemented with 5% charcoal-stripped heat-inactivated fetal bovine serum containing 700 μg/ml of aminoglycoside (30). Mixture tablet (Boehringer Mannheim, GmbH Germany) per 50 ml). R5020 (NEN Life Science Products Inc., Boston, MA). Cells were harvested in Hanks’; EDTA, cells were harvested by incubation in 10 ml of a plasmid DNA precipitate was digested with EcoRI and HindIII followed by gel isolation and purification. The cyclin D1 cDNA was a 1.1-kilobase fragment removed from the vector by restriction digest with XbaI and HindIII. The Stat5α cDNA probe consisted of a 2.5-kilobase fragment cut from the vector by EcoRI digest. Last, a glyceraldehyde-3-phosphate dehydrogenase cDNA probe representing a housekeeping gene served as a control for RNA. Fatty acid synthetase clone pG8 (30) was obtained from D. Chalbos, INSERM, Montpellier, France; the cyclin D1 cDNA in a modified puCl19 vector was obtained from A. Arnold, Massachusetts General Hospital, Boston, MA, via R. Sclafani, University of Colorado Health Sciences Center, Denver, CO; Stat5α cDNA in pcDNA3 vector was obtained from A. D’Andrea, Harvard Medical School, Boston, MA, via A. Krauf, University of Colorado Health Sciences Center, Denver, CO.

Antiphosphotyrosine Co-immunoprecipitations—Cells in 10-cm dishes were washed twice with ice-cold phosphate-buffered saline and lysed by scraping in extraction buffer (EB: 1% Triton X-100, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM sodium fluoride, 2 mM Na₃VO₄, and 1% PMSF). Lysates were clarified by centrifugation for 10 min at maximum speed in a Savant (μSpeedfuge SFR10K) bench-top centrifuge and equal amounts of protein (1 mg/ml) were immunoprecipitated with the antiphosphotyrosine monoclonal antibody 4G10 (4 μg/ml) by rotation at 4 °C for 2 h to overnight. Immuno complexes were captured by adding 30 μl of washed protein A (tinsulable formalin-fixed StaphA-derived Sorbin [Sigma]) that had been pre-equilibrated with rabbit antiserum to antibody, incubated for a constant rotation at 4 °C for an additional 2 h, then collected by centrifugation at 10,000 rpm for 3 min in a Savant bench-top centrifuge. Immunoprecipitates were washed twice in EB (1 ml), twice in PAN (10 mM PIPES (pH 7.0), 100 mM NaCl) containing 0.25% Nonidet P-40, and twice in PAN without Nonidet P-40. Washed pellets were resuspended in Laemmli sample buffer, boiled for 3 min, and analyzed by SDS-PAGE and immunoblotting.

Stable Cell Lines Expressing Flag-tagged PR and Co-immunoprecipitation of PR and Stat5—To facilitate immunoprecipitation studies, hPR1, the full-length PR-B cDNA cloned into the mammalian expression vector pSG5 from P. Chambon (Strasbourg, France) (31), was modified to disrupt the stop codon and add a carboxyl-terminal flag epitope sequence: N-termFLAG;His6A (32). The Stat5α cDNA was expressed in mammalian cells using the FLAG epitope-tagged PR (PRαfl) and expressed and functional in vivo. HeLa cells were co-transfected with PRαfl and the PRE-TATA-CAT reporter using calcium phosphate precipitation as described previously (32), and treated with the synthetic progesterone R5020. The chloramphenicol acetyltransferase activity produced by the PRαfl was equal to that of wild-type hPR1 (data not shown).

A stable cell line expressing PRαfl was established by co-transfecting HeLa cells with 4.5 μg of PRαfl plasmid and 0.5 μg of a plasmid encoding the neomycin resistance gene, pSV2neo. DNA precipitate was removed 18 h later, and cells were grown in MEM + 5% fetal bovine serum containing 700 μg/ml of the neomycin analog G418 (Life Technologies, Inc., Gaithersburg, MD) to kill non-transfected cells. Surviving neomycin-resistant colonies were expanded and cells were analyzed by immunoblotting and chloramphenicol acetyltransferase assay for clones expressing high levels of functional PRαfl.

