Differential Tyrosyl-Phosphorylation of Multiple Mitogen-activated Protein Kinase Isoforms in Response to Prolactin in Nb2 Lymphoma Cells (44129)

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Abstract. **Prolactin (PRL) stimulates mitogenesis and differentiative processes in a variety of cell types. Not all of the molecules involved in PRL signaling, which follows an initial PRL-receptor interaction, have been identified. In the present studies, PRL is shown to stimulate the differential tyrosyl phosphorylation of three isoforms (ERK-1, 2, and 4) of mitogen-activated protein kinases (MAP kinase) in a rat pre-T lymphoma cell line (Nb2). Evidence also suggests that PRL stimulates the tyrosyl phosphorylation of ERK-3, a MAP kinase isoform recently identified. When G1-arrested Nb2 cells are treated with 50 ng/ml oPRL, ERK-1 through 3 become tyrosyl phosphorylated within minutes (an indication of enzyme activation) and then become dephosphorylated within 30 min. Conversely, ERK-4 is rapidly tyrosyl phosphorylated by 5 min, and remains in this state for at least 1 hr.** [P.S.E.B.M. 1997, Vol 215]

Prolactin (PRL) is a peptide hormone whose wide range of functions includes regulation of differentiative and developmental processes in the mammary gland and certain male accessory sex glands, and regulation of specific immune responses (1). PRL induces its effects on cells by binding to its receptor, which in turn is thought to activate a cascade of intracellular signaling events. The PRL receptor (PRLR) has three forms (long, intermediate, and short), which differ primarily in the length of their cytoplasmic domains (2). These PRLR forms belong to a large family of growth factor receptors collectively known as cytokine receptors (3). Lack of intrinsic kinase activity is characteristic of cytokine receptors. This family also includes receptors for interleukin-2 and granulocyte-macrophage colony–stimulating factor, among other (4, 5).

The Nb2 cells are the only PRL target cells known to express the intermediate form of the PRLR (6). PRL is the sole growth factor required for continuous progression of Nb2 cells through the cell cycle (7). Thus, the Nb2 cell line appears to be an ideal model for studying mitogenic signaling by PRL. Recently, many mitogenic signaling molecules, common in other growth factor signaling systems, have been identified in the Nb2 cells. Kinases identified that are likely involved in PRL signaling include JAK2 (8–10), RAF-1 (11), protein kinase C (PKC, 12), and the src tyrosine kinase p59 fyn (13). More recently, other kinases were identified that are activated during Nb2 cell proliferation; these include $p44^{mapk}$ (14) and $p70^{s6k}$ (15). Further studies indicate that nonkinase mediators, including SHC and GRB (15), may also be involved in PRL signaling. Currently, there is also information suggesting the involvement of guanine nucleotide exchange factor (GEF), SOS (15), and vav-associated GEF (16) in PRL signaling in Nb2 cells.

Information in the literature suggests the involvement of the MAP kinases in a tandem sequence in the PRL mitogenic signaling pathway. The PRL signaling sequence in Nb2 cells appears to involve an initial rapid tyrosyl phosphorylation catalyzed by JAK2 and fyn, which involves recruitment of linking proteins to induce GEF activation. In turn, GEF activation of RAF-1 may indirectly cause MAP kinase activation, possibly involving a MEK (MAP kinase kinase) intermediate (17, 18). In the present study, we demonstrate the asynchronous tyrosine phosphorylation of three MAP kinase isoforms in response to PRL in Nb2 cells. The

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differing sequential phosphorylation patterns may indicate involvement of multiple MAP kinase forms in specific aspects of PRL signal transduction on proliferative processes, and may suggest a more complex signaling pathway for PRL then was earlier proposed.

Materials and Methods

Nb2 Cell Culture. A PRL-dependent Nb2 cell line was provided by Dr. C. T. Beer of the Cancer Control Agency of British Columbia (Vancouver, British Columbia, Canada). Cells were grown in Fischer's medium supplemented with 10% fetal calf serum, 10% horse serum (Sigma Chemical Co., St. Louis, MO), 1.0×10^{-4} *M* 2-mercaptoethanol, 100 IUL penicillin, and 100 μg/ml streptomycin (Eli Lilly Co., Indianapolis, IN) as previously described (7). Incubation for 24 hr in serum-free media effectively arrests growth at the G0/G1 phase of the cell cycle (19). Once serum depletion is complete, cells are harvested and treated with 50 ng/ml oPRL (NIH-P-S-17), a gift from the NIAMDD.

