Previously, our laboratory has shown that prolactin (PRL) inhibits epidermal growth factor (EGF)-induced DNA synthesis. One pathway for the initiation of DNA synthesis is EGF-receptor (EGF-R) signaling through Ras and mitogen-activated protein kinase (MAPK). To determine the effects of PRL on EGF-induced MAPK activation and phosphorylation, MAPK or phosphotyrosine (Tyr(P)) was immunoprecipitated from normal murine mammary epithelial (NMuMG) cells treated with PRL (100 ng/ml) and/or EGF (10 ng/ml) for 10-min periods. EGF-induced phosphorylation and activation were then examined by Western analysis and a myelin basic protein (MBP)-specific kinase assay. The p42 isoform of MAPK showed a distinct decrease in activity and phosphorylation when cells were treated with PRL. Concluding that PRL affects EGF signaling upstream of MAPK, we examined the effect of PRL on EGF-induced Ras activity. NMuMG cells were incubated with \(^{32}P\) orthophosphate when cells were treated with PRL. Concluding that PRL affects EGF signaling upstream of MAPK, we examined the effect of PRL on EGF-induced Ras activity. NMuMG cells were incubated with \(^{32}P\) orthophosphate, treated as described above, immunoprecipitated with an antibody specific to Ras, and nucleotides were eluted and separated by TLC. Ras activity as measured by GTP:GDP ratio was increased by EGF, but not by PRL. Additionally, PRL in combination with EGF abolished the ability of EGF to induce Ras activity. Those studies suggest that PRL alters the EGF signaling pathway upstream of Ras. Because Ras activation by EGF involves EGF-stimulated association of EGF-R with Grb2, the EGF-R was immunoprecipitated and a Western blot was probed for Grb2. As expected, we found that EGF stimulated an association of EGF-R with Grb2, PRL, however, blocked this association. When we looked at the ability of Shc to associate with the EGF-R, we found that PRL and EGF had little effect on this association. The studies demonstrate that PRL either directly or indirectly inhibits the ability of EGF to induce EGF-R association with Grb2, to activate Ras, and to activate and phosphorylate MAPK.

The lactogenic hormone PRL has previously been reported to increase EGF mRNA levels in mammary tissue and epithelial cells (Fenton and Sheffield, 1991, 1994). EGF, being a mitogenic growth factor in mammary tissue (Yang et al., 1980; Taketani and Oka, 1983a), increases DNA synthesis in NMuMG cells (Vanderboom and Sheffield, 1993). However, in the presence of PRL, EGF-induced DNA synthesis is dramatically diminished in this cell line (Fenton and Sheffield, 1994).

High affinity EGF receptors have been identified in the mammary gland (Taketani and Oka, 1983b) and shown to be developmentally regulated (Edery et al., 1985). Current dogma holds that a biological response, such as DNA synthesis, can be traced back to EGF binding to the EGF-R (Gill et al., 1987). One early consequence of EGF binding to its receptor is receptor dimerization and interchain autophosphorylation of tyrosine residues (Yarden and Schlessinger, 1987). It is believed that Grb2, a SH2 domain protein, binds the phosphorylated receptor and aids in the activation of Sos-1 (Lowenstein et al., 1992; Egan et al., 1993). Sos-1 enhances Ras protein exchange factors converting GDP to the biologically active, GTP-bound Ras (Egan et al., 1993). Upon activation, Ras assists Raf-1 localization to the membrane where it initiates a cascade of phosphorylation events including the phosphorylation and activation of the mitogen activating kinase kinase (MEK) and the mitogen activating protein kinase (MAPK) (Stokoe et al., 1994; Gardner et al., 1994).

Recently another SH2 domain protein has been shown to associate with the EGF-R. This protein, Shc, is tyrosine phosphorylated when bound to the EGF-R via a tyrosine phosphorylation site and SH2 domain interaction (Ruff-Jamison et al., 1993). Shc then has the ability to associate with Grb2 through another tyrosine phosphorylation SH2 interaction, eventually leading to Ras activation and mitogenesis (Pelici et al., 1992; Rozakis-Adcock et al., 1993). The exact binding mechanism of Shc to the EGF-R has recently been under debate. One group found a phosphotyrosine recognition domain named phosphotyrosine binding domain on the Shc protein (Kavanagh et al., 1995) which also has the ability to associate with the autophosphorylated EGF-R (van der Geer et al., 1996). It has also been discovered that a mutated EGF-R in the autophosphorylation site still retains the ability to bind tyrosine phosphorylated Shc, again leading to Ras activation (Li et al., 1994). Therefore, the functional interaction of Shc and the EGF-R has yet to be fully characterized.

