Endocrine, *vol. 4, no. 1, 65–71, February 1996* 0969-711X/96/4:65–71/$6.75

Lactogenic Hormones Rapidly Activate p21^{ras}/Mitogen-Activated Protein Kinase in Nb2-11C Rat Lymphoma Cells

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Lactogenic hormone-dependent Nb2-11C cells proliferate in response to prolactin (PRL) or human growth hormone (hGH). We have investigated the activation of p21^{ras} and mitogen-activated protein kinase (MAP-kinase) by hGH in lactogen-dependent Nb2-11C and in autonomous hormone-independent Nb2-SP rat lymphoma cells. Exposure of Nb2-11C cells to hGH resulted in a dose-dependent activation of p21^{ras} and of MAP-kinase. Activation occurs at physiological hGH concentration and with a rapid onset (~1 min) reaching maximal level at 10–20 min. In contrast, in Nb2-SP autonomous lactogen-independent cells, p21^{ras} and MAP-kinase are constitutively activated and a challenge with lactogenic hormone had a modest additional activating effect. TPA, an activator of protein kinase C, enhanced p21^{ras} and MAP-kinase activity in Nb2-11C cells but failed to induce proliferation. The mechanism of activation of p21^{ras} in Nb2-11C cells by lactogenic hormones involves both an increased binding of guanine nucleotides to p21^{ras} as well as an increase in GTP/GDP + GTP ratio. In summary, we have demonstrated here that activation of the p21^{ras}/MAP-kinase pathway follows PRL receptor activation but is not sufficient for the lactogenic hormone-dependent mitogenesis.

Abbreviations: MAP-kinase, mitogen-activated protein kinase; PKC, protein kinase C; hGH, human growth hormone; bGH, bovine growth hormone; rGH, rat growth hormone; oPRL, ovine prolactin; bPL, bovine placental lactogen; TPA, 12-0-tetradecanoylphorbol 13-acetate; ECL, enhanced chemiluminescence; JAK, janus kinase; ras-GAP, ras GTPase-activating protein; ras GNRF, ras guanine nucleotides releasing factor.

Received September 18, 1995; Revised November 2, 1995; Accepted November 20, 1995.

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Key Words: Human growth hormone; lactogenic hormones; Nb2 cells; p21^{ras}; prolactin; mitogen-activated protein kinase.

Introduction

The biological effects of lactogenic hormones, such as prolactin (PRL), placental lactogen (PL), or human growth hormone (hGH), are mediated through the membranal prolactin receptor—a member of the GH/PRL/cytokine receptor superfamily (Kelly et al., 1991). Signal transduction mediated by these family members occurs via activation of tyrosine phosphorylation through receptor-associated JAK protein tyrosine kinase, leading ultimately to the activation of numerous transcription factors (Ihle, 1994; Sato and Miyajima, 1994). A variety of growth factors and cytokines mediate proliferation by activating the p21^{ras} guanine nucleotide-binding protein. The binding of these ligands to their respective membranal receptors triggers tyrosine phosphorylation by activating intrinsic receptor protein tyrosine kinases or cytosolic protein tyrosine kinases leading to an increase in the amount of active GTP · p21^{ras} (Burgering et al., 1991; Duriono et al., 1992; Nanberg and Westermark, 1993; Sato and Miyajima, 1994). Activation of p21^{ras} results in activation of a cascade series of Ser/Thr protein tyrosine kinases such as Raf-1, mitogen-activated protein kinase and its substrate MAP-kinase (Pelech et al., 1993; Sato and Miyajima, 1994). Thus, following binding of lactogenic hormones to the PRL receptor of lymphoma cells, the currently suggested sequence of events is receptor dimerization (Elberg et al., 1990), activation of associated PTKs such as JAK2 and Fyn (Rui et al., 1994a,b; Lebrun et al., 1994; Campbell et al., 1994; Clevenger and Medaglia, 1994), and the guanine nucleotide releasing factor (GNRF)-vav (Clevenger et al., 1995) followed by Ser/Thr phosphorylation involving the kinases Raf-1 (Clevenger et al., 1994), MAP-kinase, and 40S ribosomal protein S6 kinase (Buckley et al., 1994; Carey et al., 1995). Nb2 lymphoma cell line is an excellent model for studying lactogenic hor-

