Prolonged activation of the mitogen-activated protein kinase pathway promotes DNA synthesis in primary hepatocytes from p21^{Cip-1/WAF1}-null mice, but not in hepatocytes from p16^{INK4a}-null mice

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In primary rat hepatocytes, prolonged activation of the p42/44 mitogen-activated protein kinase (MAPK) pathway is associated with a decrease in DNA synthesis and increased expression of the cyclin-dependent kinase inhibitor (CKI) proteins p21^{Cip-1/WAF1} and p16^{INK4a}. To evaluate the relative importance of these CKIs in mediating this response, we determined the impact of prolonged MAPK activation on DNA synthesis in primary cultures of hepatocytes derived from mice embryonically deleted (null) for either p21^{Cip-1/WAF1} or p16^{INK4a}. When MAPK was activated in wild-type mouse hepatocytes for 24 h, via infection with a construct to express an inducible oestrogen receptor–Raf-1 fusion protein (Δ Raf:ER), the expression of p21^{Cip-1/WAF1} and p16^{INK4a} CKI proteins increased, cyclin-dependent kinase 2 (cdk2) and cdk4 activities decreased, and DNA synthesis decreased. Inhibition of RhoA GTPase function increased the basal expression of p21^{Cip-1/WAF1} and p27^{Kip-1} but not p16^{INK4a}, and enhanced the ability of MAPK signalling to decrease DNA synthesis. Ablation of the expression of CCAATT enhancer-binding protein α (C/EBP α), but not of the expression of C/EBP β , decreased the ability of MAPK signalling to induce p21^{Cip-1/WAF1}. When MAPK was activated in p16^{INK4a}-null hepatocytes for 24 h, the ex-

pression of p21^{Cip-1/WAF1} increased, cdk2 and cdk4 activities decreased and DNA synthesis decreased. In contrast with these findings, prolonged activation of the MAPK pathway in hepatocytes from p21^{Cip-1/WAF1}-null mice enhanced cdk2 and cdk4 activities and caused a large increase in DNA synthesis, despite elevated expression of p16^{INK4a}. Inhibition of RhoA GTPase activity in p21^{Cip-1/WAF1}-null cells partly blunted both the basal levels of DNA synthesis and the ability of prolonged MAPK signalling to increase DNA synthesis. Expression of anti-sense p21^{Cip-1/WAF1} in either wild-type or p16^{INK4a}-null hepatocytes decreased the ability of prolonged MAPK signalling to increase the expression of p21^{Cip-1/WAF1}, and permitted MAPK signalling to increase both cdk2 and cdk4 activities and DNA synthesis. These results argue that the ability of prolonged MAPK signalling to inhibit DNA synthesis in hepatocytes requires the expression of p21^{Cip-1/WAF1}, and that the increased expression of p16^{INK4a} has a smaller role in the ability of this stimulus to mediate growth arrest. Our results also suggest that RhoA function can modulate DNA synthesis in primary hepatocytes via the expression of p21^{Cip-1/WAF1} and p27^{Kip-1}.

INTRODUCTION

Partial hepatectomy or dissociation followed by primary culturing triggers the entry of hepatocytes into the cell cycle [1–3]. Maximal DNA synthesis *in vivo* occurs between 12 and 36 h after two-thirds partial hepatectomy. Primary culturing *in vitro* in the presence of growth factors such as insulin, epidermal growth factor or hepatocyte growth factor stimulates hepatocyte DNA synthesis, which is maximal between 40 and 70 h after isolation [3–6]. Hepatocytes do not terminally differentiate and can enter and exit the cell cycle during cycles of liver regeneration. This is in contrast with other cell types, such as intestinal epithelial cells and keratinocytes, which undergo irreversible terminal differentiation [7,8].

Recently, two signalling pathways leading to increased DNA synthesis in primary hepatocytes have been shown to be the c-Jun N-terminal kinase (JNK) and the p38-reactivating kinase pathways [4,6]. In contrast, signalling by the p42/44 mitogen-activated protein kinase (MAPK) pathway seemed to have a smaller role in stimulating hepatocyte DNA synthesis [4], but was shown to have a prominent role in causing cell cycle arrest in these cells [9]. The ability of MAPK to cause cell cycle arrest in these studies was correlated with increased expression of the cyclin-dependent kinase inhibitor (CKI) proteins p21^{Cip-1/WAF1} and p16^{INK4a}, but not the CKI protein p27^{Kip-1} [9]. The relative abilities of p21^{Cip-1/WAF1} and p16^{INK4a} to each inhibit DNA synthesis in primary hepatocytes were not investigated.

Hepatoma cells that are incapable of increasing p21^{Cip-1/WAF1}

Abbreviations used: cdk, cyclin-dependent kinase; C/EBP, CCAATT enhancer-binding protein; CKI, cyclin-dependent kinase inhibitor; DMEM, Dulbecco's modified Eagle's medium; Δ Raf:ER, oestrogen receptor–Raf-1 fusion protein, Δ Raf:ER(301), oestrogen receptor–Raf-1 (kinase-inactive) fusion protein; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEF, mouse embryonic fibroblast; MEK, mitogen/extracellular regulated protein kinase; m.o.i., multiplicity of infection; null mouse, a mouse from which a specific gene has been embryonically deleted; Rb, retinoblastoma gene product.

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expression are known to be more tumorigenic *in vivo* than hepatoma cells that still retain the ability to express p21^{Cip-1/WAF1} [10]. This has been suggested to be due in part to the loss of p53 function, although several studies have reported that childhood hepatomas and early-stage adult liver cancers express functional p53 [11–13]. Indeed, a decrease in the ability of many cell types to increase p21^{Cip-1/WAF1} expression has also been suggested to be important in the process of transformation and differentiation [14,15]. This might be due to a loss of transcription factor function(s), or to altered signalling via other pathways that regulate p21^{Cip-1/WAF1} expression, e.g. signalling via the RhoA GTPase [16–18]. In further agreement with the importance of p21^{Cip-1/WAF1} expression in hepatocyte cell cycle control, it was recently demonstrated that inducible overexpression of p21^{Cip-1/WAF1} could blunt liver regeneration after partial hepatectomy [19–21]. Together these results suggest that regulation of p21^{Cip-1/WAF1} expression and function might have a pivotal role both in the regulation of liver regeneration and in hepatocellular transformation.

These studies were initiated to determine whether the increased expression of either p21^{Cip-1/WAF1} or p16^{INK4a}, or both CKI molecules, was required to mediate MAPK-dependent cell cycle arrest in primary mouse hepatocytes. We discovered that prolonged activation of the MAPK pathway promoted DNA synthesis in primary hepatocytes from p21^{Cip-1/WAF1}-null mice (from which the gene had been embryonically deleted), but not in hepatocytes from either wild-type or p16^{INK4a}-null mice. Inhibition of signalling from the RhoA GTPase, via the expression of dominant-negative RhoA (N19), increased the basal expression of p21^{Cip-1/WAF1}, caused a small increase in basal p27^{Kip-1} expression and caused a large decrease in DNA synthesis. Furthermore the expression of RhoA (N19) increased the ability of prolonged MAPK signalling to inhibit DNA synthesis in wild-type hepatocytes. The expression of RhoA (N19) in p21^{Cip-1/WAF1}-null hepatocytes had a decreased ability to lower basal DNA synthesis compared with wild-type cells, and partly blunted the ability of prolonged MAPK signalling to increase growth.

