Characteristics of the Prolactin Stimulation of c-*fos* mRNA Levels in Mouse Mammary Gland Explants (44001)

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Abstract. **The signal transduction pathway(s) for the prolactin (PRL) regulation of lactogenic processes in the mammary are not fully known. Recent studies indicate that the PRL occupancy of its receptor activates a tyrosine kinase (JAK-2), which is constitutively associated with the receptor. In the present studies, we have characterized the PRL stimulation of c-*fos* mRNA accumulation in mouse mammary gland explants that were precultured with insulin and cortisol for 36 hr. In time course studies, an initial effect (2-fold increase) of PRL was apparent after 15 min, a 4-fold increase occurred after 60 min, and a 2-fold increase was apparent after 2 hr. Dose-response experiments indicated an initial effect with 5 ng/ml PRL and a maximum effect with 500 ng/ml PRL. The accumulation of c-*fos* mRNA in the cultured mammary tissues was also increased by human GH, recombinant bovine PRL, cycloheximide, and phorbol 12-myristate 13-acetate (TPA). The cycloheximide and PRL responses were additive, thus indicating that cycloheximide did not "superinduce" the PRL stimulation of c-*fos* mRNA accumulation. Downregulation of protein kinase C (PKC) by pretreating mammary tissues for 1 day with TPA did not affect the magnitude of the PRL stimulation of c-*fos* mRNA accumulation. The findings from these studies are consistent with c-*fos* playing a role in the PRL stimulation of lactogenic processes in the mammary gland. PRL had no effect on c-*jun* mRNA accumulation under any of the experimental conditions employed in the c-*fos* studies.** [P.S.E.B.M. 1996, Vol 212]

Significant advances have recently been made in determining the molecular signaling mechanisms involved in the prolactin (PRL) stimulation of lactational processes. The PRL receptors belong to the cytokine family of receptors; other ligands that function through cytokine receptors include growth hormone, the interferons, erythropoietin, several interleukins, granulocyte colony–stimulating factor, and others. In contrast to other families of receptors, the cytokine receptors contain no tyrosine kinase domains or consensus ATP-binding sites (1). However, all of the cytokine receptor ligands, including PRL, do rapidly stimulate protein tyrosyl phosphorylation in target cells (2–5). The tyrosine kinase that is initially activated by PRL is Janus kinase 2 (JAK-2), which is a member of the Janus kinase family of tyrosyl kinases (6, 7). JAK-2 is constitutively associated with the PRL receptor and is activated within seconds after PRL addition to cultured mammary tissues or a PRL-responsive T-cell line (Nb_2). Genistein, a tyrosine kinase inhibitor, abolishes the effects of PRL on mitogenesis in Nb_2 cells and milk product synthesis in mammary cells (8, 9). The experimental evidence thus suggests that JAK-2 activation is the earliest event in the PRL signaling cascade.

Downstream from the initial PRL receptor signaling, PRL is known to stimulate the phospholipase C-protein kinase C pathway (10, 11), as well as the tyrosyl phosphorylation of one of the STAT proteins (STAT-5) (12). Tyrosyl-phosphorylated STAT-5 enhances the rate of transcription of several milk protein genes, and protein kinase C activation is essential for all of PRL's actions on milk product synthesis and mitogenesis.

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c-*fos* is an early response gene involved in signaling for several hormones including the cytokine hormones (13, 14). Transcription of the c-*fos* gene is known to be enhanced by agents that express responses through activation of protein kinase C. Experiments were therefore designed to characterize the prolactin regulation of c-*fos* transcription in mouse mammary gland explants.

Materials and Methods

Midpregnant (10–14 days of pregnancy) Swiss-Webster mice were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Ovine prolactin (NIDDK-oPRL-19) was a gift from the National Institutes of Health. Other materials were purchased from the following sources: cortisol from Charles Pfizer and Co. (New York, NY); medium 199 Earle's salts, restriction enzymes, and DNA random labeling kits from Gibco Laboratories (Grand Island, NY); porcine insulin, penicillin, and streptomycin from Eli Lilly Co. (Indianapolis, IN); [α-^{32}P]CTP from Dupont Corp. (Wilmington, DE); Tri reagent from Molecular Research Center, Inc. (Cincinnati, OH); all other reagents and chemicals from Sigma Chemical Co. (St. Louis, MO). The plasmids containing the mouse cDNA probes were gifts: the 1.1-kilobase cDNA fragment of mouse c-*fos* was from Dr. C. Stiles of Harvard University; the mouse c-*jun* from Dr. R. Bravo (Heidelberg, Germany), courtesy of Dr. Jessica Schwartz (University of Michigan), and mouse β-actin from Dr. David Smith of Wayne State University.

