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Epidermal growth factor induces prolactin mRNA in GH_4C_1 cells via a protein synthesis-dependent pathway

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Summary

Prolactin (PRL) gene expression is regulated through a complex network of signal transduction pathways activated by various hormones and growth factors. Estrogens regulate PRL gene transcription in vivo through both direct and indirect, protein synthesis-dependent, mechanisms. Therefore, we hypothesized that other stimulators of PRL gene transcription might also act via protein synthesis-dependent mechanisms. To test this hypothesis, we examined, in GH_4C_1 rat pituitary tumor cells, the effects of protein synthesis inhibitors on the induction of PRL mRNA by known stimulators of PRL gene transcription. Whereas induction by epidermal growth factor (EGF) was abolished by cycloheximide and puromycin, increases in PRL mRNA caused by thyrotropin releasing hormone, 12-*O*-tetradecanoylphorbol 13-acetate, forskolin, or dibutyryl cyclic AMP were unaffected. These data suggest that the induction of PRL mRNA by EGF may require the induced synthesis of an intermediary regulatory protein.

Introduction

Transcriptional regulation of the prolactin (PRL) gene is mediated through a complex network of signal transduction pathways activated by various hormones and growth factors, including estrogens, epidermal growth factor (EGF), thyrotropin releasing hormone (TRH), and dopamine (Murdoch et al., 1982, 1985; Bancroft et al., 1985; Shull and Gorski, 1986). Additionally, the pituitary-specific transcription factor, Pit-1/GHF-1 (Pit-1), is required for the expression of both the PRL and growth hormone genes (Bodner et al., 1988; Ingraham et al., 1988; Nelson et al., 1988). Pit-1 also appears to mediate the effects of certain hormones and second messengers on PRL gene transcription (Day and Maurer, 1989; Iverson et al., 1990; Yan et al., 1991).

Previous studies showed that estrogens stimulate PRL gene transcription in vivo through at least two independent pathways (Shull and Gorski, 1985). This conclusion is based on the observation of two temporally-distinct phases of enhanced PRL gene transcription following a single injection of the short-acting estrogen, 16α-estradiol. The initial phase of transcriptional stimulation coincides with estrogen receptor occupancy. Therefore, this phase is regarded to be due to direct interactions between the activated estrogen receptor and the enhancer region of the PRL gene (Shull and Gorski, 1985, 1986), which contains at least one estrogen responsive element (Maurer and Notides, 1987; Waterman et al., 1988; Lannigan and Notides, 1989). The second, protein synthesis-dependent, phase of estrogen-stimulated PRL gene transcription occurs after the number of occupied estrogen receptors returns to its basal level (Shull and Gorski, 1985). We hypothesized that this phase is a consequence of estrogen inducing the release of another hormone or growth factor, either from a second target cell or the lactotroph itself, which then stimulates PRL gene transcription through a protein synthesis-dependent mechanism.

In this study we employed GH_4C_1 rat pituitary tumor cells to examine the effects of protein synthesis inhibitors on the induction of PRL mRNA by EGF, TRH, 12-*O*-tetradecanoylphorbol 13-acetate (TPA),

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dibutyryl cAMP (db cAMP), and forskolin. Inhibition of protein synthesis by cycloheximide (CHX) blocked the induction of PRL mRNA by EGF but did not block induction by the other compounds examined, indicating that EGF differs from these other compounds in that it appears to induce PRL mRNA through a protein synthesis-dependent mechanism.

Materials and methods

Materials

The sources of the GH_4C_1 cell line and all cell culture reagents were as stated previously (Shull, 1991; Shull et al., 1992). TPA was purchased from LC Services (Woburn, MA, USA); EGF from Collaborative Research (Bedford, MA, USA); and TRH, db cAMP, forskolin, CHX, and puromycin (PUR) were from Sigma (St. Louis, MO, USA). [^{32}P]Deoxycytidine triphosphate ([^{32}P]dCTP) and [^{3}H]leucine were obtained from Amersham (Arlington Heights, IL, USA) or New England Nuclear (Boston, MA, USA). Random priming reagents were obtained from Gibco BRL Life Technologies (Gaithersburg, MD, USA). Nylon transfer membranes were purchased from Schleicher and Schuell (Nytran, Keene, NH, USA), or Micron Separations (Magna NT, Westborough, MA, USA). Rat PRL cDNA was kindly supplied by Dr. Richard Maurer, Department of Physiology and Biophysics, University of Iowa; and, β-actin cDNA by Dr. Gordon Shore, Department of Biochemistry, McGill University.