For co-immunoprecipitations, wild type HeLa and HeLa PRαfl were cultured in MEM supplemented with 5% twice charcoal-stripped heat-inactivated fetal bovine serum. Cells were treated with progesterone or R5020 (10 nM) for 1 h. Nuclear extracts were prepared according to the methods described in Ref. 33. After harvesting in Hanks’; EDTA, cells...
were washed in phosphate-buffered saline then resuspended in 5 packed cell volumes of buffer A (10 mM (pH 7.9) at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol, 0.5 mM PMSF, one complete protease inhibitor mixture tablet (Boehringer Mannheim, GmbH Germany) per 50 ml of buffer) and allowed to stand at 4 °C for 10 min. The cells were collected by centrifugation at 2,000 rpm for 10 min, then resuspended in two packed cell volumes of buffer A and lysed by 10 strokes of a Kontes all glass Doune homogenizer (B type pestle). The homogenate was centrifuged for 10 min at 2,000 rpm to pellet the nuclei. The supernatant was removed and the pellet subjected to a second centrifugation at 25,000 × g for 20 min (Beckman Optima L-80K Ultracentrifuge, 70.1 Ti rotor) to remove residual cytoplasmic material. The pellet was resuspended in 3 ml of buffer C containing 10^6 cells and re-homogenized as above. Buffer C consisted of 20 mM Hepes, 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, and protease inhibitors. The homogenate was mixed by rotation for 30 min at 4 °C then centrifuged for 30 min at 25,000 × g. The resulting clear supernatant was dialyzed at 4 °C for 5 h to overnight in 20 mM Hepes (pH 7.9), 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, and protease inhibitors. One mg of nuclear extract was incubated with 100 µl of a 50% slurry of Anti-Flag M2 Affinity Gel (Eastman Kodak, New Haven, CT) at 4 °C for 4 h. The anti-Flag resin was then washed twice with TEDG containing 0.1 mM NaCl, twice with TEDG containing 0.3 mM KCl, and twice with TEDG containing 0.1 mM NaCl and 0.1% Nonidet P-40, using 1 ml of buffer C for each wash. The PR/protein was then eluted by competition with 0.2 mg/ml Flag peptide (N-DYKDDDDK-CE (Eastman Kodak) in 200 µl of TEDG containing 0.3 mM KCl and 0.1% Nonidet P-40, for 30 min at 4 °C. Laemmli sample buffer was added to the eluate, and proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and identified by immunoblotting.

**Transient Transfections—**PR-positive breast cancer cells plated at 1 million cells per 10-cm dish in MEM supplemented with 5% fetal bovine serum were treated with 10 nM R5020 or ethanol vehicle for 48 h prior to transfection. Cells were then transiently transfected with 3 µg of plasmid DNA using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Ramsey, NJ) according to the manufacturer's instructions. The two isoforms of STAT, STAT5a and STAT5b, share 96% similarity at the protein level (Fig. 2). Aside from the non-conserved 5'- and 3'-untranslated regions, the main difference between the two isoforms is in the COOH terminus. The last 8 amino acids of the two isoforms are completely divergent, and STAT5a is 7 amino acids longer than STAT5b (37). To determine which of the two isoforms are regulated by PR, T47Dco cells were treated with or without R5020 for 8–60 h. Protein blots of whole cell lysates were probed with antibodies that recognize epitopes unique to STAT5a or STAT5b, as well as with an antibody that recognizes both isoforms (Fig. 2A). This study shows that both the longer (95 kDa) STAT5a and the shorter (92 kDa) STAT5b isoform are up-regulated by progestin treatment. STAT5a was below detectable levels in the absence of R5020 (Fig. 2A). Note that a nonspecific (NS) protein migrating just above the 95-kDa STAT5a is recognized by STAT5a antibody, but is not regulated by R5020 and serves as a loading control. Similar studies using anti-STAT3 (Fig. 2B) and anti-STAT1 (Fig. 2C) antibodies show that these STAT family members are also progestin regulated. While STAT3 is clearly up-regulated by progestin treatment, STAT1 is only slightly down-regulated. The same blot, after probing with anti-STAT1 antibody, was stripped and reprobed with antibody recognizing total STAT5, which showed that STAT5 was again strongly up-regulated as in Figs. 1 and 2A (data not shown).

