Prolactin Decreases Epidermal Growth Factor Receptor Kinase Activity via a Phosphorylation-dependent Mechanism*

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Previously, we have shown that prolactin inhibits epidermal growth factor (EGF)-induced mitogenesis in mouse mammary epithelial cells without altering the response to other growth promoting agents. This effect has been associated with reduced EGF-induced EGFR tyrosine phosphorylation, Grb-2 association, and Ras activation. Our current hypothesis is that prolactin induces an alteration in EGFR kinase activity via a phosphorylation-dependent mechanism. To test this hypothesis, we treated normal murine mammary gland cells with or without 100 ng/ml prolactin. EGFR isolated by wheat germ agglutinin affinity chromatography from non-treated cells exhibited substantial ligand-induced phosphorylation, and EGFR isolated from prolactin-treated cells displayed minimal EGF-induced EGFR phosphorylation, as well as decreased kinase activity towards exogenous substrates. The observed decrease in ligand-induced EGFR phosphorylation could not be attributed to either differential amounts of EGFR, decreased EGF binding affinity, or the presence of a phosphotyrosine phosphatase or ATPase. EGFR isolated from prolactin-treated cells exhibited increased phosphorylation on threonine. Removal of this phosphorylation by alkaline phosphatase restored EGFR kinase activity to levels observed in non-treated cells. Therefore, these results suggest that prolactin antagonizes EGF signaling by increasing EGFR threonine phosphorylation and decreasing EGF-induced EGFR tyrosine phosphorylation.

EGF and its receptor (EGFR) play a crucial role in mammary epithelial proliferation (1, 2). This effect is manifested in the context of a variety of other hormones including estrogen, progesterone, growth hormone, and insulin-like growth factor-1 (reviewed in Ref. 1). Once differentiation is completed, the once proliferative mammary gland becomes a secretory gland competent to carry out lactation (1, 2). At this stage, lactogenic hormones, such as Prl, and milk proteins may have inhibitory effects on locally acting growth factor pathways (2).

Mammogenic actions of EGF are presumed to be mediated via EGFR, which is a 1186-amino acid, 170-kDa transmembrane protein that comprises a heavily glycosylated extracellular domain, a transmembrane domain, and a cytoplasmic domain that contains a 300-amino acid sequence that is similar to the pp60c-src tyrosine kinase (3, 4). Although derived from a single gene, EGFR has been shown to be present on the membranes of most cell types, including mammary gland epithelium, as both high affinity (K_D = 0.1 nM) and a low affinity (K_D = 1 nM) sites (5, 6). The EGFR has also been shown to bind transforming growth factor-α with similar affinities (7).

Upon ligand binding, the EGFR can homo- or heterodimerize with other members of the EGFR family including erbB2, erbB3, and erbB4 (2, 8, 9). Following dimerization, the receptor undergoes inter- and intramolecular autophosphorylation on tyrosines in the C-terminal cytoplasmic domain (3, 10, 11, 12). These phosphotyrosines serve as docking sites for Src homology 2-containing proteins such as Grb2, phospholipase C-γ, phosphatidylinositol 3-kinase, and Shc (13, 14). Upon binding of Src homology 2 proteins, there is a cascade of kinase activation, which leads eventually, in the case of Grb2, to the stimulation of the serine/threonine kinase MAPK, but other Src homology 2 kinase activation mechanisms do exist (10, 15, 16). Once activated, MAPK can either phosphorylate cytoplasmic substrates such as p90rsk (16, 17) or translocate to the nucleus to induce the production of transcription factors such as c-Fos (16, 18). In addition to tyrosine phosphorylation sites needed for signal transduction, certain serine and threonine residues exist in the cytoplasmic portion of the EGFR that, upon phosphorylation, reduce EGF binding, induce receptor desensitization, or promote down-regulation (10, 19–21). While some sites have been proposed to regulate one specific event, e.g., down-regulation, some sites may participate in several mechanisms of EGFR modulation (22). Mutation of specific sites renders the receptor insensitive to homologous or heterologous regulation (reviewed in Refs. 21 and 22). The best described of these events is mediated by protein kinase C (19, 20), but others have been reported (21, 22).

