Rapid Activation of Mitogen-activated Protein Kinase and p21ras by Prolactin and Interleukin 2 in Rat Nb2 Node Lymphoma Cells

Yi-Ping Rao, Donna J. Buckley, and Arthur R. Buckley

Department of Pharmacology and Toxicology, University of North Dakota School of Medicine, Grand Forks, North Dakota 58202

Abstract

Several serine/threonine and tyrosine kinase signal transduction pathways have been recently linked to prolactin (PRL) action in lymphoid cells. Utilizing the lactogen-dependent, rat pre-T lymphoma cell line, Nb2-11, and the autonomous subline, Nb2-SFJCD1, studies were conducted to determine whether PRL- or interleukin-2 (IL-2)-stimulated Nb2 cell proliferation is coupled to the activation of p21ras and mitogen-activated protein (MAP) kinase. Stimulation of Nb2-11 cells, growth-arrested in the early G1 phase of the cell cycle, with PRL or IL-2 rapidly (5–10 min) provoked GTP binding to Ras, enhanced tyrosyl phosphorylation of MAP kinase, significantly increased its enzymatic activity, and caused its nuclear translocation. The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), which directly activates protein kinase C, similarly activated Ras and MAP kinase but failed to cause its nuclear translocation. Tyrosine kinase antagonism with genistein inhibited PRL-stimulated Ras and MAP kinase activation. In other experiments, Ras and MAP kinase were each found to be constitutively active in the Nb2-SFJCD1 line. The addition of PRL to these cultures enhanced the activity of these signaling proteins. Finally, the effects of PRL, IL-2, TPA, and phosphatase inhibition on Nb2-11 cell population density and [3H]thymidine uptake were compared. The addition of PRL, IL-2, and TPA significantly stimulated [3H]thymidine incorporation, while only the polyepitope growth factors augmented cell density. Phosphatase inhibition had no effect on either parameter. These results indicate that Nb2 cell proliferation is associated with the early activation of Ras and MAP kinase. Moreover, tyrosyl phosphorylation upstream of Ras activation appears to be required for its subsequent stimulation of mediators, which activate MAP kinase. Protein kinase C activation may be coupled to MAP kinase activation but is not sufficient for Nb2 cell proliferation.

Introduction

PRL regulates a diverse array of physiological functions including maintenance of immunocompetence (reviewed in Ref. 1). In the rat pre-T lymphoma cell line, Nb2, PRL induces the expression of a variety of growth-related genes (2, 3) and promotes mitogenesis (4). It is generally accepted that this and other actions of PRL are mediated by the initial interaction of the hormone with its cell surface receptor. Based upon the deduced amino acid sequence of its extracellular domain, the PRL receptor has been found to be a member of the cytokine/hormone receptor superfamily, which includes receptors for interleukins 2–7, 12α, erythropoietin, granulocyte/macrophage-colony stimulating factor, granulocyte-colony-stimulating factor, and growth hormone (5, 6). The receptors in this superfamily are characterized by four conserved extracellular cysteine residues and a Trp-Ser-X-Trp-Ser motif. Although lacking an intracellular tyrosine kinase domain, receptor activation induces rapid tyrosyl phosphorylation of multiple proteins (7, 8).

Using Nb2 cell lines, which express a truncated variant of the long form of the PRL receptor (9), several laboratories have implicated tyrosine and serine/threonine phosphorylation pathways as signaling mechanisms coupled to PRL receptor stimulation. This includes the tyrosine kinases, Jak2 (10, 11) and p59Fyn (FY; Ref. 12), as well as the serine/threonine-specific kinases, PKC (13), Raf-1 (14), and MAP kinase (15). Importantly, MAP kinase, or extracellular-signal regulated kinase (ERK), is viewed as an essential component of signal transduction leading to mitogenesis in many tissues and cells, including lymphocytes (16, 17). Thus, MAP kinase phosphorylates the downstream kinase, S6 II, and the nuclear proteins c-myc, c-jun, p53, and p62CCTCF (18). Each of these proteins is thought to play a functional role during proliferation, differentiation, and the development of neoplasia (19). Importantly, proliferation stimulated by growth hormone, IL-3, IL-5, erythropoietin, and granulocyte/macrophage-colony-stimulating factor requires tyrosyl phosphorylation and, as a consequence, enhanced enzymatic activity of MAP kinase family members (8, 20, 21). Although the precise molecular pathways linking cytokine and growth factor stimulation to MAP kinase activation remain to be fully clarified, several upstream regulators of the enzyme have been identified including receptor-associated tyrosine kinases (22), p21ras (23), Raf-1 (24, 25), and the serine/threonine and tyrosine kinase, MEK-1 (26).

In a previous report, we demonstrated that a maximally mitogenic concentration of PRL rapidly stimulated tyrosyl phosphorylation of MAP kinase and its subsequent translocation to the nucleus in quiescent, lactogen-dependent Nb2

Received 5/9/95; revised 7/17/95; accepted 8/4/95.