**RESULTS**

**Regulation of STAT Protein Levels by Progestosterone Treatment—**To examine the effects of progestins on STAT protein levels, PR-positive T47Dco breast cancer cells were treated with progesterone (not shown), 10 nM R5020, or ethanol vehicle (Fig. 1A) and cells were harvested 8–72 h later. Total STAT5 protein present in whole cell lysates was detected using a polyclonal antibody that cross-reacts with both STAT5a and 5b.

![FIG. 1. Stat5 up-regulation by R5020 is PR dependent. Breast cancer cells were treated with 10 nM of the synthetic progestin R5020 (+) or ethanol vehicle (−) for 8–72 h. Cells were harvested at the time points indicated, and 100 µg of whole cell extracts were resolved by SDS-PAGE and immunoblotted with an antiseraum recognizing total STAT5 (STAT5a and 5b isoforms) as indicated. Open arrows indicate the position of the 97.4-kDa standard. A, PR-positive T47Dco cells. Fold increases in STAT5 protein with R5020 treatment over paired ethanol controls are shown. B, PR-negative T47D-Y cells.

Stat5 protein levels were elevated by 8 h and remained elevated until 72 h after R5020 treatment with increases of 4–7-fold (Fig. 1A). To test the PR dependence of this effect, the experiment was repeated using PR-negative T47D-Y cells (28) (Fig. 1B). In the absence of PR, R5020 did not up-regulate Stat5.

The two isoforms of STAT5, STAT5a and STAT5b, share 96% similarity at the protein level (Fig. 2). Aside from the non-conserved 5'- and 3'-untranslated regions, the main difference between the two isoforms is in the COOH terminus. The last 8 amino acids of the two isoforms are completely divergent, and STAT5a is 7 amino acids longer than STAT5b (37). To determine which of the two isoforms are regulated by PR, T47Dco cells were treated with or without R5020 for 8–60 h. Protein blots of whole cell lysates were probed with antibodies that recognize epitopes unique to STAT5a or STAT5b, as well as with an antibody that recognizes both isoforms (Fig. 2A). This study shows that both the longer (95 kDa) STAT5a and the shorter (92 kDa) STAT5b isoform are up-regulated by progestin treatment. STAT5a was below detectable levels in the absence of R5020 (Fig. 2A). Note that a nonspecific (NS) protein migrating just above the 95-kDa STAT5a is recognized by STAT5a antibody, but is not regulated by R5020 and serves as a loading control. Similar studies using anti-STAT3 (Fig. 2B) and anti-STAT1 (Fig. 2C) antibodies show that these STAT family members are also progestin regulated. While STAT3 is clearly up-regulated by progestin treatment, STAT1 is only slightly down-regulated. The same blot, after probing with anti-STAT1 antibody, was stripped and reprobed with antibody recognizing total STAT5, which showed that STAT5 was again strongly up-regulated as in Figs. 1 and 2A (data not shown).

**PR Up-regulates STAT5 mRNA Levels—**To determine whether progesterone, via PR, up-regulates STAT5a mRNA levels, total RNA was isolated from two T47Dco breast cancer cell lines stably expressing either the PR-A or PR-B isoform (28) that had been treated with progesterone or vehicle for 12 or 24 h (Fig. 3). Northern blot analysis demonstrates hormone-dependent induction of STAT5a message at 12 and 24 h following progesterone treatment (Fig. 3). Interestingly, STAT5a appears to be more strongly induced by the PR-B than the PR-A isoform (compare lanes 2 and 4; 6 and 8). In contrast, the same Northern blot hybridized with cDNA for two genes known to be PR regulated, fatty acid synthetase (30, 38) or cyclin D1 (18, 39), shows equal induction of these genes by the two PR isoforms.
The same Northern blot hybridized with cDNA for a non-regulated gene, glyceraldehyde-3-phosphate dehydrogenase, serves as an RNA loading control.

