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Prolactin Inhibits Epidermal Growth Factor (EGF)-Stimulated Signaling Events in Mouse Mammary Epithelial Cells by Altering EGF Receptor Function

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We have previously shown that lactogenic hormones stimulate epidermal growth factor (EGF) mRNA accumulation in mouse mammary glands in vivo and in mouse mammary epithelial cells (NMuMG line). However, our in vitro studies indicate that the lactogenic hormone prolactin (PRL) completely inhibits EGF-stimulated DNA synthesis. PRL does not alter cholera toxin or insulin-like growth factor-1–stimulated cell growth, thus the inhibition appears to be specific for EGF. Our current studies are designed to evaluate the effects of PRL on EGF-stimulated signaling events in the NMuMG cell line. Cells treated with PRL for 30 min demonstrated a loss of high affinity EGF-binding ability. After long-term PRL treatment (18 h) there was a decrease in EGF receptor (R) number, as determined by [^{125}I]EGF binding. PRL treatment (8 h) also decreased EGF-R mRNA levels. An EGF-stimulated increase in EGF-R mRNA observed 2–4 h after treatment was decreased when PRL was added to the cultures. Furthermore, levels of EGF-stimulated tyrosine phosphorylation of the EGF-R (170 kDa) and phospholipase Cγ (145 kDa) are dramatically decreased in cells treated with PRL. Also of great interest was a decrease in EGF-stimulated c-*myc* mRNA in PRL-treated cells. We conclude that PRL is acting to down-regulate the EGF-R, thus limiting EGF-stimulated cell signaling in mammary tissue.

INTRODUCTION

Epidermal growth factor (EGF)[1] is a well known mammary mitogen (for review see Imagawa *et al.*, 1990). In mammary tissue, EGF (S. Fenton, unpublished data) and EGF mRNA (Brown *et al.*, 1989; Fenton and Sheffield, 1991; Snedeker *et al.*, 1991) increase dramatically with parturition and reach maximum levels by 5–10 d of lactation. In contrast, mammary EGF-receptor (R) (Edery *et al.*, 1985) and EGF-R mRNA (S. Fenton, unpublished data) are elevated during early pregnancy and fall to very low, but detectable, levels during lactation (Taketani and Oka, 1983a). It is interesting to note that during lactation, although EGF is at its highest level and the EGF-R is present, very little mammary growth takes place.

The minimal hormonal requirements for in vitro lactation of mammary glands are insulin, hydrocortisone, and prolactin (PRL). In fact, production of milk proteins in vitro is dependent primarily on the presence of PRL (for review see Borellini and Oka, 1989). Mouse serum PRL levels increase at the same time mammary gland EGF levels are rising. Serum PRL content increases appreciably in late pregnancy, reaching maximum levels by 5–10 d of lactation, which is 5- to 10-fold that of basal levels (30 ng/ml) (Sinha *et al.*, 1974). Additionally, at least two forms of PRL receptors are also present in mouse mammary glands and increase during early lactation in response to rising levels of PRL to the gland (Kelly *et al.*, 1991).

We have previously shown that EGF mRNA in the mouse mammary gland is increased by lactogenic hormones (Fenton and Sheffield, 1991) and that PRL increases EGF mRNA in the normal murine mammary gland cells (NMuMG) mammary epithelial cell line

[1] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; NMuMG, normal murine mammary gland cells; PKC, protein kinase C; PRL, prolactin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

(Fenton and Sheffield, unpublished data). However, there are reports that demonstrate antagonism of PRL effects in mammary tissue when EGF is present. Takatani and Oka (1983b,c) first demonstrated that addition of lactogenic hormones to EGF-treated cells on collagen-coated plates could not induce maximal levels of lactational proteins. Similar results were found in mouse glands cultured in vitro (Vonderhaar and Nakhasi, 1986). The addition of EGF to explants in lactogenic media inhibited induction of maximal levels of α- and κ-casein RNA. It has been argued that the EGF-induced inhibition of caseins is because of the pharmacologic levels of insulin present in vitro (Sankaran and Topper, 1987). That study was initiated because EGF does not have inhibitory effects on lactation in vivo. Although EGF and PRL in the mammary gland reach maximum levels at the same time, the lactogenic effects of PRL appear to override the growth effects of EGF in vivo. It is possible that the dramatic increase in PRL alters EGF effects in the mammary gland.