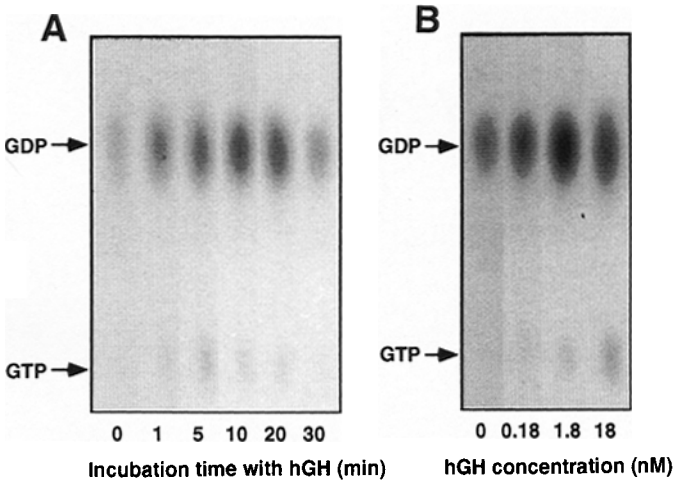

Fig. 1. Effect of hGH on activation of p21[ras]. Nb2-11C cells were exposed at 37°C to 18 n*M* hGH for different lengths of times **(A)** and to various concentrations of hGH for 5 min **(B)**, as indicated on the figure. p21[ras] labeling was performed at 4°C by cell permeabilization and incubation with [α-^{32}P]GTP for 30 min. The eluted guanosine nucleotides, GDP and GTP, from immunoprecipitated p21[ras] were separated on thin-layer chromatography and analyzed by autoradiography.

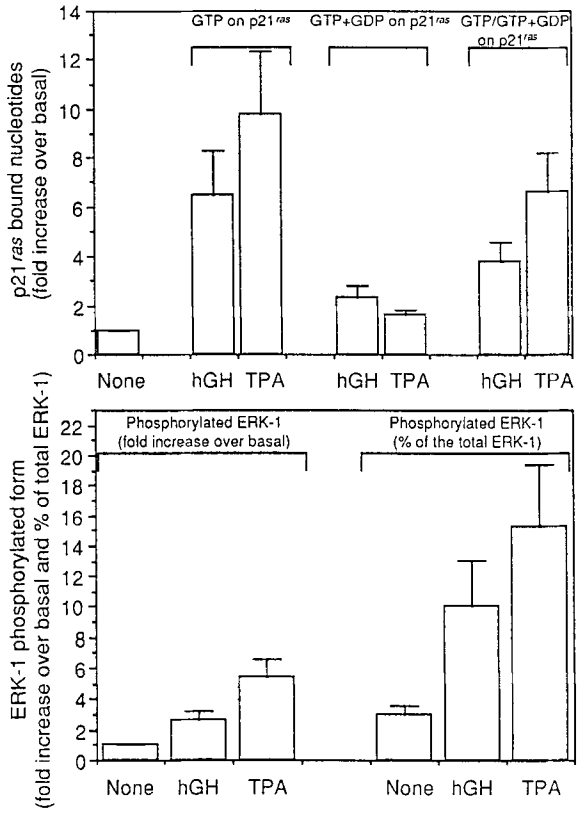

Fig. 2. Densitometry analysis of p21[ras] and MAP kinase activation by hGH and TPA. Nb2-11C cells were either unstimulated or incubated with 20 n*M* hGH for 5 min or 20 n*M* TPA for 20 min, and p21[ras] and MAP kinase activity were analyzed as described in Materials and Methods. Scanning analysis of bound nucleotides on p21[ras] and phosphorylated form of ERK-1 are presented. Data are the results of 3–4 experiments and represent the mean ± SE.