1 Supported in part by Grant DK44439 from the NIH, Grant RD-383 from the American Cancer Society, and the National Science Foundation EPSCoR program.

2 Present address: Department of Microbiology and Immunology, Medical College of Virginia, Richmond, VA 23298-0678.

3 To whom request for reprints should be addressed, at Department of Pharmacology and Toxicology, University of North Dakota School of Medicine, 501 North Columbia Road, P.O. Box 9037, Grand Forks, ND 58202-9037.

4 The abbreviations used are: PRL, prolactin; MAP, mitogen-activated protein; IL, interleukin; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; OA, okadaic acid; 2-ME, 2-mercaptoethanol.
cells (15). However, the signaling events interposed between PRL binding to its receptor and MAP kinase activation are uncertain. Herein, we show that mitogenic stimulation of lactogen-dependent Nb2-11 cultures with PRL or IL-2 rapidly activates MAP kinase, determined by tyrosyl phosphorylation and enhanced enzyme activity, prior to its nuclear translocation. Moreover, PRL enhanced p21<sup>W</sup> activity in conjunction with MAP kinase stimulation. The time course and concentration-response of mitogen-stimulated p21<sup>W</sup> and MAP kinase activation, as well as the nuclear accumulation of the enzyme, are consistent with that demonstrated for growth-related gene expression and subsequent proliferation stimulated by these cytokines (2-4). Finally, using a tyrosine kinase antagonist, we show that PRL-stimulated p21<sup>W</sup> activation requires upstream tyrosyl phosphorylation.

**Results**

**PRL, IL-2, and TPA Promote Tyrosyl Phosphorylation of MAP Kinase in Nb2 Cells.** Mitogenic stimuli activate MAP kinase by MEK-catalyzed tyrosyl and serine/threonine phosphorylation (27, 28). Therefore, activation of MAP kinase by mitogens can be assessed by determining its phosphorylation state. Previously, we demonstrated that PRL stimulates maximal tyrosyl phosphorylation of MAP kinase within 5-10 min (15). To further investigate the effect of PRL on MAP kinase activation, we evaluated the concentration dependency for PRL-provoked tyrosyl phosphorylation of the enzyme in growth-arrested Nb2-11 cells incubated with the hormone for 10 min. Lysates from PRL-treated cultures were immunoprecipitated with α-PY, resolved by SDS-PAGE, and immunoblotted with α-MAP kinase (Fig. 1A). In these experiments, mitogenic concentrations of PRL (≥ 1 ng/ml) enhanced tyrosyl phosphorylation of α MAP kinase, the expected Mr for ERK-1.

To assess antibody specificity, α-PY immunoprecipitates from PRL-treated Nb2-11, and exponentially growing Nb2-SFJC1 cells were compared to those obtained from similarly treated Nb2-11 cells that were immunoprecipitated with either normal mouse serum or an irrelevant mAb (IgG1) prior to immunoblotting with α-MAP kinase (Fig. 1B). While α-PY immunoprecipitated the M, 44,000 MAP kinase, the expected Mr for ERK-1.

**Fig. 1.** Effect of PRL on tyrosyl phosphorylation of MAP kinase in Nb2 cells. A. growth-arrested Nb2-11 cells were stimulated with the PRL concentrations indicated (ng/ml) and harvested after 10 min. Cell lysates were immunoprecipitated with α-PY and immunoblotted with α-MAP kinase. B. growth-arrested Nb2-11 cells (Lanes 1, 2, and 3) were stimulated with PRL (20 ng/ml), and similarly preincubated untreated Nb2-SFJC1 (Lane 2) cells were lysed after 10 min and immunoprecipitated with α-PY (Lanes 1 and 2), mouse sera (Lane 3), or an irrelevant mAb (Lane 4) prior to immunoblotting with α-MAP kinase as described in “Materials and Methods.” Results from a representative experiment replicated three times are presented.

**PRL, IL-2, and TPA Stimulate MAP Kinase Activity in Nb2 Cells.** Complete enzymatic activation of MAP kinase requires tyrosyl and threonine phosphorylation (28). To confirm that tyrosyl phosphorylation of MAP kinase stimulated by PRL, IL-2, and TPA results in augmented enzymatic activity, growth-arrested Nb2-11 cells were incubated with these substances for 10 min prior to determining its catalytic activity using a selective MBP synthetic peptide as a substrate (29). Mitogenic stimulation of Nb2-11 cells with PRL (20 ng/ml) increased MAP kinase activity (Fig. 3) with a time-course and concentration-response nearly identical to that demonstrated for PRL-stimulated tyrosyl phosphorylation of the enzyme (Fig. 1; Ref. 15). Enhanced MAP kinase activity was observed within 5-10 min, then returned to basal levels by 60 min (Fig. 3A). Furthermore, PRL concentrations greater than 0.1 ng/ml significantly increased its activity (Fig. 3B). Maximal enzyme activity was observed in cultures treated with ≥ 1 ng/ml of PRL, concentrations which are maximally mitogenic for these cells (4).