We suggest that PRL inhibits EGF-induced mitogenesis in the mammary gland. Recent studies in our laboratory (Fenton and Sheffield, unpublished data) have shown that EGF is able to stimulate DNA synthesis in a normal mammary epithelial cell line, but the lactogenic hormone PRL inhibits that mitogenic effect. That inhibition was specific for EGF, because PRL did not alter DNA synthesis induced by cholera toxin or insulin-like growth factor-1. It is unclear whether the PRL inhibition of EGF-stimulated DNA synthesis in these cells is via the EGF receptor or postreceptor signaling events.

Our objectives in the present study were to examine the ability of PRL to alter EGF-R number and affinity, mRNA levels, and EGF-induced cell signaling. Cells were exposed to PRL for long (8–18 h) and short (10 min–4 h) periods to better understand the time requirements for PRL inhibition of EGF effects.

MATERIALS AND METHODS

Cell Culture

NMuMG were obtained from American Type Culture Collection (Rockville, MD). These cells were developed by Owens (1974) from normal mouse mammary glands. Cultures were maintained in 100% humidity at 37°C in an atmosphere of 5% CO_2/95% air. All experiments were performed on cells between passages 15 and 17. Cells were grown on Falcon plates (Becton Dickinson, Lincoln Park, NJ) and Corning flasks (Corning, NY) in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (GIBCO, Gaithersburg, MD). When cells reached 80% confluency, they were serum-starved for 24 h in DMEM before treatment addition.

[^{125}I]EGF Binding

After serum starvation, cells (2×10^5/well on 24-well plate) were treated with DMEM (control) with or without 100 ng/ml PRL (NIDDK-oPRL-20) for 30 min or 18 h. Media was then removed, and cells were washed three times with cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Cells were incubated for 3 h at 4°C with $\sim 1 \times 10^5$ cpm [^{125}I]EGF (0.4 nM) with or without the addition of 100 ng/ml PRL. Bound [^{125}I]EGF was competed with logarithimic doses (300–0 nM) of mouse EGF (Bioproducts for Science, Indianapolis, IN). The mouse EGF was iodinated as described (Vanderboom and Sheffield, 1993) and yielded specific activities (SA) ranging from 1.5×10^8 to 2.5×10^8 cpm/μg EGF. After incubation, cells were rinsed extensively with cold PBS and 0.1% BSA. Cells were solubilized in 0.5 M NaOH with 0.1% Triton X-100 (Sigma, St. Louis, MO) and cpm [^{125}I]EGF bound were determined. Receptor affinities and number were determined using Equilibrium Binding Data Analysis and Ligand programs (Biosoft, Ferguson, MO).

RNA Preparation

Cells (1×10^6/60 mm^2 dish) were grown in DMEM, with or without EGF (10 ng/ml), PRL (100 ng/ml), or EGF and PRL for various lenths of time after serum starvation. Cytoplasmic RNA was isolated as described by Gough (1988). Total RNA (10 μg/lane) was denatured, electrophoresed in a 1.2% agarose/3% formaldehyde gel, and transferred to Zeta-Probe (Bio-Rad, Hercules, CA) as previously described (Fenton and Sheffield, 1991).

cDNA Probes

The human EGF-R cDNA probe (pHER) was obtained from American Type Culture Collection (Ullrich *et al.*, 1984). The 1.2-kilobase (kb) fragment generated by *Pst* I digestion of the plasmid was utilized. The mouse c-*myc* cDNA probe was generated by *Pst* I digestion of pSV2 (American Type Culture Collection) and the resulting 0.4-kb fragment containing the second exon of c-*myc* was subcloned into pIC-20R to generate large amounts of the probe. The probes were nick translated with [^{32}P]dCTP and loaded onto a Sephadex Nick Column (Pharmacia, Piscataway, NJ) to remove free [^{32}P]dCTP. The range of SA was 6×10^6–5×10^7 cpm/μg DNA and 5×10^7–5×10^8 cpm/μg DNA for EGF-R and c-*myc*, respectively. The probes were boiled (5–10 min) and cooled before addition to hybridization solution. Hybridization conditions were as described previously (Fenton and Sheffield, 1991) with the exception that the EGF-R probe was hybridized at 42°C. Blots were exposed to Fuji x-ray film (Fisher, Chicago, IL) at −70°C for 2 wk. Relative intensities of bands on autoradiographs or photographs of gels (18S RNA) were determined by Collage (Fotodyne, New Berlin, WI). The 18S RNA was quantitated as a basis for equal RNA loading.

