Convergence of Progesterone and Epidermal Growth Factor Signaling in Breast Cancer

POTENTIATION OF MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS*

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

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During late stages of breast cancer progression, tumors frequently acquire steroid hormone resistance with concurrent amplification of growth factor receptors; this alteration predicts a poor prognosis. We show here that following treatment with the progestin, R5020, breast cancer cells undergo a "biochemical shift" in the regulation of epidermal growth factor (EGF)-stimulated signaling pathways: R5020 potentiates the effects of EGF by up-regulating EGFR, c-ErbB2 and c-ErbB3 receptors, and by enhancing EGF-stimulated tyrosine phosphorylation of signaling molecules known to associate with activated type I receptors. Independently of EGF, R5020 increases Stat5 protein levels, association of Stat5 with phosphotyrosine-containing proteins, and tyrosine phosphorylation of JAK2 and Shc. Furthermore, progestins "prime" breast cancer cells for growth signals by potentiating EGF-stimulated p42/p44 mitogen-activated protein kinase (MAPK), p38 MAP kinase, and JNK activities. Although the levels of cyclin D1, cyclin E, and p21WAF1 are up-regulated by R5020 alone, they are synergistically up-regulated by EGF in the presence of R5020. Up-regulation of cell cycle proteins by EGF is blocked by inhibition of p42/p44 MAPK only in the presence of R5020, supporting a shift in the regulation of these cell cycle mediators from MAPK-independent to MAPK-dependent pathways. In summary, progesterone selectively increases the sensitivity of key kinase cascades to growth factors, thereby priming cells for stimulation by latent growth signals. These data support a model in which breast cancer cell growth switches from steroid hormone to growth factor dependence.

Estradiol and progesterone are involved in breast cancer development, but at the time of diagnosis only one-third of tumors are steroid hormone-dependent. As they progress, these tumors often acquire steroid hormone resistance, yet retain nuclear steroid receptors. Indeed, nuclear receptor loss or mutation accounts for only 10–20% of steroid hormone-resistant tumors (1). Thus, it has been postulated that in the majority of resistant tumors, control over growth is assumed by locally acting autocrine or paracrine peptide growth factors. As a result, invasive cancers with the worst prognosis are those that are growth factor receptor positive and steroid hormone resist-

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EGF is a major regulator of breast cancers that have become steroid hormone-resistant. EGF (c-ErbB1) expression is significantly associated with the loss of steroid hormone sensitivity in breast cancer (13), and correlates with high disease recurrence rates and decreased patient survival. The HER-2/Neu (c-ErbB2) oncogene shares extensive homology with EGF, suggesting that these receptors, although distinct, signal via similar mechanisms. The Neu receptor is stimulated by a family of closely related growth factors called heregulins or Neu differentiation factors that are present in breast cancer cell-conditioned medium and in serum (14). Gene amplification of c-ErbB2 receptors is one of the most common occurrences in breast cancers (15) and is also correlated with shortened survival (2). Heterodimerization between EGF and c-ErbB2 receptors is induced by EGF, and these heterodimers exhibit highly increased affinity for EGF compared with EGF homodimers (16). Additional members of the type I growth factor receptor family include the less well defined c-ErbB3 and c-ErbB4 receptors, which are also expressed in breast cancer (17). These receptors differ most in their C-terminal intracellular domains, predicting differences in signal transduction elicited by each receptor subtype. For example, the c-ErbB3 receptor has very weak intrinsic tyrosine kinase activity compared with the other three family members (18). However, each receptor has very weak intrinsic tyrosine kinase activity compared with the other three family members (18).