Enzyme activity was also assessed in α-MAP kinase immunoprecipitates prepared from PRL-, IL-2-, TPA-, or OA-treated Nb2-11 cells (Table 1). The addition of PRL induced a 4-fold increase in MAP kinase activity at 10 min, while IL-2 produced a 3-fold increase at the same time point. The phorbol ester, TPA, activated MAP kinase at 10 min. In addition, it also enhanced enzyme activity at 60 min, consistent with the demonstrated biphasic pattern of MAP kinase tyrosyl phosphorylation (Fig. 2B). In contrast, the phosphatase antagonist, OA, had no effect on the activity of the enzyme.
Since previous reports have implicated a role for tyrosine kinase- and PKC-mediated phosphorylation in the regulation of MAP kinase (26, 28), we assessed whether antagonism of these pathways would alter PRL-stimulated enzyme activation (Table 2). Genistein, a selective tyrosine kinase antagonist that inhibits PRL-stimulated Nb2 cell protein tyrosyl phosphorylation, growth-related gene expression, as well as proliferation (3), reduced PRL-stimulated MAP kinase activity by 88% at a concentration (20 μg/ml) that also attenuates hormone-stimulated proliferation to an equivalent extent. However, preincubation of cells with staurosporine, a potent serine/threonine kinase inhibitor, did not reduce PRL-stimulated MAP kinase activity at a concentration (5 nM) that would be expected to inhibit PKC in these cells (28). Together, these results suggest that tyrosine kinase activation is most likely interposed between PRL receptor ligation and MAP kinase activation. Moreover, PKC activation does not appear to contribute to signal transduction mediated by this pathway in PRL-stimulated Nb2 cells.

**PRL and IL-2, but not TPA, Induce Nuclear Translocation of MAP Kinase in Nb2 Cells.** PRL has been shown to stimulate tyrosyl phosphorylation of MAP kinase in Nb2 cells (15) and in rat liver (30). Moreover, the tyrosyl-phosphorylated enzyme translocates to the nucleus subsequent to PRL- and GH-stimulation (8, 15). Therefore, the time course for MAP kinase nuclear translocation after stimulation was evaluated. As shown in Fig. 4, PRL stimulated a time-dependent accumulation of tyrosyl phosphorylated MAP kinase within the Nb2-11 nucleus. Maximal accumulation occurred within 2 h, by 12 h, the level had returned toward control (Fig. 4A). Further confirmation of PRL-stimulated MAP kinase nuclear translocation was provided by cellular localization of MAP kinase by indirect immunofluorescence microscopy. Increased MAP kinase immunoreactivity in Nb2-11 nuclei was detected within 1 and maximal 2 h after PRL stimulation (data not shown), further suggesting that its nuclear translocation is delayed compared to the initial tyrosyl phosphorylation of the enzyme, detected within 1–5 min in cell lysates. These results show that a Mr 44,000 MAP kinase is tyrosyl phosphorylated, then translocated to the nucleus in response to PRL treatment. In a parallel experiment, the enzyme activity of nuclear MAP kinase was also determined (Table 3). The addition of PRL to growth-arrested Nb2-11 cells enhanced nuclear enzyme activity by nearly 3-fold after 2 h. By 12 h, the level had declined to control values. Interestingly, nuclear MAP kinase activity determined in control Nb2-SFCD1 cells was 4-fold greater than that detected in unstimulated Nb2-11 cultures. Together, these results suggest that augmented MAP kinase activity within the nucleus in PRL-stimulated Nb2-11 cells most likely reflects its upstream tyrosyl phosphorylation and subsequent movement into this compartment.

In IL-2-stimulated Nb2-11 cells, nuclear translocation of tyrosyl-phosphorylated MAP kinase was also observed (Fig. 4B), whereas by indirect immunofluorescence, it appeared more transient and less pronounced compared to that seen in PRL-treated cultures (data not shown). Notably, TPA did not cause the nuclear translocation of MAP kinase at any of the time points evaluated (Fig. 4C). Thus, while TPA enhances tyrosyl phosphorylation of the enzyme, this modification alone, does not appear to be sufficient to support its nuclear translocation.

**The MAP Kinase Isoform p44erk** Is Expressed in Nb2 Cells. The MAP kinase family is composed of several isoforms with similar in vitro substrate specificity including p44erk1 and p42erk2 (20, 27, 29, 31). To explore which isoform is activated by PRL in Nb2 cells, ERK-1-specific antisera was used to immunoprecipitate Nb2 cell lysates, followed by immunoblotting with α-MAP kinase. As shown in Fig. 5, the MAP kinase isoform expressed in whole cell and nuclear lysates is recognized by the ERK-1 antibody. PRL treatment enhanced the level of ERK-1 detected in cell lysates. Moreover, its presence within the nucleus was augmented within 2 h. These results suggest that ERK-1 is the major MAP kinase isoform activated by PRL in Nb2 cells.
Table 1  Effect of PRL, IL-2, and TPA to activate MAP kinase in Nb2-11 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MAP kinase (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-Control</td>
<td>100</td>
</tr>
<tr>
<td>PRL (20 ng/ml) 10 min</td>
<td>404 ± 27^a</td>
</tr>
<tr>
<td>IL-2 (20 IU/ml) 10 min</td>
<td>315 ± 38^a</td>
</tr>
<tr>
<td>IL-2 (20 IU/ml) 30 min</td>
<td>200 ± 40^a</td>
</tr>
<tr>
<td>TPA (20 nM) 10 min</td>
<td>184 ± 24^b</td>
</tr>
<tr>
<td>TPA (20 nM) 60 min</td>
<td>177 ± 14^b</td>
</tr>
<tr>
<td>OA (5 nM) 30 min</td>
<td>94 ± 11</td>
</tr>
</tbody>
</table>