Our results argue that p21^{Cip-1/WAF1} has a prominent role in the ability of prolonged MAPK signalling to inhibit DNA synthesis, whereas p16^{INK4a} seems to be marginally involved. Our results also argue that signalling from the RhoA GTPase inhibits the expression of p21^{Cip-1/WAF1} and that decreased RhoA signalling functions to inhibit DNA synthesis via increased p21^{Cip-1/WAF1} expression and by other mechanism(s) such as increased p27^{Kip-1} expression.

MATERIALS AND METHODS

Materials

Male C57BL/6J wild-type, p21^{Cip-1/WAF1}-null and p16^{INK4a}-null mice (30 g) had access to food and water *ad libitum*. Anti-p42^{MAP kinase} (sc-154AC), anti-(CCAATT enhancer-binding protein β) (C/EBP β ; sc-150), anti-C/EBP α (sc-61), anti-(human p53) (sc-126), anti-(rodent p53) (sc-100), anti-(cyclin-dependent kinase 2) (anti-cdk2; sc-163AC), anti-cdk4 (sc-601AC), anti-p16^{INK4a} (sc-1207), anti-p27^{Kip-1} (sc-528), anti-p21^{Cip-1} (sc-397-G and sc-817), anti-(cyclin A) (sc-596), anti-(cyclin D) (sc-753) and anti-(cyclin E) (sc-481) were from Santa Cruz Biotechnology (Santa Cruz Biotechnologies, CA, U.S.A.). [γ -³²P]ATP and [³H]thymidine were from NEN. Western immunoblotting was performed with the enhanced chemiluminescence system (Amersham). Protein preparations of other reagents were as in [4,6,9]. Plasmids containing anti-sense C/EBP α and anti-sense C/EBP β were generated from sense

plasmids described in [7,21]. Dominant-negative p53(R175H) was a gift from Dr. B. Vogelstein [22]. Dominant-negative mitogen/extracellular regulated protein kinase 1 (MEK1) S218A was a gift from Dr. C. Marshall [9]. The specific MEK1/2 activation inhibitor PD98059 [23] was a gift from Parke Davis/Warner Lambert.

Recombinant adenoviral vectors; generation and infection *in vitro*

Studies were performed with two adenoviral technologies. Replication-defective adenovirus was conjugated to poly-(L-lysine) as described in [6,24]. The DNA-conjugated virus was added to hepatocytes at a multiplicity of infection (m.o.i.) of 500 and the cells were incubated for 4 h at 37 °C. The cells were washed with medium to remove virus. Cells expressed 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 μ g of plasmid conjugated to virus particles and infected into mouse hepatocytes before plating at an m.o.i. of 500 gave 100 % infection as judged by blue coloration after incubation with 5-bromo-4-chloroindol-3-yl- β -D-galactopyranoside 24 h after infection. Secondly, we have generated recombinant adenoviruses [9,25]. To assess the effectiveness of recombinant adenoviral infection, we generated a recombinant adenovirus containing the gene for β -galactosidase. Primary mouse hepatocytes were infected with this virus after isolation *in vitro* (m.o.i. 500), and incubated at 37 °C for a further 24 h; cells were fixed and incubated with 5-bromo-4-chloroindol-3-yl- β -D-galactopyranoside. To assess infection, we performed immunoblots 10–24 h after infection [6,9]. An m.o.i. of 100 gave 100 % infection after 24 h.

Preparation of mouse hepatocytes

Mice were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the lower thorax and abdomen were 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, collagenase perfusion of the liver, and washing in Dulbecco's modified Eagle's medium (DMEM) containing 5 % (v/v) fetal calf serum as described [4]. Before plating, cells were resuspended in DMEM alone [4].

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

Mouse hepatocytes were cultured on rat-tail collagen (Vitrogen)-coated plastic dishes (12 mm $\times$ 20 mm, 2×10^5 cells) in 1 ml of DMEM in air/CO₂ (19:1) supplemented with 50 nM insulin/1 nM dexamethasone/1 nM thyroxine, and left to adhere to the dish. In ' Δ Raf-1:ER' adenoviral infection experiments medium did not contain Phenol Red indicator. At this time, cells were infected with various adenoviruses, depending on the experiment. For cells undergoing acute assay, treatments occurred 90 min after plating. For adenovirally infected cells, 4 h after infection, medium was replaced and hepatocytes were cultured in the same supplemented DMEM for 24 h. Hormonal treatments and/or protein kinase inhibitors were added 24 h after the medium change (protein kinase inhibitors were added 30 min before further treatment); 24 h after infection, hepatocytes were treated for 6 h with 50 nM 4-hydroxytamoxifen. Hepatocytes were cultured for a further 18 h without drug. The activity of p42^{MAP kinase} was determined before 4-hydroxytamoxifen addition, 20 min and 6 h after the start of treatment,

and then a further 18 h after the removal of the 4-hydroxytamoxifen. At 20 s before termination, medium was aspirated; this was followed by immediate homogenization. Cells were homogenized in 1 ml of 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 PMSF/40 µg/ml pepstatin A/1 µM microcystin-LR/0.5 mM sodium vanadate/0.5 mM sodium pyrophosphate/0.05 % sodium deoxycholate/1 % (v/v) Triton X-100/0.1 % (v/v) 2-mercaptoethanol], with trituration by using a P1000 pipette to lyse the cells. Homogenates were stored on ice before clarification by centrifugation (4 °C); clarified aliquots were subjected to immunoprecipitation. For DNA synthesis assays, hepatocytes were isolated from wild-type, p16^{INK4a}-null, and p21^{Cip-1/WAF1}-null mice and infected with an oestrogen receptor-Raf-1 fusion protein (ΔRaf:ER) or with a kinase-inactive mutant [ΔRaf:ER(301)]. At 24 h after infection, hepatocytes were treated for 6 h with 50 nM 4-hydroxytamoxifen. Hepatocytes were cultured in the presence of 10 µCi of [³H]thymidine for a further 24 h, after which cells were lysed with 0.5 M NaOH and DNA was precipitated with 12.5 % (w/v) trichloroacetic acid (final concn.). Acid-precipitable material was transferred to glass fibre filters and washed with 5 % (w/v) trichloroacetic acid, then [³H]thymidine incorporation into DNA was quantified by liquid-scintillation spectrometry [4].