Immunoprecipitation and Immunoblotting. After PRL treatment, cells (1×10^6/ml) were washed through 4× volume of PBS-orthovanadate (1 m*M*) at 4°C by centrifugation (200 *g*, 3 min) and resuspended in lysis buffer (2×10^7 cells/ml) containing 2% NP40, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After 30 min on a rocking platform the lysates were centrifuged (12,000 *g*, 30 min, 4°C). The cleared lysate (1 ml), containing 3.7 mg protein/ml, was immunoprecipitated (overnite at 2°C) with either agarose-conjugated anti-phosphotyrosine (UBI #16-101; 25 μg monoclonal Ab/ml lysate), or anti-rat MAP kinase (UBI 06-182; 1 μg/ml) followed by addition of 50 μl protein A sepharose beads (Pharmacia 17-0974-04). Proteins were solubilized in SDS-laemmli sample buffer, separated by SDS-PAGE (8%–20% linear gradient) under reducing conditions and transferred to PVDF membranes (Schleicher & Schuell). Membranes were probed with HRP-conjugated anti-phosphotyrosine (UBI 16-105; 20 mls at 1 μg/ml) for 3 hr or anti-MAP kinase (UBI 06-182; 20 mls at 1 μg/ml) for 2 hr followed by treatment with anti-rat Ig-G HRP conjugate (Amersham NA934; 25 ml at 1:10,000 dilution for 30 min). Detection was accomplished by incubation with enhanced chemiluminescence reagents (Amersham) and exposure to x-ray film.

Results

In G1-arrested Nb2 cells the temporal state of tyrosyl phosphorylation of three and perhaps four MAP kinase isoforms in response to PRL was determined. The time course of MAP kinase phosphorylation induced by 50 ng/ml oPRL is shown in Figure 1. This blot, a representation of three separate experiments, contains proteins from total lysate preparations, anti-MAP kinase immunoprecipitates, and anti-phosphotyrosine immunoprecipitates. In Figure 1, the blot was probed with an anti-phosphotyrosine antibody. PRL clearly increases the tyrosyl phosphorylation of a

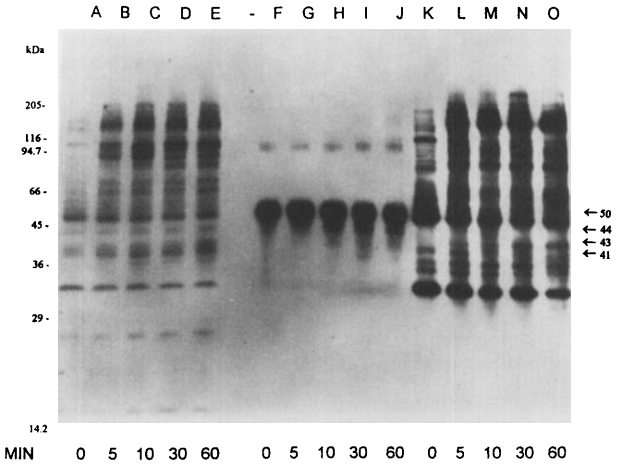

Figure 1. Time course of PRL-induced tyrosine phosphorylation of MAP kinase in Nb2 cells. G1-arrested Nb2 cells (1×10^7) were incubated in the absence or presence of oPRL (50 ng/ml) for indicated times (min). Total cell lysate preparations (TCL, Lanes A–E) were immunoprecipitated with either anti-MAP kinase R_2 (Lanes F–J) or anti-phosphotyrosine (Lanes K–O). The blot containing the three sets of SDS-PAGE separated samples was probed with anti-phosphotyrosine antibody. Molecular weight standards are indicated at left, and MAP kinase molecular weights are shown at the right.

substantial number of proteins, including Janus 2 kinase, beginning at 5 min (Lanes B–E and L–O) as was first observed in earlier studies from our laboratory and that of Carter-Su (20). Included among the tyrosyl-phosphorylated proteins are the MAP kinases which have M_r of 41–50 kDa. Although MAP kinase bands are apparent in the anti-MAP kinase precipitates (Lanes F–J), the FAB component (M_r 55 kDa) of the antibody preparation also interacts with the αPY antibody and the blots overlap with the MAP kinases.