Progestin Treatment Promotes Translocation of Stat5 into the Nucleus—Upon activation by growth factor receptors via their intrinsic tyrosine kinase activity or by cytokines via members of the JAK tyrosine kinase family, STATs are phosphorylated and translocate into the nucleus. In addition to the well characterized activation of Stat5 by prolactin (9), EGF has been shown to activate STATs in breast cancer cells (13). Since progestins sensitize breast cancer cells to the effects of EGF (Ref. 18; Lange et al. (20)) in the accompanying article and Fig. 8 herein we asked whether they influence the ability of EGF to activate and translocate Stat5 to the nucleus (Fig. 4A). T47Dco
cells were treated for 24 h with 10 nM R5020 or vehicle, then with 10 nM EGF or vehicle for 5 min. Nuclear and cytosolic extracts were prepared and equal amounts of protein were probed with antibody recognizing total Stat5 (Fig. 4A). In untreated cells, Stat5 was exclusively cytoplasmic (lane 1); no Stat5 was found in the nucleus (lane 2). As expected, EGF treatment promoted nuclear translocation of a portion of Stat5 (lanes 5 and 6). Surprisingly, R5020 not only increased total Stat5 levels (lanes 3 and 4; 7 and 8), but also promoted extensive nuclear translocation of Stat5 (compare lanes 2 and 4) independent of EGF treatment. Depending on the stringency with which nuclei are prepared, varying amounts of Stat5 are detected in the nuclear fraction in the untreated cells; however, the amount of Stat5 is consistently an average of 1.8-fold
Fig. 4. Both EGF and R5020 induce nuclear translocation of Stat5. PR-positive T47Dco cells were treated for 24 h with either 10 nM R5020 (+) or ethanol vehicle (−) followed by 10 nM EGF (+) or sterile water (−) for 5 min. Nuclear and cytosolic fractions were prepared and samples (100 μg of each) were resolved by SDS-PAGE then immunoblotted with antibody to total Stat5 as indicated. Protein levels were quantitated by Bradford assay and Ponceau S staining (not shown), and by immunoblotting with an antibody against cdc2 kinase/PSTAIR.

R5020 (24 hrs) | EGF (5 min)
---|---
| - | + |
| - | + |
| Cyt | N | Cyt | N | Cyt | N | Cyt | N

higher in the nuclear fraction of progesterone-treated cells. These results suggest that Stat5 signaling molecules, previously thought to be activated only by association with ligand-activated cell surface receptors, can be translocated to the nucleus by steroid hormone treatment.

Interaction of Stat5 and PR—To explain the surprising effects of progestins on Stat5 nuclear translocation, we speculated that Stat5 might interact with PR. This would not be unreasonable since an interaction between GR and Stat5 has recently been described (26). To determine whether PR and Stat5 can interact, co-immunoprecipitation experiments were performed using wild type HeLa cells or HeLa cells stably transfected with the Flag-tagged B-isofrom of PR (PRαF HeLa) (Fig. 5A). Both PRαF HeLa (lane 1) and wild type HeLa (lane 2) were treated with 10 nM R5020 for 1 h and nuclear extracts were immunoprecipitated with either anti-total Stat5 antibody followed by protein A-Sepharose (Fig. 5A, top) or Anti-Flag M2 Affinity Gel (Fig. 5A, bottom), then eluted and immunoblotted. The anti-Stat5 immunoprecipitates were first probed with the anti-PR antibody AB-52, followed by the anti-Stat5 antibody. The eluates obtained by competing PR and associated proteins off the Anti-Flag M2 Affinity Gel with the Flag peptide, were then probed with the anti-Stat5 antibody, followed by the anti-PR antibody. Fig. 5A, top, shows that PR-B (116–120 kDa) co-immunoprecipitated with Stat5 only from the PRαF HeLa cells (lane 1) although Stat5 is present in both cell lines (lanes 1 and 2). Fig. 5A, bottom, shows that Stat5 co-immunoprecipitates with PR only from the PRαF HeLa cells (lane 1) although both cell lines contain equal amounts of Stat5 (upper panel, lanes 1 and 2). These reciprocal studies show that PR and Stat5 are associated, either directly or in a multiprotein complex. In Fig. 5B, Stat5 was co-immunoprecipitated with PR from whole cell extracts of PRαF HeLa cells treated with either ethanol (−) or R5020 (+) for 48 h. Untreated cells contain much higher levels of PR than R5020-treated cells, which exhibit ligand-dependent PR down-regulation (40, 41). When probed with anti-Stat5 antibody, the amount of Stat5 protein corresponds to the amount of PR, suggesting that stoichiometric amounts of Stat5 co-immunoprecipitate with PR.