^a P < 0.001 versus Vehicle-Control.
^b P < 0.05 versus Vehicle-Control.

Table 2  Inhibition of PRL-stimulated MAP kinase activity by kinase inhibitors

<table>
<thead>
<tr>
<th>Preincubations</th>
<th>PRL</th>
<th>MAP kinase activity (pmol/10^6 cells)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-Control</td>
<td>-</td>
<td>50 ± 14</td>
<td>100</td>
</tr>
<tr>
<td>Vehicle-Control</td>
<td>+</td>
<td>204 ± 31^a</td>
<td>0</td>
</tr>
<tr>
<td>Genistein (20 µg/ml)</td>
<td>-</td>
<td>37 ± 7</td>
<td>100</td>
</tr>
<tr>
<td>Genistein (20 µg/ml)</td>
<td>+</td>
<td>69 ± 17</td>
<td>88</td>
</tr>
<tr>
<td>Staurosporine (5 nM)</td>
<td>-</td>
<td>55 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>Staurosporine (5 nM)</td>
<td>+</td>
<td>190 ± 24^a</td>
<td>9</td>
</tr>
</tbody>
</table>

^a P < 0.001 versus Vehicle-Control.
^b Not applicable.

PRL, IL-2, and TPA Activate p21^{ras} in Nb2 Cells. Recent reports have demonstrated that once activated, GTP-bound Ras binds the cytoplasmic serine/threonine protein kinase, Raf-1, leading to its activation through translocation to the plasma membrane (32–34). This enzyme, in turn, phosphorylates, and as a result, activates the MEKs (35), which subsequently stimulate MAP kinase. Since PRL markedly enhanced tyrosyl phosphorylation as well as MAP kinase activity and Raf-1 kinase has also been shown to be activated by PRL in Nb2 cells (14), the upstream coupling of this cascade to Ras was investigated. The activation of Ras, determined by GTP binding to p21^{ras}, was evaluated in Nb2-11 cells stimulated with PRL and in control Nb2-SFJCD1 cultures. Cells were metabolically prelabeled with [32P]orthophosphate prior to stimulation with either PRL, IL-2, TPA, or vehicle-control. Following incubation, cell lysates were immunoprecipitated with α-Ras and bound GTP/GDP was resolved by TLC. In Nb2-11 cells, PRL-stimulated maximal GTP binding to p21^{ras} by 10 min (Fig. 6A) before the levels declined toward control by 30 min. Similar to the effect of PRL, IL-2 and TPA increased GTP binding to Ras within 10 min, albeit to a lesser extent (Fig. 6B). In Nb2-SFJCD1 cultures, Ras-bound GTP was appreciably greater than that detected in control Nb2-11 cells (Fig. 6A), suggesting that it is constitutively active in this autonomous cell line. The addition of PRL to these cultures further enhanced GTP binding to Ras, demonstrating that while this subline no longer requires PRL for proliferation, it remains responsive to the hormone.

Recent evidence suggests that Ras activation by growth factors is mediated by proteins that have SH2 and SH3 domains. Furthermore, nonreceptor tyrosine kinases regulate upstream mediators of Ras in many cell systems (23, 25, 26). Thus, the tyrosine kinase antagonist, genistein, was used to determine whether tyrosyl phosphorylation is required for PRL-stimulated Ras activation. As shown in Fig. 7, genistein attenuated PRL-induced GTP binding to Ras. This observation suggests that early tyrosyl phosphorylation most likely participates upstream of Ras activation in PRL-stimulated Nb2-11 cells.