Several studies have documented transcriptional and/or proliferative synergy between EGF and progesterone or estrogen (20, 21) and have shown that progesterone up-regulates EGFR expression on the cell surface (20, 22–24). For example, progesterins but not estrogens, androgens, or glucocorticoids cause a 2–3-fold increase in EGFR number (22) and a 6-fold increase in EGF mRNA levels (25) in human breast cancer cells. In addition to increasing high affinity EGFR numbers, progesterone effects the phosphorylation state of EGF and c-ErbB2 receptors; in the presence of phosphatase inhibitors, phosphorylation of both receptors is increased severalfold by progesterone (20). Progesterone receptor (PR) levels are reportedly down-regulated by EGF, as measured by equilibrium progesterone binding studies (24), indicating bi-directional effects of each agent on the receptor of the other. Up-regulation of EGFR by progesterone in conjunction with down-regulation of PR by EGF suggests the existence of a regulatory loop in which loss of steroid hormone responsiveness parallels gain of growth factor-dependent proliferation, as is observed during breast tumor progression (13, 14). Indeed, we have recently demonstrated that progesterone pretreatment sensitizes T47D breast cancer cells to the proliferative effects of EGF (26). We therefore sought to define the molecular mechanisms involved in crosstalk between progesterone- and EGF-mediated signaling. Our data demonstrate that these two signaling pathways are integrated; progesterone enhances the effects of EGF in breast cancer cells via selective amplification of signaling cascades that are activated by type I growth factor receptors.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—Monoclonal PR-negative T47D-Y breast cancer cells and T47D-YB cells stably expressing the PR B-receptors were previously described (27). Cells were routinely seeded at 1 × 10^6 cells/dish, cultured in 10-cm dishes, and incubated in 5% CO₂ at 37°C in a humidified environment as described (27). For signal transduction experiments in which cells were treated with EGF (30 ng/ml) or heregulin (10 ng/ml), cultures were placed in serum-free media for 18–18 h prior to growth factor addition.

Antibodies for type I growth factor receptors (c-ErbB2 and c-ErbB3) were obtained from NeoMarkers (Fremont, CA). EGF receptor antisera was kindly provided by Roger Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA). Anti-phosphotyrosine (4G10) monoclonal antibody, and STAT5, JAK2, p70S6K, and cyclin D1 polyclonal antisera were purchased from Upstate Biotechnology (Lake Placid, NY); Cbl and p21WAF antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); Shc antisera was from Transduction Laboratories (Lexington, KY); phospho-specific and total MAPK antisera, phospho-specific p38, JNK, and AKT polyclonal antisera, and phospho-specific and total JNK polyclonal antisera were purchased from Cell Signaling Technology (Beverly, MA); and cyclin E polyclonal antisera was from Pharmingen (San Diego, CA). Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Human recombinant EGF was purchased from Collaborative Biomedical Products (Bedford, MA). Anti-proliferating non-proliferating cell membranes for receptor immunoblots—Cells growing on 10-cm dishes were washed with 5 ml of ice-cold phosphate-buffered saline and lysed by scraping in 1 ml of lysis buffer (20 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 1 mM EDTA, and 1 mM β-mercaptoethanol) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin). Cells in lysis buffer were further broken by passing the lysate through a 26-gauge needle, 6 times. Nuclei were cleared from the lysates by centrifugation at 1,000 rpm in a refrigerated Microfuge for 5 min. The pellet was discarded and membranes contained in the supernatant were collected by centrifugation for an additional 30 min at 15,000 rpm. The supernatant was discarded and the resulting membrane pellet was resuspended in 100–200 μl of lysis buffer and sheared through a 28-gauge needle, 6 times. Protein concentrations in each lysate were determined using the Bradford reagent and equal amounts of protein were loaded onto 8% SDS-PAGE gels and subjected to protein immunoblotting.

**Anti-phosphotyrosine Immunoprecipitations**—Cells in 10-cm dishes were washed twice with 4 ml of ice-cold phosphate-buffered saline and lysed by scraping in extraction buffer (EB: 1% (v/v) Triton X-100, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM sodium fluoride, 20 μg/ml aprotinin, 20 μM sodium fluoride, 20 μg/ml aprotinin, 20 μM sodium fluoride, 20 μg/ml aprotinin, 20 μM sodium fluoride, 20 μg/ml aprotinin, 20 μM sodium fluoride). Lysates were clarified by centrifugation for 10 min at maximum speed and equal amounts of soluble protein (1 mg/ml) were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (4G10; 4 μg/ml protein) by constant rotation at 4°C for 2 h to overnight. Immuno complexes were captured by adding 30 μl of washed protein A (insoluble formalin-fixed Staph-A derived sorbin; Sigma) that had been preincubated with rabbit anti-mouse antibody. Protein-sorbin contain-
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RESULTS

