The mitogen-activated protein (MAP) kinase cascade can either stimulate or inhibit DNA synthesis in primary cultures of rat hepatocytes depending upon whether its activation is acute/phasic or chronic

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Bailie et al. [In Vitro Cell Dev. Biol. (1992) 28A, 621–624] reported that primary cultures of rat hepatocytes possess low affinity binding sites for nerve growth factor (NGF). NGF treatment of primary cultures of rat hepatocytes with a maximally effective concentration of NGF (20 ng/ml, 0.8 nM) caused acute phasic activation of Raf-1 and p42^{MAPkinase}, and a smaller sustained activation of B-Raf. The transient increase in Raf-1 and p42^{MAPkinase} activity returned to baseline within ~30 min. NGF treatment of hepatocytes did not induce expression of cyclin dependent kinase (cdk) inhibitor proteins, but instead stimulated cdk2 activity and increased [³H]thymidine incorporation into DNA. In contrast to hepatocytes, NGF treatment of PC12 pheochromocytoma cells caused large sustained activations of B-Raf and p42^{MAPkinase}, and a lower phasic activation of Raf-1. The sustained activations of B-Raf and p42^{MAPkinase} were for more than 5 h. Treatment of PC12 cells with NGF increased p21^{Cip1/WAF1} expression, reduced cdk2 activity and inhibited DNA synthesis, the opposite to the effects of NGF treatment of hepatocytes. However when p42^{MAPkinase} was chronically

activated in hepatocytes, via infection with an inducible oestrogen receptor-Raf-1 fusion protein, expression of p21^{Cip1/WAF1} and p16^{INK4a} cdk inhibitor proteins increased, cdk2 activity decreased, and DNA synthesis decreased. Equally, treatment of hepatocytes with 50 mM ethanol elevated the basal activity of p42^{MAPkinase} and temporally extended the ability of NGF treatment to activate p42^{MAPkinase}. Ethanol and NGF co-treatment increased expression of p21^{Cip1/WAF1} and p16^{INK4a} cdk inhibitor proteins and decreased hepatocyte DNA synthesis. These data demonstrate that NGF can cause either acute/phasic or sustained activation of the MAP kinase cascade in different cell types. Acute activation of the MAP kinase cascade correlated with increased DNA synthesis. In contrast, sustained activation of the MAP kinase cascade correlated with increased expression of cdk inhibitor proteins, a reduction in cdk activity, and an inhibition of DNA synthesis. These data suggest a general mechanism exists where acute activation of the MAP kinase cascade promotes G1 progression/S phase entry and that chronic activation of the MAP kinase cascade inhibits this process.

INTRODUCTION

Partial hepatectomy or acute dissociation by collagenase treatment and primary culture of hepatocytes triggers their entry into the cell cycle [1–10]. Maximal DNA synthesis *in vivo* occurs 12–36 h post partial hepatectomy and within 5–7 days the liver has regained its original mass [1–3]. Primary culture *in vitro* in the presence of growth factors such as insulin or epidermal growth factor (EGF) stimulates hepatocyte DNA synthesis which is maximally observed 40–70 h post isolation [1–4]. Treatment of hepatocytes, isolated from either proliferating or non-proliferating livers, with either insulin or EGF has also been shown to cause an acute phasic increase of mitogen activated protein (MAP) kinase activity with a subsequent stimulation of DNA synthesis [3]. However, little is understood about the

functions or roles of signalling by the MAP kinase cascade during the proliferative response of hepatocytes either *in vitro* or *in vivo*, nor are the mechanisms understood by which hepatocytes senesce at the end of the liver-regenerative process *in vivo*. Indeed, the role of signalling by the MAP kinase cascade in terms of either cellular proliferation or cellular differentiation is currently under intensive study and recent reports using other cell types have suggested a role for chronic MAP kinase activation in causing exit from the cell cycle followed by cellular differentiation [11–16].

Nerve growth factor (NGF) has been shown to mediate survival, differentiation and proliferation in various types of neurons [17–20]. In PC12 cells, NGF has been demonstrated to chronically activate the MAP kinase cascade via the B-Raf protein kinase and to induce morphological neuronal differen-

Abbreviations used: MAP kinase, mitogen activated protein kinase; JNK, c-Jun NH₂-terminal kinase; SAP kinase, stress activated protein kinase; EGF, epidermal growth factor; NGF, nerve growth factor; cdk, cyclin dependent kinase; MBP, myelin basic protein; TAM, 4-hydroxytamoxifen; DMEM, Dulbecco's modified Eagle's medium; m.o.i., multiplicity of infection.

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tiation and cell cycle exit [17–20]. Ethanol treatment of PC12 cells has been shown capable of inducing morphological neuronal differentiation and cell cycle exit, and data by Roivainen et al. demonstrated that ethanol treatment of PC12 cells enhanced neuronal differentiation via activation of the MAP kinase cascade [21,22]. Treatment of hepatocytes either *in vitro* or *in vivo* with low concentrations of ethanol also inhibits DNA synthesis and liver regeneration, although the mechanism(s) by which this occurs are unknown [23,24]. Surprisingly, several studies have shown that NGF can also mediate effects in non-neuronal cells, suggesting other potential roles for this factor [25–28]. For example, Bailie et al. reported that primary cultures of hepatocytes possess binding sites for NGF, but these investigators did not determine whether these receptors were functional [26]. Because of this, we initially tested the ability of NGF to cause activation of the MAP kinase cascade in primary cultures of rat hepatocytes. Furthermore, since NGF has previously been shown to cause chronic activation of the MAP kinase cascade and inhibit proliferation in PC12 cells, we also tested whether chronic activation of the MAP kinase cascade in primary cultures of hepatocytes could cause an inhibition of DNA synthesis in these cells.