**Fig. 1**  Time course and concentration response for PRL-stimulated MAP kinase enzyme activity in Nb2-11 cells. A, growth-arrested Nb2-11 cells were treated with 20 ng/ml of PRL. Cells were harvested at the times indicated. Bars, SE. B, growth-arrested Nb2-11 cells were incubated for 10 min in the presence of the indicated concentrations of PRL. Cells were lysed and immunoprecipitated with α-MAP kinase; then the enzyme activity was determined using a synthetic substrate derived from myelin basic protein as described in "Materials and Methods." Results from triplicate samples from three independent experiments are presented. In B, the SE was 10% or less of mean values.
shown in Table 4, the addition of either PRL (20 ng/ml) or IL-2 (20 units/ml) stimulated significant cell proliferation, assessed by either increased population density or \(^{3}H\)thymidine incorporation. Importantly, TPA, which is a relatively weak activator of Ras and MAP kinase and does not provoke its nuclear translocation, significantly enhanced \(^{3}H\)thymidine incorporation but did not alter cell density. The phosphatase antagonists, OA and sodium O-vanadate, had no effect on either cell density or \(^{3}H\)thymidine incorporation. Therefore, the loop bundle peptide mitogens, PRL and IL-2, activate the Ras-MAP kinase cascade, an event that appears to be coupled to mitogenesis in Nb2-11 cells. Pharmacodynamic activation of PKC with TPA stimulates \(^{3}H\)thymidine incorporation but not cell proliferation. Thus, the activation of Ras-MAP kinase tyrosyl phosphorylation appears to be required but not sufficient to promote Nb2 cell proliferation.

**Discussion**

Considerable evidence supports a pivotal role for MAP kinase in the regulation of growth processes (20, 21, 26, 31, 36). In the present study, we investigated the coupling of PRL, IL-2, and TPA to the Ras-MAP kinase signaling pathway and subsequent Nb2 cell mitogenesis. The results demonstrate that PRL and IL-2 rapidly activated the MAP kinase isozyme, ERK-1. Once activated the enzyme subsequently translocated to the nucleus. Moreover, analysis of the effect of these growth factors on GTP binding to p21\(^{ras}\) demonstrated that they most likely stimulate cell proliferation through activation of the Ras-Raf-1-MAP kinase cascade.

Prolactin, IL-2, and TPA each stimulated MAP kinase in a time- and concentration-dependent manner. The enzymatic response observed in mitogen-stimulated Nb2 cells is consistent with its activation in other systems (16-18, 37). Although the precise regulation of MAP kinase remains to be determined, evidence suggests that tyrosyl and threonine phosphorylation is responsible for its activation (17, 21, 23, 37). To date, a receptor-associated tyrosine kinase that directly phosphorylates MAP kinase has not been identified. However, MAP kinase-activating factors, which are themselves regulated by phosphorylation, have been described in several systems (26, 33, 35). The helix-bundle peptides, PRL and IL-2, as well as TPA, stimulated tyrosyl phosphorylation of MAP kinase, suggesting that this modification is required for its activation by these substances. Genistein, a tyrosine kinase antagonist (3, 38), attenuated PRL-stimulated MAP kinase activation. This observation is consistent with a recent report demonstrating MAP kinase inhibition by tyrosine kinase antagonism (39). In this study, genistein inhibited MAP and S6 kinases, protein and DNA synthesis, as well as mitogenesis in Nb2 cells (39), effects similar to our previous observations (3) and the results present herein using this antagonist. Taken together, these observations support a requirement for MAP kinase activation for cytokine stimulation of cell growth.

Recently, activation of three tyrosine kinases, Jak2, Fyn, and Lck, have been separately linked to PRL action in Nb2 cells (10–12). The latter is also an IL-2 receptor-associated kinase in T lymphocytes (40). In other lymphoid systems, Lck and Src are required for Raf-1 kinase activation (34). In Nb2-11 cells, either Fyn or Lck, two members of Src kinase family, likely act as kinase intermediates for the activation of MAP kinase. However, significant tyrosyl phosphorylation of Raf-1 does not occur in PRL-stimulated Nb2 cells.
MAP activity ever, course activate activity kinase, tyrosine, phosphorylation stimulated

Fig. 5. ERK-1 is the primary MAP kinase isoform expressed in Nb2-11 cells. Growth-arrested Nb2-11 cells were stimulated with PRL (20 ng/ml) and harvested at the times indicated. Whole cell (left panel) or nuclear (right panel) lysates were immunoprecipitated with α-ERK-1, then immunoblotted with α-MAP kinase as described in "Materials and Methods." A representative experiment replicated more than three times is presented.

(14), suggesting that it may not be a direct substrate for these enzymes.

Several laboratories have implicated PKC as mediator of PRL action in target tissues, including Nb2 cells (13). Furthermore, results presented herein demonstrate that TPA-stimulated activation of PKC resulted in tyrosyl phosphorylation of MAP kinase, although, compared to PRL or IL-2, the phorbol ester was less efficacious. However, staurosporine, a PKC inhibitor in Nb2 cells (38), failed to block PRL-stimulated MAP kinase activation, suggesting that this kinase may not represent an upstream regulator of cytokine-induced enzyme activation. Furthermore, enhanced membrane-bound PKC activity occurs 20–30 min subsequent to PRL stimulation in these cells, a time when MAP kinase activity is declining toward basal levels.

Prolactin stimulation of MAP kinase tyrosyl phosphorylation suggested that protein phosphatases may also participate in the initiation and/or maintenance of this effect in Nb2 cells. Indeed, in several systems, OA, an inhibitor of protein phosphatases 1 and 2, promotes the activation of MAP kinase (41, 42). However, in Nb2 cells, OA failed to activate the enzyme. This suggests that these phosphatases most likely do not directly regulate PRL-stimulated MAP kinase phosphorylation/dephosphorylation.