Up-regulation of Type I Growth Factor Receptors by Progesterone—Because progestins can sensitize T47D breast cancer cells to the proliferative effects of EGF (26), and are known to increase expression of EGFR in certain breast cancer cell lines (22, 24, 25), we tested the effects of the progestin, R5020, on the expression of members of the type I growth factor receptor family in PR-positive T47D-YB cells and their PR-negative T47D-Y progenitors (27) (Fig. 2). T47D-YB cells were treated with a single dose of R5020 (20 nM) or vehicle for 0–72 h and the levels of EGFR, c-ErbB2, or c-ErbB3 receptors in purified membrane preparations were determined by Western blotting using specific antisera (Fig. 2A). R5020 increased the expression of all three receptors, beginning at 8 h for EGFR, and 24 h for c-ErbB2 and c-ErbB3 receptors. Peak levels, reached between 36 and 48 h, ranged from 18–20-fold over controls for EGFR, 11–14-fold for c-ErbB2, and 8–10-fold for c-ErbB3 over several independent experiments (Fig. 2B). c-ErbB4 receptor levels did not change in response to R5020 (not shown). Surprisingly, while EGFR were unresponsive to R5020 treatment in the PR-negative T47D-Y cells, the progestin increased both c-ErbB2 and c-ErbB3 levels in these cells by 2–5- and 3–4-fold, respectively, at 48 h (Fig. 2C). Nongenomic effects of progesterone are well described (28–31), although their underlying mechanisms are poorly understood.

Amplification of Phosphotyrosine-mediated Signaling by Progesterone—Since progesterone increases the levels of type I growth factor receptors, we asked whether intracellular signals originating at activated tyrosine kinase receptors were also amplified by progesterone. T47D-YB cells were treated or not with R5020 for 48 h to up-regulate type I receptors. Phosphotyrosine-containing proteins were then immunoprecipitated from control or EGF-stimulated (5 min) cells and visualized with phosphotyrosine (Tyr(P)) antisera (Fig. 3A). R5020 pretreatment, followed by short-term (3–5 min) EGF treatment, increased the tyrosine phosphorylation of several signaling proteins known to associate with activated tyrosine kinase receptors (compare lanes 2 and 4), including the SH2/SH3 domain-containing proteins, cbl (reviewed in Ref. 32), and p66 and p52 isoforms of Shc (reviewed in Ref. 33). To confirm this, tyrosine phosphorylation of the EGFR, Cbl, and p52 and p46 Shc isoforms was dependent on receptor stimulation by EGF, but was greatly increased in R5020-pretreated cells (Fig. 3, A and B). The p66 Shc isofrom was weakly detected in Shc immunoblots of Tyr(P) immunoprecipitates (not shown), but was greatly increased in R5020-pretreated cells (Fig. 3, A and B). The p66 Shc isofrom was weakly detected in Shc immunoblots of Tyr(P) immunoprecipitates (not shown), but was greatly increased in R5020-pretreated cells (Fig. 3, A and B).
tiserum containing 500 ng/ml R5020 for 15 min. Tyr(P)-containing proteins were detected in Tyr(P) immunoprecipitates from cells treated with or without R5020 for 48 h and then treated with or without EGF (30 ng/ml) for the indicated times. Activated p42 and p44 MAPKs were detected in whole cell lysates using phospho-specific MAPK antibodies. Total MAPK was also detected in the same samples using a separate antiserum; note that phosphorylated, active MAPKs undergo a slight mobility shift, thus four MAPK bands are visible in EGF (+)-treated cell lysates. Data are representative of a typical experiment (n = 6 at 5 min; n = 3 at 15 min). B, p42 and p44 MAPK activity in T47D-YB cells treated with or without R5020 and heregulin. T47D-YB cells were treated as in A with or without R5020 and/or heregulin B1 (10 ng/ml) for the indicated times (n = 3). Activated MAPKs were detected using phospho-specific antisera as in A. C, MAPK activity in PR-negative T47D-Y cells. R5020 (+) treatment for 48 h followed by no growth factor (C), EGF for 5 min (E), or heregulin B1 for 30 min (H) did not enhance MAPK activity above that of EtOH-treated controls (–). Equal amounts of protein (100 µg) was loaded onto the gels; the results of a typical experiment are shown (n = 4).