The studies reported herein demonstrate that NGF causes an acute phasic activation of the MAP kinase cascade in primary cultures of rat hepatocytes. In primary cultures of hepatocytes Raf-1 was potentially activated by NGF, whereas B-Raf was activated to a much lower extent. In both PC12 cells and primary cultures of hepatocytes acute phasic activation of the MAP kinase cascade was found to stimulate DNA synthesis, whereas chronic activation of the MAP kinase cascade in both cell types was found to inhibit DNA synthesis. Inhibition of DNA synthesis in both PC12 cells and primary cultures of hepatocytes correlated with a reduction in cyclin dependent kinase (cdk)2 activity and an increased expression of the cdk inhibitor proteins p21^{Cip-1}, and p21^{Cip-1} and p16^{INK4a} in these cells, respectively. These data suggest that a general mechanism exists whereby acute phasic activation of the MAP kinase cascade promotes DNA synthesis, while chronic activation of MAP kinase signalling inhibits G1 progression/S phase entry.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats (200 g) had access to food and water *ad libitum* [4]. Anti-Raf-1 (sc-133AC) and anti-B-Raf (sc-166-G) COOH-terminal antibodies, anti-cdk2 (sc-163), anti-p15^{INK4b} (sc-1429), anti-p16^{INK4a} (sc-1207), anti-p21^{Cip-1} (sc-397-G) and anti-p27^{Kip-1} (sc-527-G) antibodies were from Santa Cruz Biotechnology (Santa Cruz Biotechnologies, CA, U.S.A.), as were anti-Cyclin A-(sc-596), D-(sc-753) and E-(sc-481). Anti-p42^{MAPkinase} antibodies were a kind gift from Dr. M. J. Weber (University of Virginia, Charlottesville, VA, U.S.A.). Nerve growth factor (2.5S) was from Gibco-BRL. Radiolabelled [γ -³²P]ATP and [³H]thymidine were from NEN. Glutathione-S-Transferase (GST)-c-Jun (aal-169) was synthesized in *E. coli* and purified on Glutathione–Sepharose. Protein preparations of other reagents were as in [4]. The specific MEK1 activation inhibitor PD98059 [29] was from Calbiochem.

Methods

Recombinant adenoviral vectors; generation and infection *in vitro*

Studies herein are performed using two adenoviral technologies. First, replication defective adenovirus is conjugated to poly-L-

lysine as described in [30,31]. The DNA conjugated virus is added to hepatocytes at a multiplicity of infection (m.o.i.) of 250, and the cells incubated for 4 h at 37 °C. The cells are washed with media to remove virus. Cells express transduced gene products 10–24 h after infection. Using a plasmid to express β -galactosidase under control of the CMV-promoter, we determined that 1 ng of plasmid conjugated to virus particles and infected into hepatocytes prior to plating at an m.o.i. of 250 gave 100 % infection as judged by blue coloration after 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) incubation 24 h post infection. Second, we have generated recombinant adenoviruses using a novel methodology developed by Dr. Matthias Paul Wymann. In this procedure the full length recombinant adenovirus genome is cloned in a plasmid, flanked by a rare cutter (*PacI*) restriction site, and recombinant virus DNA is generated using a recombination proficient *E. coli* strain (BJ5183) [32]. To assess the effectiveness of recombinant adenoviral infection, we generated a recombinant adenovirus containing the gene for β -galactosidase. Primary cultured hepatocytes were infected with this virus immediately after isolation *in vitro* (m.o.i. 250), and incubated at 37 °C for a further 24 h; cells were fixed and incubated with X-Gal. An m.o.i. of 250 gave 100 % infection after 24 h infection. Similar data were obtained when PC12 cells were infected with the β -galactosidase virus. To assess infection of all constructs used in this manuscript we performed Western immunoblots at 10–24 h after infection. Gene products were expressed ~ 18 h after infection.

Preparation of hepatocytes

Rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the lower thorax and abdomen shaved to remove fur. A small (3 cm) vertical mid-line incision was made in the abdominal wall from just below the costal margin/xiphoid process. Hepatocytes were prepared by cannulation of the portal vein and collagenase perfusion of the liver as described [4]. Hepatocytes were placed into primary culture at 2×10^5 cells per well of a 12 well plate cultured in Dulbecco's modified Eagle's medium (DMEM).

Primary culture, hormonal treatment, and assay for DNA synthesis in cultures of hepatocytes

Hepatocytes were cultured on rat-tail collagen (Vitrogen)-coated plastic dishes (12 $\times$ 20 mm, 2×10^5 cells) in 1 ml DMEM containing the supplement in 5 % (v/v) CO₂; [100 nM Insulin, 1 nM dexamethasone, 1 nM thyroxine], and allowed to adhere to the dish. In 'ΔRaf-1:ER' adenoviral infection experiments media did not contain phenol red indicator. At this time, cells were infected with various adenoviruses as dependent upon the experiment. For cells undergoing acute assay, treatments occurred 90 min after plating. For adenovirus infected cells, after 4 h of infection media was replaced and hepatocytes cultured in the same supplemented DMEM for 24 h. Hormonal treatments, and/or protein kinase inhibitors were added 24 h after the media change (protein kinase inhibitors were added 30 min prior to further treatment). For protein kinase assays, hormones were added to give final specified concentrations and incubated for the specified times at 37 °C in a cell culture incubator. Plated cells were aspirated 20 s prior to termination followed by immediate homogenization. Cells were homogenized in 1 ml ice cold homogenization buffer A [25 mM Hepes, pH 7.4 at 4 °C, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM phenylmethyl sulphonylfluoride, 1 mg/ml soybean trypsin inhibitor, 40 μ g/ml pepstatin A, 1 μ M Microcystin-LR, 0.5 mM sodium vanadate, 0.5 mM sodium pyrophosphate, 0.05 % (w/v) sodium deoxy-

cholate, 1 % (v/v) Triton X-100, 0.1 % (v/v) 2-mercaptoethanol], with trituration using a P1000 pipette to lyse the cells. Homogenates were stored on ice prior to clarification by centrifugation (4 °C), and clarified aliquots subjected to immunoprecipitation. For DNA synthesis assays, cells were cultured in the presence of 2 μ Ci [3 H]thymidine for a further 24 h or 30 h, after which time cells were lysed with 0.5 M NaOH and DNA precipitated with 12.5 % (w/v) TCA (final). Acid precipitable material was transferred to glass fibre filters, washed with 5 % (w/v) trichloroacetic acid, and [3 H]thymidine incorporation into DNA quantified by liquid scintillation spectrometry [4].

Immunoprecipitations from homogenates

Fifty microlitres of Protein A agarose (Ag) slurry (25 μ l bead volume) were washed twice with 1 ml PBS containing 0.1 % (v/v) Tween 20, and resuspended in 0.1 ml of the same buffer. Antibodies (2 μ g, 20 μ l) or serum (20 μ l) were added to each tube and incubated (3 h, 4 °C). Clarified hepatocyte homogenates (0.5 ml, 1 mg total protein) were mixed with Protein A-Ag-conjugated antibody in duplicate using gentle agitation (2.5 h, 4 °C). Protein A-Ag was recovered by centrifugation, the supernatant discarded, and washed (10 min) sequentially with 0.5 ml buffer A (twice), PBS and buffer B [25 mM Hepes, pH 7.4, 15 mM MgCl₂, 0.1 mM Na₃VO₄, 0.1 % (v/v) 2-mercaptoethanol.