Our laboratory has previously reported Prl’s inhibition of EGF-induced DNA synthesis in a dose-dependent manner in mammary epithelial cells without affecting response to other growth-promoting agents such as insulin-like growth factor-1 or cholera toxin (23). Prl also inhibited the ability of EGF to activate the Ras-MAPK pathway, an effect that was correlated with altered EGFR receptor tyrosine phosphorylation and EGFR-Grb2 interactions (24). While these observations suggest that Prl may alter EGF receptor kinase activity (25), the results are based on altered EGFR phosphorylation state in vivo, which does not indicate whether Prl alters EGFR receptor activity directly. Therefore, the objective of the present study was to determine the effects of Prl on EGF-induced kinase activity of isolated EGFR.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal murine mammary gland epithelium (NMuMG) cells and NIH 3T3 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in Falcon
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100-mm dishes (Becton Dickinson, Lincoln Park, NJ) at 37 °C with 5% CO2, 95% air in Dubelco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (Life Technologies, Inc.).

EGF Receptor Isolation—Isolation of EGF receptor was performed as described by Yarden and Schlessinger (12) with minor modifications. Cells (100,000) in 10 15-mm borosilicate tubes were incubated with 5 × 107 cells/ml of Iodo-GEN® (Iodo-Gen; Pierce) protocol with or without 100 μg/ml of ovine prolactin (NIDDK, National Institutes of Health) in DMEM for 15 min. Cells were then washed with cold (4 °C) phosphate-buffered saline (PBS) and lysed with 2 ml of cold (4 °C) lysis buffer (50 mM HEPES, pH 7.6). Lysates were centrifuged at 50,000 × g for 4 °C, and 3 ml of the cell lysate supernatant was applied to a 2-ml gel bed of agarose-conjugated wheat germ agglutinin (Vector Laboratories, Burlingame, CA) in a 10-ml plastic column (Bio-Rad) in a 4 °C refrigerator. The column was allowed to equilibrate for 15 min by stopping the flow of supernatant over the gel bed. The full supernatant volume was then passed over the column three times. The column was then washed with 30 ml of wash buffer (50 mM HEPES, 0.1% Triton X-100, and 300 mM N-acetylglycylsine, pH 7.6). Protein content was determined either by BCA protein assay (Ferrous) or Bradford method (26). Eluted preparations were aliquoted into microcentrifuge tubes and stored at −70 °C until use. Receptor preparations were used within approximately 2 months after isolation, over which time we did not observe any decrease in kinase activity.

Receptor Kinase Assay—Receptor activity of solubilized EGF receptor was assessed as follows. Equal amounts of EGF receptor isolated from NMuMG or NIH 3T3 cells treated with or without the indicated concentrations of Prl were incubated with 1 μM [3H]EGF (with or without a 100-fold excess of unlabeled EGF) in 10 plates each treatment; 350,000 cpm of [3H]EGF was added and allowed to react for 1 h at 20 °C until use.

Preparation of 125I-EGF—Murine receptor grade EGF (Harlan, Madison, WI) was iodinated using IODO-GEN® (Pierce) protocol with some modifications. Briefly, 20 μg of Iodo-GEN® was added to the coated on the bottoms of acid-washed 12 × 75-mm borosilicate tubes, was reacted with 5 μg of EGF and 1 mCi of Na251I (NEN Life Science Products) for 3 min at room temperature. 125I-EGF was then applied to a Sephadex G25–80 column, and fractions were collected, counted, and stored at −20 °C until use.

Binding of 125I-EGF—Binding of 125I-EGF binding was performed on solubilized receptor preparations treated with or without Prl, which were incubated with or without Prl for 1 h at 20 °C. 125I-EGF was then applied to a Sephadex G25–80 column, and fractions were collected, counted, and stored at −20 °C until use.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed using Collage® image analysis system (Fotodyne, New Berlin, WI).