Western Analysis

All media and treatments added to cells (5×10^6/100 mm^2 dish) contained 1 mM Na_3VO_4 because of a previous report (McCune *et al.*, 1990) demonstrating elevated protein tyrosine phosphorylation with this phosphatase inhibitor present. After serum starvation, cells were treated for various lengths of time in DMEM with or without 10 ng/ml EGF to determine optimal conditions for EGF-R (p170) autophosphorylation. Then cells were treated with DMEM, 10 ng/ml EGF, 100 ng/ml PRL, EGF and PRL simultaneously, or PRL then EGF for 10 or 15 min each. Cells were rinsed three times with DMEM and 1 mM Na_3VO_4 on ice. Cells were solubilized and scraped from plates in sodium dodecyl sulfate (SDS)-loading dye (Laemmli, 1970) containing 1 mM Na_3VO_4. Samples were boiled for 15 min, and equal cell equivalents of protein (7.5×10^4 cells/well) were separated by 7.5 or 12.0% SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). Gels were transferred onto Immobilon-P membranes (Millipore, Bedford, MA) as described by Towbin *et al.* (1979) at 325 mA for 1.5 h at 4°C. Membranes were blocked in PBS and 2% Tween-20 (PBS-T) supplemented with 2% BSA overnight at 4°C and then incubated with 0.2 μg/ml mouse monoclonal anti-phosphotyrosine antibody (P-Tyr Ab-1, Oncogene Science, Uniondale, NY) in PBS-T containing 0.1% BSA for 1 h at 25°C. Blots were washed six times for 5 min in PBS-T with 0.1% BSA and incubated in sheep anti-mouse IgG with peroxidase conjugate for 30 min (1:1000) (Sigma, St. Louis, MO). Blots were washed again six times, and tyrosine phosphorylated proteins were detected using enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham, Arlington

Heights, IL). Exposure of membranes to Fuji x-ray film ranged from 2 to 30 min. Relative intensities of bands at 170 kDa were determined by Collage.

Anti-Phosphotyrosine Immunoprecipitation

An anti-phosphotyrosine-agarose immunoprecipitation kit that included buffers (Oncogene Science, Manhasset, NY) was utilized for this procedure. Two 150-cm^2 flasks of NMuMG cells were scraped into 1 ml of lysis buffer (1% Triton X-100, 10 mM tris(hydroxymethyl)aminomethane HCl pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM Na_3VO_4, 0.1% BSA, and 0.1% sodium azide) and 1 mM phenylmethylsulfonyl fluoride (PMSF), vortexed briefly, and allowed to lyse on ice 10 min. Lysates were centrifuged at 14 000 × *g* at 5°C for 15 min. Antiphosphotyrosine-agarose beads (25 μl) were added to 1 ml of supernatant and gently agitated for 2 h at 25°C. The suspension was centrifuged at 12 000 × *g* for 1 min, and supernatant was decanted. Beads with tyrosine-phosphorylated proteins attached were inverted in 1 ml of wash buffer (lysis buffer without BSA) containing 1 mM PMSF and centrifuged at 2000 × *g*. The wash steps were repeated three times. Finally, tyrosine-phosphorylated proteins were removed from beads with 300 μl of elution buffer (wash buffer containing 10 mM phenyl phosphate) and 1 mM PMSF, during a 2 h incubation at 25°C. The beads were pelleted, and supernatant was added to SDS-loading dye and boiled 5 min. Equal cell equivalents (5 × 10^5 cells/well) of each sample were separated by 7.5% SDS-PAGE and transferred to membranes as described. Blocked membranes were incubated with 0.5 μg/ml sheep anti-human EGF-R or mouse monoclonal phospholipase Cγ (PLCγ) antibody (UBI, Lake Placid, NY) for 2 h. Blots were then washed and incubated with anti-sheep or anti-mouse IgG (1:1000) (Sigma) for 30 min, and tyrosine phosphorylation of EGF-R and PLCγ detected by ECL as described.

Statistical Analysis

Data were analyzed using analysis of variance in Statview +512 (BrainPower, Calabasas, CA). Differences were considered significant at $p < .05$. Dunnett's t-test (Gill, 1978) was used to detect differences in RNA or protein band intensities between specific treatments and their respective controls.