Assays of Raf-1 and B-Raf activity

Assays for Raf-1 and B-Raf activity were primarily performed using 32 P-incorporation into (His)₆-MEK1 [4]. Identical results were obtained in a single experiment measuring Raf-1 and B-Raf activities in a coupled assay measuring stimulated (His)₆-MEK1 activity using kinase inactive K52R p42^{MAPkinase} as described in [4]. 32 P-incorporation in (His)₆-MEK1 or K52R p42^{MAPkinase} protein bands after SDS/PAGE was quantified by liquid scintillation spectroscopy.

Assay of p42^{MAPkinase} activity

Immunoprecipitates were incubated (final volume 50 μ l) with 50 μ l of buffer B containing 0.2 mM [γ - 32 P]ATP (5000 c.p.m./pmol), 1 μ M Microcystin-LR, 0.5 mg/ml myelin basic protein (MBP), which initiated reactions. After 20 min, 40 μ l of the reaction mixtures were spotted onto a 2 cm circle of P81 phosphocellulose paper (Whatman, Maidstone, U.K.) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid, and once with acetone, and 32 P-incorporation into MBP was quantified by liquid scintillation spectroscopy. Pre-immune controls were performed to ensure MBP phosphorylation was dependent upon specific immunoprecipitation of p42^{MAPkinase} [4].

Assay of cdk2 activity

Cdk2 activity was measured in immunoprecipitates of whole cell lysates. Approx. 100 μ g of cellular protein from whole cell homogenates (prepared as described above) was incubated sequentially with 1 μ g purified anti-cdk2 IgG, followed by 2 μ g biotinylated goat anti-rabbit IgG (Molecular probes, Eugene, OR, U.S.A.) and finally streptavidin-magnetspheres (Promega, Madison, WI, U.S.A.) on ice in 200 μ l for at least 2 h each. Immunocomplexes were washed three times with [30 mM Hepes pH 7.4, 15 mM MgCl₂, 80 mM β -glycerophosphate, 2.6 mM EGTA, 1 μ g/ml each chymostatin, leupeptin, antipain, pepstatin and soybean trypsin inhibitor, 0.5 % (v/v) NP-40 and then resuspended in 35 μ l of the same buffer without NP-40. Protein

kinase assays were conducted by assaying 10 μ l of these immunoprecipitated samples in triplicate in a total of 25 μ l containing final concentrations of [30 mM Hepes pH 7.4, 0.1 mM DTT, 25 mM MgCl₂, 30 mM β -glycerophosphate, 0.5 μ M protein kinase A inhibitor peptide (TTTADFIAGRRNAIHD), 0.02 mM [γ - 32 P]ATP (5000 c.p.m./pmol), 2 mM EGTA, and 0.5 mg/ml Histone H1. After 5–15 min of incubation at 32 °C, assays were stopped by pipetting 20 μ l of the reaction mixture onto P81 papers as per the MAP kinase assay above, and 32 P-incorporation into Histone H1 was quantified by liquid scintillation spectroscopy [33].

Assay of JNK1 activity

Immunoprecipitates were incubated (final volume 100 μ l) with 2 μ l (10 μ g) *GST-c-Jun* (aal-169), and reactions initiated with 98 μ l of buffer B containing 0.2 mM [γ - 32 P]ATP (5000 c.p.m./pmol) and 1 μ M Microcystin-LR. After 30 min, reactions were terminated with sample buffer and prepared for SDS/PAGE (10 % gel), and 32 P-incorporation into excised Coomassie blue-stained *GST-c-Jun* (aal-169) bands were quantified by liquid scintillation spectroscopy. Pre-immune control assays were performed to ensure *GST-c-Jun* (aal-169) phosphorylation was dependent upon specific immunoprecipitation of JNK1 in the assay [4].

Culture of PC12 cells

Rat pheochromocytoma (PC12) cells were cultured on poly-L-lysine coated six well plates in DMEM media supplemented with 10 % (v/v) horse serum and 5 % (w/v) fetal calf serum at 37 °C in 5 % (v/v) CO₂. PC12 cells were subjected to adenoviral infections, and the media changed 4 h after infection, and cells cultured for a further 24 h before addition of NGF. Protein kinase inhibitors were added 30 min prior to addition of 20 ng/ml NGF. For protein kinase assays, reactions were terminated after aspiration of media by ice cold homogenization buffer A and samples prepared for immunoprecipitation as described for hepatocytes. For DNA synthesis assays, DMEM media was also supplemented with 2 μ Ci of [3 H]thymidine. Cells were cultured for a further 24 h, after which time cells were lysed with 0.5 M NaOH and DNA precipitated with 12.5 % (w/v) TCA (final). Acid precipitable material was transferred to glass fibre filters, washed with 5 % (w/v) TCA, and [3 H]thymidine incorporation into DNA quantified by liquid scintillation spectrometry [34].

Data analysis

The effects of various hormone treatments were compared using one-way analysis of variance and a two-tailed *t* test. Differences with a *P*-value of < 0.05 were considered statistically significant. Results shown are either the mean of six individual points within the same experiment $\pm$ S.E.M. (hepatocytes from a single liver), or are the means of multiple separate experiments $\pm$ S.E.M. (hepatocytes from multiple livers), themselves performed at least in duplicate.

RESULTS

NGF treatment chronically activates the MAP kinase cascade in PC12 cells and acutely activates the MAP kinase cascade in primary cultures of hepatocytes

Treatment of PC12 cells with NGF induced prolonged (> 5 h) activations of B-Raf and p42^{MAPkinase} and a lower phasic activation of Raf-1 as judged by 32 P-incorporation into MEK1 in

Figure 1 Activation of the MAP kinase cascade by NGF in PC12 cells

(A) Time course of activation of Raf-1 (●) and B-Raf (○) in PC12 cells by NGF treatment. (B) Time course of activation of p42^{MAPkinase} in PC12 cells by either NGF (●) or EGF (○) treatments. PC12 cells were cultured as in Methods. Cells were treated for the indicated times with either vehicle control (PBS), 20 ng/ml NGF or 2 ng/ml EGF, lysed with ice cold homogenization buffer, and subjected to immunoprecipitation as described in Methods. Raf-1 and B-Raf activities were measured in duplicate immune-complex kinase assays versus bacterial recombinant (His)₆-MEK1. Data are expressed as -fold activation over basal activity; Raf-1 4950 ± 545 c.p.m.; B-Raf 2560 ± 205 c.p.m. Activity of p42^{MAPkinase} was measured in duplicate immune-complex kinase assays versus bovine myelin basic protein (MBP). Data are expressed as -fold activation over basal activity; p42^{MAPkinase} 22 445 ± 915 c.p.m. Data are a representative of three experiments using hepatocytes isolated from three different livers.

an immune-complex kinase assay (Figure 1). Identical data were obtained measuring Raf-1/B-Raf activities using stimulated MEK1 activity versus kinase inactive K52R p42^{MAPkinase} (data not shown). In contrast to NGF treatment, epidermal growth factor (EGF) treatment of PC12 cells induced an acute phasic activation of p42^{MAPkinase} which returned to baseline within 30 min (Figure 1B). These data are in agreement with data of several other laboratories [16,21,22,34,35]. Bailie et al. reported that primary cultures of hepatocytes possess binding sites for NGF, and we investigated whether NGF had a similar capability to modulate the MAP kinase cascade in these cells [26].