Figure 2 displays the identical blot as in Figure 1 probed with an anti-MAP kinase antibody. In Figure 2,

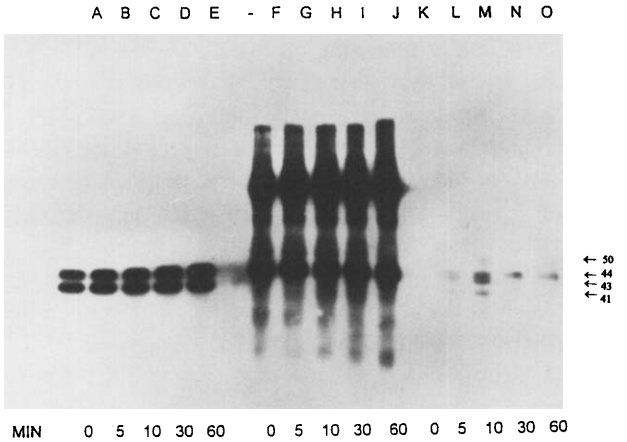

Figure 2. Time course of multiple MAP kinase isoform tyrosine phosphorylation in response to PRL. The same blot used in Figure 1 was stripped and reprobed with MAP kinase R_2 antisera. Identical samples (Lanes A–E, TCL; Lanes F–J, anti-MAP kinase immunoprecipitates; Lanes K–O, anti-phosphotyrosine immunoprecipitates) reveal PRL induced tyrosine phosphorylation of 41-, 43-, 44-, and 50-kDa MAP kinases (ERK-1, 2, 4, and 3) as indicated by arrows at right.

where the anti-phosphotyrosine immunoprecipitates were probed with anti-MAP kinase antisera, three distinct tyrosyl phosphorylated bands are apparent which migrate at 41, 43, and 44 kDa (Lanes K–O). These bands are consistent with the molecular weights of ERK-1, ERK-2, and ERK-4, respectively (21). ERK-2 and 1 were phosphorylated rapidly within 5 min, maximally at 10 min, and were completely dephosphorylated by 30 min. These temporal changes in tyrosine phosphorylation correlate directly with previously measured changes in MAP kinase activity in time course studies (22, 23). Conversely, ERK-4 remains tyrosine phosphorylated through the entire 1-h time course. Also of interest, a protein migrating at approximately 50 kDa is identified when anti-phosphotyrosine immunoprecipitates are probed with anti-MAP. This tyrosine phosphorylated protein falls well within the predicted MW range of ERK-3 (21). Furthermore, this protein has a temporal tyrosine phosphorylation pattern similar to ERK-1 and 2. The anti-MAP kinase immunoprecipitates (Lanes F–J) exhibited a high degree of nonspecific binding, probably due to the use of the same monoclonal antibody preparation for blotting and immunoprecipitation. In addition, the FAB component of the antibody preparation interacts with the anti-MAP kinase antibody and, as in Figure 1, overlaps with the MAP kinases. The results from Lanes F–J are therefore not quantifiable.

As shown in Figure 2, consistent amounts of MAP kinase protein were observed in total tissue lysates probed with anti-MAP antibody. The absence of detectable ERK-3 in the lysates is likely due to its relatively limited abundance, and it only becomes detectable after concentration through immunoprecipitation (Fig. 2). When total ERK immunoprecipitates are compared with tyrosine phosphorylated immunoprecipitates, it is clear that only a fraction of MAP kinase proteins are phosphorylated on tyrosyl residues. This is consistent with studies done by Boulton *et al.* (21, 24), who showed that ERKs may also be phosphorylated on serine or threonine residues. Even though tyrosine phosphorylation may be quantitatively limited, it may still be of vital importance for MAP kinase activation (15).