mone-mediated signaling. The proliferation of the Nb2-11C cells is absolutely dependent on lactogenic hormones (Tanaka et al., 1980). An additional important experimental tool is the lactogen-independent spontaneously proliferating Nb2-SP cell line (Gertler et al., 1985). The latter possesses an intact functional PRL-receptor (Ali et al., 1991). The events spontaneously occurring in these cells, which are ligand dependent in Nb2-11C cells, might be essential for mitogenesis. Recently, it has been shown that PRL-induced p21[ras] activation in Nb2-SP cells is mediated via the signaling proteins, SHC, growth factor receptor bound 2, and son of sevenless. However, no detectable activation of p21[ras] by PRL was found in Nb2-11C cells (Erwin et al., 1995).

In the present study, we demonstrate (for the first time) the direct activation of p21[ras] by lactogenic hormones in prolactin responsive cells.

Results

Activation of P21[ras] by hGH

Figure 1 shows that exposure of synchronized cells to hGH rapidly activated p21[ras]. Activation was evident within 1 min and occurred at hGH concentration range inducing activation of several protein kinases involved in the transduction system of the lactogenic signal (Lebrun et al., 1994; Campbell et al., 1994, Clevenger et al., 1994; Clevenger and Medaglia, 1994). Activation of p21[ras] is regulated by two mechanisms, namely through inhibition of ras-GAP and therefore increasing p21[ras] · GTP/GTP + GDP ratio (Downward et al., 1990a) or by enhancing ras-GNRF activity. This in turn will increase guanine nucleotide turnover and the amount of the active GTP bound p21[ras] (Downward et al., 1990b; Duriono et al., 1992; Nanberg and Westermak, 1993). As shown in Fig. 1, hGH-stimulus led to the rapid increase in the amount of total GDP + GTP bound p21[ras] as well as to an increased GTP/GTP + GDP ratio. This was dose dependent, and occurred at hGH concentrations ranging from 0.18 n*M* to 18 n*M*. With respect to quantitation there was a 6.5 ± 1.8-fold increase in GTP, a 2.3 ± 0.5-fold increase in GDP + GTP, and a 3.8 ± 0.8-fold increase in GTP/GTP + GDP (Fig. 2). Control experiments showed no elution of labeled nucleotides when anti-p21[ras] was replaced in immunoprecipitation procedures with normal rat IgG (not shown). Thus, these data indicate that hGH facilitates activation of p21[ras] via both regulatory pathways.

Activation of MAP-Kinase by hGH or Other Lactogenic Hormones

Activation of MAP-kinase, a downstream element of the p21[ras] signaling pathway, can be experimentally determined by the electrophoretic shift obtained on phosphorylation of the threonine and tyrosine moieties (Posada and

