Prolactin activates mammalian target-of-rapamycin through phosphatidylinositol 3-kinase and stimulates phosphorylation of p70S6K and 4E-binding protein-1 in lymphoma cells

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Abstract

Mitogens activate the mammalian target-of-rapamycin (mTOR) pathway through phosphatidylinositol 3-kinase (PI3K). The activated mTOR kinase phosphorylates/activates ribosomal protein S6 kinase (p70S6K) and phosphorylates/inactivates eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), resulting in the initiation of translation and cell-cycle progression. The prolactin receptor signaling cascade has been implicated in crosstalk with the mTOR pathway, but whether prolactin (PRL) directly activates mTOR is not known. This study showed that PRL stimulated the phosphorylation of mTOR, p70S6K, Akt, and Jak2 kinases in a dose- and time-dependent manner in PRL-dependent rat Nb2 lymphoma cells. PRL-stimulated phosphorylation of mTOR was detected as early as 10 min, closely following the phosphorylation of Akt (upstream of mTOR), but preceding that of the downstream p70S6K. PRL activation of mTOR was inhibited by rapamycin (mTOR inhibitor), LY249002, and wortmannin (P13K inhibitors), but not by AG490 (Jak2 inhibitor), indicating that it was mediated by the P13K/Akt, but not Jak2, pathway. PRL also stimulated phosphorylation of 4E-BP1 in Nb2 cells. PRL-induced phosphorylation of p70S6K and 4E-BP1 was inhibited by rapamycin, but not by okadaic acid (inhibitor of protein phosphatase, PP2A). PRL induced a transient interaction between p70S6K and the catalytic subunit of PP2A (PP2Ac) in 1 and 2 h, whereas a PP2Ac–4E-BP1 complex was constitutively present in quiescent and PRL-treated Nb2 cells. These results suggested that p70S6K and 4E-BP1 were substrates of PP2A and the inhibition of mTOR promoted their dephosphorylation by PP2A. In summary, PRL-stimulated phosphorylation of mTOR is mediated by PI3K. PRL-activated mTOR may phosphorylate p70S6K and 4E-BP1 by restraining PP2A.

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Introduction

The mammalian target-of-rapamycin (mTOR) belongs to the family of phosphatidylinositol 3-kinase (PI3K)-related kinases (Hay & Sonenberg 2004). mTOR has intrinsic serine/threonine kinase activity and may undergo autophosphorylation *in vitro* (Brown *et al.* 1995). The mTOR kinase pathway regulates initiation of translation and entry into the cell cycle in response to growth factors and nutrients. mTOR acts by directly phosphorylating/activating S6 kinase (p70S6K) and phosphorylating, but inactivating the translational repressor and eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1). The activated p70S6K phosphorylates ribosomal protein S6 to promote translation of 5′-terminal oligopyrimidine tract mRNAs (Jefferies *et al.* 1997), thus increasing the synthesis of components of the translational machinery. Concurrently, inactivation of 4E-BP1 decreases its association with eIF4E, which then becomes available for the formation of the eIF4F complex, thus initiating translation from the 5′-terminal 7-methylGTP cap of mRNAs (Thomas & Hall 1997, Cutler *et al.* 1999, Clemens 2001, Bjornsti & Houghton 2004). mTOR is sensitive to the immunosuppressive drug rapamycin, which binds its intracellular receptor, the FK506-binding protein FKBP12. The resulting complex binds to the C-terminus of mTOR to inhibit mTOR activity (Choi *et al.* 1996).

There is evidence that mTOR indirectly controls p70S6K and 4E-BP1 phosphorylation by restraining the activity of serine/threonine protein phosphatase 2A (PP2A). mTOR was shown to phosphorylate/inactivate PP2A, but rapamycin increased the activity of PP2A towards p70S6K and 4E-BP1 in Jurkat T-leukemic cells (Peterson *et al.* 1999). These findings support a model where inhibition of PP2A, by either mTOR-induced phosphorylation or specific inhibitors, would result in phosphorylation of p70S6K and 4E-BP1. In contrast, inhibition of mTOR, by rapamycin or growth factor/nutrient deprivation, would remove the restrain mTOR has on PP2A, thereby permitting dephosphorylation of p70S6K and 4E-BP1 (Peterson *et al.* 1999, Clemens 2001).