Progestins Promote Co-immunoprecipitation of Stat5 and JAK2 with Anti-Phosphotyrosine Antibody—Stat5 can be activated directly by the tyrosine kinase activity of growth factor receptors or indirectly by JAK2 (8). In both cases, activation involves tyrosine phosphorylation of Stat5. To determine the tyrosine phosphorylation state of Stat5 and JAK2 following treatment with R5020, T47Dco cells were transiently (1 h) and chronically (48 h) treated with R5020. Whole cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody (4G10) then immunoblotted with specific antisera to Stat5 or JAK2 (Fig. 6). In the absence of R5020 pretreatment, JAK2 is immunoprecipitated with the anti-phosphotyrosine antibody only following EGF treatment (lane 2). Note that JAK2 is not present in phosphotyrosine immunoprecipitates after 1 h of R5020 treatment (lane 1). However, JAK2 is immunoprecipitated with the anti-phosphotyrosine antibody as efficiently after 48 h of R5020 treatment (lane 4) as with EGF treatment (lane 5). Although less dramatic, the same results described for JAK2 were also observed with Stat5. Co-immunoprecipitation of Stat5 and JAK2 with anti-phosphotyrosine antibody following long-term R5020 treatment was also observed in independent experiments in Fig. 3 of the accompanying article (20). These results indicate that progestin treatment can influence the JAK2/Stat5 pathway and that transient progestin treatment (1 h) has different biological effects than chronic progestin treatment (48 h). Although total Stat5 levels are up-regulated by progestin treatment, JAK2 levels are unaffected (data not shown).

To confirm that progestin influences the immunoprecipitation of Stat5 with anti-phosphotyrosine 4G10 antibody, duplicate plates of T47Dco cells were treated with ethanol or R5020 for 48 h followed by 5 min of EGF treatment. Whole cell lysates were immunoprecipitated with anti-phosphotyrosine antibody (4G10) then immunoblotted with antibody to total Stat5. Fig. 6B demonstrates: 1) that more Stat5 is immunoprecipitated by the anti-phosphotyrosine antibody after progestin pretreatment, and 2) that EGF stimulates a Stat5 upshift only following progestin treatment. We therefore asked whether cytokine- or growth factor-mediated activation of Stat5 could be influenced by progestins.

Prolactin Induced Activation of Stat5 Is Progestin Dependent in T47Dco Breast Cancer Cells—Prolactin is a key cytokine influencing breast physiology that signals through the JAK2/Stat5 pathway. To determine whether progestin treatment affects the ability of prolactin to activate Stat5, T47Dco cells were first treated with R5020 or vehicle for 48 h, then treated transiently with R5020 (1 h), ethanol vehicle (1 h), or prolactin (10 min). Whole cell lysates were immunoprecipitated first with a phospho-Stat5 specific antibody, and then with antibody to total Stat5 (Fig. 7A). Stat5 was present in the cell lysates under all treatment conditions (total Stat5, lanes 1–6) and was up-regulated by the progestin treatment (total Stat5, lanes 4–6). However, Stat5 remained unphosphorylated (P-Stat5, lanes 1–5), except in the cells that were pretreated with R5020 for 48 h, prior to the 10-min prolactin treatment (P-Stat5, lane 6). Prolactin alone had no effect (lane 3) nor did transient (1 h) or chronic (48 h) R5020 treatment (lane 2). Thus, in T47Dco cells, Stat5 phosphorylation by prolactin requires progestrone pretreatment.
Fig. 5. Co-immunoprecipitation of PR and Stat5. A, HeLa cells stably expressing the PR-B isoform tagged with a Flag epitope (PRb:f HeLa, lane 1) and wild type HeLa cells (lane 2) were treated with 10 nm R5020 for 1 h and nuclear extracts were prepared. Stat5 or PR-B receptors were immunoprecipitated from 1 mg of nuclear extract protein with either anti-total Stat5 antibody followed by protein A-Sepharose (top), or with Anti-Flag M2 Affinity Gel (bottom). Immunoprecipitates were either released from the protein A-Sepharose by boiling in Laemmli sample buffer, or eluted from the Anti-Flag M2 Affinity Gel with Flag peptide followed by boiling in Laemmli sample buffer, then resolved by SDS-PAGE and transferred to nitrocellulose. Anti-Stat5 immunoprecipitates were probed first with anti-PR antibody AB52, followed by anti-total Stat5 antibody. Eluates released from the Anti-Flag M2 Affinity Gel with the Flag peptide were probed first with anti-Stat5 antibody, then with anti-PR antibody, AB-52. White arrows indicate the position of the 97.4-kDa marker. 1, PRb:f HeLa; 2, wild type HeLa cells. B, PRb:f HeLa cells were treated with either ethanol (−) or R5020 (+) for 48 h. PR were immunoprecipitated from whole cell lysates using the Anti-Flag M2 Affinity Gel, released with the Flag peptide, and co-precipitated proteins were resolved by SDS-PAGE and immunoblotted with AB-52 and Stat5 antibodies.