Cell culture

GH_4C_1 cells were maintained in culture as previously described (Shull, 1991; Shull et al., 1992). For all experiments described herein, cells were plated at a density of 42,000 cells/cm^2, in phenol red-free Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium, containing 15 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 365 mg/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and made 15% with sera (12.5% donor horse serum and 2.5% fetal bovine serum) that had been charcoal-treated as described below. Two days later, media were replaced with fresh media, and cells were pretreated for 20–30 min with either CHX (10 μM), PUR (100 μM) or their phosphate-buffered saline (PBS) vehicle. This pretreatment was followed by the addition of the following from 100 × stocks, to achieve the stated concentrations: forskolin, 1 μM; db cAMP, 1 mM; butyrate, 1 mM; TRH, 10 μM; TPA, 10 ng/ml; EGF, 25 ng/ml; or their PBS vehicle. Butyrate was used as a control for db cAMP, since butyrate is liberated from db cAMP upon its entry into the cell. Cells were harvested 18 h after treatment, since it had been previously shown that treatment of pituitary cells with CHX for this length of time did not result in cell death (Shull et al., 1987).

Removal of steroids from sera

Donor horse and fetal bovine sera were combined in a ratio of 5:1, and treated with dextran-coated charcoal to remove endogenous steroids in a manner adapted from Horwitz et al. (1975). Activated charcoal (Sigma) was rinsed with acetone, followed by triply distilled water, dried in vacuo at 80°C (up to 36 h), and stored at room temperature until use. Prior to treating sera, the acetone-washed charcoal was stirred in a solution of 0.004% Dextran, 10 mM Tris HCl (pH 8.0) for 1 h at room temperature. Buffer was removed from the charcoal by centrifugation, and sera were added (4 g charcoal/l sera) and mixed at 4°C for 30 min. Charcoal was removed from the sera by centrifugation, and the procedure was repeated twice more with freshly dextran-coated, activated charcoal. Sera were then filtered through a 1.2 μm Millipore (Bedford, MA, USA) filter to remove residual charcoal, and the pH was adjusted to 7.5. Finally, the sera were filter-sterilized using 0.2 μm Nalgene low-protein binding filter units.

Cytosolic dot blot analysis of PRL and β-actin RNAs

For preparation of total cellular RNA, culture medium was removed, and the cells were washed in 5 ml ice-cold PBS and harvested on ice by scraping twice in 0.6 ml PBS. Cell pellets were collected by centrifugation and treated on ice for 5 min with TE buffer containing 0.9% Nonidet P-40, to lyse the cell membranes. Cytosols were separated from nuclei by centrifugation at 12,000 × *g* for 2.5 min at 4°C. To measure relative mRNA levels, cytosolic dot blots were prepared by the method of White and Bancroft (1982). cDNA probes were radiolabeled by the random priming method (Feinberg and Vogelstein, 1983). Prehybridization, hybridization, and washing conditions were as previously described (Shull and Pitot, 1989). Autoradiography was performed for various times at −80°C using Kodak XAR-5 film and DuPont Cronex Lightning Plus intensifying screens. Intensity of the dots on the resulting autoradiographs was measured with a Shimadzu CS9000U densitometer, using a 0.1 × 5.0 mm light beam, which allowed the entire area of each dot to be analyzed.

Measurement of [^{3}H]leucine incorporation

The effects of CHX or PUR on protein synthesis were examined by comparing the levels of [^{3}H]leucine incorporated into trichloroacetic acid (TCA)-precipitable material, in cells treated with and without one of the protein synthesis inhibitors. 10 μCi/ml [^{3}H]leucine was added 1 h before cells were harvested, as described above. Aliquots of cytosol were spotted onto glass microfiber filters, which were allowed to air dry.

The filters were then soaked in freshly prepared 10% TCA for 15 min on ice, transferred to ice-cold 5% TCA for 10 min, then quenched in cold 95% ethanol for 5 min. The radioactivity remaining bound to the filters was measured in a Beckman LS3801 liquid scintillation counter.

Data analysis

Statistical significance was determined with a one-tailed Student's t-test, with $p < 0.05$ being considered significant.

Results

To determine whether any of several known activators of PRL gene transcription act in a protein synthesis-dependent manner, we have examined the effects of CHX on the abilities of these activators to induce PRL mRNA in GH_4C_1 cells. With no inhibition of protein synthesis, forskolin, db cAMP, TRH, TPA, and EGF all increased the level of PRL mRNA between 2-fold and 4-fold (Fig. 1*A*), whereas butyrate was without effect (data not presented). CHX, when added 20 min prior to the above compounds, completely abolished the stimulatory effect of EGF on the level of PRL mRNA, but did not affect the induction of PRL mRNA by the other compounds (Fig. 1*B*). Results similar to these were observed in at least three independent experiments. These data suggest that EGF differs from the other activators examined in the sense that only EGF appears to induce PRL mRNA through a protein synthesis-dependent mechanism.