Fig. 4. Progesterone enhances MAPK activation by EGF. A, p42 and p44 MAPK activity in T47D-YB cells treated with R5020 and/or EGF. T47D-YB cells were treated with or without R5020 for 48 h and with or without EGF (30 ng/ml) for the indicated times. Activated p42 and p44 MAPKs were detected in whole cell lysates using phospho-specific MAPK antisera. Total MAPK was also detected in the same samples using a separate antiserum; note that phosphorylated, active MAPKs undergo a slight mobility shift, thus four MAPK bands are visible in EGF (+)-treated cell lysates. Data are representative of a typical experiment (n = 6 at 5 min; n = 3 at 15 min). B, p42 and p44 MAPK activity in T47D-YB cells treated with or without R5020 and heregulin. T47D-YB cells were treated as in A with or without R5020 and/or heregulin B1 (10 ng/ml) for the indicated times (n = 3). Activated MAPKs were detected using phospho-specific antisera as in A. C, MAPK activity in PR-negative T47D-Y cells. R5020 (+) treatment for 48 h followed by no growth factor (C), EGF for 5 min (E), or heregulin B1 for 30 min (H) did not enhance MAPK activity above that of EtOH-treated controls (–). Equal amounts of protein (100 µg) was loaded onto the gels; the results of a typical experiment are shown (n = 4).

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Fig. 5. Progesterone enhances activation of p38 and JNK by EGF. A. p38 and JNK activation in R5020-treated T47D-YB cells. T47D-YB cells were treated with or without R5020 (20 nM) for 48 h and then challenged with EGF (30 ng/ml) for the indicated times. Activated p38 and activated JNK were detected in whole cell lysates using phospho-specific antisera. Equal amounts of protein (100 µg) were loaded onto the gel. B. AKT and p70S6K activation in R5020-treated T47D-YB cells. Cells were treated as in A and AKT and p70S6K activation was detected in whole cell lysates using phospho-specific antisera (AKT); active p70S6K appears as a slightly upshifted band in EGF treated (+) cell lysates. Equal amounts of protein (100 µg) was loaded onto the gels; data represent the results of a typical experiment (p38 MAPK and JNK; n = 3, AKT and p70S6K; n = 2).

Receptors via heterodimerization (37, 38) (Fig. 4B). The activity of the classical MAPKs, p42 and p44, was determined by immunoblotting whole cell extracts with highly specific antisera for the active, Thr/Tyr-phosphorylated forms of p42 and p44 MAPK (39). A separate antiserum against total MAPK was used to visualize both the active and inactive forms; phosphorylation/activation causes a slight up-shift in MAPK mobility measured using the anti-total MAPK serum (Fig. 4A). While long-term treatment with R5020 alone had little or no effect on MAPK activity, pretreatment with the progestin greatly increased the ability of EGF to stimulate MAPK activity. Specifically, in R5020-pretreated cells, EGF (5 min) caused a 16–20-fold increase in p42 activity and a 2.5–6-fold increase in p44 activity above that stimulated by EGF alone, as measured by densitometry of independent immunoblots. In contrast, the relative abundance of total MAPK remained essentially unchanged by all the treatment regimens.

Compared with EGF, the activity of MAPK in response to heregulin was minimally affected by R5020 (Fig. 4B). At most, R5020 increased p42 MAPK activity in response to heregulin at 30 and 120 min by 2–4-fold. R5020 pretreatment did not affect the magnitude of MAPK activation by either EGF or heregulin in T47D-Y cells, which lack detectable PR (Fig. 4C).

