Possible Involvement of PI3K in Prolactin-Stimulated Milk Product Formation and Iodide Transport in Mouse Mammary Explants (44329)

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Abstract. **The *in vitro* effect of wortmannin, an inhibitor of PI3 kinase, on prolactin (PRL) stimulated $P70^{S6K}$, iodide transport, and milk product synthesis were investigated in cultured mouse mammary tissues. Mouse mammary gland explants were initially incubated for 24 hr in media M199 containing 1 μg/ml insulin and 10^{-7}*M* cortisol. A subsequent treatment with wortmannin impeded, in a dose-dependent fashion, the PRL stimulation of casein, lipid, and lactose synthesis as well as the PRL stimulation of iodide transport. Rapamycin (25 ng/ml), an inhibitor of $P70^{S6K}$, also inhibited the effect of PRL on iodide transport; this drug was earlier shown to inhibit PRL effects on milk product synthesis. These results suggest the possible involvement of $P70^{S6K}$ and PI3-kinase in PRL-stimulated milk product formation and iodide transport in mouse mammary explants. Since wortmannin caused a diminished cellular content of $P70^{S6K}$ and a reduced extent of $P70^{S6K}$ migration in polyacrylamide gels (likely due to dephosphorylation), PI3-kinase likely lies upstream in the PRL signaling pathway for $P70^{S6K}$ activation.** [P.S.E.B.M. 1998, 219]

Prolactin is a member of the family of lactogens and exhibits diverse biological activities. More than 100 biological effects have been noted for prolactin (1, 2) although in mammals, it is still primarily recognized for its effects on milk production and mammary gland proliferation (2).

Exposure of resting cells to mitogens initiates a signaling cascade that leads to the phosphorylation of the S6 protein of 40S ribosomal subunits. Phosphorylation of the S6 protein is catalyzed by two S6 kinases, $P70^{S6K}$ and $P90^{RSK}$ (3, 4). $P70^{S6K}$ and its closest homolog $P90^{RSK}$ are coordinately activated within minutes after mitogen addition through Ser/Thr phosphorylation of the enzyme (5). $P90^{RSK}$ is activated by MAPK (6) and participates in a well-defined signaling network that includes *ras, raf*-1, and *MEK* 1 (7). $P70^{S6K}$ is a ubiquitous hormone-activated Ser/Thr kinase (8) that has several cellular effects, including its requirement for cells to enter S phase after mitogen stimulation (5, 9). $P70^{S6K}$ requires multiple independent inputs for activation and is activated *via* a rapamycin-sensitive pathway that possibly involves PI3 kinase (P13K) (10). Rapamycin, an immunosuppressant, blocks phosphorylation of four of seven sites on $P70^{S6K}$ that are phosphorylated upon serum stimulation.

PI3K activation has been implicated in the regulation of a number of different cellular responses. PI3K is a heterodimeric complex consisting of 85- and 110-kDa subunits (p85 and p110) (11). The p85 subunit consists of multiple domains including an SH3 domain and two SH2 domains. The two SH2 domains bind tyrosine-phosphorylated receptors and subsequently recruit the p85-p110 complex to the receptors. It is suggested that the role of p85 is to target p110 to the membrane where its lipid substrates reside (11). PI3K has been shown to be involved in the reorganization of the actin cytoskeleton (12), receptor internalization (13), histamine secretion (14), neutrophil activation (15), platelet activation (16), cell migration (17), glucose transport (18), and vesicle sorting (19). Activation of the PI3K pathway by either membrane targeting of p110, overexpression of p110

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molecules with high enzymatic activity, or a combination can trigger the stimulation of downstream responses, including stimulation of $P70^{S6K}$, but not MAPK (20).