Although EGF and other growth factors signal through a variety of pathways, the Ras/MAPK cascade has been heavily investigated. MAPKs are a family of highly conserved serine/threonine kinases that are activated by phosphorylation on both tyrosine and threonine, mediated by MEK (Boulton et al., 1991; Crews et al., 1992). MAPK phosphorylation and activation has been shown to correlate with a variety of events, including cell proliferation and differentiation (reviewed in Crews et al., 1992). In some cell lines, MAPK activation appears to be required for mitogenesis (Pages et al., 1993), but this may not be universal. Constitutive activation of the MAPK pathway (via expression of a constitutively active MEK) has been shown to induce tumorigenic transformation (Mansour et al. 1991).
MATERIALS AND METHODS

Cell Culture—NMuMG (Owens, 1974) mammary epithelial cells from American Type Culture Collection (Rockville, MD) were maintained in culture conditions of 100% humidity at 37°C with an atmosphere of 5% CO2, 95% air. Cells were grown in Dulbecco's modified Eagle's medium and 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) on Falcon and Corning plates and flasks (Becton Dickinson, Lincoln Park, NJ, and Corning, NY, respectively).

Immunoprecipitations—NMuMG cells were plated on 100-mm plates (1.5×106 cells/plate) and allowed to grow for 24 h. Cells were then serum starved in Dulbecco's modified Eagle's medium for 18 h prior to treatment. For the anti-Ras immunoprecipitation 5 ml of Dulbecco's modified Eagle's medium with 10 μM phosphor and 0.2 mM of [32P]orthophosphoric acid was added to each treatment plate for 4 h at 37°C prior to treatment application. Treatments of 100 ng/ml PRL, 10 ng/ml EGF, 100 ng/ml PRL, and 10 ng/ml EGF, or consecutive treatments of 100 ng/ml PRL then 10 ng/ml EGF were added for 10 min for the anti-Ras immunoprecipitation and for 5 min for the anti-EGF-R immunoprecipitation. (For the cells that were pretreated with PRL, they were treated for 10 min before the EGF was added for the anti-Ras immunoprecipitation and 5 min before the EGF was added for the anti-EGF-R immunoprecipitation.) Each plate was washed with ice-cold phosphate buffered saline (PBS) or Hank's balanced salt solution. Samples were then lysed with 500 μl of extraction buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 150 mM NaCl, 5% Nonidet P-40, 2 mM aprotinin, and 0.5 mM phenylmethylsulfonl fluoride) or 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.8, 1% Triton X-100, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 μM sodium fluoride, 100 μM sodium orthovanadate, 0.1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride), for anti-Ras immunoprecipitations or anti-EGF-R, anti-MAPK, or anti-Tyr(P) immunoprecipitations, respectively. The lysates were then incubated on ice for 15 min and centrifuged at 15,000 rpm for 15 min at 4°C to clear samples. The supernatants were then incubated with 5 μg of anti-Ras polyclonal antibody (UBI), Lake Placid, NY) plus 25 μl of agarose-conjugated protein A (Oncogene Science, Uniondale, NY) for 30 min, with 4 μg of anti-EGF-R sheep polyclonal antibody (UBI) plus 25 μg of agarase-conjugated protein G (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h, with 5 μg of anti-p42MAPK (UBI) plus 25 μl of agarose-conjugated protein A (Santa Cruz Biotechnology) for 2 h, or with agarase-conjugated anti-phosphotyrosine (Oncogene Science) for 2 h. Agarose beads were pelleted by centrifugation at 4,000 rpm for 3 min, washed 3 times with lysis buffer, and the pellets were resuspended in 100 μl of SDS loading dye (Laemmli, 1970) or 20 μl of 1 M KH2PO4, pH 3.3, for the anti-EGF-R and anti-MAPK or anti-Ras immunoprecipitates, respectively. The anti-Tyr(P) immunoprecipitation required an elution step with incubation of the pellet in 1 mM p-nitrophenyl phosphate in lysis buffer for 1 h and 100 μl of the supernatant was added to 100 μl of SDS loading dye.