Fig. 6. Co-immunoprecipitation of JAK 2 and Stat5 with anti-phosphotyrosine antibody. A, PR-positive T47Dco cells were pretreated for 48 h with 10 nm R5020 or ethanol vehicle, then subjected to short-term R5020 (1 h) or 10 nm EGF (5 min) treatments. Equal amounts (1 mg) of whole cell lysates were immunoprecipitated using anti-phosphotyrosine antisera (4G10). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with antibodies specific to JAK2 and total Stat5. B, duplicate plates of T47Dco cells were pretreated for 48 h with 10 nm R5020 or ethanol vehicle, then stimulated with vehicle or 10 nm EGF for 5 min. Equal amounts (1 mg) of whole cell lysates were immunoprecipitated using anti-phosphotyrosine antisera (4G10). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with antibody recognizing total Stat5. A nonspecific band is shown demonstrating equal protein loading.

The β-casein promoter, known to be induced by Stat5 when activated by prolactin via prolactin receptors and JAK2, was next used to test the function of the phosphorylated Stat5. T47D-YB cells were pretreated with either R5020 or ethanol vehicle for 48 h. Cells were then washed, transfected with a β-casein promoter-luciferase reporter, treated with either R5020 or prolactin, and harvested for luciferase assay 24 h after hormone treatment. We found that in T47D-YB breast cancer cells, prolactin alone has minimal to no significant effect on the β-casein luciferase promoter. However, after R5020 pretreatment, a 6–7-fold induction of the β-casein luciferase promoter was observed (Fig. 7B). Thus R5020 sensitized these breast cancer cells to the transcriptional effects of prolactin mediated through Stat5.

Combined Progestins and EGF Have a Synergetic Effect on Induction of p21WAF1 and c-fos Promoters—To determine whether growth factor signaling is also influenced by progestosterone, we examined the transcriptional activity from the p21WAF1 promoter and c-fos promoters. Both genes are induced by progestins and EGF and both promoters contain STAT-binding sites termed sis-inducible elements. The cell cycle regulatory gene, p21WAF1 is induced 4.5-fold by progestins (18, 34) and by EGF/STAT pathway (13, 15). The mRNA of the immediate early gene c-fos is rapidly induced by progestins (18) and by EGF/STAT (16). PR-positive T47D-YB cells were transfected with either the −320 p21-luc construct or the −357/−276 c-fos-81-TK-luc construct. The −320 p21-luc construct contains one of three sis-inducible elements at position −640. The −357/−276 c-fos-81-TK-luc construct contains a sis-inducible element at −342. Following transfection, cells were treated with EGF or R5020 alone, or simultaneously with both hormones.

Fig. 8A shows that the p21WAF1 promoter is induced 4.5-fold by progesterone, 2.4-fold by EGF, and 13.2-fold by both hormones together. The average increase in five experiments was progesterone, 3.5-fold; EGF, 2.2-fold; both hormones, 11-fold. Fig. 8B demonstrates the corresponding increases in endogenous p21 levels when T47D-YB cells are treated for 24 h with ethanol, progesterone, EGF, or both hormones. Whole cell lysates were probed by immunoblotting with antibody to p21.
hormones produced a 145-fold increase in luciferase activity. The control minimal thymidine kinase promoter linked to the luciferase reporter had 1% the activity of the c-fos containing construct, and no synergy was observed with both hormones. The experiment in Fig. 8C is also representative of five repeats in which the average fold increases were as follows: progesterone, 18-fold; EGF, 5.3-fold; and both hormones, 97-fold. The same pattern of induction of both the p21 and c-fos promoters is observed using T47Dco cells with endogenous PR, rather than stably transfected PR. Since the −2320 p21 promoter and the −357/−276 c-fos promoter lack a consensus progesterone response element (PRE), we speculate that the effect of progesterone and the synergistic effect of progesterone plus EGF are mediated through the six-inducible element, possibly via PR interactions with STATs.