Wortmannin is a fungal metabolite that is a potent inhibitor (IC50 >> 3 n*M*) of mammalian PI3K in virtually all cellular preparations tested so far (18). It irreversibly binds to the p110 catalytic subunit of PI3K and inhibits its serine kinase and lipid kinase activity in isolated rat adipocytes (21) and 3T3 L1 cells (14, 22). PI3K-induced $P70^{S6K}$ activation *in situ* is accompanied by a selective increase in the phosphorylation of $P70^{S6K}$ Thr-252; wortmannin causes selective dephosphorylation of $P70^{S6K}$ Thr-252 concomitant with inhibition of $P70^{S6K}$ activity *in situ* (23).

In this report, rapamycin and wortmannin's inhibition of PRL activation of S6 kinase formation, milk product synthesis, and iodide transport in mouse mammary gland has been examined in an attempt to discern the possible involvement of $P70^{S6K}$ and PI3K in these PRL-stimulated processes.

Materials and Methods

Incubation System. Midpregnant (10–14 days of pregnancy) Swiss-Webster mice were purchased from Harlan Laboratories (Indianapolis, IN). Ovine PRL (NIH-P-S-14) was a gift from NIH. Other substances were from the following sources: cortisol from Charles Pfizer (New York, NY); medium 199 with Earle's salts (M199) and Hanks' balanced salt solution (HBSS) from GIBCO Laboratories (Grand Island, NY); porcine insulin, penicillin, and streptomycin from Eli Lilly Co. (Indianapolis, IN); rapamycin and wortmannin from Sigma Co.; ^{3}HOH and [carboxy-^{14}C] inulin (405.8 mCi/g) from New England Nuclear (Boston, MA); ^{125}I from Amersham Life Sci., Inc. (Arlington Heights, IL); [^{3}H] leucine (53 Ci/mmol), [^{14}C] acetate (58 Ci/mmol), and [5.6-^{3}H] glucose (66.6 Ci/mmol) from NEN (Boston, MA). Methods used to culture tissues in our laboratory were described earlier (24). Briefly, mice were sacrificed by cervical dislocation, and the caudal and inguinal pair of mammary glands were removed aseptically and placed in HBSS. The glands were cut into pieces weighing 3–5 mg each and placed on siliconized lens paper floating on 6 ml M199 containing insulin (1 μg/ml) and cortisol (10^{-7} *M*) in sterile culture dishes. All incubations were carried out at 37°C in an atmosphere of 95% air-5% CO_2 (vol/vol). All explants initially underwent a 24-hr incubation in M199 containing insulin and cortisol. Experiments were then begun by adding fresh culture medium alone or plus PRL. Rapamycin and wortmannin, when used, were added 1 hr before PRL was applied.

Assays for Lactose, Lipid, and Lactose Synthesis For the casein synthesis assay, [^{3}H] leucine (0.5 μCi/ml) was added to the culture medium for the final 2 hr of incubation. The amount of [^{3}H] leucine incorporated into a casein-rich phosphoprotein fraction was then determined (25).

For the lipid synthesis assay, the tissues were exposed to [^{14}C] acetate (0.2 μCi/ml) for the final 2 hr of incubation. The tissues were weighed and homogenized in 0.4 ml distilled water. The lipids were extracted by the methods of Bligh and Dyer (26). Radioactivity incorporated into the lipid fraction was then determined.

For the lactose synthesis assay, the tissues were exposed to 1 μCi/ml [5, 6-^{3}H] glucose for the last 2 hr of incubation. Lactose was isolated *via* thin-layer chromatography, and its radioactivity was quantitated (27).

Western Immunoblotting Aliquots of concentrated total cell lysate (1 mg protein) or immunoprecipitation supernatant (from 1.5 ml total cell lysate) with 1 vol of 2× Laemmli buffer, was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was then transferred to PVDF membrane. The blotted PVDF membrane was washed twice with water and blocked in freshly prepared PBS buffer containing 3% nonfat dry milk at 22°C overnight with constant agitation. After blocking, the PVDF membrane was incubated with 1 μg/ml of S6 kinase antibody (Upstate Biotechnology Inc., Lake Placid, NY) diluted in freshly prepared PBS-milk at 25°C for 2 hr followed by incubation with secondary antibody (a goat anti-rabbit IgG linked to horseradish peroxidase 1:3000) at 25°C for 1 hr with agitation. Then the PVDF membrane was washed with water twice, PBS-0.05% Tween 20 for 5 min, and rinsed with 4–5 changes of water. Finally enhanced chemiluminescence by using ECL was employed to detect any immunoblotting.