Western Blots—To determine if equal amounts of receptor were present in assay experiments or to determine the phosphorylation state of isolated EGF receptor, equivalent amounts of protein from each receptor preparation eluted were separated on 7.5% SDS-PAGE gels and transferred to Immobilon-P membrane (Millipore, Bedford, MA). This mixture was then heated for 60 min at 110 °C. The HCl form of the 170-kDa range was excised and put in a 4-ml glass vial wrapped in plastic wrap and exposed to x-ray film at room temperature. The band in the 170-kDa range was excised and put in a 4-ml glass vial wrapped in plastic wrap and exposed to x-ray film at room temperature. Afterward, the reaction mixture was centrifuged at 320 × g for 10 min, and a portion of the supernatant was transferred to another tube. To ensure that any residual alkaline phosphatase would not interfere with subsequent kinase reactions, sodium pyrophosphate, final concentration 30 mm, was added at pH 7.8. Kinase reactions were then performed and analyzed as described above.
RESULTS

EGFR from non-Prl-treated NMuMG cells exhibited substantial EGF-induced phosphorylation of a 170,000 molecular weight protein (pp170 kDa; Fig. 1A). Prl treated NMuMG cells exhibited significantly less EGF-induced pp170 phosphorylation, which parallels our observations in intact NMuMG cells (24, 25). However, EGFR isolated from NIH 3T3 cells, which have been shown to be Prl-unresponsive (29), exhibited no reduction in EGF-induced pp170 phosphorylation (Fig. 1B). Western blot analysis indicated that the observed decrease in EGF-induced EGFR phosphorylation induced by Prl could not be attributed to differential amounts of receptor in the kinase assays (Fig. 2). EGFR immunoprecipitation studies revealed that ~70% of detectable pp170 was recovered in the pellet fraction. Phosphoamino acid analysis showed that EGF-induced EGFR phosphorylation was predominantly on tyrosines (~90%) and was substantially decreased in EGFR isolated from Prl-treated cells (not shown). Furthermore, only EGF-and transforming growth factor-α induced a similar increase in EGFR phosphorylation in nontreated NMuMG cells, whereas other growth factors examined, such as insulin-like growth factor-1, bFGF, and relaxin, failed to induce such an increase (not shown).

Time course studies indicated that receptors from NMuMG cells not treated with Prl exhibited a time-dependent EGF-induced increase in EGFR phosphorylation, reaching apparent maximal stimulation by 4 min (Fig. 3). In contrast, receptors isolated from Prl-treated cells could not overcome the decrease in EGF-induced EGFR phosphorylation even at 12 min, and overall they displayed less change in phosphorylation at all time points examined. While receptors isolated from cells not treated with Prl displayed maximal phosphorylation with 0.125 μM ATP, receptors from Prl-treated cells failed to reach levels of phosphorylation observed for the control group, even at 2 μM ATP (Fig. 4). As expected, receptors isolated from nontreated cells also exhibited a dose-dependent response to increasing concentrations of EGF, which displayed an apparent maximal phosphorylation at 10 ng/ml (1.7 nM) (Fig. 5). In contrast to receptors isolated from cells not treated with Prl, receptors isolated from Prl-treated cells showed less phosphorylation in response to EGF at all concentrations tested up to 100 ng/ml (17 nM) (Fig. 5).