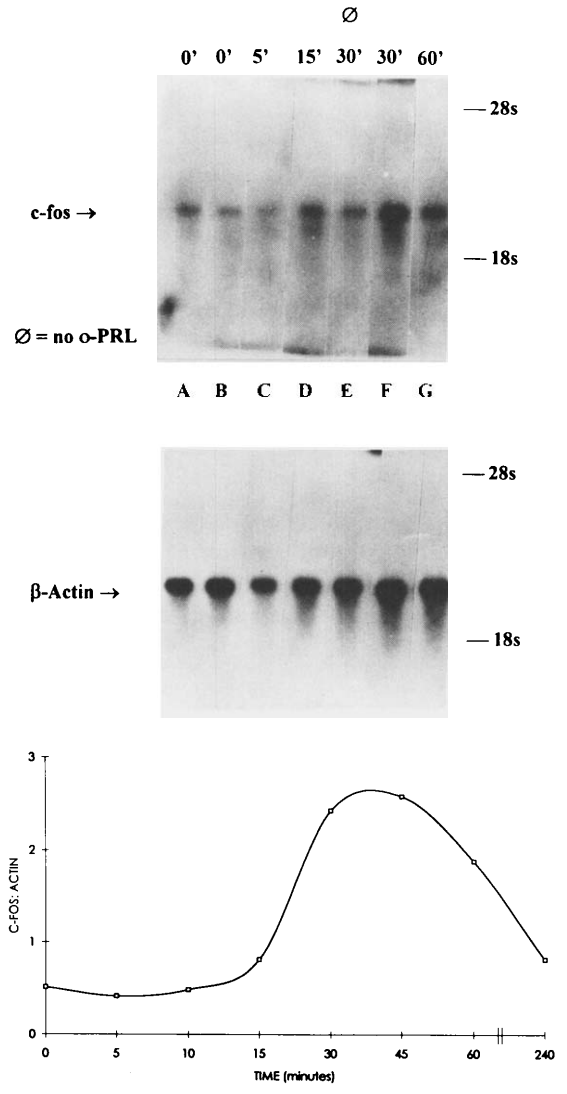

Figure 1. Time course of prolactin stimulation of c-*fos* mRNA accumulation. Mammary gland explants were cultured ± 1 μg/ml prolactin for the times indicated. Total RNA was then extracted, subjected to agarose electrophoresis and blotted to nylon filters. The filters were then hybridized to [^{32}P]-labeled cDNAs for β-actin and c-*fos* (upper panel). After autoradiography the bands were quantitated by laser densitometry and the ratio of c-*fos* to β-actin determined (lower panel). A–G, individual gel lanes.

Mice were sacrificed by cervical dislocation, and the caudal pair of mammary glands were removed and placed in medium 199 Earle's salts. Explants (3–5 mg each) were prepared as described earlier (15). Explants (16–20) from six to eight animals were randomly placed on siliconized lens paper floating on 6 ml of medium 199 Earle's salts containing 1 μg/ml insulin plus 10^{-7} *M* cortisol in 60 × 15–mm plastic petri dishes. The tissues were initially cultured for 36 hr at 37°C in a culture oven gassed with 5% CO_2/95% air. After the initial 36-hr culture, PRL and/or other agents were added to selected plates and incubation continued for times up to 48 hr.

Quantitation of mRNAs was carried out as follows. Total RNA from cultured mammary tissues was extracted by a single-step procedure employing the guanidium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (16). Tissue samples (150–200 mg) were homogenized in 2 ml of TRI reagent (a monobasic mixture of guanidium thiocyanate and phenol) in a polytron homogenizer. The phases were separated after addition of 400 μl of chloroform:isoamyl alcohol (49:1). The aqueous-guanidium thiocyanate phase was removed and the RNA precipitated by addition of isopropanol. The pellet was washed with 75% ethanol and then dissolved in diethyl pyrocarbonate-treated double distilled water. RNA concentration was spectrophotometrically determined by absorbance at a wavelength of 260 nm. At 20 μg/lane, the RNA was subjected to electrophoresis in a 1.2% agarose, 10.65 *M* formaldehyde gel. The RNA was then transferred to a nylon membrane *via* capillary elution. The immobilized RNA was hybridized overnight at 42°C with [^{32}P]-labeled cDNA probes for β-actin, c-*fos*, or c-*jun*. Labeling of the probes was accomplished with a kit purchased from Gibco BRL Life Technologies, Inc. After hybridization, the membranes were exposed to film for appropriate times at