Iodide Transport Assay. In experiments where the effects of PRL on iodide transport were to be determined, the tissues were initially cultured for 24–36 hr with 1 μg/ml insulin plus 10^{-7} *M* cortisol; PRL was then added, and incubations continued for 24 hr. For the final 2 hr of culture, the tissues were transferred to vessels containing ^{125}I (0.25 μCi/ml; 0.3 ng/ml iodide) in 4 ml of HBSS; incubations were carried out in a rotary water bath at 37°C. The tissues were then weighted and homogenized in 2 ml of 10% trichloroacetic acid (TCA) containing 0.1 m*M* NaI. After a determination of total radioactivity, the samples were centrifuged at 2000*g* for 10 min. After washing the pellet with an additional 5 ml of 10% TCA, radioactivity in the TCA-insoluble fraction was determined. The intracellular accumulation of radiolabeled iodide was calculated by subtracting the amount of radiolabeled iodide in the extracellular space from the total radioactivity in the tissue homogenates (24, 28). Results of the iodide uptake studies were expressed as a distribution ratio that represented the ratio of the intracellular specific activity divided by the extracellular specific activity of the radiolabeled iodide. The results of the incorporation studies were expressed as DPM/mg wet weight of tissues.

Statistical Analysis. Statistical comparisons were made with Student's *t* test when two means were compared, or by an analysis of variance followed by Sheffe's test for multiple comparisons. All values represent the mean ± SE of four observations.

Results

The effects of wortmannin on the PRL stimulation of milk product formation are shown in Figures 1–3. In a dose-dependent manner, wortmannin at concentrations of 5–10 μM inhibited the PRL stimulation of lipid, casein, and lactose synthesis whereas the basal rates of synthesis were not affected significantly. The effect of wortmannin and rapamycin on PRL signaling in mouse mammary tissues was further investigated by studying iodide transport regulation. As shown in Figures 4–7, total iodide uptake and iodide incorporation were increased significantly by PRL (1 μg/ml). Treatment with wortmannin at concentrations of 1 μM and above caused a complete inhibition of these PRL responses; basal levels of iodide uptake and incorporation were also decreased with the addition of 5 μM wortmannin. Treatment with rapamycin at concentrations of 25 ng/ml and above attenuated the PRL effect on iodide transport whereas basal levels of uptake were not significantly affected.

The cellular content and phosphorylation state of the $P70^{S6K}$ were assessed by migration patterns on polyacrylamide gels and subsequent visualization on Western blots. Figure 8 shows an altered migration pattern of $P70^{S6K}$ in SDS/polyacrylamide gels depending on the concentration of wortmannin added. At 500 nM or above, wortmannin caused $P70^{S6K}$ to shift to a faster migrating hypophosphorylated species; meanwhile, the content of $P70^{S6K}$ was decreased in proportion to the concentration of wortmannin treatment. In Figure 9, all the experimental conditions were the same as those in Figure 8, except the second incubation with PRL was extended to 19 hr instead of 12 hr. Figure 9 shows that wortmannin induced a decrease in the cellular content of $P70^{S6K}$ in a dose-dependent manner. At wortmannin concentrations of 700 nM or above, there was a significant decrease in the $P70^{S6K}$ level compared to the