Immunoprecipitations from homogenates

Protein A-agarose slurry (50 µl; 25 µl bead volume) was washed twice with 1 ml of PBS containing 0.1 % (v/v) Tween 20, and resuspended in 0.1 ml of the same buffer. Antibodies (2 µg, 20 µl) or serum (20 µl) were added to each tube and incubated for 3 h at 4 °C. Clarified hepatocyte homogenates (1.0 ml, 1 mg of total protein) were mixed with Protein A-agarose-conjugated antibody in duplicate with gentle agitation for 2.5 h at 4 °C. Protein A-agarose was recovered by centrifugation; the supernatant was then discarded and washed (10 min) sequentially with 0.5 ml of 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].

Assay of p42^{MAP kinase} activity

Immunoprecipitates were suspended in a final volume of 50 µl of buffer B containing 0.2 mM [^γ-³²P]ATP (2000 c.p.m./pmol), 1 µM microcystin-LR and 0.5 mg/ml myelin basic protein (MBP), which initiated reactions; the mixture was incubated at 37 °C. After 20 min, 40 µl of the reaction mixture was spotted on 2 cm circles of P81 phosphocellulose paper (Whatman, Maidstone, Kent, 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 ³²P incorporation into MBP was quantified by liquid-scintillation spectroscopy. Preimmune controls were performed to ensure that MBP phosphorylation was dependent on specific immunoprecipitation of p42^{MAP kinase} [4].

Assay of cdk2 and cdk4 activities

cdk2 and cdk4 activities were measured in immunoprecipitates of whole-cell lysates 24 h after treatment of cells with 4-hydroxytamoxifen. Approx. 100 µg of cellular protein from whole-cell homogenates (prepared as described above) was incubated with agarose-conjugated anti-cdk2 IgG or anti-cdk4 IgG on ice in 200 µl 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 µg/ml chymostatin/1 µg/ml leupeptin/1 µg/ml antipain/1 µg/ml pepstatin/1 µg/ml soybean trypsin inhibitor/0.5 % (v/v) Nonidet P40] and then resuspended in 35 µl of the same buffer without Nonidet P40. Protein kinase assays were conducted by assaying 10 µl of immunoprecipitated samples in triplicate in a total of 25 µl containing final concentrations of 30 mM Hepes (pH 7.4)/0.1 mM dithiothreitol/25 mM MgCl₂/30 mM β-glycerophosphate/0.5 µM protein kinase A inhibitor peptide (TTTAD-FLAGRRNAIHD)/0.1 mM [^γ-³²P]ATP (10000 c.p.m./pmol)/2 mM EGTA containing 0.5 mg/ml histone H1 (for cdk2 assays) or 2 µg of glutathione S-transferase (GST)-retinoblastoma (Rb) protein (residues 379–928) (for cdk4 assays). For cdk2 assays, after 15 min of incubation at 37 °C, assays were stopped by pipetting 20 µl of the reaction mixture on P81 papers as in the MAPK assay described above, and ³²P incorporation into histone H1 was quantified by liquid-scintillation spectroscopy [9]. For cdk4 assays, reactions were terminated by the addition of SDS/PAGE sample buffer followed by SDS/PAGE [10 % (w/v) gel]. GST-Rb protein was revealed by staining with Coomassie Blue, and ³²P incorporation into excised GST-Rb bands was determined by liquid-scintillation spectroscopy.

Data analysis

Comparison of the effects of various treatments was done with one-way analysis of variance and a two-tailed *t*-test. Differences with *P* < 0.05 were considered statistically significant. Experiments shown are the means ± S.E.M. for multiple individual points from multiple separate experiments (with hepatocytes from different animals).

RESULTS

Prolonged activation of the MAPK cascade increases p21^{Cip-1/WAF1} and p16^{INK4a} expression in primary mouse hepatocytes

Hepatocytes were isolated from wild-type, p16^{INK4a}-null and p21^{Cip-1/WAF1}-null mice and infected with a construct to express an inducible ΔRaf:ER, or with ΔRaf:ER(301). Cells were treated with 4-hydroxytamoxifen as described in the Materials and methods section and the activity of p42^{MAP kinase} was determined. Treatment of wild-type hepatocytes expressing ΔRaf:ER with 4-hydroxytamoxifen increased MAPK activity approx. 6-fold after 6 h, which fell 18 h after the removal of drug to approx. 5-fold above basal levels (Table 1). MAPK activation was blocked by incubation with the specific inhibitor of MEK1/2, PD98059 (50 µM), or by coexpression of dominant-negative MEK1 (results not shown) (in agreement with [9]). This prolonged MAPK activation caused an increase in p16^{INK4a} and p21^{Cip-1/WAF1} protein expression (Figure 1A). No increase in MAPK activity or in p16^{INK4a} and p21^{Cip-1/WAF1} protein expression was observed in 4-hydroxytamoxifen-treated hepatocytes infected with ΔRaf:ER(301) (results not shown, and Figure 1A) [9].

Although MAPK-induced p21^{Cip-1/WAF1} expression was blocked by incubation with 50 µM PD98059 and by the expression of anti-sense C/EBPα or dominant-negative MEK1, it was not decreased by the expression of either dominant-negative p53 (R175H) or anti-sense C/EBPβ (Figure 1A, and results not shown) (in agreement with [9,18]). The expression of either anti-sense C/EBPα, dominant-negative p53 (R175H) or anti-sense C/EBPβ did not alter the ability of ΔRaf:ER to activate the MAPK pathway. The infection of constructs to express either anti-sense C/EBPα or anti-sense C/EBPβ decreased p42 C/EBPα

Table 1 Treatment of mouse hepatocytes expressing Δ Raf:ER with 4-hydroxytamoxifen for 6 h causes activation of MAPK, which is maintained for 18 h after the removal of 4-hydroxytamoxifen

Hepatocytes were infected with either Δ Raf:ER poly-(L-lysine) adenovirus or Δ Raf:ER(301) (results not shown) poly-(L-lysine) adenovirus (each at 500 m.o.i.), followed by culturing as described in the Materials and methods section. As indicated, and in addition to Δ Raf:ER infection, cells were also infected with null recombinant adenovirus, a recombinant adenovirus expressing p21^{Cip-1} anti-sense mRNA or a recombinant adenovirus expressing p21^{Cip-1} sense (each at 100 m.o.i.). After 24 h to allow protein/mRNA expression, hepatocytes were treated with either vehicle control or 50 nM 4-hydroxytamoxifen for 6 h, followed by washing with warm medium and further culturing for an additional 18 h in medium without 4-hydroxytamoxifen (total time in culture 48 h). Cells were assayed for MAPK activity before the addition of 4-hydroxytamoxifen (0 min) and at 20 min, 360 min and 1440 min after the addition of 4-hydroxytamoxifen. Results are means $\pm$ S.E.M. for triplicate determinations from three separate experiments/animals and are expressed as c.p.m. incorporated into MBP above background (250 ± 50 c.p.m.) in the standard MAPK assay (as described in the Materials and methods section). No activation of MAPK was observed in cells infected with Δ Raf:ER(301) treated with 4-hydroxytamoxifen (results not shown). No activation of MAPK was observed in cells infected with Δ Raf:ER treated with matched vehicle control (DMSO) (results not shown). The addition of 50 μ M PD98059 to the culture medium abolished the activation of MAPK by Δ Raf:ER in all the cells examined above (results not shown), in agreement with [9].