Treatment of primary cultures of hepatocytes with NGF activated the MAP kinase cascade (Figure 2). In contrast to NGF treatment of PC12 cells, NGF treatment of hepatocytes

caused acute phasic activations of Raf-1 and p42^{MAPkinase}, whereas B-Raf was activated to a much lesser extent (Figures 2A and 2B). Identical data were obtained measuring Raf-1/B-Raf activities using stimulated MEK1 activity versus kinase inactive K52R p42^{MAPkinase} (data not shown). Treatment of hepatocytes with EGF also induced an acute phasic activation of the MAP kinase cascade (Figure 2B), in agreement with data in [36]. In dose-response studies, maximal activation of the MAP kinase cascade in primary cultures of hepatocytes by NGF treatment occurred at 10–20 ng/ml (~0.8 nM) with a half maximal activation at ~3 ng/ml (~0.25 nM) (Figure 2C). Analysis of the NGF dose-response/MAP kinase activation curve suggested that a single population of receptors was present, in agreement with data of Bailie et al. [26] examining the binding of radiolabelled NGF to its plasma membrane receptor in these cells (data not shown). In neither PC12 cells nor hepatocytes did NGF treatment activate JNK1 (data not shown).

NGF inhibits DNA synthesis in PC12 cells and stimulates DNA synthesis in primary cultures of hepatocytes

We next investigated the abilities of NGF and EGF to stimulate or inhibit DNA synthesis in both PC12 cells and primary cultures of hepatocytes (Table 1). NGF inhibited the ability of PC12 cells to synthesize DNA, which was partly reversed either by the specific inhibitor of MEK1 activation (PD98059, 50 µM) or by infection of these cells with a recombinant adenovirus to express dominant negative MEK1 (Table 1). Both treatments reduced the ability of NGF to activate p42^{MAPkinase} by ~80% (data not shown). EGF treatment of PC12 cells stimulated DNA synthesis, in agreement with data of others [34]. In marked contrast to our observations in PC12 cells, NGF treatment of hepatocytes stimulated the ability of these cells to synthesize DNA, which was also blocked by either PD98059 treatment or by expression of dominant negative MEK1 (Table 1). In a parallel experiment, EGF treatment also stimulated the ability of primary cultures of hepatocytes to synthesize DNA, in agreement with data of others [3].

Thus NGF treatment of PC12 cells causes a chronic elevation of MAP kinase activity and leads to a reduction in DNA synthesis, whereas EGF treatment of PC12 cells causes an acute phasic activation of the MAP kinase cascade and stimulates DNA synthesis. Similarly, either NGF or EGF treatment of primary cultures of hepatocytes causes an acute phasic activation of the MAP kinase cascade and stimulates DNA synthesis. These data suggest that acute phasic activation of the MAP kinase cascade signals an increase DNA synthesis, whereas chronic activation of the MAP kinase cascade signals a reduction of DNA synthesis. In addition, and of particular note, PD98059 treatment and expression of dominant negative MEK1 both caused a significant reduction in basal DNA synthesis in PC12 cells but not in hepatocytes, suggesting basal MAP kinase activity may play a greater role in maintaining basal DNA synthesis in established cultures of PC12 cells versus primary cultures of hepatocytes. We next investigated potential downstream mechanisms by which chronic activation of the MAP kinase cascade may inhibit DNA synthesis.

NGF induces p21^{Cip-1/WAF1} in PC12 cells, but not in hepatocytes, via a p42^{MAPkinase} dependent mechanism

Several studies have suggested that chronic activation of protein kinase C and the MAP kinase cascade can lead to increased expression of cyclin dependent kinase (cdk) inhibitor proteins, in particular p21^{Cip-1} and p27^{Kip-1} [11,12]. To examine further the mechanism(s) by which NGF inhibits DNA synthesis in PC12

Figure 2 Activation of the MAP kinase cascade by NGF in primary cultures of hepatocytes

(A) Time course of activation of Raf-1 (○) and B-Raf (●) in primary cultures of rat hepatocytes by NGF treatment. (B) Time course of activation of p42^{MAPkinase} in primary cultures of rat hepatocytes by either NGF (○) or EGF (●) treatments. Primary cultures of rat hepatocytes were cultured as in Methods. Cells were treated for the indicated times with either vehicle control (PBS), 20 ng/ml NGF or 2 ng/ml EGF, lysed with ice cold homogenization buffer, and subjected to immunoprecipitation as described in Methods. Raf-1 and B-Raf activities were measured in duplicate immune-complex kinase assays versus bacterial recombinant (His)₆-MEK1. Data are expressed as -fold activation over basal activity; Raf-1 2550 ± 200 c.p.m.; B-Raf 1200 ± 155 c.p.m. Activity of p42^{MAPkinase} was measured in duplicate immune-complex

cells and promotes DNA synthesis in hepatocytes, we immunoblotted NGF-treated cells to examine the expression of the cdk inhibitor proteins p15^{INK4b}, p16^{INK4a}, p21^{Cip-1/WAF1}, and p27^{Kip-1} (Figure 3). NGF treatment of PC12 cells induced p21^{Cip-1/WAF1} expression after 24 h, but did not increase expression of p27^{Kip-1} (Figure 3). We could not detect by immunoblotting either p15^{INK4b} or p16^{INK4a} in PC12 cells under any of the conditions used for these studies (data not shown). NGF treatment also did not significantly alter immunoblotting levels of either cyclins A, D or E (data not shown). Induction of p21^{Cip-1/WAF1} by NGF was dependent upon activation of the MAP kinase cascade as judged by the abilities of both PD98059 and dominant negative MEK1 to block induction (Figure 3, and data not shown). Treatment of PC12 cells with PD98059 alone also decreased basal p21^{Cip-1/WAF1} expression, suggesting that under basal conditions, MAP kinase signalling can directly modulate p21^{Cip-1/WAF1} expression. In contrast to data in PC12 cells, NGF treatment of primary cultures of hepatocytes did not induce p21^{Cip-1/WAF1} (Figure 3). In general agreement with these data and those in Table 1, treatment of PC12 cells with NGF inhibited cdk2 activity by 39 ± 5 %, whereas NGF treatment of hepatocytes caused a stimulation in cdk2 activity by 31 ± 3 % (± S.E.M.; n = 3, in agreement with data in [11,12]). Since chronic activation of the MAP kinase cascade inhibited DNA synthesis in PC12 cells, we next investigated whether a similar mechanism existed in primary cultures of hepatocytes.

