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Activation of extracellular signal-regulated kinase (ERK) in G_2 phase delays mitotic entry through *p21^{CIP1}*

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Abstract. Extracellular signal-regulated kinase activity is essential for mediating cell cycle progression from G_1 phase to S phase (DNA synthesis). In contrast, the role of extracellular signal-regulated kinase during G_2 phase and mitosis (M phase) is largely undefined. Previous studies have suggested that inhibition of basal extracellular signal-regulated kinase activity delays G_2- and M-phase progression. In the current investigation, we have examined the consequence of activating the extracellular signal-regulated kinase pathway during G_2 phase on subsequent progression through mitosis. Using synchronized HeLa cells, we show that activation of the extracellular signal-regulated kinase pathway with phorbol 12-myristate 13-acetate or epidermal growth factor during G_2 phase causes a rapid cell cycle arrest in G_2 as measured by flow cytometry, mitotic indices and cyclin B1 expression. This G_2-phase arrest was reversed by pretreatment with bisindolylmaleimide or U0126, which are selective inhibitors of protein kinase C proteins or the extracellular signal-regulated kinase activators, MEK1/2, respectively. The extracellular signal-regulated kinase-mediated delay in M-phase entry appeared to involve *de novo* synthesis of the cyclin-dependent kinase inhibitor, *p21^{CIP1}*, during G_2 through a p53-independent mechanism. To establish a function for the increased expression of *p21^{CIP1}* and delayed cell cycle progression, we show that extracellular signal-regulated kinase activation in G_2-phase cells results in an increased number of cells containing chromosome aberrations characteristic of genomic instability. The presence of chromosome aberrations following extracellular signal-regulated kinase activation during G_2-phase was further augmented in cells lacking *p21^{CIP1}*. These findings suggest that *p21^{CIP1}* mediated inhibition of cell cycle progression during G_2/M phase protects against inappropriate activation of signalling pathways, which may cause excessive chromosome damage and be detrimental to cell survival.

INTRODUCTION

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein (MAP) kinase family and are involved in a variety of cellular functions including

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proliferation, differentiation, apoptosis and motility (Lewis *et al.* 1998; Pearson *et al.* 2001). ERK protein activation most often occurs sequentially through Ras G-proteins, Raf kinases, and MAP/ERK kinases 1 and 2 (MEK1/2), which are the only known activators of ERK1/2 (Lewis *et al.* 1998). Upon activation, the ERK pathway promotes cell proliferation by indirect regulation of the activity of cyclin-dependent kinases (Cdk) at specific times during the cell cycle. For example, Ras G-protein activity couples growth factor receptor signalling to ERK proteins, which promote cyclin D1 expression, Cdk2 activity, and G_1/S-phase progression (Liu *et al.* 1995; Aktas *et al.* 1997). Ras also promotes progression through G_1-phase by increasing cyclin E/Cdk2 activity in cooperation with the ERK substrate, c-Myc (Leone *et al.* 1997). Activated Cdk proteins phosphorylate the retinoblastoma (Rb) tumour-suppressor protein, which acts as a cell cycle repressor of transcription during G_1-phase in its hypophosphorylated form (Hatakeyama & Weinberg 1995). Thus, Ras, indirectly through the activation of Cdk proteins, causes Rb phosphorylation and inhibition of Rb repression of genes required for entry into S phase (Mittnacht *et al.* 1997; Peeper *et al.* 1997). As expected, proteins downstream of Ras, including Raf, MEK, and ERK, have also been shown to play a central role in promoting entry into S-phase progression by regulating the expression of cyclins and activation of Cdk proteins (Weber *et al.* 1997; Cheng *et al.* 1998; Lents *et al.* 2002).

The ERK pathway may also promote cell cycle progression by regulating the expression of Cdk inhibitors such as $p21^{CIP1}$ (Sewing *et al.* 1997; Kivinen & Laiho 1999; Coleman *et al.* 2003). For example, many growth factors that require Ras signalling through ERK to increase cell proliferation also induce $p21^{CIP1}$ expression (Leone *et al.* 1997; Kivinen & Laiho 1999). Similarly, pharmacological inhibition of MEK1/2 with PD98059 blocks growth factor-mediated G_1/S-phase progression as well as $p21^{CIP1}$ expression (Kivinen & Laiho 1999). Although it may appear counter productive for the Ras/Raf/MEK/ERK pathway to promote the expression of both cyclin D proteins and the $p21^{CIP1}$ inhibitor, studies using cells that do not express the $p21^{CIP1}$ gene show a reduced ability to activate cyclin D/Cdk complexes and promote G_1/S-phase progression (Cheng *et al.* 1999). Given that increased $p21^{CIP1}$ expression is observed in more that 90% of breast cancer tissues that also over-express cyclin D proteins (Russell *et al.* 1999), elevated $p21^{CIP1}$ levels have been proposed to function in delaying cell cycle progression in order to protect cancer cells against DNA damaging agents that would otherwise promote apoptosis (Gartel & Radhakrishnan 2005). Thus, tumour cells containing oncogenic Ras or Raf proteins may regulate the expression of cyclins and cell cycle inhibitors, which may simultaneously provide cells with a proliferative advantage and protection against damaging agents during cell cycle progression.