RESULTS

We recently found (Fenton and Sheffield, unpublished data) that EGF-induced DNA synthesis in NMuMG cells was prevented by 18 h PRL treatment. To discern the mechanism by which PRL blocks EGF mitogenic function, the role of PRL on EGF-R abundance and activity was examined. The [^{125}I]EGF binding was used as the basis for determining EGF-R number and binding affinities after PRL treatment. NMuMG cells contain two affinity classes of EGF-R (0.1 nM and 2 nM). NMuMG cells treated with PRL for 18 h (the same amount of time as for DNA synthesis) demonstrated a significant 50% decrease in the number of high affinity EGF-R present (Table 1). However, when cells were treated with PRL for 30 min there was no significant change in receptor number, but a decrease in the affinity of the high affinity class of receptor from 0.1 nM to 0.5 nM was noted (Table 2). These studies were also performed in the constant presence of 100 ng/ml PRL. Binding affinities and EGF-R abundance were the same as in Tables 1 and 2 after inclusion of PRL with the radiolabeled EGF, indicating that PRL does not physically hinder EGF binding to its receptor.

Table 1. Scatchard analysis of EGF receptor after 18 h of treatment with and without PRL

	Receptor class			
	High		Low	
Treatment	Affinity	Receptor #	Affinity	Receptor #
None	0.1 nM[a]	23 408[a]	1.5 nM[a]	62 421[a]
PRL	0.2 nM[a]	11 733[b]	2.5 nM[a]	42 523[a]

[a,b] Values with different superscripts within a column are different ($p < 0.05$).

Table 2. Scatchard analysis of EGF receptor after 30 min treatment with and without PRL

	Receptor class			
	High		Low	
Treatment	Affinity	Receptor #	Affinity	Receptor #
None	0.1 nM[a]	17 064[a]	2.5 nM[a]	43 164[a]
PRL	0.5 nM[b]	14 042[a]	2.6 nM[a]	44 210[a]

[a,b] Values with different superscripts within a column are different ($p < 0.05$).

The decrease in EGF-R number seen after long-term (18 h) PRL treatment was substantiated when EGF-R mRNA levels were examined over various lengths of time. There was a biphasic effect on EGF-induced EGF-R mRNA levels. EGF significantly increased EGF-R mRNA levels (2.5-fold) after short-term treatment (4 h), yet also caused significant downregulation of the receptor mRNA to less than one-half basal level production by 16 h (Figure 1). PRL also increased EGF-R mRNA slightly by 2 h but then caused a time-dependent decrease in EGF-R mRNA levels with the maximal decrease seen by 8 h. When PRL was added to EGF-stimulated cells, it attenuated the spike in EGF-R mRNA at 4 h and accelerated the decrease in EGF-R mRNA levels so that the maximal decrease (30%) was seen at 8 h, instead of 16 h.

Binding of EGF to its receptor causes a rapidly enhanced protein kinase activity, because of autophosphorylation of tyrosine residues on the C-terminal end of the receptor (Ushiro and Cohen, 1980; Downward *et al.*, 1984). To more clearly understand the role of PRL on basal and EGF-activated EGF-R tyrosine phosphorylation, cells were first treated with EGF (10 ng/ml) or PRL (100 ng/ml) for various lengths of time to optimize ligand-induced autophosphorylation (p170) in these cells. Maximal levels of EGF-stimulated p170 tyrosine

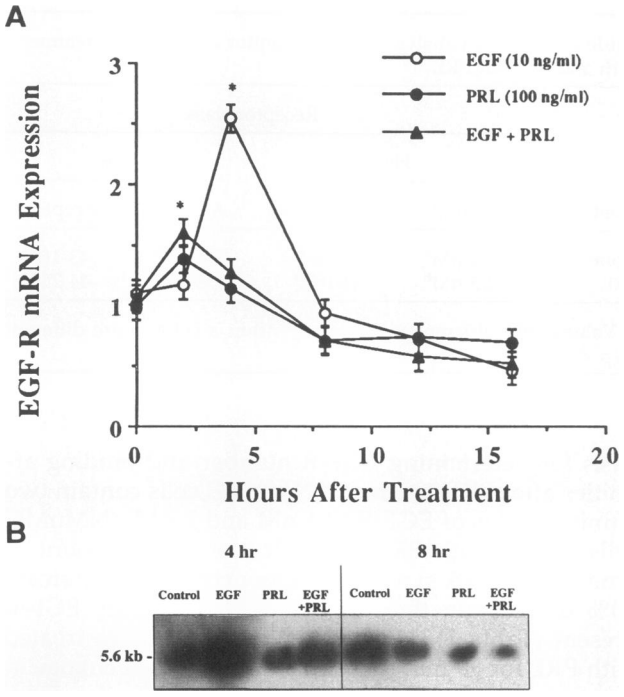

Figure 1. (A) EGF-R mRNA expression relative to control (DMEM = 1.0) in cells treated with 10 ng/ml EGF, 100 ng/ml PRL, or EGF and PRL for various lengths of time. Each point is the mean intensity (±SE) of the EGF-R band from five separate experiments. *, significantly different from 0 h of the same treatment ($p < 0.05$). (B) A representative autoradiograph of EGF-R mRNA (5.6 kb) after 4 and 8 h EGF treatment.

phosphorylation were seen after 10 min of EGF treatment (Figure 2). PRL failed to tyrosine phosphorylate any proteins in the 170-kDa range at all times examined (2–60 min).