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The activation of mTOR by growth factors is mediated by the P13K signaling pathway (Hay & Sonenberg 2004). P13K activates serine/threonine protein kinase Akt (also called protein kinase B, PKB), which then activates mTOR. The TSC1/TSC2 proteins are negative regulators of mTOR. Inhibition of mTOR by the TSC1/TSC2 complex was shown to inactivate p70S6K and activate 4E-BP1 (Inoki *et al.* 2002). Furthermore, insulin-activated Akt was shown to directly phosphorylate and inactivate TSC2, causing destabilization of TSC2 and disrupting its interaction with TSC1 (Inoki *et al.* 2002).

Our previous studies have implicated crosstalk between the mTOR pathway and the prolactin receptor (PRLR) signal-transduction cascade. We showed that a component of the mTOR pathway, the α4 phosphoprotein, was downregulated by prolactin (PRL) in the PRL-dependent rat Nb2 lymphoma cells (Boudreau *et al.* 2002). Rapamycin inhibited PRL-stimulated Nb2 cells proliferation and growth inhibition was maximal at 50%. We also showed that transient overexpression of α4 in COS-1 cells inhibited PRL stimulation of interferon regulatory factor-1 (IRF-1) promoter activity (Boudreau *et al.* 2002). IRF-1 is an immediate-early PRL-responsive gene in lymphoid cells (Yu-Lee *et al.* 1990).

PRL signals primarily through the PRLR-associated Jak2 tyrosine kinase (for reviews, see Clevenger *et al.* 1998, 2003). PRL-induced activation of Jak2 results in phosphorylation of Jak2 and the PRLR, the latter provides docking sites for the Stat family of transcription factors. Consequently, Stat dimerization, nuclear translocation, and Stat binding to γ-interferon-activated sequences result in the upregulation of PRL-responsive genes, such as IRF-1 in Nb2 cells. PRL also activates the SHC/GRB2/Ras/Raf/MAPK pathway to directly stimulate cell proliferation and modulation of Stat activity. Furthermore, PRL activation of the P13K/Akt(PKB) pathway mediates the anti-apoptotic effect of PRL on Nb2 cells (Al–Sakkaf *et al.* 2000).

The present study further examined the role of PRL in the activation of the mTOR pathway. We showed that PRL stimulated the phosphorylation of mTOR and this was mediated by P13K/Akt. Our studies also showed that rapamycin inhibited the phosphorylation of p70S6K and 4E-BP1, both of which interacted with PP2A in the Nb2 cells.

Materials and Methods

Antibodies and inhibitors

The antibodies used and their sources were as follows: polyclonal anti-mTOR, phospho-mTOR (Ser2448), Akt, phospho-Akt, Jak2, and phospho-Jak2 (Tyr1007/1008) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA); anti-p70S6K and phospho-p70S6K (Thr412) antibodies were from Upstate USA, Inc. (Charlottesville, VA, USA); and anti-4E-BP1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Zymed Laboratories, Inc. (San Francisco, CA, USA). Monoclonal anti-catalytic subunit of PP2 A (PP2Ac) was from Transduction Laboratories (Lexington, KY, USA). Polyclonal anti-Stat5b and monoclonal anti-phospho-Tyr were both from Santa Cruz Biotechnology, Inc. The horseradish peroxidase (HRP)-conjugated secondary antibodies used were donkey anti-rabbit IgG (Amersham Biosciences, Inc.) and goat anti-mouse IgG (Bio-Rad Laboratories, Inc.).

AG490 was purchased from Research Biochemicals, Inc. (Natick, MA, USA). Rapamycin, okadaic acid, and wortmannin were from Sigma-Aldrich Canada Ltd; LY294002 was from Calbiochem (San Diego, CA, USA).