It is also possible that the synergistic effects of progesterone plus EGF are due to enhanced STAT activation via tyrosine phosphorylation. To test this, T47D-YB cells were treated for 24 h with 10 nM R5020 or ethanol vehicle, then subjected to short-term 10 nM R5020 (1 h) (+), prolactin (10 min) (+), or ethanol (−) treatments. Phosphorylated (P) Stat5 and total Stat5 were detected in whole cell extracts of T47Dco cells by immunoblotting first with an antibody specific for Stat5 phosphorylated on tyrosine 694, followed by an antibody that recognizes total Stat5. B, triplicate dishes of T47D-YB cells stably expressing the PR B-isoform, were pretreated for 48 h with 10 nM R5020 or ethanol vehicle, then transfected with the −2300/+490 β-casein promoter fused to a luciferase reporter and treated with either R5020 or prolactin for an additional 24 h. Luciferase units are shown on the y axis and standard errors are indicated. 1, R5020; 2, prolactin.

Since both progesterone and STATs are key molecules in the growth and differentiation of the normal breast, we have studied their relationship in breast cancer. We show here, using breast cancer cells, that progestins regulate the levels of STAT proteins in a PR-dependent manner. Additionally, we document that Stat5 can interact with PR and that progesterin treatment induces Stat5 translocation to the nucleus. Stat5 and JAK2 are present in phosphotyrosine immunoprecipitates following chronic progesterin treatment. This surprising result implies that progestins may activate a kinase that mediates the tyrosine phosphorylation of JAK2, or that JAK2 and Stat5 associate with, and coimmunoprecipitate with, another phosphotyrosine-containing protein following long-term progesterin treatment. We have not addressed the role of a potential progesterin initiated autocrine loop; however, we do not observe constitutive activation of EGFR or mitogen-activated protein kinases by progestins alone, as shown in Fig. 3–5 of the accompanying article (20). These data led to the hypothesis that progestins sensitize breast cancer cells to signaling by cytokines and growth factors. Indeed, we show that progesterin pretreatment is necessary for prolactin signaling to the β-casein promoter via Stat5. Additionally, we observe transcriptional synergy between progesterone and EGF on the promoters of two growth regulatory genes containing STAT sites.

Finally, we show that progesterone enhances the ability of EGF to activate Stat5 through phosphorylation of tyrosine 694. Together, these results suggest novel pathways for the integration of progesterone and cytokine/growth factor signaling.

PR-mediated Regulation of Stat5—Knock-out studies have confirmed that Stat5 is essential for normal mammary gland development (6). Additionally, Stat5a-deficient mice are unable to lactate due to failure of the gland to differentiate appropriately during pregnancy (6). In fact, in wild-type animals, Stat5 mRNA and protein levels increase during pregnancy, peaking just prior to parturition, then declining during lactation (10, 37). This rise and fall parallels changes in progesterone levels, suggesting a STAT-regulatory role for this pregnancy hormone. We now demonstrate the up-regulation of STAT mRNA and proteins by progestins in a PR-dependent manner in breast cancer cells.
cancer cells (Figs. 1–3). The PR-B isoform appears to be more potent in this regard (Fig. 3). In contrast, two other PR-regulated genes, cyclin D1 and fatty acid synthetase, are equally up-regulated by both PR isoforms. The PR-B isoform is a stronger transcriptional activator than PR-A on some transiently expressed promoters in vitro models and of the flavin-containing monooxygenase 5 gene in breast cancer cells (42). Whether STAT promoters contain a progesterone response element remains to be determined. Fold increases in luciferase activity over ethanol vehicle controls are shown above the bars. Representative results of five independent experiments are shown. B, T47D-YB cells were treated for 24 h with ethanol vehicle, 10 nM progesterone, 10 nM EGF, or both hormones together. Whole cell lysates were probed by immunoblotting with an antibody recognizing p21WAF1. C, triplicate dishes of T47D-YB cells were transfected with the −357/−276 c-fos-81TK-luciferase promoter/reporter or a control 81-TK-luciferase construct. Cells were treated with ethanol vehicle, progesterone, EGF, or both progesterone and EGF, then harvested 24 h later. Fold increases in luciferase activity over ethanol vehicle controls are indicated above the bars. Luciferase units are shown on the y axis and standard errors are indicated. Representative results of five independent experiments are shown.