To determine if the Prl-induced decrease in EGF-induced EGFR phosphorylation was due to reduced EGFR affinity for EGF, we performed 125I-EGF binding analysis of solubilized EGFR from cells treated with or without Prl (Table I). As

![Fig. 1. Prl decreases EGF-induced EGFR phosphorylation. EGFR from NMuMG (A) or NIH3T3 (B) cells treated with or without Prl were incubated with 1 μM [γ-32P]ATP (specific activity, 300 Ci/mmol), 1 mM MgCl2, with or without 10 ng/ml EGF. Reaction mixtures were separated by SDS-PAGE, fixed, dried, and exposed to x-ray film. Autoradiograms are representative of three separate experiments. Bar graphs are presented as mean pp170 density relative to control (−Prl/−EGF) of three separate experiments ± S.E. *p < 0.05 compared with control.](image)

![Fig. 2. EGFR Western blot. EGFR from NMuMG cells treated with or without Prl were separated on a 7.5% SDS-PAGE gel, transferred to membranes, blocked overnight, and probed with anti-EGFR. The Western blot is representative of three separate experiments. The bar graph is presented as mean pp170 density relative to control (−Prl) of three separate experiments ± S.E.](image)
reported in intact NMuMG cells (25), we observed high (2 × 10^{-11} to 4 × 10^{-11} M) and low (8 × 10^{-10} to 9 × 10^{-10} M) EGF binding affinities from cells treated with or without Prl. Although Prl-treated cells displayed numerically higher dissociation constants \( K_d \) for both high and low affinities compared with control cells, they were not significantly different from nontreated cells. Paralleling our Western blot data (Fig. 2), there was no significant difference in the concentration of receptors between the treatment groups \((p > .05)\).

| Receptor class | High affinity \( K_d \) | Low affinity \( K_d \) |
|----------------|------------------------|----------------------|
| Control        | 2 × 10^{-11} \( M \)   | 2 × 10^{-11} \( M \)  |
| Prl            | 4 × 10^{-11} \( M \)   | 2 × 10^{-11} \( M \)  |
|                | 8 × 10^{-10} \( M \)   | 1 × 10^{-10} \( M \)  |
|                | 9 × 10^{-10} \( M \)   | 1 × 10^{-10} \( M \)  |

To determine if the observed decrease in EGF-induced EGFR phosphorylation in Prl-treated NMuMG cells was specific to Prl, NMuMG cells were incubated with or without 100 ng/ml of Prl, bovine growth hormone, or mouse U5 anti-prolactin receptor (not shown). Receptors isolated from bovine growth hormone-treated NMuMG cells exhibited EGF-induced EGFR phosphorylation approximately equal to that of nontreated cells. As in our previous studies (24, 25), Prl inhibited EGF-induced EGFR phosphorylation. Additionally, the U5 anti-Prl receptor antibody, which was previously shown to act as a Prl agonist by presumably inducing receptor dimerization (30), mimicked the effect of Prl in decreasing EGF-induced EGFR phosphorylation. However, these effects were only observed if Prl was added to intact cells. The addition of Prl to isolated EGFR failed to decrease EGF-induced EGFR phosphorylation (not shown).

To ascertain whether the Prl-induced decrease in EGFR kinase activity is restricted to EGF-induced autophosphorylation, we examined the activity of EGFR to phosphorylate peptide substrates. While we determined that EGFR \( K_m \) for angiotensin II in isolates from NMuMG cells not treated with Prl was approximately 1.6 mM, a \( K_m \) for EGFR isolated from Prl-treated cells could not be determined due to the failure to detect an...
increase in peptide phosphorylation (not shown). As shown in Fig. 6, EGF induced an increase in angiotensin II (\(\text{Fig. 6A}\)) and Src peptide phosphorylation (\(\text{Fig. 6B}\)) from EGFR isolated from NMuMG cells not treated with Prl. However, paralleling our autophosphorylation data, Prl decreased EGF-induced EGFR phosphorylation of both angiotensin II and Src peptides to similar extents.

To determine if Prl induces a phosphatase that could be involved in heterologous regulation of EGFR kinase activity, we assessed phosphotyrosine phosphatase activity in our EGFR isolates by the previously described malachite green method (31). We observed no Prl-induced increase in dephosphorylation of either Src-autophosphorylated site, representing amino acids 412–422 of v-Src (RRLIEDAEpYTARG) (where pY represents phosphorylated Tyr), or an alternative tyrosine-phosphorylated peptide (RRLIEDAEpYAARG, not shown). Furthermore, using silica gel thin layer chromatography, we detected no changes in ATPase activity in EGFR isolates from Prl-treated cells (not shown). Therefore, these results suggest that the observed changes were probably due to altered EGFR kinase activity and not due to an increase in EGFR dephosphorylation or decrease in ATP content in the assay.