Based upon the present observations, together with those reported in the literature, Raf-1 appears to be a likely upstream mediator of PRL-stimulated MAP kinase; the time course for its activation by PRL is nearly identical to that observed for MAP kinase in this system (14, 15). In addition, Raf-1 phosphorylation and activation of MAP kinase have been causally linked in other cell systems (17, 26). However, the elucidation of a role for Raf-1 in MAP kinase stimulation required investigation of the coupling of PRL and IL-2 to p21^{ras}, a receptor proximal intermediate in this pathway (23, 24, 26).

Analysis of the effect of PRL, IL-2, and TPA on p21^{ras} activity demonstrated that each of these substances activated this regulatory factor with kinetics that paralleled MAP kinase activation. Thus, it appears that p21^{ras} participates in MAP kinase activation stimulated by these agents.

Importantly, genistein attenuated PRL-stimulated p21^{ras} activity, suggesting that tyrosyl phosphorylation may be required for its downstream kinase modulation. To date, Jak2 is the only demonstrated PRL receptor-associated tyrosine kinase that exhibits a kinetic pattern of activation consistent with that required for p21^{ras} and MAP kinase stimulation. This kinase is extremely sensitive to PRL and exhibits a rapid onset of activation following lactogen receptor ligation (10, 11).

The nuclear actions of MAP kinase have been an area of considerable interest. Several nuclear proteins serve as substrates for MAP kinase, leading to the suggestion that this enzyme may play a regulatory role in this compartment (18). Therefore, the subcellular distribution of MAP kinase was determined in PRL-stimulated Nb2 cells. Activated MAP kinase was found within the nucleus and cytosol subsequent to mitogenic stimulation. The significant increase in nuclear MAP kinase activity appeared to reflect transient translocation of the tyrosyl phosphorylated form. The MAP kinase family includes several isoforms, and it is possible that each isoform may exhibit a distinct subcellular pattern of distribution (16, 27). However, in previous studies, the relative distribution of individual isoforms has not been clearly addressed (29, 43). Results from the present study indicate that activated p44^{ras-1} is the primary MAP kinase isoform expressed in Nb2 cell nuclei.

The role played by MAP kinase within the nucleus is presently unclear. Previously, MAP kinase-mediated phosphorylation of the nuclear proteins, c-myc, c-jun, p53, and p62^{nrs}, has been demonstrated in vitro and in vivo (8, 43, 44). The available evidence strongly suggests that MAP kinase and these proteins participate in mediating a growth response (43, 44). In Nb2 cells, PRL induces the expression of several immediate early, growth-related genes, such as interferon regulatory factor 1, c-myc (2), heat shock protein 70 (3), and pim-1 (45). Thus, either transcription factors that regulate expression of these genes or the proteins themselves may serve as substrates for nuclear MAP kinase-mediated phosphorylation.

In addition to lactogen-dependent Nb2-11 cells, p21^{ras} and activated MAP kinase were also evaluated in the PRL-independent subline, Nb2-SF1CD1. Results from these experiments indicated that MAP kinase and p21^{ras} are each

Unpublished observation.
active in this autonomous subline in the absence of mitogen stimulation. Whether growth factor-independent activation of this signal transduction cascade in these cultures represents an epiphenomenon or the cause of PRL autonomy remains to be determined.

The precise role played by MAP kinase in PRL-stimulated Nb2 cell mitogenesis is unknown. Interestingly, the rapid onset of PRL-stimulated MAP kinase activity is comparable to the demonstrated time course for its induction of c-myc, c-fos, and pim-1 mRNAs (2, 39, 45). The concentration of PRL required to activate MAP kinase is well within the range that stimulates Nb2 cell proliferation (3, 4) and is in good agreement with those which stimulate tyrosine kinase activation (10–12). A recent report demonstrated that expression of antisense constructs of p44rsk-1 decreased growth factor activation of MAP kinase and inhibited cell growth (31), suggesting that mitogenesis requires MAP kinase-mediated phosphorylation. However, TPA significantly activated p44rsk-1 in Nb2 cells but failed to induce Nb2 cell proliferation. Moreover, TPA did not induce MAP kinase nuclear translocation. These results suggest that MAP kinase activation in Nb2 cells is likely required but not sufficient to support proliferation similar to other systems (47). The results from the present study also indicate that the effects produced by nuclear MAP kinase may be required for mitogenesis. Therefore, based upon the demonstrated effects of PRL and IL-2 to activate the Ras-MAP kinase pathway, we suggest that, in addition to tyrosyl phosphorylation, the signal transduction cascade leading to the nuclear translocation of the active form of MAP kinase is a salient component in Nb2 cell proliferation. Further work is required to precisely delineate the relationships among the numerous enzymatic components of this key growth regulatory pathway and its role in Nb2 cell proliferation.