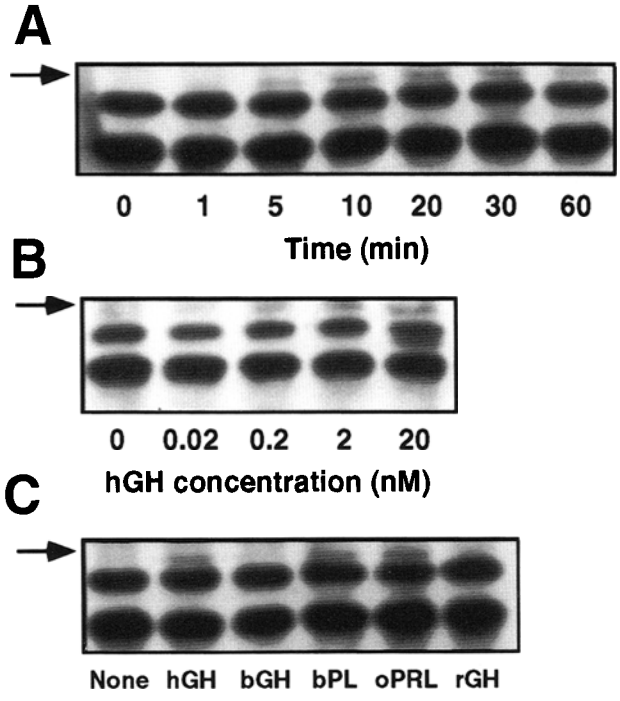

Fig. 3. Immunoblot analysis of the activation of MAP-kinase induced by lactogenic hormones in Nb2-11C cells. Cells were exposed to 20 n*M* hGH for different lengths of time **(A)**, to various concentrations of hGH for 20 min **(B)** and to 20 n*M* of various lactogenic or somatogenic hormones for 20 min **(C)**. The cytosolic extracts of the cells were prepared and subjected to SDS-PAGE and immunoblotting with anti-MAP-kinase antibody. Arrows on the left side indicate the slight shift of MAP-kinase induced by lactogenic hormones.

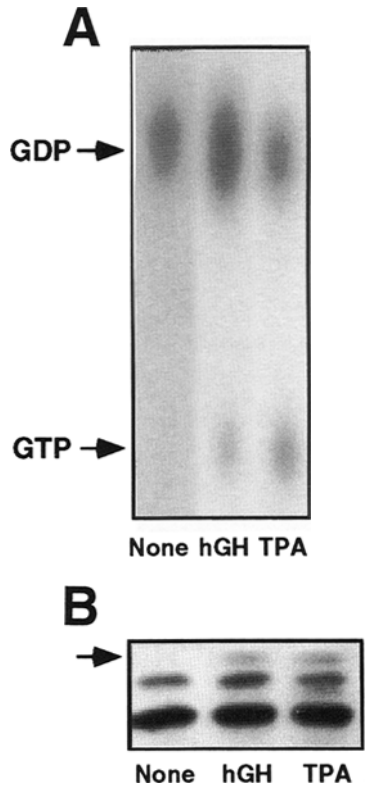

Fig. 4. Activation of p21[ras] and MAP-kinase by TPA and hGH in Nb2-11C cells. Cells were exposed to 18 n*M* hGH for 5 min before p21[ras] bound nucleotide analysis, for 20 min before MAP-kinase analysis and to 20 n*M* TPA for 20 min in both cases. p21[ras] activation was analyzed following cell permeabilization and exposure to [α-^{32}P]GTP for 30 min at 4°C. The eluted GTP and GDP from immunoprecipitated p21[ras] were separated on thin-layer chromatography and analyzed by autoradiography **(A)**. Activation of MAP-kinase was analyzed by immunoblotting of cytosolic extracts with anti-MAP-kinase antibody. The arrows indicate the slight electrophoretic shift of MAP-kinase induced by hGH or TPA **(B)**.

Cooper, 1992; Seger et al., 1994). To examine whether the activation of p21[ras] in lactogen stimulated Nb2-11C cells is associated with increased MAP-kinase activity, cells were exposed to hGH for different lengths of time and to various concentrations (Fig. 3). In unstimulated cells, the two forms of MAP-kinase, ERK-1 (M_r = 44 kDa) and ERK-2 (M_r = 42 kDa) were detected. Exposure of Nb2-11C cells to hGH induced the appearance of an additional band in the upper region of the ERK-1 protein (arrow) corresponding to an electrophoretic shift of MAP-kinase. Activation of MAP-kinase was detected at 1 min, reaching a maximal level at 10 min and sustained for approx 30 min. The activation was dose dependent and occurred at hGH concentrations ranging from 0.2 to 20 n*M* (Fig. 3B). Other lactogenic hormones such as bPL and oPRL, which are mitogenic in Nb2-11C cells, also stimulated MAP- kinase activity. In contrast, the somatogenic hormones bGH and rGH, which lack mitogenic effect in these cells (Tanaka et al., 1980), had no effect on activation of MAP-kinase activation (Fig. 3C). Treatment of Nb2-11C cells with cholera toxin or with 8-bromo-cAMP did not alter the effect of hGH at all in activating MAP-kinase (not shown). A 2.7 ± 0.5-fold increase in the phosphorylated form of ERK-1 is detected in Nb2-11C cells treated with hGH and comprise 10 ± 3% of the total enzyme (Fig. 2).