Time (min)	Radioactivity incorporated into MBP (c.p.m.)					
	Wild-type	p16 ^{INK4a} -null	p21 ^{Cip-1} -null	p16 ^{INK4a} -null p21 ^{Cip-1} anti-sense	Wild-type p21 ^{Cip-1} anti-sense	p21 ^{Cip-1} -null p21 ^{Cip-1} sense
0	1425 $\pm$ 120	1550 $\pm$ 160	1430 $\pm$ 125	1600 $\pm$ 130	1500 $\pm$ 150	1445 $\pm$ 105
20	6260 $\pm$ 350	6715 $\pm$ 290	7110 $\pm$ 270	6135 $\pm$ 325	6350 $\pm$ 400	6845 $\pm$ 375
360	9325 $\pm$ 500	9220 $\pm$ 725	9040 $\pm$ 900	8605 $\pm$ 525	8760 $\pm$ 350	8390 $\pm$ 610
1440	7880 $\pm$ 325	7525 $\pm$ 700	8590 $\pm$ 650	7565 $\pm$ 500	7130 $\pm$ 355	7360 $\pm$ 405

Figure 1 Prolonged activation of the MAPK cascade results in increased expression of the CKIs p21^{Cip-1} and p16^{INK4a}

(A) Wild-type hepatocytes; (B) p16^{INK4a}-null hepatocytes; (C) p21^{Cip-1}-null hepatocytes; (D) p16^{INK4a}-null hepatocytes plus p21^{Cip-1} anti-sense; (E) wild-type hepatocytes plus p21^{Cip-1} anti-sense; (F) p21^{Cip-1}-null hepatocytes plus p21^{Cip-1} sense. Hepatocytes were infected with either Δ Raf:ER(301) or Δ Raf:ER poly-(L-lysine) adenoviruses (all at 500 m.o.i. each), followed by culturing as described in the Materials and Methods section. In some experiments and in addition to Δ Raf:ER infection, cells were also infected with either a null recombinant adenovirus, a p21^{Cip-1} anti-sense mRNA recombinant adenovirus, or a p21^{Cip-1} sense recombinant adenovirus (at 100 m.o.i. each). In (A), in addition to Δ Raf:ER infection, some cells were also infected with either anti-sense C/EBP β poly-(L-lysine) adenovirus or with anti-sense C/EBP α poly-(L-lysine) adenovirus (at 500 m.o.i. each). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 50 nM 4-hydroxytamoxifen for 6 h, followed by washing with warm medium and further culturing for an additional 18 h in medium without 4-hydroxytamoxifen (total time in culture 48 h). Protein expression of p21^{Cip-1} and p16^{INK4a} was determined by immunoblotting 24 h after addition of 4-hydroxytamoxifen. There was equal protein loading (200 μ g) in each lane; exposures of X-ray film for the enhanced chemiluminescence immunoblots shown were from 30 s to 1 min. Lane 1, Δ Raf:ER(301) plus 4-hydroxytamoxifen; lane 2, Δ Raf:ER plus vehicle control; lane 3, Δ Raf:ER plus 4-hydroxytamoxifen; lane 4, Δ Raf:ER plus 4-hydroxytamoxifen plus 50 μ M PD98059; lane 5, Δ Raf:ER plus 4-hydroxytamoxifen plus anti-sense C/EBP β ; lane 6, Δ Raf:ER plus 4-hydroxytamoxifen plus anti-sense C/EBP α . A representative experiment for each cell type/condition is shown ($n = 6$).

Figure 2 Prolonged activation of the MAPK cascade decreases cdk2 and cdk4 activities in hepatocytes expressing p21^{Cip-1}, but not in hepatocytes where p21^{Cip-1} expression has been blocked

(A) Wild-type hepatocytes; (B) p16^{INK4a}-null hepatocytes; (C) p21^{Cip-1}-null hepatocytes; (D) p16^{INK4a}-null hepatocytes plus p21^{Cip-1} anti-sense; (E) wild-type hepatocytes plus p21^{Cip-1} anti-sense; (F) p21^{Cip-1}-null hepatocytes plus p21^{Cip-1} sense. Hepatocytes were infected with either ΔRaf:ER(301) poly(L-lysine) adenovirus or ΔRaf:ER poly(L-lysine) adenovirus (all at 500 m.o.i. each), followed by culturing as described in the Materials and Methods section. In some experiments, and in addition to ΔRaf:ER infection, cells were also infected with either null recombinant adenovirus, p21^{Cip-1} anti-sense recombinant adenovirus, or p21^{Cip-1} sense adenovirus (at 100 m.o.i. each). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or 50 nM 4-hydroxytamoxifen (TAM) for 6 h, followed by washing with warm medium and further culturing for an additional 18 h in medium without 4-hydroxytamoxifen. Cells were assayed for either cdk2 activity (against histone H1) or for cdk4 activity (against GST-Rb protein residues 379–928) 1440 min after the addition of 4-hydroxytamoxifen. Results are means ± S.E.M. for quadruplicate experiments from three animals expressed as the fold alteration in cdk2 or cdk4 activity. The basal activity of cdk2/cdk4 in ΔRaf:ER(301) cells without treatment with 4-hydroxytamoxifen was defined as 1.00. The basal activities were as follows: in (A), wild-type hepatocytes cdk2 8812 ± 1500 c.p.m., cdk4 716 ± 68 c.p.m.; in (B), p16^{INK4a}-null hepatocytes cdk2 16880 ± 2025 c.p.m., cdk4 634 ± 107 c.p.m.; in (C) p21^{Cip-1}-null hepatocytes cdk2 11387 ± 1413 c.p.m., cdk4 423 ± 96 c.p.m.; in (D) p16^{INK4a}-null hepatocytes plus p21^{Cip-1} anti-sense cdk2 12850 ± 2919 c.p.m., cdk4 442 ± 128 c.p.m.; in (E) Wild-type hepatocytes plus p21^{Cip-1} anti-sense cdk2 11597 ± 1674 c.p.m., cdk4 442 ± 110 c.p.m.; in (F) p21^{Cip-1}-null hepatocytes plus p21^{Cip-1} sense, parallel p21^{Cip-1}-null hepatocytes without exogenous p21^{Cip-1} expression cdk2 11433 ± 1333 c.p.m., cdk4 459 ± 104 c.p.m. ($P < 0.05$). Symbols: *, decrease compared with ΔRaf:ER(301) cells plus 4-hydroxytamoxifen control; †, increase compared with ΔRaf:ER(301) cells plus 4-hydroxytamoxifen control.

and p30 C/EBP β immunoblottable protein levels by more than 75% and more than 90% respectively, 24 h after infection (results not shown).