Chronic activation of the MAP kinase cascade in primary cultures of hepatocytes by 'ΔRaf-1:ER' induces p21^{Cip-1/WAF1} and p16^{INK4a} expression and inhibits DNA synthesis

NGF treatment of primary cultures of hepatocytes caused an acute phasic activation of the MAP kinase cascade (Figure 2), and did not induce p21^{Cip-1/WAF1} expression (Figure 3). To mimic in hepatocytes the effect of chronic MAP kinase cascade activation as induced by NGF in PC12 cells, hepatocytes were infected with a poly-L-lysine conjugated adenovirus to express a modified oestrogen receptor-Raf-1 ('ΔRaf-1:ER') fusion protein, which has been shown in fibroblasts to be regulatable by oestradiol treatment [11,13]. The oestrogen receptor portion of our constructs contains a modified form of the hormone binding domain of the oestrogen receptor that has become non-responsive to oestradiol, and is now activated by the anti-oestrogen, 4-hydroxy-tamoxifen. Activation of the receptor portion of the construct by 4-hydroxytamoxifen alters the conformation of the whole protein, and results in activation of the 'Raf' protein kinase domain, which in turn leads to activation of the MAP kinase cascade. Control experiments used a form of 'ΔRaf-1:ER' (ΔRaf-1:ER 301) which had the active site lysine mutated to a methionine and was thus kinase inactive.

Hepatocytes were treated for 6 h with or without 50 nM 4-hydroxytamoxifen (TAM) 24 h after infection with either the 'ΔRaf-1:ER' or 'ΔRaf-1:ER 301' construct, and the ability of

kinase assays versus bovine MBP. Data are expressed as -fold activation over basal activity; p42^{MAPkinase} 6100 ± 290 c.p.m. Data are a representative of three experiments using hepatocytes isolated from three different livers. (C) Dose response activation curve of p42^{MAPkinase} by NGF treatment in primary cultures of rat hepatocytes. Primary cultures of rat hepatocytes were cultured as in Methods. Cells were treated for 5 min with 0, 0.2, 0.4, 0.8, 3.0, 10.0, 30.0 and 100 ng/ml NGF, lysed with ice cold homogenization buffer, and subjected to immunoprecipitation as described in Methods. Activity of p42^{MAPkinase} was measured in duplicate immune-complex kinase assays versus bovine MBP. Data shown are means of hexatuplicates ± S.E.M. from a single representative experiment. Identical data were obtained in two further experiments using hepatocytes isolated from different livers.

Table 1 Nerve growth factor treatment inhibits DNA synthesis in PC12 cells and stimulates DNA synthesis in primary cultures of hepatocytes, which is blocked in both cell types by inhibition of the MAP kinase cascade

PC12 cells and hepatocytes were infected with either control recombinant replication adenovirus or with a recombinant replication defective adenovirus to express a *CMV* promoter driven dominant negative MEK1 24 h prior to hormonal treatment [45]. A portion of the control virus infected PC12 cells and hepatocytes was treated with either vehicle (DMSO) or 50 μ M PD98059 30 min prior to hormonal addition. Cells were treated with vehicle control (PBS), NGF (20 ng/ml) or EGF (2 ng/ml), and 2 μ Ci [3 H]thymidine added to the media. Cells were cultured for a further 24 h after which they were lysed and [3 H]thymidine incorporation into DNA determined as in Methods. Data are the means of [3 H]thymidine incorporation into DNA from hepatocytes isolated from eight livers/eight separate passages of PC12 cells, each individual experiment being performed in quadruplicate. $P < 0.05$ compared to: * decrease from control value; † increase from control value; ‡ increase from NGF treated PC12 cells; § decrease from value treated with NGF alone.

3 H]thymidine incorporation into DNA (c.p.m.)	Control	NGF	EGF	NGF + PD	NGF + MEK1 ⁻	PD	MEK1 ⁻
PC12 cells	5170 $\pm$ 380	2240 $\pm$ 310*	9810 $\pm$ 980†	3810 $\pm$ 400‡	3200 $\pm$ 205‡	2400 $\pm$ 360*	3200 $\pm$ 200*
Hepatocytes	3330 $\pm$ 290	6800 $\pm$ 550†	7225 $\pm$ 800†	3670 $\pm$ 525§	3310 $\pm$ 400§	2975 $\pm$ 260	3700 $\pm$ 420

Figure 3 NGF treatment induces p21^{Cip-1} expression in PC12 cells, but not in primary cultures of hepatocytes, in which it is blocked by the MEK1 inhibitor PD98059

PC12 cells and hepatocytes were cultured as described in Methods. Thirty minutes prior to NGF treatment, a portion of the PC12 cells was incubated with either vehicle control (DMSO) or 50 μ M PD98059. Cells were treated with 20 ng/ml NGF and cultured for a further 24 h after which media was removed and the cells snap frozen. Cells were thawed/lysed with 100 μ l 5 $\times$ SDS/PAGE sample buffer, diluted to 250 μ l with distilled water, and placed in a 100 $^{\circ}$ C dry bath for 15 min. One hundred microlitre aliquots of each time point were subjected to SDS/PAGE on 14% gels. Gels were transferred to nitrocellulose and Western blotting versus anti-p21^{Cip-1} or anti-p27^{Kip-1} performed as in Methods (a representative experiment from determinations made on three separate livers and three separate passages of PC12 cells). For PC12 cells versus anti-p21^{Cip-1/WAF1} treatments were Lane 1, control; Lane 2, NGF; Lane 3, NGF + PD98059; Lane 4, PD98059. For PC12 cells versus anti-p27^{Kip-1} treatments were Lane 5, control; Lane 6, NGF. For primary cultures of hepatocytes versus p21^{Cip-1/WAF1} treatments were Lane 1, control; Lane 2, NGF.