Activation of p21[ras] and MAP-Kinase by TPA

To further explore the role of the p21[ras]/MAP-kinase signaling pathway in the lactogen-dependent Nb2-11C cells, we treated the cells with the protein kinase C activator, TPA. This agent has no mitogenic effects in these cells but was shown to enhance mitogenesis induced by lactogenic hormones (Gertler et al., 1985). As demonstrated in Fig. 4, TPA stimulated the activities of both p21[ras] and of MAP-kinase. Densitometric analysis is shown in Fig. 2.

p21[ras] and MAP-Kinase Activity in Nb2-SP Cells

It has been shown recently that Raf-1 (located downstream of p21[ras] and upstream of MAP-kinase on the same signaling pathway) is constitutively phosphorylated in the lactogen-independent Nb2-SP cells (Clevenger et al., 1994). To examine whether p21[ras] and MAP-kinase are also excessively activated in Nb2-SP cells, we determined their activities with or without exposure to hGH. Figure 5 shows that p21[ras] and MAP-kinase are substantially active

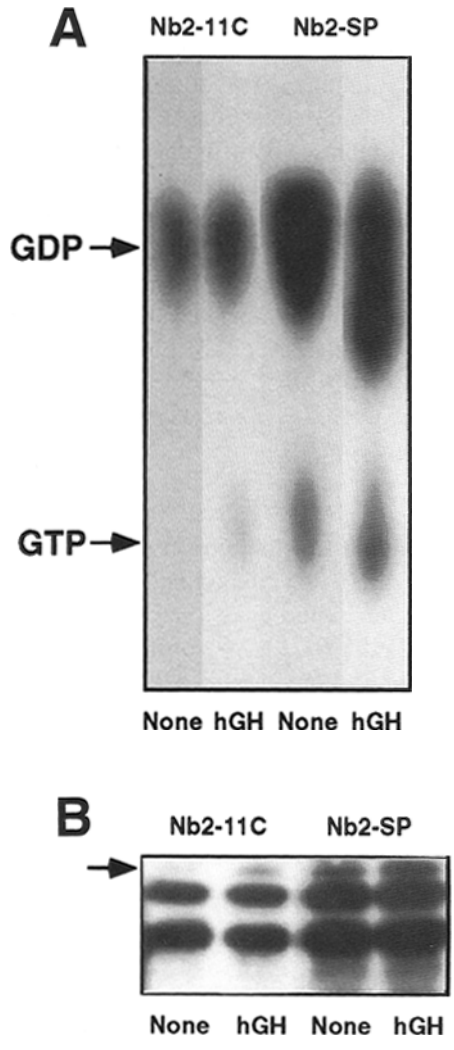

Fig. 5. Comparison of p21[ras] and MAP-kinase activity in nonstimulated and hGH-stimulated Nb2-11C and Nb2-SP cells. p21[ras] bound guanosine nucleotide analysis **(A)** and immunoblot analysis of MAP-kinase **(B)** were performed as described in the legend of Fig 4. Nb2-SP cells contain approx twofold more proteins than synchronized Nb2-11C cells.

in the nonstimulated cells. This was not owing to a higher p21[ras] and MAP-kinase expression in the proliferative Nb2-SP cells, since samples were equalized for protein levels and the amount of p21[ras] and MAP-kinase as detected by Western blotting was comparable in Nb2-SP and Nb2-11C cells (not shown). A two- to threefold increase in GTP bound p21[ras] and phosphorylated form of ERK-1 were observed following exposure to hGH. Thus, in the spontaneously proliferative cell line, p21[ras]MAP-kinase pathway is turned on in a permanent fashion.