Figures 3–6 show changes in the tyrosyl phosphorylation status of the MAP kinase isoforms as quantitated using a scanning laser densitometer. Intensity of phosphorylation levels are represented in arbitrary units (a.u.). The data represent the mean of three experiments ± standard error of the mean. Figure 3 displays a 7-fold increase in tyrosyl phosphorylation of ERK-3 (50 kDa), which is maximal in 10 min and returns to basal levels within 30 min of PRL exposure. A 14-fold peak increase in phosphorylation of ERK-4 (44 kDa) occurs at 10 min (Fig. 4). It is noteworthy to mention that ERK-4 remains tyrosyl phosphorylated at a level seven times higher than basal through 60 min of PRL treatment. Figure 5 shows a 9-fold maximum increased phosphorylation of ERK-2 (43 kDa) at 10 min. Interestingly, a significant decrease belwo the basal tyrosyl phosphorylation level occurs at 60 min. Finally, an 8-fold increase in phosphorylation of ERK-1 (41 kDa) is evident after a 10-min PRL treatment. Initial tyrosyl phosphorylation levels of ERK-1 are reestablished within 30 min.

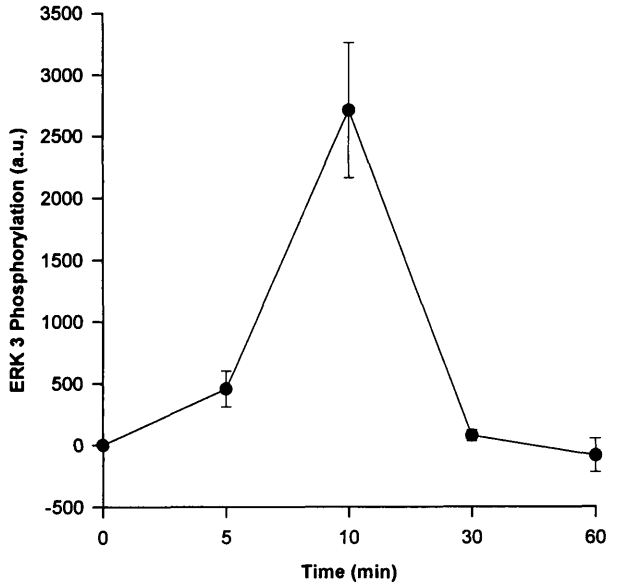

Figure 3. Time course of ERK-3 tyrosyl phosphorylation. G1-arrested Nb2 were incubated in the absence or presence of 50 ng/ml oPRL for the indiated times prior to immunoprecipitation with anti-MAP kinase R_2. Quantitation of the autoradiographs were performed using a scanning laser densitometer. Fluctuations in tyrosyl phosphorylation levels are presented in arbitrary units (a.u.).

DISCUSSION

Several aspects of the PRL regulation of the MAP kinases in Nb2 cells have been uncovered. A proposed sig-

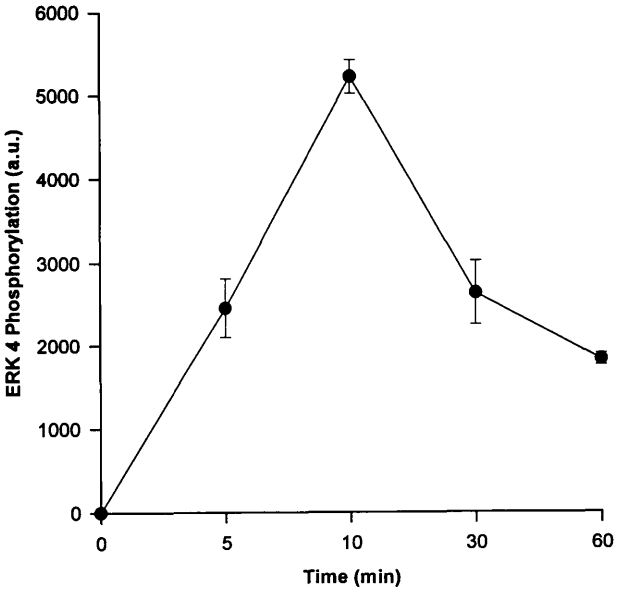

Figure 4. Time course of ERK-4 tyrosyl phosphorylation. G1 arrested Nb2 cells were incubated in the absence or presence of 50 ng/ml oPRL for the indicated times prior to immunoprecipitation with anti-MAP kinase R_2. Quantitation of the autoradiographs were performed using a scanning laser densitometer. Fluctuations in tyrosyl phosphorylation levels are presented in arbitrary units (a.u.).