Cell culture and drug treatment

Suspension cultures of PRL-dependent rat Nb2 lymphoma cells were maintained in Fischer's medium containing 10% fetal bovine serum (as lactogenic source) and 10% lactogen-free horse serum (HS). Confluent cells were made quiescent in medium containing 10% HS for 18–24 h as previously described (Too *et al.* 1987), prior to treatment with PRL. In inhibition studies, cells were incubated with AG490 (10 μM), LY249002 (1 μg/ml), wortmannin (100 nM), rapamycin (100 nM), or okadaic acid (5–10 nM for specific inhibition of PP2A) for 15 min prior to the addition of PRL. Cells were viable at the end of treatment, as assessed by the Celltiter 96 AQueous non-radioactive cell proliferation assay (Promega).

Immunoprecipitation and Western blotting

Total cell lysates from about 30 to 50×10^6 cells/treatment were prepared for immunoprecipitation in RIPA buffer containing protease inhibitors as previously described (Dodd *et al.* 2000). Immunocomplexes or cell lysates were used for SDS-PAGE (4–20% gels) and followed by western analysis. The primary antibody dilutions used were as follows: anti-mTOR, anti-phospho-mTOR, anti-Akt, anti-phospho-Akt, anti-Jak2, and anti-phospho-Jak2 (all at 1:1000); anti-p70S6K (1·5 μg/ml), anti-phospho-p70S6K (0·5 μg/ml), anti-4E-BP1 (1:1000 from Santa Cruz Biotechnology, Inc. or 3 μg/ml from Zymed Laboratories, Inc.); and monoclonal anti-PP2Ac (1:2500). HRP-conjugated secondary antibodies were as follows: donkey anti-rabbit IgG (1:2000–1:5000) and goat anti-mouse IgG (1:1250). Skim milk (10%) in Tris-buffered saline containing 0·05% Tween-20 was used for all blocking procedures. Immunoreactive signals were detected with Super Signal ULTRA Kit (Pierce Chemical Co., Rockford, IL, USA) or Immobilon Kit (Millipore Corp., Billerica, MA, USA).

Results

PRL stimulates mTOR phosphorylation through the PI3K/Akt pathway

To determine if PRL activates mTOR, quiescent Nb2 cells were treated with PRL. Western analysis of total cell lysates

showed that PRL stimulated the phosphorylation of mTOR in a dose- and time-dependent manner (Fig. 1). In the time-dependent studies, PRL (100 ng/ml) also stimulated the phosphorylation of Jak2, Akt, and p70S6K (Fig. 1B). PRL stimulation of Jak2 occurred rapidly within 10 min. The phosphorylation of mTOR was also detected in 10 min, closely following the phosphorylation of upstream Akt (10 min), but preceding that of downstream p70S6K (15 min). PRL-stimulated tyrosine phosphorylation of Jak2 has been reported as early as 30 s in rat Nb2 cells (Campbell *et al.* 1994) and of p70S6K in 30 min in murine W53 lymphoid cells (Dominguez-Caceres *et al.* 2004).

PRL-stimulated phosphorylation of mTOR was inhibited by the mTOR inhibitor rapamycin (Fig. 2A), and the P13K inhibitors (LY294002, Fig. 2A; wortmannin, Fig. 2C), but not by the Jak2 inhibitor AG490 (Fig. 2A). The efficacy of AG490 was demonstrated by its inhibition of PRL-induced tyrosine phosphorylation of Stat5b (Fig. 2B). These results indicated that PRL-stimulated phosphorylation of mTOR in Nb2 cells was mediated by the PI3K/Akt pathway, but not by Jak2.

PRL-stimulated phosphorylation of p70S6K is inhibited by rapamycin

The mTOR inhibitor rapamycin was reported to abrogate p70S6K enzyme activity in Nb2 cells (Carey & Liberti 1995), but its effect on mTOR itself has not been demonstrated in these cells. To further examine the role of mTOR in p70S6K phosphorylation, Nb2 cells were treated with PRL in the presence or absence of rapamycin. As shown in Fig. 3, PRL-stimulated phosphorylation of p70S6K was inhibited by rapamycin, indicating that this was the mechanism underlying the abrogation of p70S6K activity reported earlier (Carey & Liberti 1995).