Our studies were then expanded to ascertain the role of PRL on EGF-stimulated p170 tyrosine phosphorylation. Cells were treated with DMEM, EGF (10 ng/ml), PRL (100 ng/ml), EGF and PRL simultaneously, or PRL then EGF for 15 min each. A representative autoradiograph from these experiments is shown in Figure 3. As expected, EGF enhanced p170 tyrosine phosphorylation, and similar phosphorylation was demonstrated when cells were treated with EGF and PRL concomitantly. Of great interest, however, was the dramatic reduction in EGF-stimulated p170 tyrosine phosphorylation when cells were first treated with PRL for 15 min.

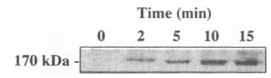

Figure 2. A representative autoradiograph demonstrating the time-dependent increase in tyrosine phosphorylation of 170-kDa band after EGF treatment. Similar results were obtained from two separate experiments.

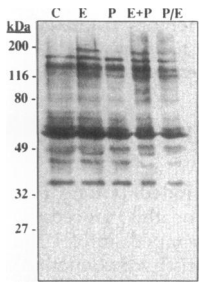

Figure 3. Representative autoradiograph of protein tyrosine phosphorylation after treatment of cells with DMEM, EGF (10 ng/ml), PRL (100 ng/ml), EGF and PRL concomitantly (EGF + PRL), or PRL then EGF (PRL/EGF) for 15 min each. Molecular weight standard sizes (kDa) are shown.

These results were repeated in five separate experiments as summarized in Figure 4. The average induction of p170 phosphorylation by EGF or EGF and PRL was nearly 10-fold, whereas PRL pretreatment of the cells reduced the effect of EGF to <40% of its original level.

The identity of tyrosine phosphorylated p170 was determined after immunoprecipitation of tyrosine-phosphorylated proteins. The proteins from cells treated as described above were separated on SDS-PAGE, and tyrosine-phosphorylated EGF-R was detected with Western blotting using a polyclonal anti-EGF-R. We demonstrate that p170 is indeed the EGF-R. Figure 5 depicts (similar to results in Figure 4) an increase in autophosphorylation of EGF-R (p170) after treatment of cells with EGF or EGF and PRL. EGF-R activation was inhibited by 15 min pretreatment with PRL.

Autophosphorylation of EGF-R has been shown to induce PLCγ tyrosine phosphorylation (Wahl *et al.*, 1989; Huckle *et al.*, 1990; Margolis *et al.*, 1990). Amount of tyrosine-phosphorylated PLCγ in NMuMG cells treated with or without PRL and EGF was determined using a mouse monoclonal antibody to PLCγ. There was a substantial decrease in EGF-stimulated tyrosine

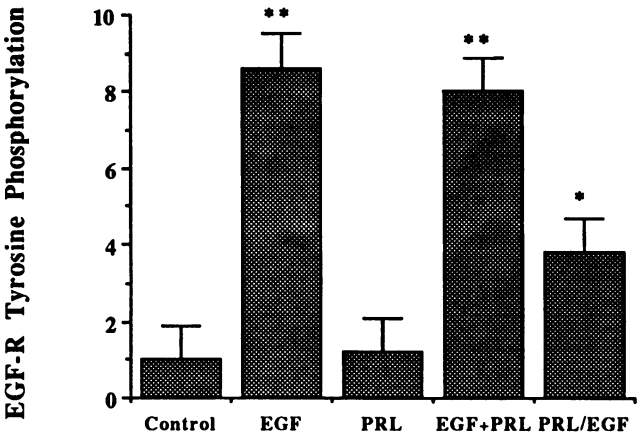

Figure 4. PRL inhibits EGF-induced tyrosine phosphorylation of p170-kDa protein. Presented is a summary of changes in tyrosine phosphorylation intensity of 170-kDa band after 15 min treatments. Each bar is the average (±SE) band intensity of p170 from five separate experiments. *, significantly greater than control levels; **, significantly greater than PRL then EGF (PRL/EGF)-treated cells.