Based on the assumption that the β-actin gene is constitutively expressed in GH_4C_1 cells, we examined the level of β-actin mRNA as an indicator of the effects of each treatment on cell number and the biosynthetic status of the cells. None of the above stimulators of PRL mRNA accumulation caused any change in the level of β-actin mRNA (data not shown), suggesting that none of the treatments affected cell number over the time course examined, and that the effects of the treatments on PRL mRNA were gene-specific.

To obviate the possibility that CHX inhibition of the EGF effect on PRL mRNA levels was due to a side-effect of CHX, we examined the effects of another inhibitor of protein synthesis, PUR, on the induction of PRL mRNA by EGF, TRH, and TPA (Fig. 2). CHX inhibits protein synthesis by inhibiting the peptidyl transferase function of the large ribosomal subunit, whereas PUR acts as an aminoacyl-tRNA analog that, once incorporated into the nascent peptide, precludes elongation, resulting in premature termination. When the concentration of puromycin greatly exceeds that of the tRNAs, it acts as an effective inhibitor of protein synthesis. Results of experiments using PUR were similar to those with CHX: protein synthesis inhibition by PUR abolished only the induction of PRL mRNA by EGF, while having no effect on the ability of either TRH or TPA to increase the level of PRL mRNA. Collectively, these results strongly suggest that the induction of PRL mRNA by EGF is mediated through a protein synthesis-dependent pathway.

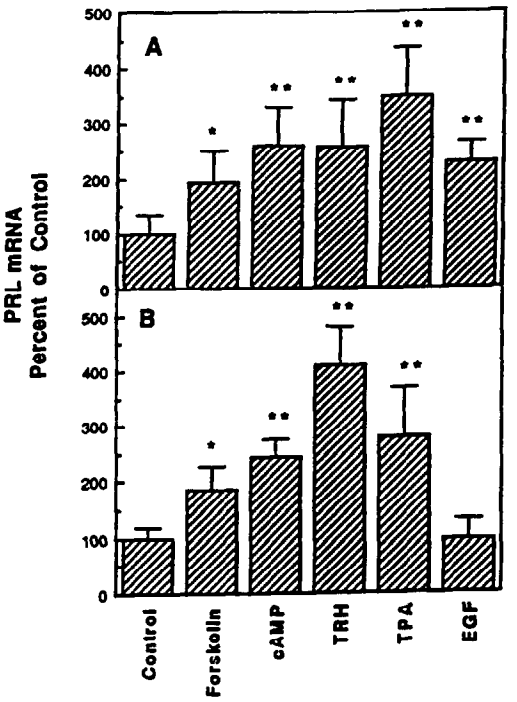

Fig. 1. Effects of CHX on the induction of PRL mRNA by activators of PRL gene transcription. Cells were plated at a density of 42,000 cells/cm^2, in phenol red-free DMEM/F12 medium, made 15% with sera (12.5% donor horse serum and 2.5% fetal bovine serum) that had been charcoal-treated to remove endogenous steroids. Two days later, media were replaced with fresh media, and cells were incubated for 20–30 min with either PBS (*A*) or 10 μM CHX (*B*). This was followed by the addition of the indicated stimulatory agents. Cells were harvested 18 h later, and PRL mRNA was measured by the cytosolic dot blot procedure of White and Bancroft (1982) as described in the Materials and methods section. This technique was chosen to allow replicate assay of multiple RNA samples from each treatment group. Cytosolic dot blots were probed with PRL cDNA, and the hybridized signals were quantified by scanning densitometry. Each bar represents triplicate determinations of duplicate or triplicate cytosols, from individual representative experiments (±SEM). Experiments were repeated at least 3 times. *A*: Cells were treated with PBS, 1 μM forskolin, 1 mM db cAMP, 10 μM TRH, 10 ng/ml TPA, or 25 ng/ml EGF in the absence of CHX. *B*: Cells were incubated with 10 μM CHX for 20–30 min before administration of the above compounds. * $p < 0.01$; ** $p < 0.001$.

Incorporation of [^{3}H]leucine into TCA-precipitable material in GH_4C_1 cells was measured to ensure that protein synthesis was inhibited to a similar extent in all treatment groups. Both CHX (Fig. 3) and PUR (data not shown) inhibited [^{3}H]leucine incorporation by 85–95%, regardless of whether the cells were treated concurrently with the various activators of PRL gene transcription.