Furthermore, p70S6K was shown to be dephosphorylated by PP2A, but mTOR restrained the action of PP2A in Jurkat-1 cells (Peterson *et al.* 1999). If p70S6K were a substrate of PP2A, the inhibition of PP2A should not abolish PRL-stimulated phosphorylation of p70S6K. To test this, Nb2 cells were treated with PRL in the presence or absence of okadaic acid (inhibitor of PP2A). As seen in Fig. 3, PRL-stimulated phosphorylation of p70S6K was not affected by okadaic acid. These results are consistent with the findings of Peterson *et al.* (1999), who showed that inhibition of mTOR lifted its restraint on PP2A, which then dephosphorylated p70S6K. However, when PP2A was inhibited, mitogen-stimulated phosphorylation of p70S6K was maintained (Peterson *et al.* 1999).

PRL-stimulated phosphorylation of 4E-BP1 is inhibited by rapamycin

The 4E-BP1 is another immediate downstream target of mTOR. PRL treatment of Nb2 cells stimulated phosphorylation (i.e. inhibition) of 4E-BP1 with the appearance of a higher molecular weight, hyperphosphorylated band in 30–90 min (Fig. 4A). The apparent increase in 4E-BP1 protein levels after PRL treatment was not consistently reproducible and was likely due to variable protein loading. Similar to the effects on p70S6K (Fig. 3), PRL-induced phosphorylation of 4E-BP1 in the Nb2 cells was inhibited by rapamycin, but not by okadaic acid (Fig. 4B). These results were again consistent with the model (Peterson *et al.* 1999) suggesting that the mitogen-activated mTOR acted to restrain PP2A activity.

It is noted that in other studies, 4E-BP1 has also been shown to appear as a single immunoreactive band in SDS-PAGE gels, with the phosphorylated form having a decreased gel mobility and/or an increased proportion in relation to the unphosphorylated protein (Seal *et al.* 2005). 4E-BP1 may also appear as a triplet of α, β, and γ bands, with the unphosphorylated α form having a faster mobility than the intermediate β and hyperphosphorylated γ forms (Lin & Lawrence 1997, Gingras *et al.* 1999). These differences are likely due to the sources of the antibodies and the percentage of gel used.

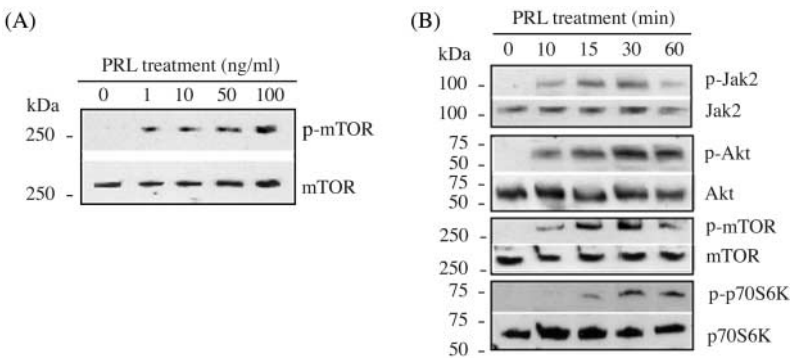

Figure 1 PRL activates Jak2, Akt, mTOR, and p70S6K. Quiescent Nb2 cells were treated with (A) increasing doses of PRL for 60 min, or (B) PRL (100 ng/ml) for various times. Total cell lysates (25 μg protein/lane) were used for SDS-PAGE and Western analysis. In (A) and (B), each sample was run in duplicate for Western blotting of the indicated protein or its phosphorylated (p) form. Representative data from three experiments are shown.