Discussion

In this study we have demonstrated the activation of the p21[ras]/MAP-kinase signaling pathway by hGH in the lactogen-dependent Nb2-11C cells and the constitutive activation of this pathway, in the hormone-independent, spontaneously proliferating Nb2-SP cells. Taken together, the data indicate the involvement of these signals, both in receptor-mediated and in the autonomous proliferative response of Nb2 cells. Our data receive further support from a recent report demonstrating the constitutive activation of Raf-1 in Nb2-SP cells (Clevenger et al., 1994b). The latter is located downstream of p21[ras] on the same signaling pathway (Koide et al., 1993). Activation of p21[ras] and MAP-kinase by TPA and hGH resembled each other in Nb2-11C cells; however, TPA is not mitogenic in these cells and down regulation of PKC does not significantly affect prolactin-mediated mitogenesis (Meyer et al., 1992). TPA has been shown to stimulate several rapid post-PRL receptor events in Nb2-11C cells, including activation of the Na^+/H^+ antiport system (Too et al., 1987), an increase in cytosolic Ca^{2+} (Cohen, 1993), and Nb29 gene expression (Horn et al., 1994). However, at least 4 h of exposure of Nb2-11C cells to lactogenic hormones is necessary to induce mitogenesis (Walker and Gertler, 1985). Thus early and late receptor-mediated events are required. Activation of p21[ras] is preceded by activation of protein tyrosine kinase in several cellular systems (Burgering et al., 1991; Nanberg and Westermak, 1993; Sato and Miyajima, 1994). In Nb2-11C cells following binding, PRL-receptor dimerizes and activates JAK2 and Fyn (Rui et al., 1994a,b; Lebrun et al., 1994; Clevenger and Medaglia, 1994). Inhibition of these tyrosine kinases blocks stimulation of MAP-kinase and the proliferative response (Carey et al., 1995). Thus, early events leading to mitogenesis appear to involve a PRL-dependent increase in tyrosine phosphorylation. Our results indicate that activation of p21[ras]/MAP-kinase pathway alone by cell treatment with TPA, bypassing tyrosine kinases, is not sufficient to simulate lactogenic-dependent mitogenesis in these cells. The increase in p21[ras]/MAP kinase activity (Fig. 2) was small but consistent with previous reports (Burgering et al., 1991; Wintson and Bertics, 1992; Yang and Farese, 1993; Rapoport et al., 1993; Nanberg and Westermark, 1993; Erwin et al., 1995). However, this was achieved at hGH concentrations, which were at least one order of magnitude higher than the concentration required for mitogenesis (Tanaka et al., 1980; Gertler et al., 1985). Lower hGH concentration (20 p*M*) resulted in inconsistent activation of this pathway (data not shown). This indicates that full stimulation of mitogenesis may be achieved at hormone concentrations that fail to affect p21[ras] or MAP kinase. It may also argue that stimulation of this pathway may not be related to lactogen mitogenesis. Such dissociation between cellular proliferation and activation of MAP kinase was reported in cytokine responsive cells (Miura et al., 1994). However, it is also possible that undetectable activation of p21[ras] and MAP kinase in Nb2 cells may occur at mitogenic hGH concentrations. This is supported by the ability of anti-p21[ras] monoclonal antibody to block growth factor stimulated cellular proliferation even in the absence of detectable increase in p21[ras] activity (Mulcahy et al., 1985; Yu et al., 1988).