Treatment of either p16^{INK4a}-null or p21^{Cip-1/WAF1}-null hepatocytes expressing Δ Raf:ER with 4-hydroxytamoxifen also caused an approx. 5-fold increase in MAPK activity after 6 h that remained elevated 24 h after removal of the drug (Table 1). Elevated MAPK activity increased p21^{Cip-1/WAF1} expression, but not p16^{INK4a} expression, in p16^{INK4a}-null hepatocytes (Figure 1B). Elevated MAPK activity increased p16^{INK4a} expression, but not p21^{Cip-1/WAF1} expression, in p21^{Cip-1/WAF1}-null hepatocytes (Figure 1C). Over multiple experiments the basal expression levels of p21^{Cip-1/WAF1} and p16^{INK4a} were not significantly altered when wild-type cells are compared with either p21^{Cip-1/WAF1} or p16^{INK4a}-null cells (results not shown).

In experiments identical with those above, MAPK activity also remained elevated 24 h after drug removal in p16^{INK4a}-null hepatocytes expressing p21^{Cip-1/WAF1} anti-sense mRNA (Table 1), in wild-type hepatocytes expressing p21^{Cip-1/WAF1} anti-sense mRNA (Table 1) and in p21^{Cip-1/WAF1}-null hepatocytes overexpressing p21^{Cip-1/WAF1} protein (Table 1). The expression of full-length human p21^{Cip-1/WAF1} anti-sense mRNA blunted the ability of mouse hepatocytes to increase mouse p21^{Cip-1/WAF1} protein levels (Figures 1D and 1E). The constitutive expression of p21^{Cip-1/WAF1} in p21^{Cip-1/WAF1}-null cells did not alter the ability of MAPK to induce p16^{INK4a} (Figure 1F). The cross-species effectiveness of the anti-sense p21^{Cip-1/WAF1} probably relates to the extensive nucleic acid sequence conservation between mouse and human p21^{Cip-1/WAF1} genes [26]. These results demonstrate that we can elevate MAPK activity via Δ Raf:ER and that we can manipulate p21^{Cip-1/WAF1} and p16^{INK4a} CKI protein expression in primary mouse hepatocytes via both —/—-null mouse and anti-sense mRNA technologies.

Prolonged MAPK activation inhibits cdk2 and cdk4 activities in wild-type and p16^{INK4a}-null hepatocytes but not in p21^{Cip-1/WAF1}-null hepatocytes

Experiments parallel to those described above were performed to examine the ability of prolonged MAPK signalling to alter the activities of cdk2 and cdk4 in wild-type, p16^{INK4a}-null and p21^{Cip-1/WAF1}-null hepatocytes 24 h after treatment with 4-hydroxytamoxifen (Figure 2). Prolonged activation of MAPK decreased cdk2/cdk4 activities in wild-type hepatocytes (Figure 2A) and in p16^{INK4a}-null hepatocytes (Figure 2B) but increased cdk2/cdk4 activities in p21^{Cip-1/WAF1}-null hepatocytes (Figure 2C).

The expression of anti-sense p21^{Cip-1/WAF1} mRNA in both p16^{INK4a}-null hepatocytes (Figure 2D) and wild-type hepatocytes (Figure 2E) permitted prolonged activation of the MAPK pathway to stimulate cdk2/cdk4 activities in these cells. The ability of prolonged MAPK signalling to increase cdk2/cdk4 activities in p21^{Cip-1/WAF1}-null hepatocytes was blocked by overexpression of p21^{Cip-1/WAF1} (Figure 2F). Prolonged MAPK activation did not significantly alter the protein expression of cdk2, cdk4, cyclin A, and cyclin E in wild-type, p16^{INK4a}-null or p21^{Cip-1/WAF1}-null hepatocytes (results not shown). However, prolonged activation of MAPK caused a 5-fold increase in the expression of cyclin D1, which was inhibited by 75 $\pm$ 11% (n = 3) when cells were incubated with 50 μ M PD98059 (results not shown). These results demonstrate that the loss of p21^{Cip-1/WAF1} expression permits prolonged MAPK signalling to increase the activities of cdk2 and cdk4 above basal levels. Because the activities of cdk2 and cdk4 are important in the ability of cells to progress through G₁ and to enter S phase, we next examined the

ability of prolonged MAPK signalling to regulate DNA synthesis in wild-type, p16^{INK4a}-null and p21^{Cip-1/WAF1}-null mouse hepatocytes.

Prolonged MAPK activity promotes DNA synthesis in the absence of p21^{Cip-1/WAF1} expression

Hepatocytes were isolated from wild-type, p16^{INK4a}-null and p21^{Cip-1/WAF1}-null mice and infected with Δ Raf:ER or Δ Raf:ER(301). The ability of treatment with 4-hydroxytamoxifen to alter [³H]thymidine incorporation into DNA was determined as described in the Materials and methods section. In agreement with cdk2/cdk4 activity results in Figure 2, prolonged MAPK activity decreased DNA synthesis in wild-type and p16^{INK4a}-null hepatocytes (Table 2). The ability of prolonged MAPK signalling to inhibit DNA synthesis in p16^{INK4a}-null hepatocytes was statistically less than its ability to do similarly in wild-type hepatocytes (P < 0.02). These results argue that expression of p16^{INK4a} has a modest role in the MAPK-mediated inhibition of DNA synthesis in primary hepatocytes. Also in agreement with cdk2/cdk4 activity results in Figure 2, prolonged MAPK activity increased DNA synthesis in p21^{Cip-1/WAF1}-null hepatocytes (Table 2). These results demonstrate that the ability of prolonged MAPK signalling to inhibit DNA synthesis in primary hepatocytes requires p21^{Cip-1/WAF1} expression.

To confirm that p21^{Cip-1/WAF1} was a more important mediator of MAPK-induced DNA synthesis inhibition than p16^{INK4a}, we performed experiments in which wild-type and p16^{INK4a}-null hepatocytes were infected with the construct to express p21^{Cip-1/WAF1} anti-sense mRNA. Expression of p21^{Cip-1/WAF1} anti-sense mRNA in both p16^{INK4a}-null hepatocytes and in wild-type hepatocytes blocked the ability of prolonged MAPK activation to increase p21^{Cip-1/WAF1} expression (Table 1, Figures 1D and 1E) but permitted the ability of this stimulus to increase DNA synthesis (Table 2). In addition, exogenous overexpression of p21^{Cip-1/WAF1} protein in p21^{Cip-1/WAF1}-null hepatocytes blocked the ability of prolonged MAPK signalling to increase DNA synthesis (Table 2). These results support the view that the most important molecular mechanism by which prolonged MAPK signalling inhibits DNA synthesis in primary cultures of mouse hepatocytes is via increasing protein levels of p21^{Cip-1/WAF1} and not those of p16^{INK4a}.