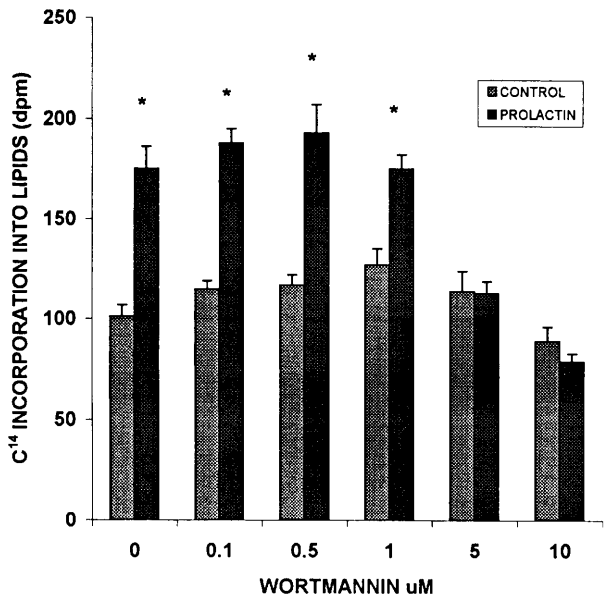

Figure 1. Wortmannin inhibition of PRL stimulated lipid synthesis. Explants were incubated for 24 hr in culture media containing insulin (1 μg/ml) and cortisol (10^{-7} M). After preincubation with wortmannin for 1 hr, 1 μg/ml PRL was added, and incubation was continued for 24 hr. Lipid synthesis was determined as described in the "Materials and Methods." *Significantly greater than corresponding control with $p < 0.05$.

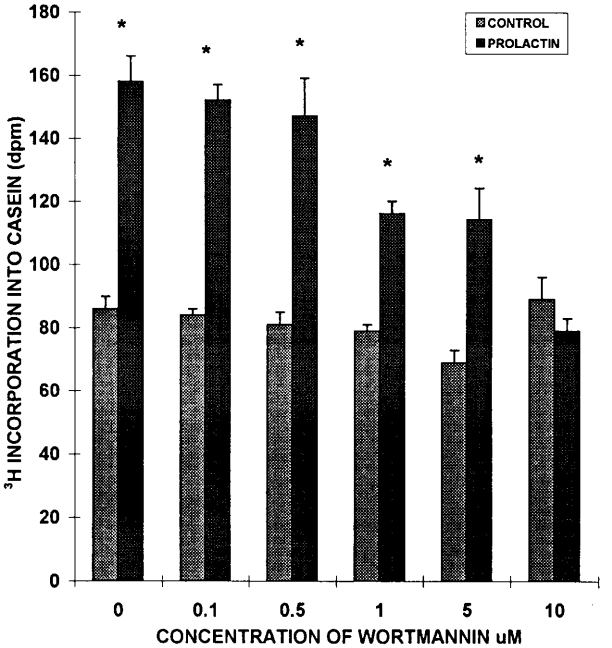

Figure 2. Wortmannin inhibition of PRL stimulated casein synthesis. Explants were incubated for 24 hr in culture media containing insulin (1 μg/ml) and cortisol (10^{-7} M). After preincubation with wortmannin for 1 hr, (1 μg/ml) PRL was added, and incubation was continued for 24 hr. Casein synthesis was determined as described in the "Materials and Methods." *Significantly greater than corresponding control with $p < .05$.