Sensitization of MAP Kinase Family Members, p38 and JNK, by Progesterone—Although activation of the MAPK-related enzymes, p38 and JNK, by stress-producing agents and growth factors including EGF has been well documented (8, 9) the role of these kinases in the determination of cellular responses to these agents remains controversial (10) and effects of steroid hormones on the activity of these enzymes have not been described. To determine if progesterone can alter the ability of EGF to stimulate p38s and JNKs (several isoforms exist), T47D-YB cells were pretreated with R5020 for 48 h and then treated with EGF for 5–30 min (Fig. 5A). R5020 markedly increased the activation of the p38 kinase in response to EGF; the activity of JNK isoforms 1 and 2 were also modestly increased by R5020. Thus, in independent experiments, R5020 enhanced the ability of EGF to activate p38 MAP kinase 6–8-fold, and JNK 3–4-fold, above that in EtOH/EGF-treated controls (15 min). In contrast to activation of p42 and p44 MAPKs by EGF (Fig. 4A), which peaked at 5 min, the peak of p38 and JNK activity occurred 15 min after exposure to EGF (Fig. 5A), and was relatively transient, declining substantially by 30 min.

Interestingly, neither phosphatidylinositol 3-kinase (not shown) nor its downstream mediators, AKT and p70S6K, were influenced by R5020 pretreatment (Fig. 5B). These data indicate that there is specificity in R5020-mediated sensitization of “kinase-cascade” signaling by type I growth factor receptors. Thus, while MAPK family members (p42, p44, p38, and JNKs) become hyperactivated by EGF following progestin exposure, phosphatidylinositol 3-kinase-dependent signaling cascades remain unaffected.

Regulation of Cell Cycle Mediators by Progesterone and EGF—Expression of the cyclin regulatory subunits of cell cycle-dependent protein kinase complexes (CDKs) and their CDK inhibitors are important readouts of cell cycle progression (40–42). Indeed, multiple mitogenic pathways converge at the regulation of cyclin D1-CDK (43). We previously found that levels of the cell cycle modulators, cyclin D1, cyclin E, and the CDK inhibitor, p21WAF1, are regulated biphosphorally in response to a single dose of progesterone, leading to one round of cell division in the initial 24 h, followed by growth arrest at G1/S of the second cell cycle between 24 and 96 h (26). During the progesterone-arrested state, cells acquire sensitivity to the proliferative effects of EGF (26). We therefore asked whether regulation of cell cycle proteins by EGF is influenced by prior exposure to progestins. Since R5020 pretreatment amplifies MAPK activation by EGF (Fig. 4A), the MAPK dependence of EGF-induced changes in protein expression was monitored by including the MEK inhibitor (PD98059), which selectively blocks the action of p42 and p44 MAPKs (44) (Fig. 1). To ensure that the inhibitor is effective in breast cells, we added PD98059 (20 µM) to T47D-YB cell cultures 60 min prior to a 5-min challenge with EGF (30 ng/ml); this resulted in greater than 95% inhibition of p42 and p44 MAPK activities (Fig. 6A). Therefore, T47D-YB cells were first pretreated with R5020 (20 nM) for 48 h. The MEK inhibitor or vehicle control was then added to cultures 60 min prior to EGF (30 ng/ml) challenge for an additional 6–8 h. Cyclin D1, cyclin E, and p21WAF1 levels, as well as Stat5 levels, were examined in immunoblots of cell lysates (Fig. 6B). Forty-eight hours of progesterin treatment increases the levels of cyclin D1 6.0-fold, cyclin E 3.5-fold, and p21WAF1 12.4-fold (lanes 1 versus 5). In the absence of R5020 (lanes 1–4), EGF has more subtle effects on protein levels, increasing cyclin D1 4.6-fold and p21WAF1 3.1-fold, while having no effect on cyclin E (lanes 1 versus 2). However, in the presence of R5020 (lanes 5–8), EGF increases the expression of cyclin D1 15.2-fold, cyclin E 7.2-fold, and p21WAF1 39-fold (lane 6). Notably, cyclin E levels are up-regulated by EGF only after exposure of cells to progesterone (lanes 1 and 2 versus lanes 5 and 6). Thus, as with the signaling molecules, EGF-induced regulation of cell cycle proteins was strongly enhanced by progestin pretreatment. A nonspecific protein band present in immunoblots serves as a loading control, demonstrating the specificity of the observed changes in cyclin D1, cyclin E, and p21WAF1 protein levels.