The fact that many investigators have reported various hormones that can regulate EGFR kinase activity by serine and/or threonine phosphorylation prompted us to examine the phosphorylation state of the EGFR that were isolated from cells treated with or without Prl. While Western blot analysis revealed that receptors isolated from cells not treated with Prl exhibited low but detectable threonine phosphorylation, receptors isolated from Prl-treated cells displayed a significant increase (\(\text{Fig. 7A}\)) but no detectable serine phosphorylation (not shown). To conclusively show that Prl does indeed induce phosphorylation of EGFR, we employed \(^{32}\)P metabolic labeling followed by thin layer electrophoresis. As shown in Fig. 8, while there were detectable levels of serine phosphorylation in control and Prl-treated cells, NMuMG cells treated with Prl exhibited a clear increase in threonine phosphorylation. However, there was no phosphotyrosine observed in either treatment group.

To determine if the removal of the observed threonine phosphorylation would cause the return of kinase activity of EGFR isolates from Prl-treated cells, we dephosphorylated isolated EGFR and examined kinase activity in response to EGF. Incubation of EGFR isolated from Prl-treated cells with alkaline phosphatase efficiently reduced detectable levels of threonine phosphorylation (Fig. 9A) and resulted in the return of EGF-induced EGFR phosphorylation that was equivalent to cells not treated with Prl (Fig. 9C).
band in the 170-kDa range was excised and hydrolyzed in 6N HCl at acid. Cells were treated with or without Prl and lysed, and EGFR was threonine, which results in either desensitization or down-

been reported to phosphorylate the EGFR on serine and/or downstream event cannot be determined from those results. In this study, we have also shown that receptors isolated from NMuMG cells treated with Prl exhibit a decrease in EGFR phosphorylation as well as a decrease in the ability of EGFR to phosphorylate angiotensin II or Src peptides, an effect that could not be attributed to differential EGFR concentrations, increasing phosphotyrosine phosphatases, ATPase activity, or varying binding affinities for EGF. NIH 3T3 cells, which have been shown to be unresponsive to Prl (29), presumably because they have no Prl receptors, however, failed to exhibit a Prl-induced decrease in EGF-induced EGFR phosphorylation. The effect of Prl in the present study appeared to be specific, insofar as the U5 anti-prolactin receptor, which has been shown to stimulate Prl receptor signaling (30), also decreased EGF-induced EGFR phosphorylation to levels observed in the Prl-treated group. However, this was not observed in EGFR that were isolated from NMuMG cells treated with bovine growth hormone, which has been reported not to bind to the Prl receptor (32). Increasing concentrations of EGF, ATP, or reaction time did not allow the EGFR isolated from Prl-treated cells to overcome the observed decrease in EGF-induced EGFR phosphorylation. These results suggest that EGFR isolated from Prl-treated cells contain an inhibitory constraint that inhibits kinase activity in response to ligand binding.