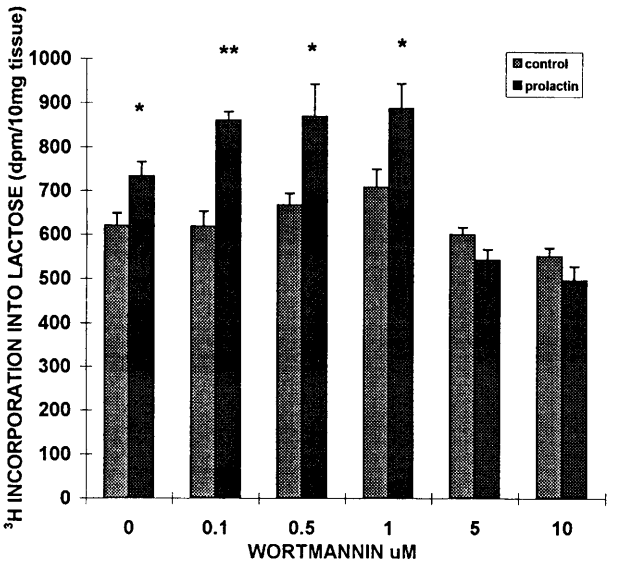

Figure 3. Wortmannin inhibition of PRL stimulated lactose synthesis. Explants were incubated for 24 hr in culture media containing insulin (1 μg/ml) and cortisol (10^{-7} M). After preincubation with wortmannin for 1 hr, 1 μg/ml PRL was added, and incubation was continued for 24 hr. Lactose synthesis was determined as described in the "Materials and Methods." *Significantly greater than relative control with $p < 0.05$, **Significantly greater than corresponding control with $p < 0.01$.

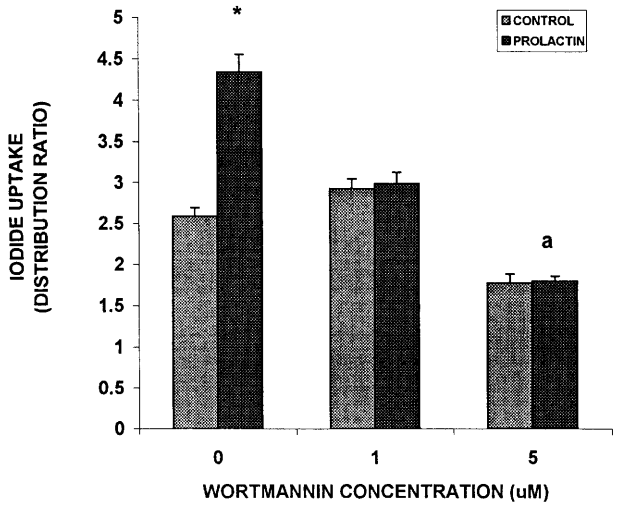

Figure 4. Effect of wortmannin on PRL stimulation of iodide uptake. Explants were cultured for 24 hr with 10^{-7} *M* cortisol plus 1 μg/ml insulin; tissues were then treated with various concentrations of wortmannin for 1 hr followed by an additional 24-hr incubation with or without 1 μg/ml PRL. ^{125}I (0.25 μCi/ml, 3 ng/ml) was present during the final 1 hr of culture. *Significantly greater than relative control with $p < 0.05$. [a]Significantly less than in the absence of wortmannin ($p < 0.05$).

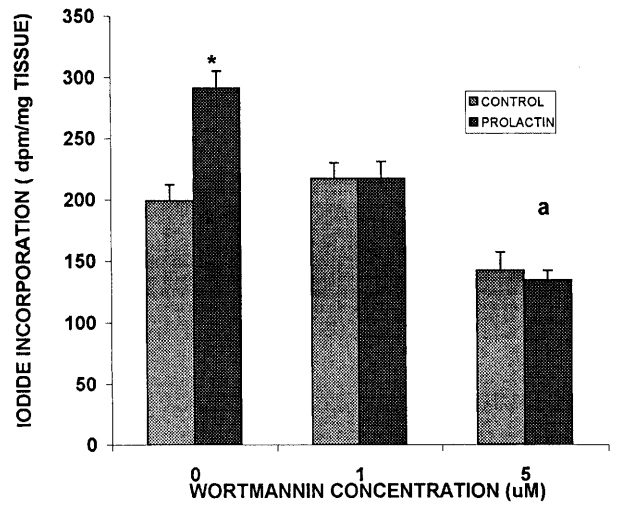

Figure 5. Effect of wortmannin on PRL stimulation of iodide incorporation. Explants were cultured for 24 hr with 10^{-7} *M* cortisol plus 1 μg/ml insulin; tissues were then treated with various concentrations of wortmannin for 1 hr followed by an additional 24-hr incubation with or without 1 μg/ml PRL. ^{125}I (0.25 μCi/ml, 3 ng/ml) was present during the final 1 hr of culture. *Significantly greater than relative control with $p < 0.05$. [a]Significantly less than in the absence of wortmannin ($p < 0.05$).