**Fig. 8.** Progesterone and epidermal growth factor synergize on the c-fos and p21WAF1 promoters. A, triplicate dishes of T47D-YB cells were transfected with −2320 p21-luciferase promoter/reporter. Cells were treated with ethanol vehicle (■), progesterone (□), EGF (▲), or both (■) progesterone and EGF, then harvested 24 h later. Luciferase units are shown on the y axis and standard errors are indicated. Fold increases in luciferase activity over ethanol vehicle controls are shown above the bars. Representative results of five independent experiments are shown. B, T47D-YB cells were treated for 24 h with ethanol vehicle, 10 nM progesterone, 10 nM EGF, or both hormones together. Whole cell lysates were probed by immunoblotting with an antibody recognizing p21WAF1. C, triplicate dishes of T47D-YB cells were transfected with the −357/−276 c-fos-81TK-luciferase promoter/reporter or a control 81-TK-luciferase construct. Cells were treated with ethanol vehicle, progesterone, EGF, or both progesterone and EGF, then harvested 24 h later. Fold increases in luciferase activity over ethanol vehicle controls are indicated above the bars. Luciferase units are shown on the y axis and standard errors are indicated. Representative results of five independent experiments are shown.

**PR Regulates Stat5 Expression and Activity**

and it will be important to determine whether STAT expression correlates with PR positivity in such tumors.

**Progesterin Regulation of Stat5 Localization through PR Binding?**—Cytoplasmic Stat5α and 5β are substrates for tyrosine phosphorylation by growth factor tyrosine kinases such as EGFR, or by cytoplasmic protein tyrosine kinases of the JAK family activated by cytokines such as PRL. Phosphorylation of the conserved COOH-terminal tyrosine residue on Stat5 (tyrosine 694) leads to homo- or heterodimerization followed by nuclear translocation through unknown mechanisms (8, 12). Recently, however, Ali and Ali (44), showed that Stat5 tyrosine phosphorylation can be dissociated from nuclear translocation. In addition, Stat1, mutated at the critical tyrosine phosphorylation site, retains activity and may act in its monomeric state
ticoids and prolactin. Stat5-dependent transcription from the prolactin Stat5 on tyrosine 694.

with 10 nM progesterone or vehicle, followed by 10 nM EGF for 5 min.

site, and GR (26). Since the interaction between Stat5, bound to its consensus DNA-binding site, and GR (26), and that STATs, perhaps as monomers, can enter the cytoplasm and nucleus (49, 50). This raises the possibility that progestin-induced synergism does not require the binding of PR to the promoter region, where the receptors bind to Sp1 and CBP/p300, rather than directly to DNA (34). Interestingly, maximal activation of the ICAM promoter requires a physical interaction between Sp1 and Stat1 in which Sp1 is thought to recruit Stat1 to the promoter and/or serves to link Stat1 to the basal transcription complex (53). Taken together these data suggest mechanisms for cross-talk between STATs and PR in which one or the other transcription factor is tethered to DNA indirectly.

Finally, we show here that progesterone pretreatment enhances the ability of EGF to activate Stat5 through phosphorylation of tyrosine 694. This raises the possibility that progestin-induced synergism does not require the binding of PR to the promoter. Instead, progestin treatment may enhance the amount of activated Stat5 capable of binding to the promoter.

In summary, our findings that progestins regulate STATs, foster protein-protein interactions between PR and Stat5, promote translocation of Stat5 to the nucleus, enhance the ability of prolactin and EGF to induce phosphorylation of Stat5, and synergize with EGF to activate transcription of growth regulatory genes, demonstrate that steroid and growth factor/cytokine signaling pathways can converge at multiple levels.

Acknowledgments—We gratefully acknowledge Dany Chalbos for the gift of fatty acid synthetase clone pG8; Robert Sclafani and Andrew Kraft, for cyclin D1 and Stat5a cDNA clones, respectively. The –2320-base pair p21WAF1 promoter construct, a gift of Andrew Kraft and Joseph Biggs, was cloned into a pA3-LUC vector obtained from William Wood, and the c-fos-81TK-luc was kindly provided by Arthur Gutierrez-Hartmann.

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