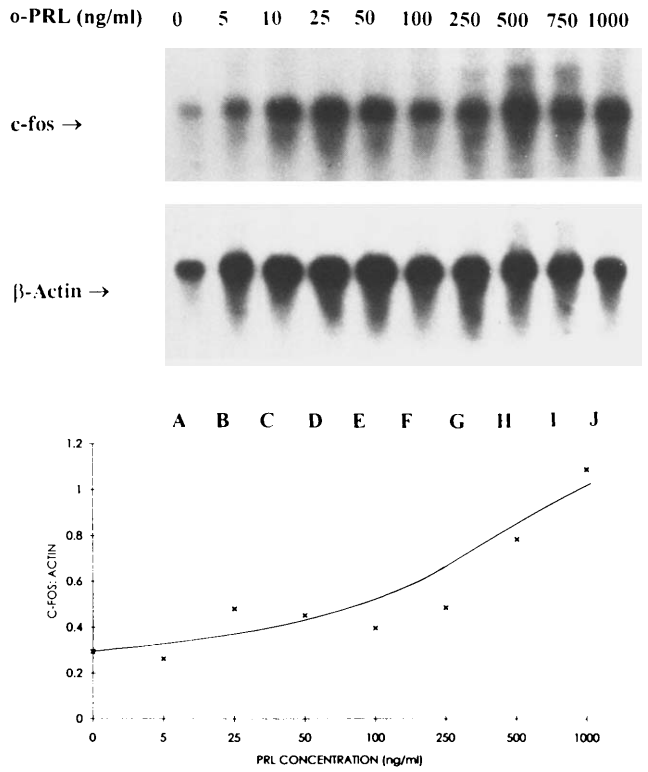

Figure 2. Dose response of prolactin stimulation of c-*fos* mRNA accumulation. Mammary gland explants were cultured ± 0–1 μg/ml prolactin for 30 min and then treated as in Figure 1. A–J, individual gel lanes.

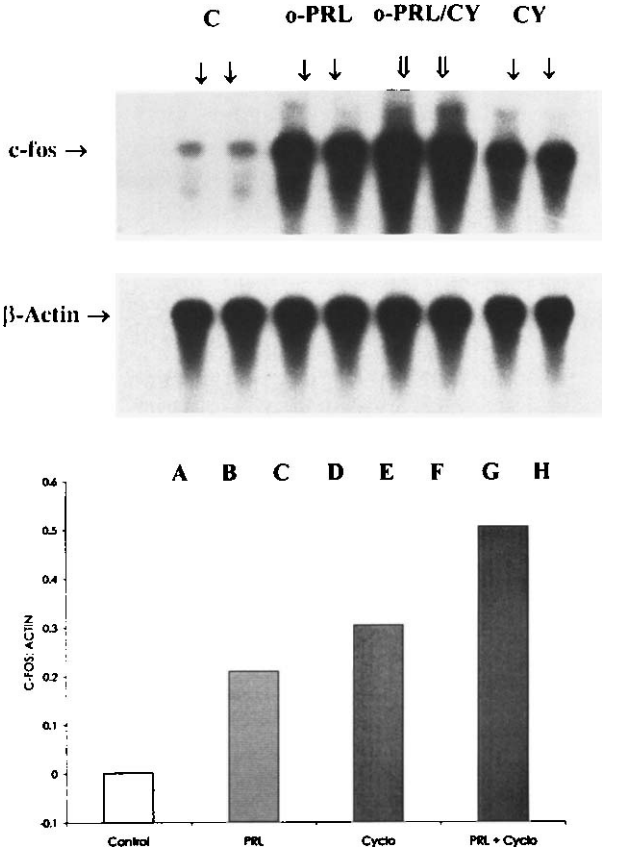

Figure 3. Effect of cycloheximide on the prolactin stimulation of c-*fos* accumulation. Mammary tissues were incubated with 1 μg/ml prolactin and/or 10 μg/ml cycloheximide for 30 min. c-*fos* mRNA was then measured as detailed in Figure 1. A–H, individual gel lanes.

−80°C. The films were then developed and the bands quantitated *via* laser densitometry. Results are expressed as a ratio of the densities of the c-*fos* band to that of the β-actin band. All experiments presented in the results section are selected from at least three experiments where similar results were observed.