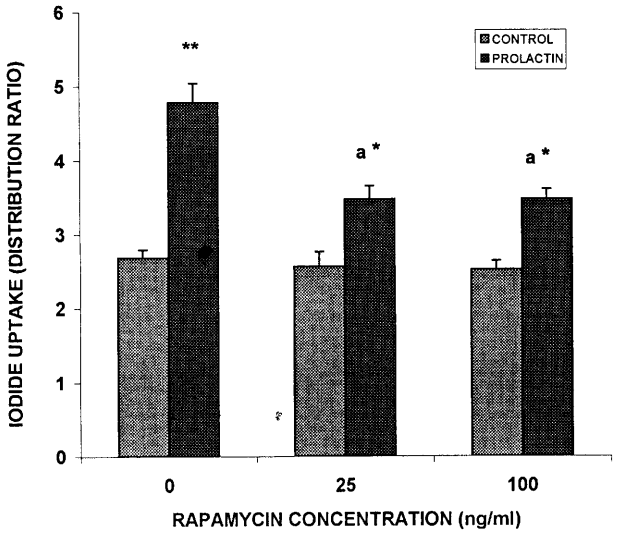

Figure 6. Effect of rapamycin on PRL stimulation of iodide uptake. Explants were cultured for 24 hr with 10^{-7} *M* cortisol plus 1 μg/ml insulin; tissues were then treated with various concentrations of rapamycin for 1 hr followed by an additional 24-hr incubation with or without 1 μg/ml PRL. ^{125}I (0.25 μCi/ml, 3 ng/ml) was present during the final 1 hr of culture. *,**Significantly greater than relative control with $p < 0.05$, $p < 0.01$ respectively. [a] Significantly less than in the absence of wortmannin ($p < 0.05$).

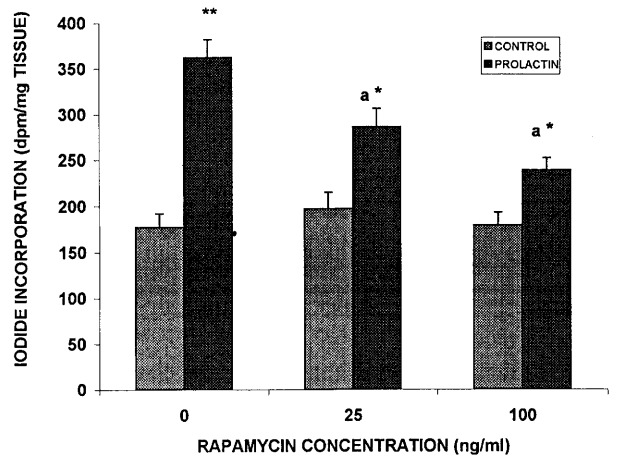

Figure 7. Effect of rapamycin on PRL stimulation of iodide incorporation. Explants were cultured for 24 hr with 10^{-7} *M* cortisol plus 1 μg/ml insulin; tissues were then treated with various concentrations of wortmannin for 1 hr followed by an additional 24-hr incubation with or without 1 μg/ml PRL. ^{125}I (0.25 μCi/ml, 3 ng/ml) was present during the final 1 hr of culture. *Significantly greater than relative control with $p < 0.05$. [a]Significantly less than in the absence of wortmannin ($p < 0.05$).

control group. In contrast, no detectable migration pattern changes were observed in Figure 9. Figure 10 shows that PRL at all concentrations tested (0.01 μg/ml–1.0 μg/ml) did not induce a detectable mobility shift of the $P70^{S6K}$ molecule, nor did it significantly alter its intracellular content. Figure 11 shows that rapamycin, a specific $P70^{S6K}$ inhibitor, also did not cause a detectable alteration in the molecular weight of $P70^{S6K}$, as it did not induce a detectable mobility shift of the molecule.