Materials and Methods

Materials. Ovine PRL (oPRL-19) was obtained through the NIH Pituitary Hormone and Antisera Program (Bethesda,
MD), and recombinant human IL-2 was obtained from R & D Systems (Minneapolis, MN). [125I]Orthophosphate (500 mCi/ml) was from ICN (Irvine, CA). An anti-phosphotyrosine mAb (α-PY) and the MAP kinase substrate (APRTPG-GRR) were obtained from Upstate Biotechnology (Lake Placid, NY). The α-MAP kinase mAb, which reacts with ERK-1 (p43, p42MAPK) and ERK-2 (p41, p42MAPK), was obtained from Zymed Laboratories, Inc. (San Francisco, CA). The mAb to v-H ras and ERK-1 antisera were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant protein G-Sepharose was obtained from GIBCO-BRL (Gaithersburg, MD) and genistein from Calbiochem (La Jolla, CA). All other reagents were of molecular biological grade and obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

**Nb2 Lymphoma Cell Cultures.** The PRL-dependent rat T lymphoma cell line, Nb2-11, originally cloned by Dr. H. G. Freisen (Winnipeg, Manitoba, Canada) and a PRL-independent subline, designated Nb2-SFJCD1, developed from the parental line by lactogen starvation and cloning of surviving cells, were generously provided by Dr. P. W. Gout (Vancouver, British Columbia, Canada). Nb2-11 cells were maintained at 37°C in Fischer’s medium containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) as a source of lactogen, 10% horse serum (BioWhittaker), 2-ME, (10⁻⁴ M), penicillin (50 units/ml), and streptomycin (50 µg/ml) as described by Gout et al. (4). For various experiments, the cells were growth arrested in the early G1 phase of cell cycle by an 18-24 h preincubation in lactogen-free medium, i.e., Fischer’s medium supplemented with 2-ME, antibiotics, and 10% nonmitogenic gelatin serum (ICN, Irvine, CA; assay medium). When used for comparative purposes, Nb2-SFJCD1 cultures were similarly preincubated in assay medium for 18-24 h prior to commencement of the experimental procedures.

**Isolation of Nb2 Nuclei.** Nuclei were isolated from Nb2 cells using a modification of a procedure described previously (15, 38). Briefly, cells were incubated in a hypotonic buffer [10 mM Tris-HCl, (pH 7.4), 1 mM EDTA, 3 mM CaCl₂, 10 mM KCl, 50 µg/ml leupeptin, and 10 µg/ml aprotinin at 4°C] for 10 min. After the addition of hypotonic buffer containing 1.6 M sucrose, the swollen cells were centrifuged at 750 x g for 10 min. The cells were disrupted in a buffer containing 94 mM K₂PO₄ (pH 7.4), 2.5 mM MgCl₂, 0.32 M sucrose, 0.5 mM EDTA, and 50 µg/ml leupeptin using a glass dounce homogenizer, then centrifuged at 750 x g. The nuclear pellet was washed with a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 25% glycerol, and 25 µg/ml leupeptin.

Purity of the nuclear preparations was verified by biochemical means. Three different markers, two enzymes specific for plasma membrane (5'-nucleotidase and phosphodiesterase I) and one for microsomes (NADPH-cytochrome c reductase), were examined as described (48). Phosphodiesterase I was undetectable, while 5'-nucleotidase and NADPH-cytochrome reductase were present at extremely low levels (Table 5). If extrapolated to the entire cell population, the activities of these enzymes in the nuclear preparations represent 1% or less of total enzyme activity. Therefore, nuclear contamination cannot account for the level of tyrosyl phosphorylated MAP kinase observed in this compartment subsequent to PRL and IL-2 stimulation (Figs. 4 and 5).

**Immunoprecipitation Procedures.** Immunoprecipitation of various proteins was performed as described previously (15, 47). Cell extracts were prepared in HNTG buffer containing 50 µM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 100 µM sodium orthovanadate, 10 µg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 10,000 x g for 10 min and precleared by incubation with protein G prior to the addition of 0.1 µg/ml of α-MAP kinase or a 1:2000 dilution of α-ERK-1 antisera to the resulting supernatant. The samples were incubated overnight at 4°C with continuous mixing. Protein G-Sepharose was added, and the incubation continued for an additional 60 min with mixing. Immune complexes were washed 5x with HNTG buffer. For direct SDS-PAGE, immunoprecipitated proteins were eluted by boiling in 25 µl of SDS sample buffer containing 10% 2-ME. For the determination of phosphotransferase activity, the immunoprecipitates were washed with kinase buffer (120 mM HEPES (pH 7.2), 5 mM MgCl₂, 1 mM EDTA, 5 mM 2-ME, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of aprotinin and leupeptin) prior to the analysis of enzyme activity.