Results

Figure 1 shows the results of an experiment in which the time course of the effect of PRL on the accumulation of c-*fos* mRNA was determined. The upper panel illustrates the autoradiography banding patterns that were generated and subsequently quantitated using laser densitometry. The resulting ratio of c-*fos*:actin mRNA is presented in the lower panel. PRL expresses a transient effect on c-*fos* mRNA accumulation with the initial effect occurring between 15 and 30 min after hormone addition. A peak response (5-fold increase) occurs after 30–45 min of PRL treatment, and the effect abates and is no longer apparent after 4 hr. In studies similar to those described above, PRL was found to have no effect on the tissue accumulation of c-*jun* mRNA (data not included). Other lactogenic hormone preparations including human growth hormone and recombinant bovine PRL stimulated c-*fos* mRNA accumulation similar to the responses elicited with ovine PRL (data not included).

In dose-response studies, the PRL response was assessed after a 30-min treatment period (Fig. 2). The lowest PRL concentration that elicited a significant response was 25 ng/ml (increases were observed in three separate experiments), and a maximum effect was observed with 500–1000 ng/ml PRL. These PRL concentrations are within the range of those present in the plasma under a variety of physiological states (17).

Cycloheximide has been reported to cause a "superinduction" of c-*fos* and c-*jun* mRNA accumulation in response to a number of growth factors including growth hormone (14). Figure 3 shows that cycloheximide does stimulate c-*fos* mRNA accumulation in cultured mammary tissues; in fact, the response to cycloheximide is greater than that elicited with PRL. However, when cycloheximide and PRL were tested together, a clearly additive response is observed. Superinduction by cycloheximide of the PRL stimulation of c-*fos* mRNA accumulation is therefore not apparent in the mammary gland.

A number of earlier studies suggest the involvement of protein kinase C activation in the PRL stimulation of its target cells. Figure 4 shows that TPA, an activator of protein kinase C, stimulates c-*fos* mRNA

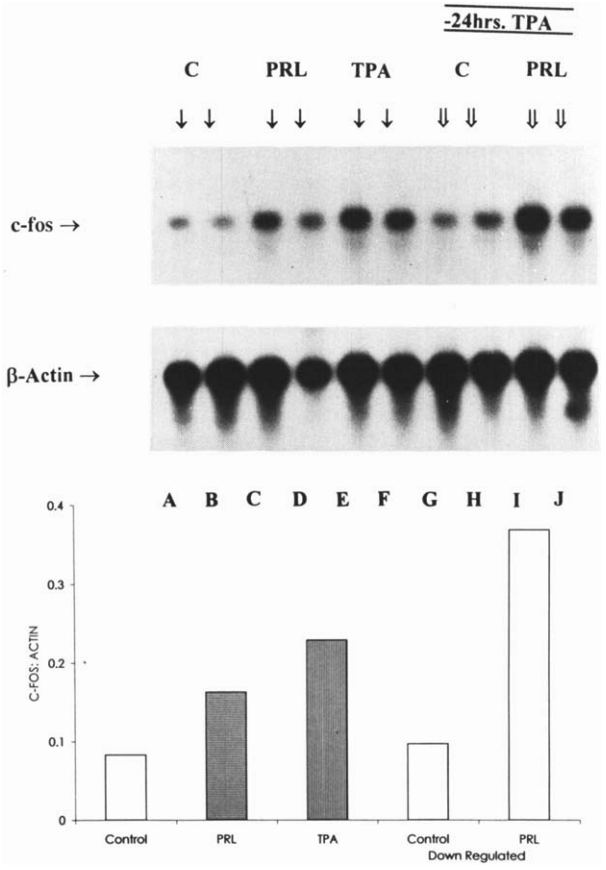

Figure 4. Effect of PKC downregulation on prolactin stimulation of c-*fos* mRNA accumulation. PKC downregulation was accomplished by culturing the tissues with 10 μg/ml TPA for 24 hr. The mammary tissues were then cultured for 30 min with 1 μg/ml prolactin or 10 μg/ml TPA as indicated after which c-*fos* mRNA was measured by procedures indicated in Figure 1. A–J, individual gel lanes.

accumulation during a 30-min treatment period. However, when PKC is "downregulated" by pretreating mammary tissues for 24 hr with TPA, the magnitude of the PRL stimulation of c-*fos* mRNA accumulation is greater than that observed in tissues in which protein kinase C is not "downregulated." This is clearly an unexpected response and will be discussed later.

Other agents that have been shown to express PRL-like responses in cultured mouse mammary tissues include phospholipase C and arachidonic acid. Both of these substances (Fig. 5) also stimulate c-*fos* mRNA accumulation in cultured mammary explants.