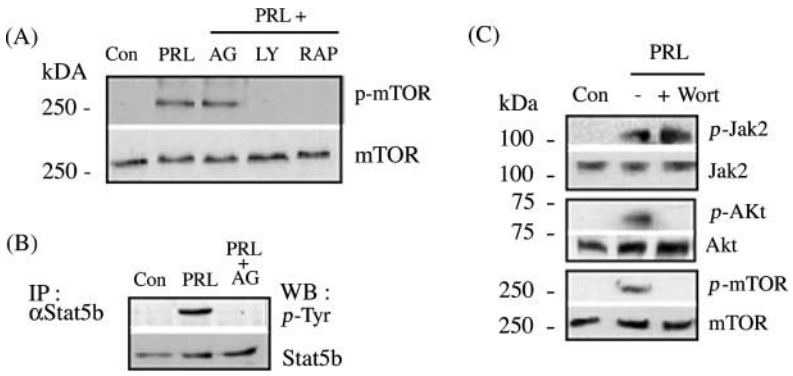

Figure 2 mTOR is inhibited by rapamycin and PI3K inhibitors. Quiescent Nb2 cells (Con) were treated with PRL (100 ng/ml) for 1 h. In inhibition studies, control cells were given rapamycin (RAP, 100 nM), AG490 (AG, 10 μM), LY294002 (LY, 1 μg/ml), or wortmannin (Wort, 100 nM) for 15 min prior to the addition of PRL. In (A) and (C), total cell lysates were used for Western blotting (WB) (25 μg/lane) as indicated. In (B), anti-Stat5b antibodies (αStat5b) were used for immunoprecipitation (IP), followed by Western blotting for phospho-Tyr (p-Tyr) and then for Stat5b. The blot was stripped between each immunoblotting. Representative data of two to three experiments are shown.

PRL stimulates interaction of PP2A with p70S6K and 4E-BP1

PP2A regulation of p70S6K and 4E-BP1 (Figs 3 and 4) suggested the possibility of a physical interaction between PP2A and its two substrates in the Nb2 cells. Co-immunoprecipitation studies showed that PRL stimulated the formation of a transient interaction between p70S6K and the catalytic subunit of PP2A (PP2Ac) in 1 and 2 h, but not later in 3–4 h. The p70S6K–PP2Ac complex was not detected in quiescent cells (Fig. 5A). The presence of p70S6K and the equal loading of proteins in each of the samples were demonstrated in Fig. 5B.

Co-immunoprecipitation studies also showed that PP2Ac interacted with 4E-BP1 (Fig. 6). The PP2Ac–p70S6K complex was transiently induced by PRL (Fig. 5), the PP2Ac–4E-BP1 complex was detected in both quiescent and PRL-treated Nb2 cells (Fig. 6).

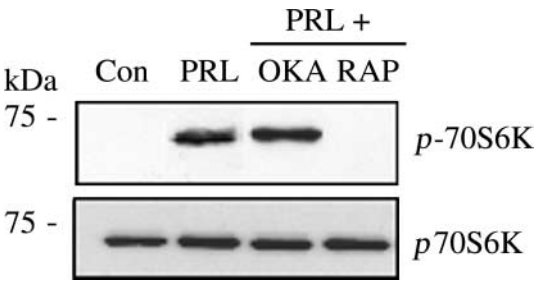

Figure 3 PRL-induced phosphorylation (p) of p70S6K is inhibited by RAP. Quiescent Nb2 cells (controls, con) were treated with PRL (100 ng/ml) for 60 min. Alternatively, quiescent cells were treated with okadaic acid (OKA, 10 nM), or rapamycin (RAP, 100 nM) for 15 min prior to the addition of PRL. Total cell lysates were used for western analysis (25 μg protein/lane). Representative data of three experiments are shown.

Discussion

An interaction between the PRLR and mTOR signaling cascades in lymphoid cell lines has been implicated in a number of studies, but a direct activation of mTOR by PRL has not been reported. The present study demonstrated for the first time that PRL stimulated the phosphorylation of mTOR in a time- and dose-dependent manner. In the rat Nb2 lymphoma cells, PRL stimulation of mTOR was inhibited by rapamycin, LY294002, and wortmannin, but not by AG490, indicating that it was mediated by the PI3K, but not the Jak2, pathway. The downstream targets of mTOR are p70S6K and 4E-BP1, and both proteins were phosphorylated by PRL treatment of Nb2 cells.