these cells to activate the MAP kinase cascade, to induce expression of p15^{INK4b}, p16^{INK4a}, p21^{Cip-1/WAF1} or p27^{Kip-1}, to alter cdk2 activity, or to synthesize DNA was examined after a further 24 h of culture (Figure 4). Treatment of ‘ Δ Raf-1:ER’ infected hepatocytes with TAM for 6 h caused chronic activation of the MAP kinase cascade for up to 24 h (Figure 4A) and induced expression of both p21^{Cip-1/WAF1} and of a more novel nature p16^{INK4a}, but did not induce expression of either p15^{INK4b} or p27^{Kip-1} (Figure 4B). No activation of the MAP kinase cascade nor induction of p21^{Cip-1/WAF1} and p16^{INK4a} was observed in ‘ Δ Raf-1:ER 301’ control cells (Figures 4A and 4B). Inhibition of ‘ Δ Raf-1:ER’/MAP kinase cascade signalling in hepatocytes by either PD98059 treatment or expression of dominant negative MEK1 blocked the expression of both p21^{Cip-1/WAF1} and p16^{INK4a} (Figure 4B), as was previously observed for NGF signalling via the MAP kinase cascade in PC12 cells (Figure 3). Co-expression

of dominant negative p53 (R175H) in Δ Raf-1:ER+TAM hepatocytes did not block the ability of chronic MAP kinase signalling to increase p21^{Cip-1} protein levels (data not shown). Since little p21^{Cip-1/WAF1} was expressed in primary cultures of hepatocytes it was not possible to determine whether PD98059 treatment also reduced basal p21^{Cip-1/WAF1} expression in hepatocytes. Treatment of ‘ Δ Raf-1:ER’ infected hepatocytes with TAM had reduced cdk2 activity after 24 h by 64% as judged by 32 P-incorporation into Histone H1 from 27353 $\pm$ 598 c.p.m. to 9962 $\pm$ 189 c.p.m. ($n = 3 \pm$ S.E.M.) and caused a reduction in hepatocyte DNA synthesis (Table 2). In contrast, treatment of ‘ Δ Raf-1:ER 301’ hepatocytes with TAM did not reduce cdk2 activity (32 P-incorporation into Histone H1 was 24355 $\pm$ 610 c.p.m. in untreated and 22681 $\pm$ 909 c.p.m. in treated). Treatment of ‘ Δ Raf-1:ER’ infected hepatocytes with TAM also increased the amount of p21^{Cip-1} co-immunoprecipitating with cdk2 in these cells (Figure 4C). TAM treatment of ‘ Δ Raf-1:ER’ hepatocytes did not alter immunoblottable levels of either cyclins A, D or E, which was similar to that observed for NGF treatment of PC12 cells (data not shown). Together, the data presented in Figures 2–4 strongly suggest that prolonged activation of the MAP kinase cascade in both PC12 cells and in primary cultures of hepatocytes inhibits DNA synthesis by differential increases in the expression of multiple cdk inhibitor proteins.

To confirm by an independent methodology that chronic activation of the MAP kinase cascade inhibits DNA synthesis in primary cultures of hepatocytes, we examined the ability of ethanol to modify the ability of NGF to signal through the MAP kinase cascade in these cells (Figure 5). Several studies have demonstrated that chronic elevation of plasma ethanol levels into the $\sim$ 50 mM range inhibits hepatocyte proliferation/liver regeneration *in vivo* [23,24]. Furthermore, ethanol treatment of PC12 cells has been shown to inhibit DNA synthesis and to potentiate the ability of NGF to increase neurite outgrowth, which were shown to be dependent upon signalling via the MAP kinase cascade [21,22]. Other workers examining NGF function have shown that neurite outgrowth in PC12 cells caused by treatment with this factor is dependent upon signalling via the MAP kinase cascade, which we have confirmed (data not shown) [36]. These data suggested that ethanol treatment may be able to enhance growth factor signalling through the MAP kinase cascade.

Treatment of hepatocytes with 50 mM ethanol caused a $\sim$ 1.5-fold increase in the basal activity of p42^{MAPkinase} and dramatically potentiated the ability of NGF treatment to cause chronic activation of the MAP kinase cascade (Figure 5A). In agreement

Figure 4 Chronic activation of the MAP kinase cascade by Δ Raf-1:ER increases protein levels of p21^{Cip-1} and p16^{INK4a}, and inhibits cdk2 activity

(A) Isolated hepatocytes were infected with an SV40 promoter driven Δ Raf-1:ER plasmid conjugated via poly-L-lysine to a replication defective adenovirus (250 m.o.i.) and cultured for 24 h as in Methods [1,46]. As a control, identical aliquots of hepatocytes were infected in a similar manner using an SV40 promoter driven plasmid to express a kinase-inactive form of Δ Raf-1:ER (301 kinase inactive mutant). After 24 h hepatocytes were treated with 50 nM TAM for 6 h before removal of drug, washing of the cells with warm media, and further culture for another 18 h. The activity of p42^{MAPkinase} was determined in immune complex kinase assays immediately after TAM treatment (6 h incubation) and 24 h after start of TAM treatment and is shown as a -fold increase over basal control untreated matched cell p42^{MAPkinase} activity. At 6 h after treatment basal p42^{MAPkinase} activity in untreated Δ Raf-1:ER cells 6650 $\pm$ 290 c.p.m. and Δ Raf-1:ER (301) cells 5970 $\pm$ 410 c.p.m. At 24 h after treatment basal p42^{MAPkinase} activity in untreated Δ Raf-1:ER cells was 7440 $\pm$ 320 c.p.m. and Δ Raf-1:ER (301) cells 6205 $\pm$ 415 c.p.m. Data shown are a representative experiment (three experiments, three separate livers) showing the means of hexaplicates of p42^{MAPkinase} activity $\pm$ S.E.M. (B) Isolated hepatocytes were infected with SV40 promoter driven Δ Raf-1:ER/ Δ Raf-1:ER (301 mutant) plasmids conjugated via poly-L-lysine to a replication defective adenovirus (250 m.o.i.) as in (A). A portion of these infected cells were also co-infected with a recombinant replication defective adenovirus to express a CMV promoter driven dominant negative MEK1 (m.o.i. 250). Thirty minutes prior to TAM addition, a portion of the single infected Δ Raf-1:ER hepatocytes were treated with 50 μ M PD98059. Hepatocytes were treated with 50 nM TAM for 6 h as described above, and 24 h after TAM treatment cells were lysed and subjected to SDS/PAGE on 14% gels. Gels were transferred to nitrocellulose and Western blotting versus either anti-p21^{Cip-1} or anti-p16^{INK4a} performed as in Methods. For p21^{Cip-1/WAF1} and p16^{INK4a} immunoblots treatments were Lane 1, kinase inactive Δ Raf-1:ER 301 + TAM; Lane 2, Δ Raf-1:ER + TAM; Lane 3, Δ Raf-1:ER; Lane 4, Δ Raf-1:ER + TAM + PD98059; Lane 5, Δ Raf-1:ER + TAM + dominant negative MEK1; Lane 6, PD98059; Lane 7, dominant negative MEK1. A representative experiment is shown. Identical data were obtained in four further experiments using hepatocytes isolated from different livers. (C) A representative experiment is shown of p21^{Cip-1/WAF1} which co-immunoprecipitated with cdk2 in assays to determine cdk2 activity (see text). Identical data were obtained in two further experiments using hepatocytes isolated from different livers. SDS/PAGE and Western blotting were performed as in Methods. Treatments were Lane 1, kinase inactive Δ Raf-1:ER 301; Lane 2, kinase inactive Δ Raf-1:ER 301 + TAM; Lane 3, Δ Raf-1:ER; Lane 4, Δ Raf-1:ER + TAM.