Inhibition of RhoA signalling increases the basal expression of p21^{Cip-1/WAF1} and potentiates the ability of prolonged MAPK signalling to decrease DNA synthesis

It was recently demonstrated in Swiss 3T3 cells that signalling from the Rho GTPase exerts a negative regulatory effect on the ability of v-Ha-Ras (V12) to increase p21^{Cip-1/WAF1} protein levels [16]. Because v-Ha-Ras (V12) can modulate the activities of many downstream pathways, e.g. the MAPK and JNK pathways, and because of our results (Tables 1 and 2, Figures 1 and 2), we next examined the ability of dominant-negative RhoA (N19) to alter the ability of Δ Raf:ER, via the MAPK pathway, to modulate p21^{Cip-1/WAF1} expression and DNA synthesis in wild-type and p21^{Cip-1/WAF1}-null hepatocytes.

In either wild-type or p21^{Cip-1/WAF1}-null hepatocytes, the expression of dominant-negative RhoA (N19) did not alter the ability of Δ Raf:ER to activate the MAPK pathway (results not shown, in agreement with Table 1). The expression of RhoA (N19) in wild-type cells increased in the basal expression of p21^{Cip-1/WAF1} caused a small increase in the basal expression of p27^{Kip-1} and did not alter the expression of p16^{INK4a} (Figure 3A). Expression of RhoA also reduced cyclin E expression by approx. 50% (results not shown). Expression of RhoA (N19) in

Table 2 Prolonged activation of the MAPK pathway inhibits DNA synthesis in hepatocytes expressing p21^{Cip-1} but not in hepatocytes in which p21^{Cip-1} protein expression has been blocked

Hepatocytes were infected with either Δ Raf:ER(301) poly-(L-lysine) adenovirus or Δ Raf:ER poly-(L-lysine) adenovirus (each at 500 m.o.i.), followed by culturing as described in the Materials and methods section. In some experiments and in addition to Δ Raf:ER infection, cells were also infected with null recombinant adenovirus, p21^{Cip-1} anti-sense recombinant adenovirus (p21^{Cip-1} as) or p21^{Cip-1} sense adenovirus (each at 100 m.o.i.). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or 50 nM 4-hydroxytamoxifen (TAM) for 6 h, followed by washing with warm medium and further culturing for 18 h in medium without 4-hydroxytamoxifen (total time in primary culture 48 h). In some experiments, before 4-hydroxytamoxifen addition, cells were treated with 50 μ M PD98059. Hepatocytes under all conditions were continually incubated with 10 μ Ci [³H]thymidine throughout the final 24 h of culturing, after which they were lysed and [³H]thymidine incorporation into DNA was determined as described in the Materials and methods section. Results are the means $\pm$ S.E.M. (c.p.m. [³H]thymidine incorporated into DNA) for octuplicate experiments from seven separate animals. Symbols: *, decrease compared with Δ Raf:ER(301) cells plus 4-hydroxytamoxifen control ($P < 0.05$); †, increase compared with Δ Raf:ER(301) cells plus 4-hydroxytamoxifen control ($P < 0.02$); ‡, increase compared with wild-type hepatocyte Δ Raf:ER cells plus 4-hydroxytamoxifen ($P < 0.02$); §, decrease compared with p21^{Cip-1}/WAF1-null cells not constitutively expressing p21^{Cip-1}/WAF1 ($P < 0.001$).

Infection/treatment	Radioactivity in DNA (c.p.m.)					
	Wild-type	p16 ^{INK4a} -null	p21 ^{Cip-1} -null	p16 ^{INK4a} -null p21 ^{Cip-1} as	Wild-type p21 ^{Cip-1} as	p21 ^{Cip-1} -null p21 ^{Cip-1} sense
Δ Raf:ER(301) + TAM	10760 $\pm$ 1230	10560 $\pm$ 1810	10660 $\pm$ 1695	10095 $\pm$ 1520	10885 $\pm$ 1340	550 $\pm$ 25§
Δ Raf:ER + vehicle	11285 $\pm$ 2300	10140 $\pm$ 2220	9410 $\pm$ 1600	10930 $\pm$ 2150	11270 $\pm$ 1605	695 $\pm$ 25§
Δ Raf:ER + TAM	1450 $\pm$ 230*	4795 $\pm$ 695*‡	37370 $\pm$ 4600†‡	32690 $\pm$ 4600†‡	29895 $\pm$ 1960†‡	720 $\pm$ 30§
Δ Raf:ER + TAM + PD98059	8000 $\pm$ 955*‡	7960 $\pm$ 1370*‡	13230 $\pm$ 1950‡	12780 $\pm$ 1855#	13200 $\pm$ 2100‡	640 $\pm$ 50§
Δ Raf:ER + PD98059	8210 $\pm$ 1100*‡	7255 $\pm$ 1085*‡	8305 $\pm$ 1245*‡	8250 $\pm$ 1450*‡	8100 $\pm$ 1305*‡	700 $\pm$ 40§

Figure 3 Inhibition of RhoA function increases basal levels of p21^{Cip-1} and p27^{Kip-1} in primary hepatocytes

(A) Ability of RhoA (N19) to alter the expression of p16^{INK4a}, p21^{Cip-1} and p27^{Kip-1} in wild-type and p21^{Cip-1}-null hepatocytes; (B) ability of Δ Raf:ER to alter p21^{Cip-1} expression in the presence or absence of RhoA (N19) co-expression in wild-type and p21^{Cip-1}-null hepatocytes. Hepatocytes were infected with either Δ Raf:ER(301) or Δ Raf:ER poly-(L-lysine) adenoviruses (all at 500 m.o.i. each), followed by culturing as described in the Materials and Methods section. In addition to Δ Raf:ER infection, cells were also infected where indicated with either null poly-(L-lysine) adenovirus or RhoA (N19) poly-(L-lysine) adenovirus (at 500 m.o.i. each). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 50 nM 4-hydroxytamoxifen for 6 h, followed by washing with warm medium and further culturing for an additional 18 h in medium without 4-hydroxytamoxifen (total time in culture 48 h). Protein expression of p16^{INK4a}, p21^{Cip-1} and p27^{Kip-1} was determined by immunoblotting 24 h after the addition of 4-hydroxytamoxifen. There was equal protein loading (200 μ g) in each lane; exposures of X-ray film for the enhanced chemiluminescence immunoblots shown were 2 min (A) and 6 min (B). (A) Cell type as indicated: lane 1, null plasmid-infected cells; lane 2, RhoA (N19)-infected cells. (B) Cell type as indicated: lane 1, Δ Raf:ER plus 4-hydroxytamoxifen plus 50 μ M PD98059; lane 2, Δ Raf:ER(301) plus 4-hydroxytamoxifen; lane 3, Δ Raf:ER plus vehicle control; lane 4, Δ Raf:ER plus 4-hydroxytamoxifen. A representative experiment for each cell type/condition is shown ($n = 3$).