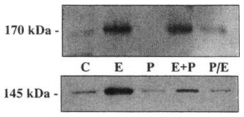

Figure 5. Identification of p170 as the EGF-R (top) and PRL inhibition of EGF-induced PLCγ tyrosine phosphorylation (bottom). Anti-phosphotyrosine was immunoprecipitated from cells after treatments shown on these representative autoradiographs. Blots were then incubated with EGF-R or PLCγ antibody. Similar results were obtained from three separate experiments.

phosphorylation of PLCγ after pretreatment with PRL (Figure 5). However, unlike the EGF-R, there was a detectable decrease, below EGF-induced levels, in tyrosine phosphorylation of PLCγ in cells treated concomitantly with EGF and PRL.

EGF-stimulated c-*myc* expression also follows activation of the EGF-R. Induction of c-*myc* gene expression is a general response to mitogenic stimuli (such as EGF) in a variety of tissues, and it has been suggested that an increase in c-*myc* is required for DNA synthesis (Rollins and Stiles, 1988). A recent study in our laboratory (Nass and Sheffield, 1993) using c-*myc* anti-sense oligomer treatment of NMuMG cells demonstrated a requirement for c-*myc* activity for EGF-induced DNA synthesis. In the present study, levels of c-*myc* mRNA were chosen as the endpoint to further clarify the time requirement and specificity of PRL inhibition of EGF-stimulated events. EGF (10 ng/ml) stimulated c-*myc* mRNA expression in a time-dependent fashion (Figure 6) with a significant increase by 4 h. EGF-stimulated c-*myc* mRNA reached maximal levels (threefold) by 8 h of treatment and returned to basal levels by 20 h. PRL (100 ng/ml) treatment alone did not alter control level c-*myc* mRNA abundance (Figure 7). However, when PRL was added to EGF-stimulated cells, there was a dramatic inhibition of EGF-stimulated c-*myc* mRNA levels after 8 h; the time at which EGF was found to cause maximal increases in c-*myc* mRNA.

DISCUSSION

We have demonstrated that PRL has the ability to regulate the EGF-R in several ways. The events that cause a cellular response after the binding of PRL to its receptor are not well understood, however. The majority of the studies on PRL mechanism of action have utilized the Nb_2 rat lymphoma cell line or rat liver for mitogenic effects and mammary gland explants for differentiation or secretion of milk proteins (Kelly *et al.*, 1991). A number of signal transduction pathways have been examined. Although cAMP, G proteins, Na+/H+ exchange, polyamines, and others have been considered, the most popular possible mode of action is via protein kinase C (PKC) (for review see Kelly *et al.*, 1991). We also believe that one of the mechanisms by which PRL modifies

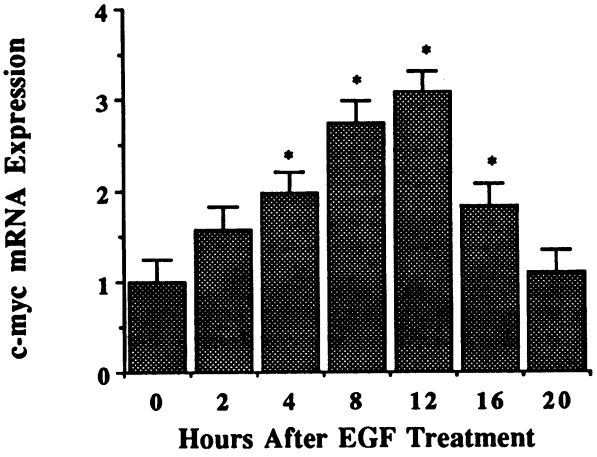

Figure 6. Time-dependent induction of EGF-induced c-*myc* expression. Each bar represents the mean intensity (±SE) of the c-*myc* mRNA band from six separate experiments. *, significantly different from 0 h of the same treatment ($p < 0.05$).

intracellular activities is through PKC. This idea is based on previous studies addressing the effect of PKC on EF cell signaling and the similarity of those studies to results in the present study.