Discussion

Previous studies reported that PRL stimulates milk casein, lipids, and lactose synthesis as well as iodide transport (7, 28, 29) in cultured mammary tissues. Our data presented here and earlier (30) clearly show that wortmannin and rapamycin inhibit all of these effects of PRL in a dose-dependent manner. This is consistent with an involvement of $P70^{S6K}$ and PI3K in the PRL signaling pathway for the

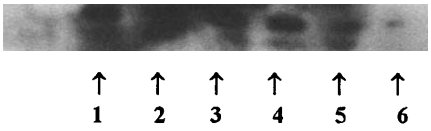

Figure 8. Dose-response wortmannin effect on PRL stimulated $p70^{S6K}$ activity. Tissues were preincubated in M199 containing insulin (1 μg/ml) and cortisol (10^{-7} *M*) for 24 hr. The tissues then were treated with or without wortmannin (WM) at the indicated concentrations (0.1 μ*M*–5.0 μ*M*) for 1 hr followed by PRL (1 μg/ml) for another 12 hr. Tissues were collected and homogenized, and total lysate supernatants were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-$p70^{S6K}$ antibody. 1-control; 2-PRL; 3-PRL + 0.1 μ*M* WM; 4-PRL + 0.5 μ*M* WM; 5-PRL + 1.0 μ*M* WM; 6-PRL + 5.0 μ*M* WM.

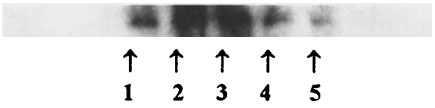

Figure 9. Concentration-response of wortmannin effect on PRL stimulated $p70^{S6K}$ activity. Tissues were preincubated in M199 containing insulin (1 μg/ml) and cortisol (10^{-7} *M*) for 24 hr. The tissues then were treated with or without wortmannin (WM) at the indicated concentrations (350–1200 n*M*) for 1 hr followed by PRL (1 μg/ml) for another 19 hr. Tissues were collected and homogenized, and total lysate supernatants were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-$p70^{S6K}$ antibody. 1-control; 2-PRL; 3-PRL + 350 n*M* WM; 4-PRL + 700 n*M* WM; 5-PRL + 1200 n*M* WM.

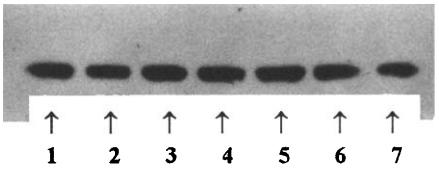

Figure 10. Concentration-response effect of PRL on $p70^{S6K}$. Tissues were preincubated in M199 containing insulin (1 μg/ml) and cortisol (10^{-7} *M*) for 24 hr. The tissues then were treated with or without PRL at the indicated concentrations (0.01–1.0 μg/ml) for 12 hr. Tissues were collected and homogenized, and total lysate supernatants were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-$p70^{S6K}$ antibody. 1-Control; 2-0.01 μg/ml PRL; 3-0.05 μg/ml PRL; 4–0.1 μg/ml PRL; 5–0.5 μg/ml PRL; 6–1.0 μg/ml PRL.

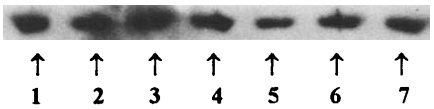

Figure 11. Concentration-response effect of rapamycin on PRL stimulated $p70^{S6K}$ activity. Tissues were preincubated in M199, containing insulin (1 μg/ml) and cortisol (10^{-7} *M*), for 24 hrs. The tissues then were treated with or without rapamycin (RAP) at the indicated concentrations (10–500 ng/ml) for 1 hr followed by PRL (1 μg/ml) for another 12 hr. Tissues were collected and homogenized, and total lysate supernatants were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-$p70^{S6K}$ antibody. 1-Control; 2-PRL; 3-PRL + 10 ng/ml RAP; 4-PRL + 50 ng/ml RAP; 5-PRL + 100 ng/ml RAP; 6-PRL + 500 ng/ml RAP; 7-Control.

stimulation of milk product formation in the mammary gland.