Two mechanisms are presently known to activate p21^{ras}. In lymphoid cells, the first one involves inhibition of ras-GAP activity mediated through the T-cell receptor, whereas the second one involves the activation of p21^{ras}-GNRF's following cell exposure to IL-2 (Izquierdo et al., 1995). Our results indicate that, in Nb2-11C cells, the up-regulation of p21^{ras} by the PRL receptor is achieved by a dual mechanism, i.e., activation of Ras nucleotide exchange factor and releasing p21^{ras} from the inhibitory effect of ras-GAP, resulting in higher GTP + GDP binding to p21^{ras} and an increased GTP/GTP + GDP ratio, respectively. It is of interest to note that a similar increase in total guanine nucleotides and GTP/GTP + GDP bound to p21^{ras} was also observed in TPA-stimulated Nb2-11-C cells (Fig. 2), suggesting that PKC and hGH may utilize the same regulatory pathway in activating p21^{ras}.

Recent data suggested that elevation of cellular cAMP level can block ligand-dependent activation of Raf-1 and subsequently MAP-kinase (Graves et al., 1993; Wu et al., 1993; Cook and McCormick, 1993; Hodijk et al., 1994). This may explain the inhibitory effect of cholera toxin and cell-permeable cAMP analogs on mitogenesis of Nb2-11C (Pines et al., 1988; Larsen and Dufau, 1988). Moreover, Nb2-SP cells contain minor amounts (~5%) of cholera-toxin dependent sensitive Gs-protein, as compared to Nb2-11C (Too et al., 1990). This may imply that the decreased function of adenylate cyclase in Nb2 cells allows the spontaneous increase in p21^{ras}/MAP-kinase activity. However, MAP-kinase activation by hGH in Nb2-11C cells was not altered by intracellular cyclic AMP elevating agents and cAMP act downstream p21^{ras} (Graves et al., 1993; Cook and McCormick, 1993). Thus, the high level of p21^{ras}- activity in Nb2-SP cannot be explained by this rationale and appears to be unrelated to the reduced function of adenylate cyclase in these cells.

In conclusion, our data suggest that the p21^{ras}/MAP-kinase signaling pathway is an essential entity of the proliferative machinery being triggered by hGH, in the lactogen-dependent and autonomously activated in the lactogen-independent cell lines. It is also suggested that this signaling pathway acts in concert with additional signaling pathways, and by no means sufficient alone, to promote proliferation. Further studies are required to identify the additional upstream elements, participating in prolactin receptor-dependent mitogenesis.

Materials and Methods

Materials

Recombinant hGH was a gift from General Biotechnology (Rehovot, Israel). Bovine growth hormone (bGH), ovine prolactin (oPRL), and rat growth hormone (rGH) were obtained from the National Pituitary Program (located at the University of Maryland, School of Medicine). Bovine placental lactogen (bPL) was kindly provided by Monsanto (St. Louis, MO). The anti-p21^{ras} rat monoclonal antibody, Y13-259, and rabbit anti-MAP kinase polyclonal antibody (#7884) were donated by G. Mills (Toronto General Hospital) and R. Seger (Weizmann Institute of Science, Rehovot, Israel). [α-^{32}P]GTP, ECL Western blot detection system, and protein A conjugated with horseradish peroxidase were purchased from Amersham (Buckinghamshire, UK), 12-O-tetradecanoylphorbol 13-acetate (TPA), and goat antirat antibody coupled to agarose were purchased from Sigma (St. Louis, MO). Streptolysin O was purchased from Wellcome Diagnostics (Greenville, NC). All other chemicals were of analytical grade.

Nb2 Lymphoma Cell Culture

Nb2 lymphoma cell lines were cultured as described elsewhere in RPMI-1640 medium supplemented with 5% (v/v) fetal calf serum for Nb2-11C cells or 5% (v/v) lactogen-free horse serum for Nb2-SP cells. Synchronization of the Nb2-11C cells was performed in medium containing horse serum for 20 h (Elberg et al., 1990).