Table 3 Expression of dominant-negative RhoA decreases basal DNA synthesis and potentiates the ability of prolonged MAPK signalling to inhibit DNA synthesis in wild-type hepatocytes

Hepatocytes were infected with either Δ Raf:ER(301) poly-(L-lysine) adenovirus or Δ Raf:ER poly-(L-lysine) adenovirus (each at 500 m.o.i.), followed by culturing as described in the Materials and methods section. In addition to Δ Raf:ER infection, cells were also infected with either null poly-(L-lysine) adenovirus or dominant-negative RhoA (N19) poly-(L-lysine) adenovirus (each at 500 m.o.i.). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or 50 nM 4-hydroxytamoxifen (TAM) for 6 h, followed by washing with warm medium and further culturing for 18 h in medium without 4-hydroxytamoxifen (total time in primary culture 48 h). In some experiments, before the addition of 4-hydroxytamoxifen, cells were treated with 50 μ M PD98059. Hepatocytes under all conditions were continually incubated with 10 μ Ci [3 H]thymidine throughout the final 24 h of culturing, after which they were lysed and [3 H]thymidine incorporation into DNA was determined as described in the Materials and methods section. Results are means $\pm$ S.E.M. (c.p.m. [3 H]thymidine incorporated into DNA) for octuplicate experiments from three separate animals. Symbols: *, decrease compared with wild-type cells Δ Raf:ER(301) cells plus 4-hydroxytamoxifen control ($P < 0.05$); †, decrease compared with wild-type Δ Raf:ER cells plus 4-hydroxytamoxifen ($P < 0.05$); ‡, increase compared with wild-type RhoA (N19) Δ Raf:ER(301) cells plus 4-hydroxytamoxifen control ($P < 0.05$); §, increase compared with wild-type hepatocyte Δ Raf:ER cells plus 4-hydroxytamoxifen ($P < 0.05$); ||, decrease compared with wild-type hepatocyte Δ Raf:ER cells plus 4-hydroxytamoxifen ($P < 0.05$).

Infection/treatment	Radioactivity in DNA (c.p.m.)			
	Wild-type	Wild-type RhoA (N19)	p21 ^{Cip-1} -null	p21 ^{Cip-1} -null RhoA (N19)
Δ Raf:ER(301) + TAM	10950 $\pm$ 1150	4510 $\pm$ 925*	11415 $\pm$ 1190	6590 $\pm$ 920*‡
Δ Raf:ER + vehicle	12005 $\pm$ 1650	4620 $\pm$ 1020*	10100 $\pm$ 1600	6530 $\pm$ 1055
Δ Raf:ER + TAM	1890 $\pm$ 310*	695 $\pm$ 70*†	35850 $\pm$ 3700§	15620 $\pm$ 2105§
Δ Raf:ER + TAM + PD98059	7870 $\pm$ 990	4060 $\pm$ 655	12910 $\pm$ 1950	4640 $\pm$ 795
Δ Raf:ER + PD98059	8400 $\pm$ 1510	3125 $\pm$ 910	8915 $\pm$ 1260	3265 $\pm$ 820

p21^{Cip-1/WAF1}-null cells also caused a small increase in the basal levels of p27^{Kip-1} (Figure 3A). Inhibition of RhoA GTPase function further increased the ability of MAPK signalling to elevate p21^{Cip-1/WAF1} expression in wild-type cells (Figure 3B). Increased MAPK signalling did not alter expression levels of p27^{Kip-1} in either wild-type or p21^{Cip-1/WAF1}-null cells (results not shown) (in agreement with data [9]). These results argue that RhoA function negatively regulates the protein expression of p21^{Cip-1/WAF1}, and to a smaller extent p27^{Kip-1}, in primary hepatocytes (in agreement with [16,17,27]).

Because the inhibition of RhoA function increased the expression of CKI proteins, we next investigated whether this impinged on the ability of hepatocytes to synthesize DNA. The expression of RhoA (N19) significantly decreased basal DNA synthesis in both wild-type and p21^{Cip-1/WAF1}-null hepatocytes, although the ability of RhoA (N19) to inhibit DNA synthesis was partly decreased in p21^{Cip-1/WAF1}-null cells compared with wild-type cells (Table 3; $P < 0.05$). The expression of RhoA (N19) increased the ability of MAPK signalling to block DNA synthesis in wild-type cells and decreased by 25 % the ability of MAPK signalling to increase DNA synthesis in p21^{Cip-1/WAF1}-null cells (Table 3; 3.2-fold compared with 2.4-fold; $P < 0.05$). Thus inhibition of RhoA function increases the expression of the CKI molecules p21^{Cip-1/WAF1} and p27^{Kip-1}, which can each function to inhibit hepatocyte DNA synthesis [16,27]. Our results also demonstrate that combined inhibition of the MAPK and RhoA pathways decreases basal DNA synthesis by approx. 70 % in wild-type and p21^{Cip-1/WAF1}-null cells, suggesting that activations of stress pathways and the MAPK pathway are critical in the mitogenic responses of primary hepatocytes *in vitro* [4,6,9].

DISCUSSION

Expression of the CKI p21^{Cip-1/WAF1} has been proposed to have an important role in the control of the cell cycle in many cell types, including hepatocytes *in vivo* and *in vitro* [9,10,13–21]. We recently demonstrated that prolonged activation of the MAPK pathway increases the expression of two CKI proteins, p16^{INK4a} and p21^{Cip-1/WAF1}, in primary rat hepatocytes and causes a decrease in DNA synthesis [9]. These studies were initiated to determine whether the increased expression of p21^{Cip-1/WAF1} or

p16^{INK4a}, or both CKI proteins, was responsible for the observed decrease in DNA synthesis in primary hepatocytes caused by prolonged MAPK activation.

We demonstrated that prolonged activation of the MAPK pathway inhibited cdk2/cdk4 activities and decreased DNA synthesis in both wild-type and p16^{INK4a}-null mouse hepatocytes. The ability of MAPK to inhibit DNA synthesis was statistically less in p16^{INK4a}-null hepatocytes than in wild-type hepatocytes. However, in p21^{Cip-1/WAF1}-null hepatocytes, prolonged activation of the MAPK pathway potentially increased cdk2/cdk4 activities and stimulated DNA synthesis. Anti-sense neutralization of p21^{Cip-1/WAF1} protein expression in either wild-type or p16^{INK4a}-null hepatocytes permitted prolonged activation of the MAPK pathway to stimulate cdk2/cdk4 activities and increase DNA synthesis. These results indicate that in response to prolonged MAPK activation, increased expression of p21^{Cip-1/WAF1} has a more important role than increased expression of p16^{INK4a} in blunting hepatocyte DNA synthesis.

It was recently demonstrated that signalling from the Rho GTPase negatively regulated the v-Ha-Ras (V12)-induced expression of p21^{Cip-1/WAF1} in fibroblasts [16]. We have confirmed and extended these observations in epithelial cells by using a specific activator of the MAPK pathway, Δ Raf:ER. Our results also demonstrate that positive signalling from the RhoA pathway negatively regulates the expression of p27^{Kip-1}, in agreement with results in [17,27]. This suggests that co-ordinated signalling via the MAPK and RhoA pathways regulates expression of the CKIs p16^{INK4a}, p21^{Cip-1/WAF1} and p27^{Kip-1}. Further studies will be needed to understand the signalling processes downstream of MAPK and RhoA by which they regulate hepatocyte DNA synthesis via CKI expression.