Western Analysis—Samples from the immunoprecipitations were separated by 14, 12, or 7.5% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA) as described by Towbin et al. (1979) at 150 volts for 1 h at 4°C. Membranes were blocked in PBS and 3% Tween 20 PBS-T (PBS containing 0.05% sodium azide and 0.5% Nonidet P-40) for 1 h, and membranes were incubated with 1 μg/ml anti-Grb2 monoclonal antibody (UBI), 0.2 μg/ml anti-Shc polyclonal antibody (Santa Cruz Biotechnology), 0.5 μg/ml anti-human EGF-R sheep polyclonal antibody (UBI), 5 μg/ml anti-MAPK (UBI; recognizing 42- and 44-kDa forms), 5 μg/ml anti-Erk2 (42 kDa specific; UBI), 10 μg/ml anti-phosphotyrosine (Sigma), or 100 μg/ml anti-phosphotyrosine monoclonal antibody (Oncogene Science) in PBS-T containing 2% bovine serum albumin for 1 h at room temperature. The membranes were then washed 6 times for 5 min each, with PBS-T containing 0.1% bovine serum albumin. The secondary antibody for all but the EGF-R and Shc antibodies was anti-mouse IgG peroxidase conjugate (Sigma) at a dilution of 1:1,000. For the EGF-R antibody an anti-sheep IgG peroxidase conjugate (Sigma) and for the Shc antibody an anti-rabbit IgG peroxidase conjugate (Sigma) were used at the same dilution as above. The membranes were incubated with the corresponding secondary antibody for 30 min at room temperature and washed again as above. The proteins were then detected using enhanced chemiluminescence as described by the manufacturer (DuPont, Boston, MA).

MAPK Activity—For determination of MAPK activity, p42MAPK immunoprecipitates were suspended in 30 μl of assay buffer (50 mM Tris, pH 7.2, 10 mM MgCl2, 1 mM sodium orthovanadate). To this suspension, 10 μl of myelin basic protein (1 mg/ml) was added and reactions started by adding [γ-32P]ATP (10 μl of a 500 μM solution containing 10 μCi of [32P]). Reactions were continued for 5 min. Reactions were stopped by adding 50 μl of SDS loading buffer and heating samples in bolling water for 5 min. Samples were then separated by SDS-PAGE, gels dried, and autoradiography performed using calcium tungstate intensifying screens and pre-flashed Fuji x-ray film. The Caliper® image analysis system (Fotodyne, New Berlin, WI) was used to determine relative densities of the myelin basic protein bands.

Ras Activity—Samples from anti-Ras immunoprecipitates were analyzed using thin layer chromatography (TLC). PEI-cellulose F plates (EM Science, Gibbstown, NJ) were spotted with 10 μl of each sample and 10 μl of GTP and GDP standards (Sigma). The TLC was developed with 1 M KH2PO4, pH 4.5. The plates were dried and exposed to x-ray film. The film was then developed and analyzed using a computer-aided densitometry program (Caliper®). Densities were corrected for different specific activities of GTP and GDP, and reported as ratio of GTP:GDP.

Statistical Analysis—All experiments were replicated at least three times. Quantitative data were analyzed by two-way analysis of variance. Matels were compared by planned comparisons as described under “Results” (Gill, 1978). Unless otherwise stated, significance level was at least p < 0.05 for all comparisons deemed significant.

RESULTS

MAPK Phosphorylation—Preliminary time course studies indicated that EGF elevates MAPK phosphorylation at a maximum level between 5 and 10 min (data not shown). Therefore 10 min was chosen as a time point in the following MAPK studies. Immunoprecipitates of tyrosine-phosphorylated proteins probed with anti-MAPK (Fig. 1) indicated that EGF increased phosphorylation of both 42- and 44-kDa forms of MAPK. PRL increased phosphorylation of the 44-kDa form, but not the 42-kDa MAPK. Interestingly, PRL appeared to inhibit the ability of EGF to induce tyrosine phosphorylation of the 42-kDa MAPK. In subsequent experiments (Fig. 2), p42MAPK was immunoprecipitated and probed with anti-phosphotyrosine and that PRL blocked this effect. Similar results were also observed for thromine phosphorylation of MAPK.