p21^{ras} Assay

The assay was performed as a modification of the method described by Downward et al. (1990a). 5–8 × 10^7 synchronized Nb2-11C or Nb2-SP cells maintained in 0.5 mL phosphate buffered saline containing 5 μM $MgCl_2$ and 10 μM $CaCl_2$ were treated at 37°C with hGH or TPA at concentrations and lengths of time specified in legends. Subsequent steps were performed at 4°C. Cell permeabilization and labeling was carried out using streptolysin O (0.8 U) and [α-^{32}P]GTP 5 μCi for 30 min. Cells were then collected by centrifugation and lysed in 50 mM HEPES buffer (pH 7.4), containing 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM EGTA, and 1 mM sodium vanadate. Lysates were then treated with goat antirat IgG coupled to agarose (20 μL) and centrifuged at 15,000g. The supernatant was diluted twice with solution containing 150 mM NaCl, 0.5% (w/v), deoxycholate, and 0.05% (w/v) SDS. Immunoprecipitation of p21^{ras} was performed by adding anti-p21^{ras} rat monoclonal antibody (Y13-259, 10 μg/mL) or normal rat IgG as control for 1 h. Goat antirat IgG coupled to agarose was then added. The precipitates were washed five times with 50 mM HEPES buffer (pH 7.5) containing 0.5M NaCl, 5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, and 0.005% (w/v) SDS. Nucleotides were eluted at 70°C for 20 min with buffer containing 2 mM EDTA, 5 mM dithiothreitol, 1 mM GDP, 1 mM GTP, and 0.2% (w/v) SDS. Nucleotides were resolved by thin-layer chromatography on polyethyleneimine-cellulose plates, developed in 1M KH_2PO_4 (pH 3.4). Plates were autoradiographed for 1–2 wk.

Determination of MAP Kinase Activity

Nb2-lymphoma cells (about 10^6 cells/treatment) were frozen at –70°C, thawed and homogenized in 50 μL 50 mM β-glycerophosphate buffer (pH 7.3) containing 1.5 mM

EGTA, 1 m*M* EDTA, 1 m*M* dithiothreitol, 0.1 m*M* sodium vanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin A. The homogenates were centrifuged at 15,000*g* for 20 min, and the supernatants (cytosolic extracts) were supplemented with fivefold concentrated sample buffer (300 m*M* Tris-HCl, pH 6.8, 10% [w/v] SDS, 25% [v/v] glycerol, and 0.025% [w/v] pyronine y). A gel shift assay was performed by applying the cytosolic extract on SDS-PAGE according to Laemmli (1970) using polyacrylamide (10% w/v) and bisacrylamide (0.1% w/v) to obtain optimal separation of the phosphorylated and nonphosphorylated forms of MAP-kinase. The proteins were transferred onto nitrocellulose membranes (Towbin et al., 1979) and reacted with rabbit polyclonal antibodies to MAP-kinase (dilution 1:2000). The enzyme was detected with ECL kit according to the manufacturer's instructions.

Densitometric Analysis

Western blot and thin-layer chromatography autoradiograms were quantitated by densitometric analysis (Computing Densitometer, Molecular Dynamics, Model 300A; Eugene, OR). The activation of p21[ras] and MAP kinase in Nb2-11C cells with TPA or hGH was compared to basal values.

Acknowledgments

We gratefully thank R. Seger from The Weizmann Institute of Science (Rehovot, Israel) for the generous gift of rabbit anti-MAP-kinase polyclonal antibodies, A. Levanon from Biotechnology General (Rehovot, Israel) for hGH, R. J. Collier and J. Byatt from Monsanto Co. (St. Louis, MO) for bPL, S. Moshonov for critical reading of this manuscript, and R. Levin and M. Kopelowitz for typing the manuscript. We also thank the National Hormone Pituitary Program (University of Maryland, School of Medicine) for the gift of ovine prolactin, rat, and bovine growth hormones. Yoram Shechter is the incumbent of C. H. Hollenberg Chair in Metabolic and Diabetes Research, established by the Friends and Associates of C. H. Hollenberg of Toronto, Canada. Gerard Elberg is a recipient of the Levi Eshkol Postdoctoral Fellowship from the Ministry of Science and Technology in Israel. Micha J. Rapoport is a recipient of grant No. 138-741 from the Rekanati Foundation, Tel Aviv University, Israel.

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