with data presented in Figures 4 and 5, and Table 2, ethanol and NGF co-treatment increased expression of both p21^{Cip-1} and p16^{INK4a} in a MAP kinase dependent manner (Figure 5B) and reduced cdk2 activity (data not shown). However, ethanol treatment alone appeared to have little ability to increase expression of either p21^{Cip-1} or p16^{INK4a} (Figure 5B). Co-treatment of hepatocytes with ethanol and NGF reversed the ability of NGF to stimulate DNA synthesis as judged by a reduction in [³H]thymidine incorporation into DNA [Control, 3136 $\pm$ 265 c.p.m. + 20 ng/ml; NGF, 6782 $\pm$ 460 c.p.m. + 50 mM; ethanol, 2784 $\pm$ 160 c.p.m. 50 mM; ethanol + 20 ng/ml NGF, 3068 $\pm$ 320 c.p.m.]. In agreement with the data in Figure 5B showing that ethanol treatment alone did not significantly induce expression of either p21^{Cip-1} or p16^{INK4a}, we found that ethanol treatment alone did not significantly inhibit the basal level of DNA synthesis. These data again strongly argue, by an independent non-molecular method, that chronic activation of the MAP kinase cascade in hepatocytes increases expression of cdk inhibitor proteins which lead to a decrease in DNA synthesis and cell cycle exit.

DISCUSSION

Several studies have demonstrated that cells as diverse as neurons, hepatocytes, skeletal muscle, pancreatic beta cells and mast cells can either specifically bind to or respond to NGF [25–28]. This study was initially designed to test whether binding sites for NGF, reported to be present on the surface of hepatocytes by Bailie et al. [26] were functional for the activation of the MAP kinase cascade in these cells. Our studies demonstrated that NGF treatment of both PC12 cells and primary cultures of rat hepatocytes resulted in rapid activations of the MAP kinase cascade. However, and to our surprise, we discovered that, whereas NGF caused chronic stimulation of MAP kinase cascade activity in PC12 cells, NGF caused acute phasic stimulation of MAP kinase cascade activity in primary cultures of hepatocytes. NGF treatment of PC12 cells caused a strong activation of B-Raf in comparison to that of Raf-1. In contrast, NGF treatment of hepatocytes caused a strong activation of Raf-1 in comparison to that of B-Raf. The degree to which MAP kinase activity was increased by NGF treatment was also different between the two types of cell. In contrast to the chronic activation of the MAP kinase cascade induced by NGF in PC12 cells, a more modest acute activation of the MAP kinase cascade was induced in NGF treated hepatocytes. Thus the intracellular signalling components utilized by NGF and the degree to which these components are activated in response to NGF can differ depending upon the cell type being examined.

The reason why we see a difference in NGF signalling towards Raf-1/B-Raf and p42^{MAPkinase} between PC12 cells and hepatocytes is unclear. PC12 cells express both high affinity (TrkA) and low affinity (p75) NGF receptors. Data from Bailie et al. examining both the K_d and B_{max} values for NGF receptor binding in hepatocytes suggested that these cells possess only the low affinity, p75, NGF receptor [26]. Our data also suggested that the ability of NGF to activate the MAP kinase cascade in hepatocytes was due to a single low affinity receptor sub-type. Thus one possible explanation for the differences between the cell types could therefore be due to differential expression of high and low affinity Trk/NGF receptors with different signalling properties to downstream signal transduction components. This possibility has been suggested by others for the differential capabilities of the low and high affinity NGF receptors to protect cells from apoptosis [37,38]. A further possible explanation for

Table 2 Chronic activation of the MAP kinase cascade by Δ Raf-1:ER + TAM decreases DNA synthesis in primary cultures of rat hepatocytes

Isolated hepatocytes were infected with an SV40 promoter driven Δ Raf-1:ER plasmid conjugated via poly-L-lysine to a replication defective adenovirus (250 m.o.i.) and cultured for 24 h as in Methods [1,46]. As a control, identical aliquots of hepatocytes were infected in a similar manner using an SV40 promoter driven plasmid to express a kinase-inactive form of Δ Raf-1:ER (kinase inactive 301 mutant). After 24 h hepatocytes were treated with 50 nM TAM for 6 h before removal of drug, washing of the cells with warm media, and further culture for another 30 h in identical media containing 2 μ Ci [3 H]thymidine after which they were lysed and [3 H]thymidine incorporation into DNA determined as in Methods. Data are the means of [3 H]thymidine incorporation into DNA from hepatocytes isolated from eight livers, each individual experiment being performed in quadruplicate. $P < 0.05$ compared to * decrease from control treatment value.

	Δ Raf-1:ER (301)	Δ Raf-1:ER (301) + TAM	Δ Raf-1:ER	Δ Raf-1:ER + TAM
[3 H]thymidine (c.p.m.) incorporation into hepatocyte DNA	3520 $\pm$ 470	3110 $\pm$ 150	3680 $\pm$ 230	1910 $\pm$ 320*

the difference could be due to the relative expression levels of downstream signalling components within PC12 cells and hepatocytes. For example, Western blotting analysis of PC12 cells and hepatocytes demonstrates that expression of B-Raf versus Raf-1 is much lower in hepatocytes than in PC12 cells, and in PC12 cells the predominant 'Raf' kinase activated after NGF treatment is B-Raf and not Raf-1 (data not shown, Figure 1A). Thus the differential ability of NGF to activate Raf-1 versus B-Raf or p42^{MAPkinase} in PC12 cells versus hepatocytes may be due to the relative protein levels of the receptor-proximal 'Raf' kinases. However, the reason why hepatocytes or other non-neuronal cells possess NGF receptors is unclear.