Activation of PKC by stimulation of cells with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) has been re-

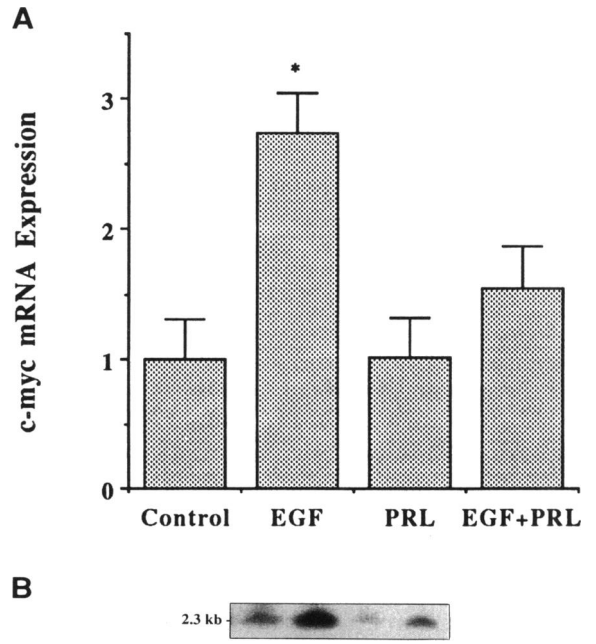

Figure 7. (A) PRL inhibition of EGF-induced c-*myc* mRNA expression after 8 h of treatment. Each bar represents the mean intensity (±SE) of the c-*myc* mRNA band from four separate experiments. *, significantly different from control level c-*myc* expression ($p < 0.05$). (B) A representative autoradiograph of c-*myc* mRNA (2.3 kb).

ported to attenuate EGF-induced biological responses such as DNA synthesis (Decker, 1984; Bjorge and Kudlow, 1987). Those findings are in agreement with our previous work that demonstrates PRL inhibition of EGF-stimulated DNA synthesis (Fenton and Sheffield, unpublished data). It has been reported that PKC phosphorylates Thr^{654} of the EGF-R that effectively inactivates the receptor (Hunter *et al.*, 1984; Lund *et al.*, 1990). Reduced EGF-R affinity and inactivation via Thr^{654} phosphorylation (Brown *et al.*, 1979; Shoyab *et al.*, 1979; McCaffrey *et al.*, 1984; Lund *et al.*, 1990) may explain decreased cell growth. We demonstrate in our present study that PRL caused a decrease in high affinity EGF binding after only a 30 min treatment period. However, long-term PRL treatment of cells initiated a reduction in the total number of available EGF-R when compared to control cells. EGF is still able to interact with its receptor after long-term exposure of cells to TPA, even though ^{125}I[EGF] binding is inhibited by >50% (Brown *et al.*, 1979; Shoyab *et al.*, 1979; Decker, 1984). Although total EGF binding is decreased, PKC has not been previously shown to decrease total receptor number after long-term TPA treatment. In fact, studies in our laboratory also demonstrate elimination of high affinity EGF binding without loss of total receptor number after TPA treatment (S. Fenton, unpublished data).

EGF is able to increase EGF-R mRNA levels after 4 h of treatment in several cell types (Clark *et al.*, 1985; Earp *et al.*, 1986, 1988; Thompson and Rosner, 1989). Our studies confirm that by 4 h NMuMG cells treated with EGF demonstrate a significant elevation of EGF-R mRNA. PRL decreased ligand-induced EGF-R mRNA content of these cells. That finding strengthens our hypothesis that PRL acts on the EGF-R to alter EGF-induced cell signaling by decreasing the amount of EGF-R present to respond to its ligand. Because previous studies have demonstrated an increase in EGF-R mRNA after stimulation of PKC by TPA (Clark *et al.*, 1986; Earp *et al.*, 1988), our findings indicate a mechanism of PRL action in addition to PKC. It is also possible that EGF itself may be the cause of EGF-R mRNA and protein downregulation. We have shown in our previous studies that PRL stimulates an increase in EGF mRNA levels (Fenton and Sheffield, unpublished data). Thus, the ability of PRL to induce EGF and PKC may both be affecting the abundance and activity of the EGF-R. The reduction in receptor mRNA expression and number found in our present studies may not be satisfactory to explain complete inhibition of EGF-induced DNA synthesis that we have previously reported (Fenton and Sheffield, unpublished data). Therefore, a rapid PRL-induced decrease in EGF-R activity may also be required to inhibit EGF effects.