Discussion

These studies clearly indicate that PRL, at physiological concentrations, stimulates a transient 5-fold increase in c-*fos* mRNA accumulation in cultured mouse mammary tissues. In contrast, no effect of PRL was observed on the tissue accumulation of c-*jun* mRNA. The transient PRL effect on c-*fos* mRNA accumulation is not unlike that elicited by a number of other growth factors, including growth hormone, in

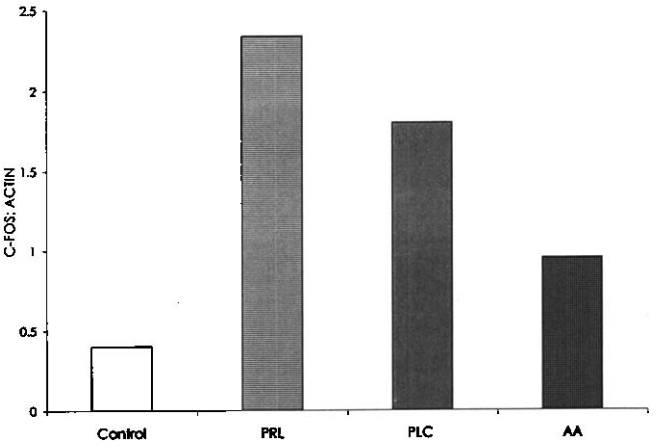

Figure 5. Effects of phospholipase C and arachidonic acid on c-*fos* mRNA accumulation. Mammary gland explants were cultured for 30 min with 50 μg/ml bee venom phospholipase C, 10 μg/ml arachidonic acid, or 1 μg/ml PRL. c-*fos* mRNA was then measured as detailed in Figure 1.

other types of cells (14). The rapid effect of PRL on c-*fos* mRNA accumulation is consistent with a functional role in the early PRL signaling processes. Accordingly, it is of great interest that the time courses for the PRL stimulation of protein kinase C activity and the tissue content of inositol triphosphate (IP_3) are virtually the same as that for c-*fos* mRNA accumulation in cultured mouse mammary tissues (10, 18). Since TPA, a stimulator of PKC, stimulates c-*fos* mRNA accumulation as does PRL, it seems likely that the PRL stimulation of protein kinase C may be causally related to the PRL stimulation of c-*fos* mRNA accumulation. Although the studies in which protein kinase C was downregulated do not support this thesis, it is important to point out that extended treatment (1–3 days) of mammary tissues with TPA to "downregulate" protein kinase C only reduces protein kinase C activity by about 50% (10). Accordingly, the remaining protein kinase C activity may be adequate for PRL to express its effects on c-*fos* mRNA accumulation, but may be inadequate for the PRL effects on milk product formation which are abolished by "downregulation" of protein kinase C (10). It was further shown in these studies that other agents including phospholipase C and arachidonic acid, which have been shown in earlier studies to stimulate protein kinase C activity in mammary cells (14), also stimulate c-*fos* mRNA accumulation. These observations further suggest that protein kinase C activation is in the signaling pathway for the PRL stimulation of c-*fos* mRNA accumulation.

It remains to be shown if and how the c-*fos* and/or the c-*jun* proteins participate in the signaling pathway for the PRL stimulation of milk product synthesis. It is possible that the milk product genes have AP-1 enhancer sites (13) which are regulated by the c-*fos* and c-*jun* proteins.

The cycloheximide, a protein synthesis inhibitor, stimulation of c-*fos* mRNA accumulation in a variety of cells is a consistent response. Although cycloheximide has been reported to "superinduce" the effects of several growth factors on c-*fos* mRNA expression, the PRL and cycloheximide responses in mammary tissues were additive. Cycloheximide therefore clearly does not "superinduce" the PRL effect on c-*fos* accumulation in cultured mammary tissues. The significance of these pharmacological observations is not currently known.

In earlier studies from Ebner's laboratory (19, 20), PRL was reported to stimulate c-*myc* but not c-*fos* expression in a PRL-responsive T-cell line (Nb_2 cells), however, protein kinase C activators including TPA and phospholipase C stimulated c-*fos* expression in the Nb_2 cells. In experiments not presented, we have also been unable to observe a PRL effect on c-*fos* mRNA accumulation in Nb_2 cells, thus confirming the observations published by Ebner's group. PRL therefore clearly has differing effects on c-*fos* mRNA accumulation in mammary cells versus Nb_2 cells. The difference may reside in the fact that the Nb_2 cells are neoplastic, or for some other reason yet to be identified.

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