The abilities of both acute phasic and chronic MAP kinase cascade activation to either stimulate or inhibit proliferation were tested in both PC12 cells and in primary cultures of hepatocytes. Our data demonstrated that the temporal extent to which the MAP kinase cascade could be activated by either NGF, EGF, ' Δ Raf-1:ER' or NGF and ethanol correlated with the ability of each cell type either to synthesize DNA or to arrest. We discovered that acute phasic activation of the MAP kinase cascade in both cell types resulted in a stimulation of DNA synthesis. In contrast, we found that chronic activation of the MAP kinase cascade in PC12 cells induced p21^{Cip-1} expression, inhibited cdk2 activity, and reduced DNA synthesis. Similarly, we also demonstrated that chronic activation of the MAP kinase cascade in primary cultures of hepatocytes by either Δ Raf-1:ER/4-hydroxytamoxifen treatment or ethanol/NGF treatment induced the cdk inhibitor proteins p21^{Cip-1}, and of a more novel nature p16^{INK4a}, inhibited hepatocyte cdk2 activity, and reduced hepatocyte DNA synthesis. In neither cell type did chronic activation of the MAP kinase cascade increase p27^{Kip-1} expression, which is different from data presented by Frey et al. for intestinal epithelial cells [39]. This, together with our own data, suggests that different cell types can induce different cdk inhibitor proteins to cause cell cycle arrest via similar stimuli. Our data strongly suggest that a general cellular mechanism exists where acute short-term activation of the MAP kinase cascade leads to a stimulation of DNA synthesis, whereas chronic activation of the MAP kinase cascade leads to an inhibition of DNA synthesis, via induction of the cdk inhibitor proteins. These data also demonstrate that one potential mechanism by which ethanol inhibits liver regeneration is via potentiation of MAP kinase cascade activation by growth factors, leading to induction of the cdk inhibitor proteins p21^{Cip-1} and p16^{INK4a}, with a reduction in hepatocyte DNA synthesis.

In PC12 cells chronic inhibition of the MAP kinase cascade by PD98059 or expression of dominant negative MEK1 reduced basal expression of p21^{Cip-1/WAF1} (Figure 3), and inhibited DNA synthesis (Table 1). In contrast, neither PD98059 nor expression

of dominant negative MEK1 caused any significant reduction in hepatocyte DNA synthesis (Table 1). These data suggest that basal MAP kinase activity plays a more important role in maintaining basal DNA synthesis in PC12 cells versus hepatocytes. These data also suggest that in PC12 cells, either chronic activation or chronic inhibition of the MAP kinase cascade can reduce DNA synthesis. Since chronic inhibition of the MAP kinase cascade reduced p21^{Cip-1/WAF1} expression, we must conclude that the reduction of MAP kinase activity in PC12 cells blocks DNA synthesis by either reducing cyclin levels or by blocking cdk dephosphorylation [43,44]. These possibilities are currently under investigation.

We observed that chronic activation of the MAP kinase cascade in both PC12 cells and primary cultures of hepatocytes did not significantly alter the levels of cyclins A, D or E. These data would suggest that alterations in the levels of cyclin proteins are not the prime mechanism by which chronic MAP kinase signalling inhibits cdk2 activity. The possibility does exist, however, that chronic activation of the MAP kinase cascade may result in altered phosphorylation/dephosphorylation of cdk2 potentially via the Cdc25 protein phosphatase. It is possible that chronic activation of the MAP kinase cascade may blunt Cdc25 activity, leading to a reduction of cdk2 dephosphorylation, and resulting in an inhibition of its activity. This is also being examined in our ongoing studies.

Several publications have documented that both oncogenic Ras and oncogenic Raf can stimulate proliferation and suppress differentiation in SV40 virus immortalized hepatocytes [9,10]. Since both oncogenic Ras and oncogenic Raf will cause potent activation of the MAP kinase cascade, these data would initially appear to be in conflict with our results. However, because these hepatocyte cell lines were immortalized with SV40 virus the function of p53 may have been compromised, and p53 function has been demonstrated to be one mechanism by which cells can induce expression of p21^{Cip-1} [40–42]. Thus it is probable that SV40 transformation of these hepatocyte cell lines may have made these cells incompetent to induce p21^{Cip-1} via chronic activation of the MAP kinase cascade. In contrast, inhibition of p53 function in primary hepatocytes did not block MAP kinase-induced p21^{Cip-1} expression. These data suggest that primary hepatocytes and hepatocyte cell lines use different mechanisms downstream of MAP kinase to induce p21^{Cip-1}.

In a previous study we demonstrated that proliferating hepatocytes isolated from regenerating livers 20–24 h after partial hepatectomy have lower basal levels of p42^{MAPkinase} activity than do hepatocytes isolated from non-proliferating livers [4]. Hepatocytes 20–24 h after partial hepatectomy *in vivo* are at the end of G1 and most cells are in S phase, whereas hepatocytes cultured *in vitro* for 20–24 h are mostly in G1 [3]. Based on the studies

Figure 5 Ethanol treatment of hepatocytes potentiates the ability of NGF to activate the MAP kinase cascade and to increase protein levels of p21^{Cip-1} and p16^{INK4a}

Ethanol treatment (A) temporally extends the ability of NGF to activate the MAP kinase cascade and (B) increases expression of the cdk inhibitor proteins p16^{INK4a} and p21^{Cip-1}. (A) Primary cultures of rat hepatocytes were cultured as in Methods. Cells were treated for the indicated times with either vehicle control (PBS), 20 ng/ml NGF (open triangles), 50 mM ethanol (closed circles), or both NGF and ethanol (closed squares). Cells were lysed with ice cold homogenization buffer, and subjected to immunoprecipitation as described in Methods. Activity of p42^{MAP kinase} was measured in duplicate immune-complex kinase assays versus bovine myelin basic protein (MBP). Data are expressed as -fold activation over basal activity; p42^{MAP kinase} 14323 ± 590 c.p.m. Data are a representative experiment. Similar data were obtained using hepatocytes isolated from three different livers. (B) Primary cultures of rat hepatocytes were cultured as in Methods. A portion of the isolated hepatocytes were infected with either a control virus or a recombinant replication defective adenovirus to express a CMV promoter driven dominant negative MEK1 (m.o.i. 250) and cultured for 24 h as in Methods. Thirty minutes prior to NGF/ethanol addition, another portion of the hepatocytes were treated with 50 μM PD98059. Hepatocytes were then treated with either media control, NGF (20 ng/ml), ethanol (EtOH, 50 mM) or NGF and EtOH and 24 h after treatment cells were lysed with 100 μl 5 × SDS/PAGE sample buffer, diluted to 250 μl with distilled water, and placed in a 100 °C dry bath for 15 min. Western blotting versus either anti-p16^{INK4a} (upper panel) or anti-p21^{Cip-1} (lower panel) was performed as in Methods. Treatments of cells with control, PD98059, or dominant negative MEK1 are as indicated. Data are representative of three experiments performed using hepatocytes isolated from different livers.

reported herein, one possible explanation for the observed reduction in basal hepatocyte MAP kinase activity during G1/S phase *in vivo* and *in vitro* is that its reduction may be essential to lower the levels of cdk inhibitor proteins present in non-

proliferating hepatocytes or so as to decrease the ability of MAP kinase signalling to reach the threshold level sufficient to induce cdk inhibitor protein expression. This would permit cyclin dependent kinases to function and thus allow progression of hepatocytes through G1 and entry into S phase.

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