MAPK Activation—As expected from the MAPK phosphorylation studies, p42MAPK activity was increased 16-fold by EGF treatment (Fig. 3). PRL appeared to give a slight increase in...
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**Ras Activity**

**FIG. 2.** Verification of EGF and PRL effects on p42MAPK tyrosine phosphorylation. The 42-kDa form of MAPK was immunoprecipitated (IP) and Western blots probed with anti-phosphotyrosine (panel A), anti-MAPK (panel B), or anti-phosphothreonine (panel C). C, control; E, EGF, 10 ng/ml for 10 min; P, PRL, 100 ng/ml for 10 min; EP, simultaneous treatment with EGF and PRL; PE, sequential treatment with PRL, then EGF. Representative of three blots.

**FIG. 3.** Effect of EGF and PRL on MAPK activity. p42MAPK was immunoprecipitated and myelin basic protein kinase activity determined as described under “Materials and Methods.” C, control; E, EGF, 10 ng/ml for 10 min; P, PRL, 100 ng/ml for 10 min; EP, simultaneous treatment with EGF and PRL; PE, sequential treatment with PRL, then EGF. Mean of three determinations. Means with different letters represent a significant difference between treatment at p < 0.05.

**FIG. 4.** Effect of EGF and PRL on Ras activity. NMuMG cells were labeled with 0.2 mCi/treatment [32P]orthophosphoric acid, treated with EGF and/or PRL, and immunoprecipitated with anti-Ras antibody. GTP and GDP were isolated and separated via thin layer chromatography (TLC). The TLC plate was exposed to x-ray film and analyzed via Collage®. C, control; E, EGF, 10 ng/ml for 10 min; P, PRL, 100 ng/ml for 10 min; EP, simultaneous treatment with EGF and PRL; PE, sequential treatment with PRL, then EGF. This experiment was repeated three times and a two-way ANOVA presentation of the statistics was used in analysis. *, p < 0.05.

MAPK activity (approximately double control activity), which may be due to either a small PRL activation of p42MAPK or to MAPK activity (approximately double control activity), which was indicated that the PRL antagonism of p42MAPK phosphor- than that observed with EGF alone.

Ras Activity—Time course studies were also used in the Ras activity assays to determine the optimal time of EGF induced Ras activation. Treatment for 10 min with EGF appeared to give maximal stimulation of Ras (not shown) and was therefore the time point used in subsequent studies. EGF increased the amount of GTP bound to Ras over 6-fold from untreated cells, as measured by Ras immunoprecipitation from [32P]-labeled cells, followed by TLC analysis of GTP:GDP ratios (Fig. 4). PRL alone did not significantly increase the amount of GTP associated with Ras. In addition, PRL inhibited the ability of EGF to increase GTP bound to Ras. Since Ras is active when binding GTP, it follows that EGF stimulates Ras activity while PRL alone and in combination with EGF (simultaneously or sequentially) fail to increase Ras activity. Ras activation is part of a major pathway for cell proliferation in mammary epithelial cells (Mulchahy, 1985). These data suggest that EGF increased stimulation of the Ras pathway, whereas PRL blocks the ability of EGF to activate Ras.

EGF Receptor-Grb2 Association—Because EGF activation of Ras is thought to occur via EGF induced association of the EGFR with Grb2, we examined the effect of PRL on EGFR association with Grb2. Immunoprecipitates of EGF receptor probed with anti-Grb2 indicated that EGF increased Grb2 association with the receptor, and that this association was decreased by PRL treatment (Fig. 5A). These changes did not appear to be associated with changes in the amount of EGFR immunoprecipitated, but did appear to associate with changes in receptor tyrosine phosphorylation (Fig. 5B and C).

EGF Receptor-Shc Association—Recently Shc has been shown to be involved with EGF induced signaling. We examined the effect of PRL and EGF in EGFR association with Shc. Immunoprecipitates of EGFR receptor probed with anti-Shc indicated that Shc is associated with the receptor constitutively and that neither EGF nor PRL affected this association in any reproducible manner (Fig. 6). Since Shc itself is tyrosine phosphorylated, we also looked at the tyrosine phosphorylation of Shc with EGF and PRL stimulation and again observed no distinct change in phosphorylation levels (data not shown).