**SDS-PAGE and Immunoblotting.** Samples were resolved by SDS-PAGE using 12% gels, then immunoblotted as described previously (15, 47). Resolved proteins were electrophoretically transferred to ImmunoMite membrane (BioRad, Richmond, CA). Membranes were blocked by an 18-h incubation at 4°C with 5% nonfat dry milk in Tris-buffered saline. Following extensive washing, the membranes were incubated for 3 h at 25°C with α-PY (0.1 µg/ml), α-MAP kinase (0.1 µg/ml), or α-ERK-1 (1:2000). Goat α-mouse or α-rabbit IgG, conjugated to alkaline phosphatase, were used as secondary antibodies (1:3000; Bio-Rad). Proteins were detected using a chemiluminescent reaction, followed by exposure to X-ray film.

**Determination of MAP Kinase Activity.** The enzymatic activity of MAP kinase in cell lysates and immunoprecipitates was assessed using a nine-amino-acid synthetic peptide derived from the sequence of bovine myelin basic protein as described (27, 29). Briefly, 10-µl aliquots from Nb2 cell lysates or eluted immunoprecipitates were incubated in a final volume of 25 µl with 25 µg of synthetic peptide, in the presence of 50 µM [γ-³²P]ATP (10 Ci/mmol; Dupont, NEN, Boston, MA), in a buffer containing 25 mM HEPES (pH 7.4) and 10 mM MgCl₂ for 20 min at 25°C. Control incubations were performed without the addition of the substrate. The reaction was terminated by the addition of 10 µl of 125 mM ATP in 45% (v/v) formic acid. A 15-µl aliquot was spotted onto phosphocellulose discs (What-

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**Table 5** Nb2 nuclear preparation characterization

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Nuclei</th>
<th>Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase (nmol/min/mg protein)</td>
<td>0.1</td>
<td>7.9</td>
</tr>
<tr>
<td>Relative enrichment&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphodiesterase I (pmol/min/mg protein)</td>
<td>nondetectable</td>
<td>25.9</td>
</tr>
<tr>
<td>Relative enrichment&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>NADPH-cytochrome C reductase (nmol/min/mg protein)</td>
<td>0.15</td>
<td>16.9</td>
</tr>
<tr>
<td>Relative enrichment&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.009</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity/mg protein nucleus/mg protein in homogenate.
man, Hillsboro, OR) and washed 2x with 1 m acetic acid containing 4 mM sodium pyrophosphate. The incorporation of [14C]phosphate into the substrate was determined by counting the discs in a Beckman liquid scintillation counter.

**Determination of p21** Activation. Growth-arrested Nb2-11 cells were washed 2x with phosphate-free RPMI 1640 (GIBCO) supplemented with 2% dialyzed gelling serum and 20 mM HEPES (labeling medium), then metabolically labeled at a density of 5 x 10^8 cells/ml by incubation for 2 h with 0.5 mCi/ml [32P]orthophosphate. The prelabeled cultures were then stimulated with PRL or IL-2. In some experiments, genistein, a tyrosine kinase antagonist, was added 20–30 min prior to mitogen stimulation. The cells were harvested by centrifugation at 4°C at various time intervals, washed with ice-cold PBS, and lysed in HNTG buffer. The lysates were centrifuged at 10,000 X g for 10 min, and the supernatants were incubated with α-p21 overnight at 4°C. The immune complexes were collected by the addition of recombinant protein G-Sepharose and washed 8x in a buffer containing 50 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, and 0.005% SDS. Bound nucleotides were eluted by incubation at 68°C for 20 min in a solution containing 2 mM EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP (added as TLC standards). Radiolabeled GTP and GDP were resolved on polyethyleneimine-cellulose TLC plates (Alltech, Deerfield, IL) developed in 1.2 mM ammonium formate/0.8 mM HCl. The standards were visualized by UV irradiation as described by Downward et al. (36). The plates were washed 2x with ddH2O, air dried, and subjected to autoradiography and/or counting of the excised nucleotides in a scintillation counter.

**Effect of Growth Factors, Kinase Activators, and Phosphatase Inhibitors on Nb2 Cell Proliferation.** Various dilutions of PRL, IL-2, TPA, OA, or sodium O-vasenate (40, 41) were added to triplicate cultures of growth-arrested Nb2-11 cells. The cultures were incubated for 44 h at 37°C in an atmosphere of 5% CO2/air and then pulse-labeled (4 h) with 0.5 mCi/well of [3H]thymidine (specific activity, 71 Ci/mmol; Dupont, NEN). Cells were harvested after 48 h using a PHD cell harvester (Cambridge Technology, Watertown, MA), precipitated with ice-cold 10% trichloroacetic acid, and washed with ethanol. Radioactivity of the TCA insoluble material was determined by liquid scintillation spectroscopy. Population density in parallel triplicate cultures was determined by cell counting using a Coulter counter (Hialeah, FL).

**Data Analysis.** All experiments were replicated three times unless otherwise noted. Results are expressed as mean ± SE. When appropriate, statistical differences between treatment groups were determined by ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons.

**Acknowledgments**

We thank Kay Gunn Boushee for excellent secretarial assistance.

**References**


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