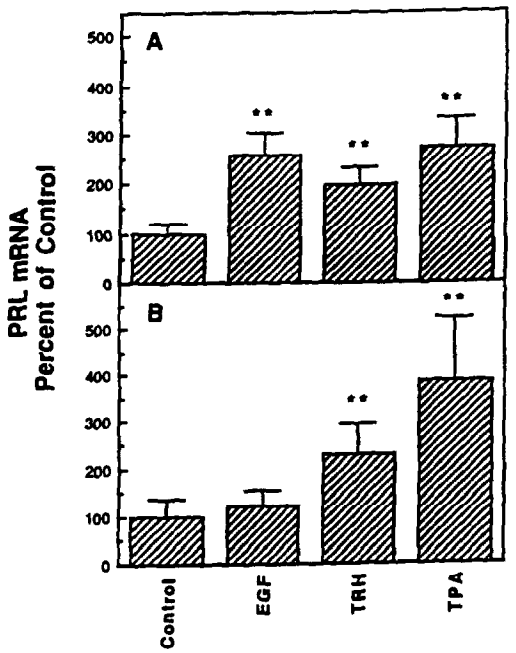

Fig. 2. Effects of PUR on the induction of PRL mRNA by activators of PRL gene transcription. Cells were cultured, treated, and harvested as in Fig. 1, except that 100 μM PUR was substituted for CHX. Cytosolic dot blots were probed with PRL cDNA, and the hybridized signals were quantified by scanning densitometry. Each bar represents triplicate determinations of triplicate cytosols, from an individual representative experiment ($\pm$SEM). Similar results were observed in two independent experiments. *A*: Cells were treated with PBS, 25 ng/ml EGF, 10 μM TRH, or 10 ng/ml TPA in the absence of PUR. *B*: Cells were pretreated for 20 min with 100 μM PUR before administration of the above compounds. $**p < 0.001$.

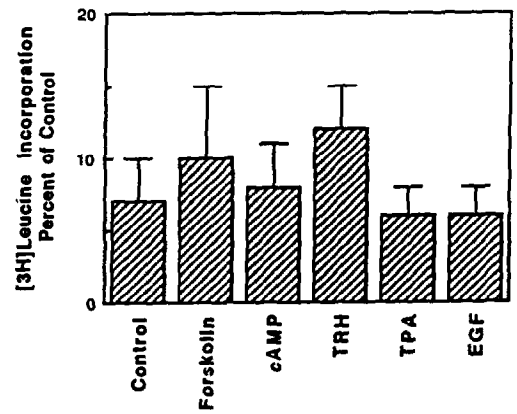

Fig. 3. Effects of CHX on incorporation of [^{3}H]leucine into acid-precipitable material by cells treated with activators of PRL gene transcription. Cells were cultured, treated, and harvested as in Fig. 1, except that cells were incubated with [^{3}H]leucine for 1 h before harvesting, and radioactivity incorporated into acid-precipitable material was measured. Each data bar represents the level of [^{3}H]leucine incorporated by cells pretreated with CHX, expressed as a percentage of the level incorporated by cells pretreated with the PBS vehicle ($n = 3$, $\pm$SEM). Similar results were seen in at least three independent experiments.

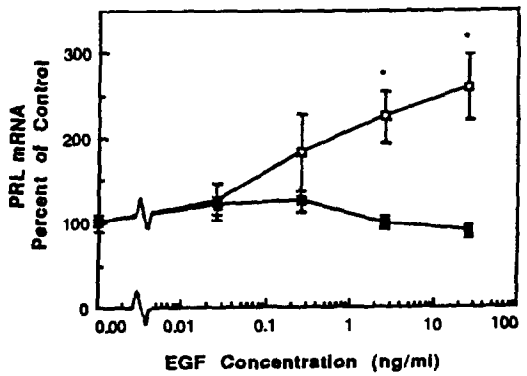

Fig. 4. Effects of CHX on the induction of PRL mRNA by various concentrations of EGF. Cells were cultured as in Fig. 1, and treated with 0.025, 0.25, 2.5, or 25 ng/ml EGF following a 30 min incubation with 10 μM CHX (■) or its PBS vehicle (□). Cytosolic dot blots were probed with PRL cDNA, and the hybridized signals were quantified by scanning densitometry. Each point represents triplicate determinations from triplicate cytosols, from an individual representative experiment ($\pm$SEM). Similar results were seen in two independent experiments. $* p < 0.01$.