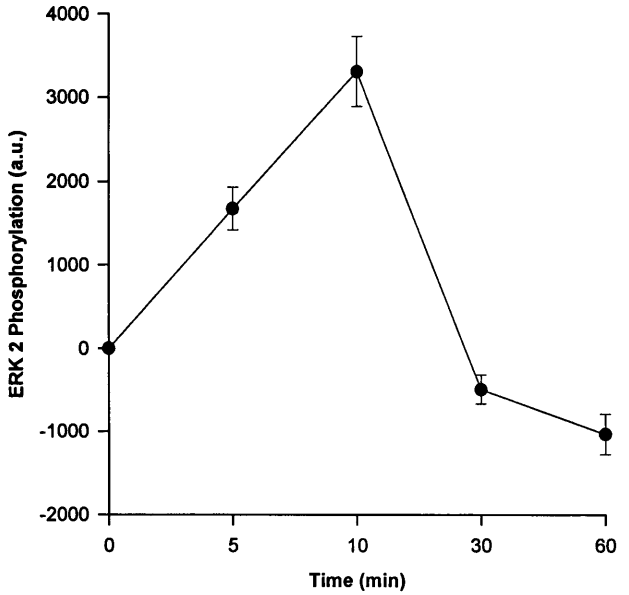

Figure 5. Time course of ERK-2 tyrosyl phosphorylation. G1-arrested Nb2 cells were incubated in the absence or presence of 50 ng/ml oPRL for the indicated times prior to immunoprecipitation with anti-MAP kinase R_2. Quantitation of the autoradiographs were performed using a scanning laser densitometer. Fluctuations in tyrosyl phosphorylation levels are presented in arbitrary units (a.u.).

naling sequence for PRL actions in Nb2 cells is as follows: PRL initially binds to its receptor and activates rapid tyrosyl phosphorylation through JAK2 (8–10) and fyn (13). Once this occurs, recruitment of linking proteins (SHC and GRB) (15) activates a GEF, which may be SOS (15) or vav associated (16). The GEF then causes activation of RAF-1 (11) through direct RAS interaction (25, 26), which then activates MEK (a tyrosine/threonine kinase) (27). This in turn activates MAP kinase by tyrosyl phosphorylation (14, 15). Finally, MAP kinase may play a role in activating the S6 kinases (15) either to regulate translation, or to translocate into the nucleus (14) to regulate transcriptional processes.

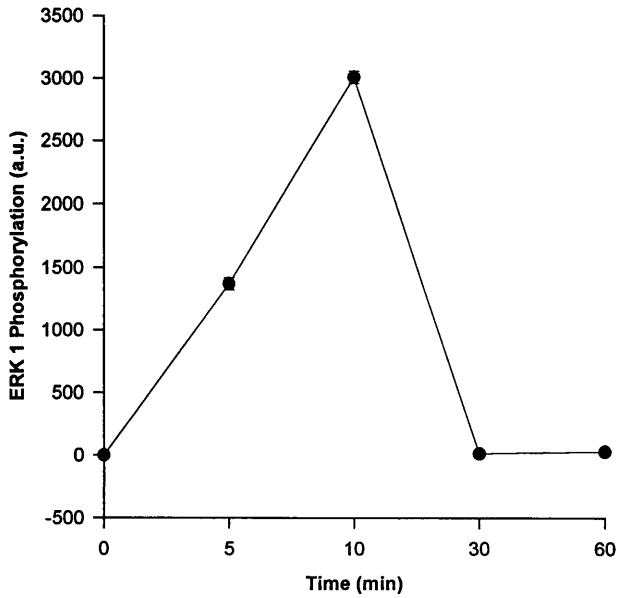

Figure 6. Time course of ERK-1 tyrosyl phosphorylation. G1-arrested Nb2 cells were incubated in the absence or presence of 50 ng/ml oPRL for the indicated times prior to immunoprecipitation with anti-MAP kinase R_2. Quantitation of the autoradiographs were performed using a scanning laser densitometer. Fluctuations in tyrosyl phosphorylation levels are represented in arbitrary units (a.u.).

Earlier work by Boulton *et al.* (21) reported that ERKs are tissue, development, and hormone specific. Furthermore, ERK-1, 2, and 4 are phosphorylated in response to growth factors, and increased MAP kinase activity coincides with increases in tyrosine phosphorylation of ERK-1 and 2. Subsequently, several laboratories have measured MAP kinase activity with specific studies focusing on ERK-1 and 2 (22, 28, 29). There have been no previous studies focusing on the possible direct involvement of ERK-3 and 4 in growth factor signaling.