Addition of the MEK inhibitor prior to EGF treatment raised the baseline levels of all three cell cycle proteins (lanes 1 versus 3). Therefore, to aid interpretation of the data shown in Fig. 6B, we corrected for basal-expression changes (Fig. 6C). Up-regulation of cyclin D1 and p21WAF1 by EGF was only minimally suppressed by the MEK inhibitor in R5020-naive cells (lanes 3 and 4 in Fig. 6B and C). Thus, in the absence of R5020 pretreatment, cyclin D and p21 are up-regulated by EGF even in the presence of the MEK inhibitor, suggesting that their regulation is largely MAPK-independent. However, following
regulation of cyclin D1, cyclin E, and p21WAF1 by EGF is shown. Since EGF can also signal through STAT proteins, changes in Stat5 protein levels induced by R5020 or EGF in T47D-YB cells were monitored in whole cell lysates using an antiserum that recognizes Stat5a and Stat5b (Fig. 6B). Stat5 is constitutively expressed in most cell types and its levels are not known to be regulated by either steroid hormones or growth factors (12). EGF did not alter Stat5 levels regardless of R5020 pretreatment (lanes 1 versus 2, 5 versus 6) (36). Surprisingly, R5020 increased the levels of Stat5 protein by 8-fold (lane 1 versus 5). Recall that Stat5 is associated with Tyr(P)-containing proteins following exposure of cells to progestin alone (Fig. 3B). This suggests an entirely novel mechanism for progestosterone-mediated regulation of Stat5 signaling, which is explored in greater detail in the accompanying article (36).

**DISCUSSION**

Our data support an entirely new function for progestins as integrators of growth signaling pathways. We have previously shown that progestins can sensitize breast cancer cells to the mitogenic effects of EGF (26). We now show that progestins can also increase the sensitivity of downstream cytoplasmic kinase (Figs. 4 and 5) and STAT (Figs. 3 and 6B, and Ref. 36) signaling molecules to the effects of growth factors. Potentiation of growth factor-activated pathways by progesterone is reflected in a shift in the regulation of the cell cycle mediators, cyclin D1, p21WAF1, and cyclin E toward MAP kinase-dependence (Fig. 6). We postulate that through this shift, control of breast cancer cell proliferation is transferred from progesterone regulation, to one involving EGF- or other growth factor-mediated signaling cascades. Thus, progesterone "primes" cells by up-regulating key signaling molecules, thereby sensitizing cells to subsequent growth factor stimulation. Such cross-talk may explain the acquisition of growth factor responsiveness by breast cancer cells that have become resistant to steroid hormones.

**Progesterone and Type I Growth Factor Receptors—**All four type I receptor tyrosine kinase family members are expressed in normal and malignant breast epithelial cells (17), and are under steroid hormone regulation (45, 46). Here, we show that the levels of at least three family members (EGFR, c-ErbB2, and c-ErbB3) are up-regulated by progesterone (Fig. 2). Little is known about the selectivity of signaling pathways by different type I receptor homo- versus heterodimer pairs (19). c-ErbB3 receptor activation has been implicated in anchorage-independent growth of breast tumor cell lines (47). In normal cells or pre-malignant states, PR and ER are directly correlated with EGFR and c-ErbB2 receptor expression (48), while an

blockage of the MEK inhibitor. Independent experiments and the results shown in part B were quantified by densitometric analysis and corrected for changes in base-line levels (in the absence of EGF) by subtraction from EGF-stimulated levels for either EGF-treated (solid bars) or EGF plus the MEK inhibitor (striped bars). Error bars represent the mean of duplicate measures. Induction of cell cycle proteins by EGF in R5020-pretreated cells was blocked by inhibition of p42/p44 MAPK.
inversely relationship exists in malignancies (49). The retention and up-regulation of type I growth factor receptors in steroid receptor negative tumors is associated with a more aggressive phenotype (13, 14). Related to this, Sarup et al. (24) reported that EGF can down-regulate PR, suggesting a feedback mechanism for the acquisition of steroid hormone resistance, concurrent with increased growth factor sensitivity. We have not examined the effects of growth factors on PR directly, but have preliminary data that expression of a constitutively active MEKK1, an upstream activator of MAP kinases (6), diminishes PR-mediated transcriptional activity on a minimal PRE-containing promoter.2