**DISCUSSION**

Initial results of these studies indicated that PRL decreased the ability of EGF to phosphorylate and activate p42MAPK (Erk2). These results were anticipated as a previous study (Fenton and Sheffield, 1993) demonstrated that EGF and PRL altered phosphorylation of 40–50-kDa proteins when total phosphorylation was examined. However, the exact identity of the proteins was not established in that study. These results presented here clearly identify the proteins as MAPKs.

MAPK phosphorylation and activation have previously been shown to be induced by a variety of growth factor signaling via tyrosine kinase receptors, including EGF (reviewed in Crews et al. (1992)). In at least some cases, increased MAPK activity appears to be necessary for growth factor-induced mitogenesis.
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NG/10 min; 44-kDa forms of MAPK, PRL alone had no effect on p42MAPK phosphorylation, but increased p44MAPK phosphorylation. The reason for this is unclear, since most studies to date have reported similar patterns of activation of 42- and 44-kDa forms of MAPK.

Because Ras activation appears to play a critical role in EGF-induced MAPK activation in systems studied to date, we examined the effects of EGF and PRL on Ras activation. These studies indicated that PRL lacks the ability to stimulate the Ras signaling pathway. Thus, PRL activation of p44MAPK phosphorylation would appear to proceed via a Ras-independent pathway. Recently, Erwin et al. (1995) showed PRL treatment doubled Ras activity when compared to that of the untreated NMuMG cells. However, in other cases MAPK activation appears to be associated with cellular differentiation. In PC12 cells, this difference has been attributed to different temporal patterns of MAPK inactivation (Traverse et al., 1992). However, to the best of our knowledge, this hypothesis has not been examined in other model systems.

In addition to tyrosine kinase receptors, cytokine receptors, such as those for growth hormone and for PRL, have been shown to increase phosphorylation and activation of MAPKs (Moller et al., 1992). Interestingly, the present study indicated that, while EGF increased phosphorylation of both 42- and 44-kDa forms of MAPK, PRL alone had no effect on p42MAPK phosphorylation, but increased p44MAPK phosphorylation. This process was associated with a decrease in EGF-R tyrosine phosphorylation. Thus, these studies support a model in which PRL induces a modification in EGF-R or an early signaling event that leads to reduced EGF-R autophosphorylation. This autophosphorylation reduction leads to a decrease in Grb2 association with the EGF-R, which in turn reduces or eliminates Ras activation and consequent activation of the MAPK pathway.

Interestingly, PRL and EGF had little or no effect on EGF-R/Grb2 association. Together with the data in this paper and previous studies showing that Shc can associate with a mutant EGF-R that is not autophosphorylated (Li et al., 1994) but Shc could not be tyrosine phosphorylated by this mutant (Gotoh et al., 1995) leads us to believe that Shc is not playing a major role in the PRL sensitive EGF signaling pathway we are studying. If Shc is involved it could be activated through PRL since it was found that PRL, via JAK2 activation, has the ability to recruit Shc to the membrane and that the total amount of Shc protein found in the membrane fraction increases with PRL stimulation (Erwin et al., 1995). So perhaps PRL is recruiting Shc to the membrane to be phosphorylated by an undefined kinase, which then allows Shc to associate with the EGF-R.

The PRL-induced pathway which alters EGF-R signaling remains unclear. A wide variety of signaling mechanisms have been postulated for PRL, including the JAK-STAT pathway (Lebrun et al., 1994), Src-related kinases (Clevenger and Medaglia, 1994), and activation of protein kinase C (Banerjee and Vonderhaar, 1994). Clearly these pathways are not mutually exclusive, and PRL action may involve a multifaceted signaling system. Previous studies have suggested that protein kinase C phosphorylates the EGF-R and decreases its tyrosine kinase activity (Hunter et al., 1984). Thus, while prolactin modulation of EGF signaling via phosphorylation of the EGF-R appears likely, the exact enzymology of the pathway remains unclear. Studies in our laboratory are now underway to clarify the involvement of PRL-stimulated PKC in down-regulation of EGF-R signaling.

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Julie L. Johnson, Suzanne Fenton and Lewis G. Sheffield

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