It has been shown that various kinases are activated either directly or indirectly in the EGFR signaling pathway (reviewed in Refs. 15 and 33). In addition to phosphorylating cellular substrates that elicit a biological response, these kinases have been reported to phosphorylate the EGFR on serine and/or threonine, which results in either desensitization or down- regulation of the receptor (10, 19–21) or activation of specific tyrosine phosphatases (34). While the latter has been shown not to be a probable mechanism in our current study, two alternatively proposed modes of EGFR autokinase regulation (22) are more consistent with our data. One model is that upon Ser/Thr phosphorylation there is a conformational change in the receptor disallowing further kinase activity, whereas the second model proposes that the Ser/Thr phosphorylation inhibited EGFR function by preventing the receptor from interacting with other biomolecules within the usual signaling complexes. Researchers have determined that the EGFR is under extensive modulation by other growth factor receptors (transmodulation), such as nerve growth factor (35), platelet-derived growth factor (28), insulin (36), and basic fibroblast growth factor (35), modifying EGFR autokinase activity, EGFR binding, or down-regulation. These have been proposed to be via phosphorylation (35) but may also involve phosphorylation-independent mechanisms (28). Although Western blotting and phosphoamino acid analysis results reported here show that EGFR isolated from Prl-treated cells have increased threonine phosphorylation, they do not conclusively show that such a phosphorylation event can decrease EGFR kinase activity. However, the restoration of kinase activity of EGFR isolated from Prl-treated cells by dephosphorylation lends evidence to the hypothesis that threonine phosphorylation of EGFR in response to Prl causes a decrease in EGF-induced EGFR kinase activity.

Prl has been proposed to signal through a variety of mechanisms including Ras (37), MAPK (38), heterotrimeric G proteins (39), protein kinase C (40), and Jak-STAT (41) pathways. Transmodulation of the EGFR by several of these pathways, most notably protein kinase C and MAPK, have been previously reported. In fact, mechanisms of EGFR phosphorylation leading to altered biochemical activity have been loosely categorized as protein kinase C-dependent and -independent. Treatment of cells with phorbol esters decreases EGFR signaling (3, 10, 19, 42) and decreases high affinity binding (10, 20, 43) but fails to affect dimerization (22). While in vivo studies have shown that protein kinase C can phosphorylate Thr-654 (19, 20, 22), other phosphorylation sites have also been described. EGFR has been shown to be phosphorylated on Thr-669 after treatment with EGF, phorbol esters, or thapsigargin (44–46). This effect has been shown to be mediated by MAPK (45, 46), and investigators have reported that phosphorylation at this site causes a slight reduction in EGF-induced EGFR kinase activity (19). In recent years, several researchers have described serine kinases that may be involved in regulating EGFR function. Kuppuswamy et al. (21) have reported that Cdc2 is capable of phosphorylating EGFR in vitro on serine 1002, an effect that resulted in a time-dependent decrease in kinase activity. Furthermore, the calcium-calmodulin dependent kinase II has been shown to phosphorylate EGFR on serine 1046/1047 and subsequently decrease EGFR kinase activity (22). While a flurry of research has tried to delineate which residue is involved in specific EGFR regulatory functions, e.g. desensitization or down-regulation, mutational analysis studies have reported that neither Thr-654 nor Thr-669 is sufficient to desensitize EGFR to MAPK or protein kinase C (19, 22); therefore, other mechanisms of regulating EGFR function must exist. However, serine 1046/1047 may be involved in regulation of EGFR kinase activity (22), while Thr-654/669 may be involved in regulating high affinity binding or EGFR numbers (20, 22, 42). In the present study, we observed low levels of basal serine phosphorylation that was unaffected by Prl. In contrast, Prl induced a readily detectable increase in threonine phosphorylation. This finding, combined with the observation...
FIG. 9. Alkaline phosphatase restores EGF kinase activity. EGF from NMuMG cells treated with or without PRL were incubated with or without alkaline phosphatase, separated by SDS-PAGE, transferred to membranes, and probed with anti-phosphothreonine (A) or anti-EGRF (B). C, alkaline phosphatase-treated EGF from PRL-treated and nontreated cells were incubated with 1 μM [γ-32P]ATP (specific activity, 300 Ci/mmol), 1 mM MgCl₂, and 10 ng/ml EGF. Reaction mixtures were separated by SDS-PAGE gel, fixed, dried, and exposed to x-ray film. Blots and autoradiograms are representative of three separate experiments. Bar graphs are presented as mean p170 density relative to control (A and B; −PRL/−ALP) or mean pp170 density relative to control (C; −PRL/EGF−ALP) of three separate experiments ± S.E. *, p < 0.05 compared with control.
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