Most of our studies focused on the interactions between MAPK signalling and the expression of p21^{Cip-1/WAF1} and p16^{INK4a}. It was recently shown in passaged mouse embryonic fibroblasts (MEFs) that prolonged activation of the MAPK pathway inhibited DNA synthesis in wild-type fibroblasts but stimulated DNA synthesis in p21^{Cip-1/WAF1} $-/-$ cells [28–31]. Furthermore, and in agreement with an inability of dominant-negative p53(R175H) to block MAPK-induced expression of p21^{Cip-1/WAF1} in primary hepatocytes, these studies also demonstrated that MAPK signalling induced p21^{Cip-1/WAF1} expression in p53 $-/-$ MEFs [18,30]. The ability of prolonged activation

of the MAPK pathway to increase p16^{INK4a} expression in these studies was not determined.

Other groups have examined the ability of oncogenic v-Ha-Ras to promote DNA synthesis and colony formation in p16^{INK4a}-null MEFs [32–34]. These studies found that expression of oncogenic v-Ha-Ras (V12), which can cause constitutive activation of the MAPK pathway, increased DNA synthesis and colony formation ability in p16^{INK4a}-null MEFs but not in wild-type cells. The ability of oncogenic v-Ha-Ras (V12) to increase p21^{Cip-1/WAF1} in these studies was not determined. Our results in primary hepatocytes suggest a greater role for p21^{Cip-1/WAF1} than p16^{INK4a} in mediating growth arrest, which correlates more closely with results in [28–31] than with those in [32–34]. Differences between our results and those in [28–34] might depend on the use of epithelial rather than fibroblast cells, the use of unpassaged primary adult cells rather than passaged embryonic cells, or the presence of serum in the culture medium.

That p21^{Cip-1/WAF1}-null primary hepatocytes do not arrest their growth in response to prolonged MAPK signalling was unexpected because they possess a functional Rb protein and can express p16^{INK4a} (Figure 1C). Increased expression of p16^{INK4a} would be expected to inhibit cdk4 activity, blocking both Rb phosphorylation and its inactivation. Our results suggest that p21^{Cip-1/WAF1} has a more prominent role in regulating cdk4 activity and cell cycle progression in hepatocytes, which has been suggested by others [19,20]. Another possibility is that the amount of p16^{INK4a} synthesized by prolonged MAPK signalling in primary hepatocytes is insufficient to inhibit cdk4 activity fully *in vivo* and cause arrest in the absence of p21^{Cip-1/WAF1} expression. This possibility might be true because prolonged MAPK signalling was statistically less efficacious in blunting DNA synthesis in p16^{INK4a}-null hepatocytes in comparison with wild-type cells, which suggests that increased p16^{INK4a} expression has a partial effect on the regulation of DNA synthesis in primary hepatocytes (Table 2).

Results from several laboratories have suggested that MAPK signalling can induce p21^{Cip-1/WAF1} expression by both p53-dependent and p53-independent mechanisms [28–31,35]. The ability of MAPK signalling to increase p21^{Cip-1/WAF1} expression in primary mouse hepatocytes was independent of p53, which was similar to our results in primary rat hepatocytes [9]. In general agreement with these results, Serfas et al. [18] demonstrated that the ability of CCl₄ to increase p21^{Cip-1/WAF1} expression in hepatocytes *in vivo* was p53-independent. Recently an alternatively spliced gene product from the p16^{INK4a} gene, termed p19^{ARF}, was demonstrated to have an important role in enhancing p53 activity, including the ability of p53 to induce p21^{Cip-1/WAF1} [22,36,37]. In general agreement with the lack of p53-dependence in our system, primary hepatocytes from p16^{INK4a}-null mice, which were therefore also null for p19^{ARF}, were still capable of MAPK-induced expression of p21^{Cip-1/WAF1} (Figure 1B). However, in other hepatocyte-derived cell types, e.g. HepG2 hepatoma cells, the expression of p21^{Cip-1/WAF1} seems to be p53-dependent [38]. These results are consistent with the possibility that p53-independent mechanisms downstream of MAPK and upstream of the p21^{Cip-1/WAF1} protein, which are functional in primary hepatocytes, might be non-functional in hepatoma cells. It is tempting to speculate that a decreased ability to express p21^{Cip-1/WAF1} might represent an early event in the process of hepatocellular transformation.

Several other transcription factors besides p53 might be able to link MAPK signalling to the regulation of the p21^{Cip-1/WAF1} promoter and/or protein expression. In particular, members of the C/EBP family of transcription factors have been proposed to regulate p21^{Cip-1/WAF1} protein levels in hepatocytes and in other

cell types [7,39–43]. Anti-sense C/EBP β reduced protein levels of C/EBP β by approx. 90% and did not block the ability of prolonged MAPK signalling to increase p21^{Cip-1/WAF1} protein levels (Figure 1A). This suggests that C/EBP β is not an essential mediator of MAPK signalling towards p21^{Cip-1/WAF1} in primary hepatocytes, although it is possible that the remaining small amounts of C/EBP β could still mediate MAPK signalling. This seems unlikely on the basis of results presented elsewhere [7,42]. Recent studies have suggested that the ability of C/EBP β to regulate p21^{Cip-1/WAF1} expression is dependent on the function of the cAMP-dependent protein kinase [7,42]. We have demonstrated that an elevation of cAMP levels inhibited both DNA synthesis and MAPK signalling in primary rat hepatocytes but that these two events were not causally related [4]. These findings suggest that hepatocytes might possess both cAMP-dependent and MAPK-dependent mechanisms to cause growth arrest by increasing the expression of CKI molecules.

Other investigators have suggested that the CCAAT/enhancer binding protein family member, C/EBP α , might also have a role in regulating the promoter and protein levels of p21^{Cip-1/WAF1} in primary hepatocytes and in hepatoma cells [18,39–41,43]. For example, Serfas et al. [18] demonstrated via immunohistochemistry that p21^{Cip-1/WAF1} expression and nuclear C/EBP α protein expression were co-localized in hepatocytes *in vivo*. In agreement with this view, we found that anti-sense C/EBP α blunted the ability of prolonged MAPK signalling to increase p21^{Cip-1/WAF1} protein expression. Thus results from our own and several other laboratories suggest that C/EBP family members might have a key role in mediating MAPK signalling towards the p21^{Cip-1/WAF1} protein levels in hepatocytes.

Further studies, outside the scope of this paper, will be required to determine fully the molecular mechanism(s) downstream of MAPK signalling by which it regulates p21^{Cip-1/WAF1} promoter function and protein levels in primary cultures of mouse hepatocytes.

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