P13K is an upstream regulator of mTOR (Hay & Sonenberg 2004). Thus, in earlier studies, the inhibition of PRL-stimulated cell proliferation by P13K inhibitors and/or

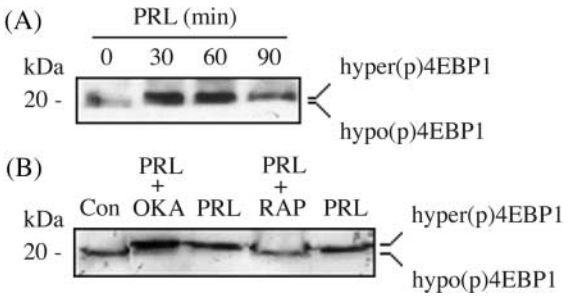

Figure 4 PRL-induced phosphorylation of 4E-BP1 is inhibited by RAP. In (A), quiescent Nb2 cells were treated with PRL (100 ng/ml) for the indicated times and total cell lysates were used for western analysis. In (B), quiescent Nb2 cells, treated with PRL ± okadaic acid (OKA) or rapamycin (RAP), were used for western analysis as described in Fig. 3. Lower and upper immunoreactive bands are hypo- and hyperphosphorylated 4E-BP1 respectively. Representative of three experiments are shown.

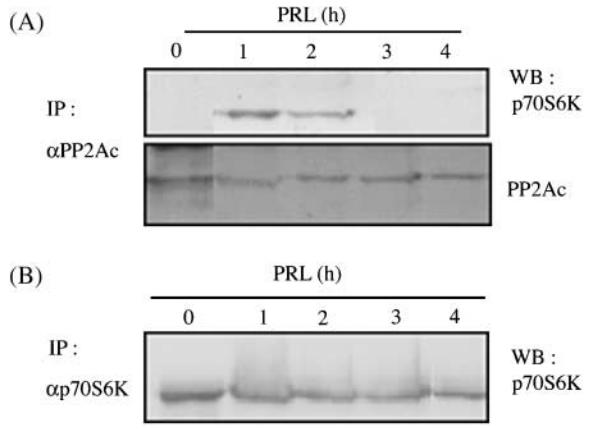

Figure 5 PRL induces transient PP2A–p70S6K interaction. Quiescent Nb2 cells were treated with PRL (100 ng/ml) for the indicated times. The total cell lysate from each treatment was equally divided for immunoprecipitation (IP) with antibodies to (A) PP2Ac, or (B) p70S6K. Western blotting was performed as indicated. Representative data of three experiments are shown.

by rapamycin pointed to a possible interaction between the PRLR and mTOR pathways. We have shown that rapamycin partially inhibited Nb2 cell proliferation, but crosstalk between the PRL and mTOR pathways was further supported by our observation that overexpressed α4 phosphoprotein, a component in the mTOR pathway, inhibited PRL-stimulated IRF-1 promoter activity in transfected COS-1 cells (Boudreau *et al.* 2002). In the murine L2 clonal T-cell line, which requires both interleukin 2 (IL2) and PRL for proliferation, rapamycin, and PI3K inhibitors (wortmannin and LY294002), markedly inhibited IL2-induced cell proliferation and nuclear translocation of PRL

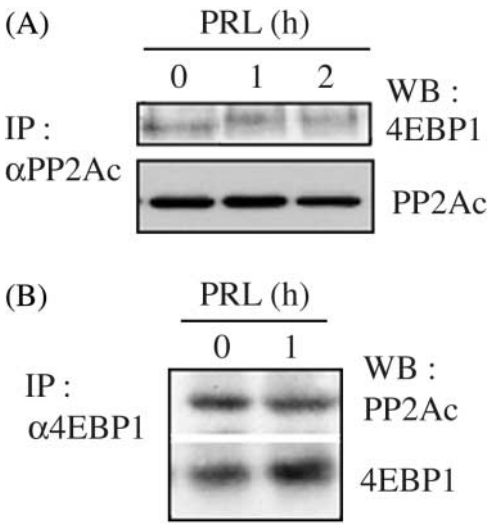

Figure 6 4E-BP1 interacts with PP2A. Quiescent Nb2 cells were treated with PRL (100 ng/ml) for 1 and 2 h or left untreated. Total cell lysates were prepared for immunoprecipitation (IP) with antibodies to (A) PP2Ac, or (B) 4EBP1, followed by Western blotting as indicated. Representative data of two experiments are shown.