In previous studies with Nb2 cells, PRL stimulation of ERK-1 tyrosine phosphorylation was reported (14). PRL stimulation of MAP kinase activity has also been reported, but has been attributed primarily to ERK-1 activation (23). It is evident from the results of our studies, however, that at least three ERK isoforms are present in Nb2 cells, and PRL regulates the tyrosyl phosphorylation of each of these with differing temporal patterns. Differences in our result from those published earlier may be explained by enhanced peptide resolution on SDS-PAGE 8%–20% linear gradient gels (employed in our studies) as well as the recognition specificity of the antibodies employed.

It is noteworthy that kinase active ERK-1 and 2 are transiently tyrosine phosphorylated, while ERK-4 remains tyrosine phosphorylated for more than 1 hr following PRL stimulation. A functional explanation for this difference could be that ERK-4 may represent a kinase inactive MAP kinase still capable of interacting with other molecules to become tyrosyl phosphorylated. Furthermore, the lack of enzyme activity may be associated with its lack of dephosphorylation. In contrast, ERK-1 and 2 are known kinase-active MAPK isoforms which are turned on and off by rapid tyrosyl phosphorylation and dephosphorylation. A similar situation maintains with MEK-3, the kinase-inactive form of the MEK family (30). MEK-3 is able to interact with, but not phosphorylate or activate, its substrate (MAPK). Also, MEK-3 does become phosphorylated, although it does not autophosphorylate; hence, as suggested with MEK-3, ERK-4 may play a regulatory function in PRL signaling, where it competes for upstream activators or downstream substrates (30). Our results also show for the first time that PRl stimulates the rapid tyrosine phosphorylation of what is likely ERK-3. The transient phosphorylation of ERK-3, like that of ERK-1 and 2, suggests it may also be an active form of MAP kinase.

The involvement of multiple isoforms of signaling molecules in hormone transduction pathways suggest that the signaling pathways are far more complex than was originally proposed. Clearly, future studies need to focus on

distinguishing roles for specific mediator isoforms of MAP kinases in specific hormone signaling pathways.