Progestins are known to increase EGFR at the transcriptional level (22, 25), but less is known about the regulation of c-ErbB2 and c-ErbB3 receptors by steroid hormones. Surprisingly, R5020 modestly increased c-ErbB2 and c-ErbB3 receptor levels, but not EGFR, in PR-negative T47D-Y cells (Fig. 2C), indicating that these receptor subtypes are differentially regulated. Progesterone may influence the levels of these receptors via its nongenomic effects (28–31). A high affinity progesterone-binding membrane protein complex of about 200 kDa present in a variety of different mammalian tissues has been recently discovered (50). Progesterone binds directly to the G-protein coupled oxytocin receptor and this interaction inhibits peptide-mediated signaling by this receptor (51). Similarly, ER-independent regulation of c-ErbB2 receptor activity by estrogens has been reported (52).

**Type I Growth Factor Tyrosine Kinase Signaling Cascades: STATs and MAPKs**—While gene amplification or up-regulation of type I growth factor receptor mRNA has been well documented in human breast cancers (13, 14), our study has uniquely addressed the effects of long-term progestin exposure on the regulation of downstream signaling molecules coupled to these receptors. The contribution of increased receptor numbers to the amplified signal transduction that we observe is difficult to assess. Our cumulative data (26 and data herein) suggest that the progestosterone-induced increase in EGFR receptors is necessary for this amplification, and that sensitization of downstream signaling molecules by progesterone is also required. It is clear that the activation of signaling pathways by progestins and EGF is selective, suggesting some level of specificity in their regulation. We find that, independently of EGF, progestin treatment leads to the increased tyrosine phosphorylation of both JAK2 and Shc, and the increased abundance and association of Stat5 with Tyr(P)-containing proteins (Fig. 3). Association of STATs with JAKs is well documented (12), and association of Stat5 with Tyr(P)-containing proteins (Fig. 5) coupled with the progestin-induced amplification of JAK2/Stat5 signaling (Fig. 3 and Ref. 36) may serve to push progesterone-arrested breast cancer cells into a mitogenic program. Thus, proliferation resulting from increased sensitivity to EGF after exposure to progestins may in part be explained by threshold effects in receptor-activated MAPK signaling.

**Progesterone and Regulation of the Mitotic Cell Cycle**—Regulation of the cyclin D-CDK-retinoblastoma protein (pRb) controlled G1 checkpoint appears to lie at the convergence of mitogenic signaling cascades initiated by diverse classes of receptors including EGF, which signal through STATs and MAPKs; ER and PR, which bypass cytoplasmic signaling cascades; and G-protein-coupled thyrotropin receptors, which signal via cAMP-dependent mechanisms (43). The influence of progestins (26, 60) and EGF (36, 61) on cell cycle proteins, including p21WAF1 and G1/S cyclins, in breast cancer cells illustrates the potential for integration of these pathways. Our data suggest that progestin pretreatment shifts the EGF-dependent regulation of cyclin D1, cyclin E, and p21WAF1 toward MAPK-dependent pathways. Thus, EGF up-regulation of these proteins is entirely sensitive to the MEK inhibitor only after progestin pretreatment (Fig. 6C). Interestingly, there appear to be basal MAPK-dependent as well as MAPK-independent components to regulation of cell cycle proteins by EGF in the absence of progestins (Fig. 6B). Consistent with this finding, exposure of T47D-YB cells growing in 5% serum to the MEK inhibitor for 0–24 h reduces basal growth rates by 5–15%, but does not block the initial proliferative burst characterized by up-regulation of cyclin D following short-term exposure to progestins (Ref. 26, and data not shown). This suggests that R5020-induced cell proliferation and cyclin D regulation is largely MAPK-independent, and supports our thesis of a shift toward MAPK dependence after prolonged exposure to progestins. Little is known about how signaling pathways elicited by either growth factors or steroid hormones bridge to alter cyclin-CDK activities. Clues from yeast indicate a clear link between the MAPK pathway and cell cycle regulatory molecules; the yeast pRb-like transcription factor, Far1, is directly regulated by yeast MAPK (Fus3) (62). Precisely how MAPK pathways are linked to the control of the more complex mammalian cell cycle is less well delineated. Cyclin D1 expression is

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\[^{2}\text{C. A. Lange and K. B. Horwitz, unpublished results.}\]
regulated positively by p42/p44 MAPK and negatively by p38 MAPK in CCL39 fibroblast cells (63). Growth factor-mediated transcriptional activation of the p21 \(^{WAF1}\) promoter is MAPK-dependent in HeLa and PC12 cells (64).