To investigate further the effects of EGF on the level of PRL mRNA, we examined the effects of CHX on the induction of PRL mRNA by various concentrations of EGF. In the absence of CHX, the level of PRL mRNA increased in a dose-dependent manner in response to EGF, whereas CHX effectively blocked induction at all concentrations of EGF (Fig. 4). The EGF concentrations employed in these studies covered the range of responsiveness previously described for EGF-induced PRL secretion from GH_4C_1 cells (Schonbrunn et al., 1980).

Discussion

The effects of protein synthesis inhibitors on the induction of PRL mRNA by several known stimulators of PRL gene transcription have been examined. This study was undertaken in response to earlier observations that estrogens stimulate PRL gene transcription in vivo through at least two independent pathways, one of which appears to require de novo protein synthesis (Shull and Gorski, 1985). We hypothesized that estrogens may act either on a second target cell or on the lactotroph itself to alter the release of one or more hormones or growth factors that subsequently regulate PRL gene transcription through a protein synthesis-dependent mechanism. We report that CHX completely blocked the EGF-induced increase in PRL mRNA, whereas it had no effect on induction by either TRH, TPA, forskolin, or db cAMP. Therefore, EGF appears to act upon the PRL gene through a mechanism distinct from the other compounds examined, possibly through the induced synthesis of an intermediary regulatory protein.

Murdoch et al. (1985) reported that a 2–4 h pretreatment of GH cells with CHX had no inhibitory effect on the stimulation of PRL gene transcription by EGF. However, data supporting this statement were not presented by these authors, and the time points at which PRL gene transcription was examined were not indicated. Consequently, it is difficult to reconcile the previously reported failure of CHX to block induction of PRL gene transcription by EGF (Murdoch et al., 1985) with the data presented herein indicating the CHX blocks the induction of PRL mRNA by this growth factor. In an earlier study, Murdoch et al. (1982) observed that: (1) transcriptional stimulation of the PRL gene by EGF follows burst-attenuation kinetics, falling rapidly from an immediate 9-fold stimulation to less than 3-fold by 20 h; and (2) cytoplasmic PRL mRNA continues to be elevated for several days without a sustained increase in PRL gene transcription. Taken together, these data suggest that EGF may act at multiple points to induce PRL mRNA.

Previous studies suggested that EGF, TRH, and TPA act similarly to activate PRL gene transcription (Supowit et al., 1984; Murdoch et al., 1985). Deletion analyses showed that a short segment of the PRL gene, extending approx. 40 bp upstream of the transcription start site and containing the most proximal Pit-1 binding site, confers responsiveness to EGF, TRH, and TPA (Elsholtz et al., 1986; Yan et al., 1991). In addition, these compounds, as well as cAMP, cause the same basic chromatin-associated protein to be phosphorylated (Murdoch et al., 1982, 1983, 1985). The data presented herein demonstrate that EGF induces PRL mRNA via a protein synthesis-dependent pathway, whereas TRH, TPA, and db cAMP do not. In light of the demonstrated role of the Pit-1 transcription factor in mediating the stimulatory effects of certain hormones and second messengers on PRL gene transcription (Day and Maurer, 1989; Iverson et al., 1990; Yan et al., 1991), and a report indicating that both cAMP and TPA induce Pit-1 phosphorylation and alter its ability to bind to *cis*-acting elements associated with the PRL gene (Kapiloff et al., 1991), we hypothesized that the protein synthesis-dependent mechanism through which EGF induces PRL mRNA might involve the induction of Pit-1 synthesis. Although we have not tested this hypothesis exhaustively, we have observed that EGF has no significant effect on the level of Pit-1 mRNA over an 18 h time course (unpublished data).

Previous studies revealed that pimozide, a dopamine antagonist, stimulates PRL gene transcription in vivo through a protein synthesis-dependent mechanism (Shull and Gorski, 1990). This raises the possibility that dopamine inhibits PRL gene transcription by decreasing the level of the same intermediary regulatory protein that EGF induces. This regulatory protein could then stimulate PRL gene transcription either by interacting directly with the promoter/enhancer region of the PRL gene, or by facilitating interactions between proteins already bound to regulatory sites. Alternatively, an EGF-induced regulatory protein might function instead to stabilize the PRL mRNA, thus increasing its steady state level. The data presented herein clearly demonstrate that EGF induces PRL mRNA through a pathway distinct from those initiated by TRH, TPA, or cAMP. However, the protein synthesis-dependent pathway(s) linking EGF, estrogen, and dopamine to the PRL gene remain to be elucidated.

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