1. Horseman ND. Famine to feast-growth hormone and prolactin signal transducers. (editorial) Endocrinology **135:**1289–1291, 1994.
2. Kelly PA, Boutin J, Jolicoeur C, Okamura H, Gagnon J, Edery M, Sirota M, Banville D, Dusanter-Fourt I, Djiane J. Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell **53:**69–77, 1988.
3. Chao TO, Tonks NK, Rapp UR, Reed JC. Interleukin-2 regulates Raf-1 kinase activity through a tyrosine phosphorylation-dependent mechanism in a T-cell line. Proc Natl Acad Sci U S A **90:**5544–5548, 1993.
4. Morrison DK, Kaplan DR, Escobedo IA, Rapp UR, Roberts TM, Williams LT. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF B-receptor. Cell **58:**649–657, 1989.
5. Isumi T, Tamemoto H, Nagao M, Kadowaki T, Takaku F, Kasuga M. Insulin and platelet-derived growth factors stimulate phosphorylation of the c-RAF product at serine and threonine residues in intact cells. J Biol Chem **266:**7933–7939, 1991.
6. Ali S, Pellegrini I, Kelly PA. A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. J Biol Chem **266:**20110–20117, 1991.
7. Gout PW, Noble RL, Beer CT. Cultured Nb rat lymphoma cells in endocrine and cancer research. Biochem Cell Biol **64:**659–666, 1985.
8. Rui H, Kirken RA, Farrar WL. Activation of receptor-associated tyrosine kinase JAK2 by prolactin. J Biol Chem **269:**5364–5367, 1994.
9. Campbell GS, Argetsinger LS, Ihle JN, Kelly PA, Rillema JA, Carter-Su C. Activation of JAK tyrosine kinase by prolactin receptors in Nb2 cells and mouse mammary gland explants. Proc Nat Acad Sci U S A **91:**5232–5236, 1994.
10. Lebrun JJ, Ali S, Sofer L, Ullrich A, Kelly PA. Prolactin-induced proliferation of Nb2 cells incolces tyrosine phosphorylation of the prolactin receptor and its associated tyrosine kinase JAK2, J Biol Chem **269:**14021–14026, 1994.
11. Clevenger CV, Torigoe T, Reed JC. Prolactin induces rapid phosphorylation and activation of prolactin receptor-associated Raf-1 kinase in a T-cell line. J Biol Chem **269:**5559–5565, 1994.
12. Buckley AR, Montgomery DW, Kibler R, Putnam CW, Zukoski CF, Gout PW, Beer CT, Russell DH. Immunopharmacology **12:**37–51, 1986.
13. Clevenger CV, Medaglia MV. The protein tyrosine kinase p59fyn is associated with prolactin (PRL) receptor and is activated by PRL stimulation of T-lymphocytes. Mol Endocrinol **8:**674–681, 1994.
14. Buckley AR, Rao Y, Buckley DJ, Gout PW. Prolactin-induced phosphorylation and nuclear translocation of MAP kinase in Nb2 lymphoma cells. Biochem Biophys Res Commun **204:**1158–1164, 1994.
15. Erwin RA, Kirken RA, Malabarba MG, Farrar WL, Rui H. Prolactin activates RAS via signaling proteins Shc, growth factor receptor bound 2, and Son of Seven less. Endocrinology **136:**3512–3518, 1995.
16. Clevenger CV, Ngo W, Sokol DL, Luger SM, Gerwirtz AM. VAV is necessary for prolactin-stimulated proliferation and is translocated into the nucleus of a T-cell line. J Biol Chem **27:**13246–13253, 1995.
17. Zheng CF, Guan KL. Cloning and characterization of two distinct human extra-cellular signal-regulated kinase activator kinases, MEK1 and MEK2. J Biol Chem **268:**11435–11439, 1993.
18. Zheng CF, Guan KL. Activities of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. EMBO J **13:**1123–1131, 1994.
19. Rillema JA, Tarrant TM, Linebaugh BE. Studies on the mechanism by which prolactin regulates protein, RNA, and DNA synthesis in Nb2 node lymphoma cells. Biochim Biophys Acta **1014:**78–82, 1989.
20. Rillema JA, Campbell GS, Lawson DM, Carter-Su C. Evidence for a rapid stimulation of tyrosine kinase activity by prolactin in Nb2 rat lymphoma cells. Endocrinology **131:**973–975, 1992.
21. Boulton T, Nye S, Robbins D, Ip N, Radziejewska E, Morgenbesser S, DePinho R, Panayotatos N, Cobb M, Yancopoulos G. ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell **65:**663–675, 1991.
22. Anderson GN. Growth hormone activates mitogen-activated protein kinase and S6 kinase and promotes intracellular tyrosine phosphorylation in 3T3-F442A preadipocytes. Biochem J **284:**649–652, 1992.
23. Rao Y, Buckley DJ, Buckley AR. Rapid activation of mitogen-activated protein kinase and $p21^{ras}$ by prolactin and interleukin 2 in rat Nb2 node lymphoma cells. Cell Growth Differ **6:**1235–1244, 1995.
24. Boulton T, Gregory J, Cobb M. Purigication and properties of extracellular signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. Biochem **30:**278–286, 1991.
25. Zhang X, Settleman J, Kuriais J, Takekuchi-Suzuki E, Elledge S, Marshall M, Bruder J, Rapp UR, Acruch J. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature **364:**308–313, 1993.
26. Ishikawa F, Sakai R, Ochiai M, Takaku F, Sigimura T, Nagao M. Identification of a transformation activity suppressing sequence in the c-Raf oncogene. Oncogene **3:**653–658, 1988.
27. Force T, Bonventre J, Heidecker G, Rapp UR, Avruch J, Kuriakis J. Enzymatic characteristics of the c-Raf-2 protein kinase. Proc Natl Acad Sci U S A **91:**1270–1274, 1994.
28. Piccoletti R, Maroni P, Bendinelli P, Bernelli-Zazzera A. Rapid stimulation of mitogen-activated protein kinase of rat liver by prolactin. Biochem J **303:**429–433, 1994.
29. Pages G, Lenormand P, L'Allemain G, Chambard J, Meloche S, Pouyssegur J. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci U S A **90:**8319–8323, 1993.
30. Zheng C, Guan K. Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. J Biol Chem **268:**23933–23939, 1993.