The effects of progesterins on cyclin E are of particular interest. Recall that EGF has no effect on cyclin E expression in progestin-naïve cells, but up-regulates cyclin E following progestin pretreatment (Fig. 6). Cyclin E is required for entry into S phase, and in contrast to cyclin D1, cyclin E can promote G1/S transition even in cells lacking functional pRb protein (65). Accumulation of active cyclin E/Cdk2 complexes is controlled by Myc and Ras (66). Progestins are known to increase the expression of c-Myc (67) and the human c-myc promoter contains a progestin response element (68). This may explain how EGF, via Ras activation, can regulate cyclin E only in progestin-pretreated cells, where c-Myc is also up-regulated (Fig. 6 and Ref. 64). Similarly, only progestin-pretreated breast cancer cells undergo proliferation in response to EGF, despite low levels of hypophosphorylated pRb (26). We speculate that EGF-stimulated cell cycle re-entry of cells that are growth arrested following progestin treatment may be mediated by a cyclin E-dependent, pRb-independent, pathway.

The role of p21 \(^{WAF1}\) in the dual control of breast cancer cell growth by progestins and EGF is also of interest. In T47D cells, increased p21 \(^{WAF1}\) expression is associated with cell growth arrest in response to progestins alone (26). However, following release from this arrest by EGF, p21 \(^{WAF1}\) levels increase even further (Fig. 6, B and C), and progestrone plus EGF synergize to enhance p21 \(^{WAF1}\) promoter-driven transcription (36). This effect of progesterone plus EGF appears to be paradoxical, given the classical CDK-inhibitory/differentiative properties of p21 \(^{WAF1}\). It is now recognized, however, that p21 \(^{WAF1}\) has multifunctional actions (69) that can contribute to anti-differentiative, anti-apoptotic, or proliferative cellular responses. For example, forced expression of p21 \(^{WAF1}\) inhibits terminal differentiation of primary mouse keratinocytes, a function that is separable from the ability of p21 \(^{WAF1}\) to inhibit cyclin-CDK complexes (70). Furthermore, p21 \(^{WAF1}\) binds to and inactivates JNK (71), a mediator of apoptosis in certain systems (9, 10) (Fig. 1). This function of p21 may explain the modest activation of JNKs by EGF in progestin-pretreated T47D-YB cells compared with that of p38 MAPK (Fig. 5). Similarly, elevated levels of p21 \(^{WAF1}\) contribute to androgen-independent growth of prostatic carcinoma cells and are believed to play an anti-apoptotic role in the development of steroid hormone resistance in these cells (72). Additional proliferative actions of p21 \(^{WAF1}\) may be explained by the finding that at low concentrations, p21 \(^{WAF1}\) acts as a nucleation factor that assembles and activates cyclin CDK kinase complexes, while higher concentrations of p21 \(^{WAF1}\) inhibit a constant level of cyclin-associated CDK activity (73). Since progestin plus EGF treatment up-regulates G1/S phase cyclins (Ref. 26 and Fig. 6B), concomitantly up-regulated p21 \(^{WAF1}\) may participate in cyclin-CDK activation (73). Thus, in breast cancer cells, multiple complex functions of p21 \(^{WAF1}\) may contribute to restarting the cell cycle machinery in response to secondary growth signals initiated by EGF, following progestin pretreatment and cell cycle arrest.

Here, and in the accompanying paper by Richer et al. (36), we have demonstrated the convergence of PR and growth factor-activated signaling pathways. The traditional view of signal transduction pathways as linear and independent modes of cellular communication has gradually given way to the more current understanding that, while they represent a series of modular, yet integrated systems. Other surprising interactions between what were previously thought to be separate signaling path-
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