Wortmannin, a PI3K inhibitor, had a concentration-dependent effect on $P70^{S6K}$ expressed in a time-dependent manner. After a 12-hr incubation period, where PRL exerts a maximal stimulation of S6 kinase activity (30), wortmannin induced not only a sizeable change in the cellular content of $P70^{S6K}$ but also a differential migration pattern on polyacrylamide gels. When the incubation time was extended to 19 hr, where PRL's effect on S6 kinase activity was shown to be diminished (30), the changes in migration pattern were diminished although a similar decrease in the cellular level of $P70^{S6K}$ was retained. It is thus suggested that wortmannin may cause the dephosphorylation of $P70^{S6K}$ and accordingly may inhibit the PRL effects on milk product synthesis. Although the downstream effects of PI3K have not been elucidated clearly, PI3K activity has been correlated with an increase in $P70^{S6K}$ activity in PDGF-stimulated HepG2 cells (31), IL-2 stimulated CTLL T cells (32), and CD28-stimulated T cells (33, 34). Our studies, which suggest that dephosphorylation of $P70^{S6K}$ along with its diminished cellular content were induced by wortmannin, demonstrate a possible causal link between the activation of PI3K and $P70^{S6K}$. However, recent studies (35) on insulin-signaling have implied the existence of other wortmannin-sensitive targets such as inhibition of the catalytic subunit of DNA dependent kinase (36). As suggested from the polyacrylamide gel migration studies, wortmannin may cause dephosphorylation of $P70^{S6K}$ after a 12-hr PRL treatment; this effect abates after a 19-hr PRL treatment. The most hyperphosphorylated and hence the most activated state of $P70^{S6K}$ peaks around 12 hr of incubation with PRL whereas this stimulation effect of PRL decreases about 50% after 19 hr of PRL treatment. It might be suggested that only at its peak activation state can a wortmannin inhibitory effect be detected, or alternatively a specific configuration of the $P70^{S6K}$ molecule is required for wortmannin to exert its inhibition.

It is well established that unlike $P90^{RSK}$, which is clearly activated *via* the MAPK signaling network (6), $P70^{S6K}$ activation requires a distinct activation pathway (37). The specific target of wortmannin, PI3K, may be uniquely associated with $P70^{S6K}$ activation. There are also other enzymes that have been shown to be inhibited by wortmannin, such as DNA-dependent protein kinase (36) which is related to PI3K, but the role of this enzyme in signaling pathways is not yet well defined.

This study shows that although PRL has a stimulatory effect on $P70^{S6K}$ activity (30), but does not induce a detectable altered migration of $P70^{S6K}$ on polyacrylamide gels; this suggests that its phosphorylation state is not altered to the extent that the altered migration is detectable on gels. Rapamycin, a specific inhibitor of $P70^{S6K}$ was reported earlier to inhibit the stimulatory effect of PRL on $P70^{S6K}$ activity (30), but it also had no effect on the migration pattern of $P70^{S6K}$ in polyacrylamide gels. It is accordingly possible that the activation of $P70^{S6K}$ by PRL and inactivation by rapamycin in mouse mammary tissue may not be directly correlated to the phosphorylation/dephosphorylation of the $P70^{S6K}$ molecule. On the other hand, if the phosphorylation/dephosphorylation of $P70^{S6K}$ by PRL/rapamycin in-

volves only one amino residue, this subtle change may not be detectable in the migration pattern on gels. It is also possible that a conformational change of the $P70^{S6K}$ molecule may be responsible for our experimental findings. A similar pattern of PRL and rapamycin effects on milk product synthesis and iodide transport in mouse mammary gland is consistent with the conclusion that $P70^{S6K}$ is in the signaling pathway for the PRL stimulation of milk product synthesis and iodide transport in mouse mammary tissues.

Altogether, we suggest that $P70^{S6K}$ may be an upstream regulator of milk product synthesis and iodide transport, and at least in part, $P70^{S6K}$ activation mediates the effects of PI3K on these processes.

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