(Belkowski *et al.* 1999). In murine W53 lymphoid cells, PRL activation of PI3K was necessary for c-Myc mRNA expression and cell proliferation, and both events were abolished by LY294002 (Dominguez-Caceres *et al.* 2004). Although rapamycin had little or no effect on either event, it reinforced PRL activation of an alternate pathway, i.e. through Erk1/2 to stimulate c-Fos mRNA expression and cell proliferation in these cells (Dominguez-Caceres *et al.* 2004).

The mTOR pathway probably plays a supporting role in PRLR signal transduction, acting to promote the initiation of translation to complement the signal-transduction cascades emanating from the PRLR to stimulate cell proliferation. We have observed that rapamycin alone did not completely abolish, but only partially inhibited the PRL stimulation of Nb2 cell proliferation by 50% (Boudreau *et al.* 2002). PRL has also been reported to stimulate the activity of MAPK before that of S6K (i.e. p70S6K) in Nb2 cells and rapamycin was shown to block completely the enzyme activity of S6K, but not MAPK (Carey & Liberti 1995). Although rapamycin had no effect on MAPK or PRLR-associated tyrosine kinases, it delayed the effects of these kinases on DNA and protein synthesis and cell proliferation (anabolism), leading to the conclusion that these kinase cascades regulated cell anabolism, whereas p70S6K only played an ancillary role (Carey & Liberti 1995). Bifurcation of the PRLR-signaling cascades, one of which involves the P13K/Akt pathway, has also been reported in other PRL-responsive cell lines. The PRL-stimulated c-Src/PI3K and c-Src/Fak/Erk1/2 pathways were shown to control cell proliferation and/or the expression of c-Myc and cyclin D1 in T47D and MCF7 breast cancer cells (Acosta *et al.* 2003). In these cells, LY294002 abolished cell proliferation and activation of Akt and p70S6K, but it had no effect on PRL-dependent activation of Erk1/2 (Acosta *et al.* 2003).

Hormone-inducible phosphorylation (inactivation) of 4E-BP1 has previously been reported in mammary cells. PRL and insulin acted synergistically to rapidly induce phosphorylation of PHAS-1 (i.e. 4E-BP1) within minutes, leading to PHAS-1 dissociation from the eIF-4E translation initiation factor in differentiating mammary epithelial CID-9 cells (Barash 1999). The rapid induction of PHAS-I phosphorylation matched the rate for JAK2/STAT5a activation and STAT5a–DNA binding, but complete phosphorylation of the PHAS-I protein occurred later at ~120 min. Both wortmannin and rapamycin abrogated PHAS-I phosphorylation. These inhibitors caused a reciprocal shift between the fully phosphorylated PHAS-I γ form and its non-phosphorylated α form, but the partly phosphorylated PHAS-I β form was not significantly affected, suggesting that more than one kinase may mediate the synergistic effect of PRL and insulin on PHAS-I phosphorylation (Barash 1999).

The mTOR protein was shown to control phosphorylation of 4E-BP1 and p70S6K by indirectly restraining PP2A activity in Jurkat-1 cells (Peterson *et al.* 1999). In this model, PP2A might be predicted to interact physically with its substrates and, indeed, PP2A formed complexes with p70S6K or 4E-BP1 (Peterson *et al.* 1999, Clemens 2001). Consistent with this model, in the present

study using Nb2 cells, the inhibition of mTOR abrogated PRL-stimulated phosphorylation of p70S6K and 4E-BP1, whereas the inhibition of PP2A promoted their phosphorylation. The present study also showed that PP2A interacted transiently with p70S6K, but constitutively with 4E-BP1 in the Nb2 cells.

In summary, PRL stimulated the phosphorylation of mTOR through the PI3K, but not Jak2, pathway. PRL also stimulated the phosphorylation of p70S6K and 4E-BP1, both of which interacted with PP2A. PRL-stimulated phosphorylation of p70S6K and 4E-BP1 was inhibited by rapamycin, but not okadaic acid, suggesting that mTOR acted to restrain PP2A activity in the Nb2 cells.

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