Upon EGF binding, autophosphorylation events occur on the tyrosine residues in the extreme C-terminus of the EGF-R (Ushiro and Cohen, 1980; Downward *et al.*, 1984; Gill *et al.*, 1987), resulting in a fully activated receptor kinase. The activated receptor then acts as a tyrosine protein kinase, initiating a cascade of tyrosine phosphorylation events and biological responses (mechanism reviewed by Gill *et al.*, 1987). PLCγ has been identified as a substrate for ligand-induced EGF-R kinase activity in a variety of cell lines (Wahl *et al.*, 1989; Huckle *et al.*, 1990; Margolis *et al.*, 1990). EGF has been shown to cause increased cellular phosphotyrosine content (Cochet *et al.*, 1984; Decker, 1984), as well as rapid tyrosine phosphorylation of its own receptor, demonstrated through utilization of an anti-phosphotyrosine antibody (McCaffrey *et al.*, 1984; McCune *et al.*, 1990). Using an anti-phosphotyrosine antibody, we have shown that pretreatment of cells with PRL drastically reduced ligand-induced EGF-R tyrosine autophosphorylation. However, this effect was not seen when PRL and EGF were added simultaneously. In addition, EGF-induced PLCγ tyrosine phosphorylation was decreased in cells pretreated with PRL. Because previous studies report that activation of PKC after TPA treatment also reduces EGF-R ligand-stimulated tyrosine phosphorylation and kinase activity (Cochet *et al.*, 1984; Decker, 1984; McCaffrey *et al.*, 1984; Huckle *et al.*, 1990), we suspect that PRL may induce PKC that would downregulate the EGF-R, thus decreasing EGF-R and PLCγ tyrosine phosphorylation. Interestingly, we found an inhibition of PLCγ tyrosine phosphorylation when cells were treated with PRL, EGF and PRL, or PRL then EGF below levels of control, or EGF-stimulated PLCγ tyrosine phosphorylation, respectively. We are uncertain of the mechanism by which PRL caused the changes in tyrosine phosphorylation of PLCγ. We have not determined whether the changes are because of PRL induction of specific tyrosine phosphatases, activation PLC isozymes other than γ, reduction in basal level tyrosine kinase activity, or a change in physical association between the EGF-R and PLCγ.

Reduction of EGF-induced c-*myc* mRNA after 8 h PRL treatment does not come as a surprise in the present study. Nass and Sheffield (1993) have recently demonstrated that treatment of NMuMG cells with a c-*myc* anti-sense oligomer caused drastic decreases in EGF-induced DNA synthesis. Thus, it seems likely that elevated c-*myc* is required during EGF-stimulated mitogenesis of mammary cells, and the c-*myc* levels must be reduced for differentiation of mammary epithelium to take place. In fact, there is a reported negative association of c-*myc* levels and cell differentiation. Several studies demonstrate that high expression of c-*myc* (constitutive or induced) blocked or decreased cellular differentiation and by repressing c-*myc*, differentiation was able to proceed (Coppola and Cole, 1986; Langdon *et al.*, 1986; Freytag, 1988; Holt *et al.*, 1988).

We conclude that PRL regulates EGF-induced cell signaling events through an inhibition of EGF-R function. The mechanism(s) of PRL action are unknown at this time, but we are currently investigating the ability

of PRL to activate PKC in these cells. It has been reported that regulation of EGF-R is through PKC-dependent and independent pathways (reviewed by Gill *et al.*, 1987), so there are alternative means to down-regulate the EGF-R. In fact, preliminary studies in our laboratory suggest that PKC may not be the only mediator of PRL inhibition of EGF-R function in mammary epithelial cells. We believe that PRL may be exerting its effects in NMuMG cells through a tyrosine kinase pathway, also. This theory is supported by the appearance of a PRL-stimulated tyrosine phosphorylated band at 120–125 kDa (see Figure 5) and by changes in tyrosine phosphorylated proteins at 42–46 kDa (Fenton and Sheffield, unpublished data). Similar bands have recently been reported in PRL-treated rat Nb_2 lymphoma cells (Rillema *et al.*, 1992; Rui *et al.*, 1992). Furthermore, it has been demonstrated that EGF induces mitogen-activated protein kinases to phosphorylate Thr^{669} of the EGF-R, by a PKC-independent mechanism (Northwood and Davis, 1990). Phosphorylation of Thr^{669} on the EGF-R probably functions as a negative feedback control, as does PKC-stimulated phosphorylation of Thr^{654}. Phosphorylation of these sites on the EGF-R after PRL treatment are now under investigation.

Regardless of the mechanism of PRL action, we provide data that suggests that PRL controls EGF-stimulated cell events when it is present at physiological levels. We believe this to be the case in the mammary gland of periparturient mice, i.e., PRL increases mammary EGF mRNA and protein levels (Fenton and Sheffield, 1991) and at the same time causes a decrease in EGF-R receptor mRNA (S. Fenton, unpublished data) and protein content (Edery *et al.*, 1985). Furthermore, because EGF is still able to bind to EGF-R in lactating tissue (Taketani and Oka, 1983a), PRL may be working not only to decrease the amount of available receptor but also to inhibit EGF-induced EGF-R function, thus allowing the mammary gland to differentiate into a secretory organ.

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