Despite its important role in regulating G_1/S phase, the function of the ERK pathway in regulating somatic cell cycle progression through G_2 phase and mitosis (M phase) is still under investigation. Inhibition of basal ERK pathway activity, with pharmacologic agents, RNA interference, or by over-expression of dominant negative mutants of MEK1, causes a delay in G_2 and M-phase progression (Wright *et al.* 1999; Roberts *et al.* 2002; Liu *et al.* 2004). Several proteins of the ERK pathway have been shown to have increased catalytic activity during the mitotic phase. For example, a cytoplasmic form of Raf-1 has been shown to be activated through a Ras-independent mechanism and is uncoupled from MEK1/2 in total protein lysates isolated from cells arrested in mitosis with nocodazole (Laird *et al.* 1995; Ziogas *et al.* 1998; Laird *et al.* 1999). However, Raf-1–dependent activation of MEK1 has been linked to the regulation of mitotic Golgi apparatus fragmentation (Colanzi *et al.* 2003). Thus, it is possible that localized Raf-1/MEK/ERK signalling modules may be more relevant to cells progressing through G_2 and M phases. Localized ERK pathway activation is supported by several studies demonstrating the presence of active forms of MEK and ERK proteins that are localized to the nucleus, chromosome

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kinetochores, centrosomes, microtubules, and the Golgi complex during G_2 and M-phase transitions (Acharya *et al.* 1998; Shapiro *et al.* 1998; Zecevic *et al.* 1998; Cha & Shapiro 2001; Willard & Crouch 2001). However, regulatory functions of the ERK pathway proteins and of the mitotic substrates at these intracellular locations remain largely unknown.

Extracellular signal-regulated kinase pathway regulation during G_2/M-phase progression may involve protein kinase C (PKC) isoforms. Earlier studies have suggested that PKC proteins activate Raf-1 through direct phosphorylation of serines 259 and 499 (Kolch *et al.* 1993). However, others have suggested that these phosphorylation sites are not required for Raf-1 activation (Barnard *et al.* 1998; Schonwasser *et al.* 1998). Alternatively, PKC may indirectly activate Raf-1 by phosphorylating and inactivating the Raf kinase inhibitory protein (RKIP) (Corbit *et al.* 2003). These studies proposed that phosphorylated RKIP dissociates from Raf-1, which allows Raf-1 activation to occur.

The classical PKC isoforms (α, βI, βII, and γ) are activated by diacylglycerol (DAG) and calcium, whereas activation of the novel PKC isoforms (δ, ε, η, and θ) only requires DAG (Ron & Kazanietz 1999). DAG analogues such as phorbol esters, are potent activators of the classical and novel PKC isoforms (Ron & Kazanietz 1999). Long-term exposure (> 12 h) to phorbol 12-myristate-13-acetate (PMA) has been reported to induce G_2-phase arrest through a mechanism involving phospholipid metabolites (Kaszkin *et al.* 1991) or G_1-phase arrest through inhibition of cyclin dependent kinases and activation of $p21^{CIP1}$ and $p27^{KIP1}$ expression (Hamada *et al.* 1996; Frey *et al.* 1997). Depending on the cell line, PKC isoforms may promote or inhibit G_2/M-phase progression. For example, PKC activity has been reported to decrease in mitotic glioma cells (Soma *et al.* 1994), but is elevated in promyelocytic leukaemia cells and has been involved in mitotic nuclear envelope breakdown (Goss *et al.* 1994). MCF-7 breast cancer cells treated with PMA immediately after release from aphidicolin-induced S-phase arrest have shown increased $p21^{CIP1}$ expression and subsequent accumulation of cells in the G_2 phase (Barboule *et al.* 1999). However, the signalling events that mediate the G_2-phase arrest in response to PMA have not been determined.

The goal of the current study was to examine the consequence of ERK activation in regulating G_2/M-phase progression. Our findings demonstrate that ERK activation during the G_2 phase delays cell cycle progression into mitosis through a mechanism involving *de novo* synthesis of $p21^{CIP1}$. In addition, ERK activation during G_2 phase causes an increase in the hallmarks of chromosome instability, a phenomenon that is exacerbated in cells lacking $p21^{CIP1}$. These data suggest that ERK-mediated induction of $p21^{CIP1}$ during G_2 phase and cell cycle arrest may protect cells from excessive DNA damage that might occur during mitotic transitions and result in compromised cell survival.

MATERIALS AND METHODS

Cell culture and reagents

HeLa cells (#CCL-2, ATCC, Manassas, VA), human retina epithelial cells that stably express human telomerase reverse transcriptase (hTERT-RPE cells, #CRL-4000; ATCC, Manassas, VA), HCT116 parental, or HCT116 $p21^{-/-}$ cells (kindly provided by Dr Bert Vogelstein, Johns Hopkins University) were cultured in a complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml) from Invitrogen (Carlsbad, CA). Epidermal growth factor (EGF) and PMA were purchased from Sigma (St. Louis, MO) and used at final concentrations of

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0.01–100 ng/ml and 0.01–0.1 µM, respectively. The MEK1/2 inhibitor, U0126, and the PKC inhibitor, bisindolylmaleimide (Bis) I, were purchased from Calbiochem (La Jolla, CA) and used at final concentrations of 10 µM and 1 µM, respectively. The proteasome inhibitor, MG115, and protein synthesis inhibitor, cyclohexamide, were purchased from Calbiochem and used at final concentrations of 10 µg/ml and 10 µM, respectively.

Antibodies specific for cyclin B1 (sc-245) and $p21^{CIP1}$ (sc-397) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for phosphorylated ERK1/2 (M-8159) and α-tubulin (T-6557) were purchased from Sigma. Immunoblotting analysis was performed as previously described (Dangi *et al*. 2003).

Cells were synchronized at the G_1/S-phase boundary using a double thymidine block as previously described (Dangi *et al*. 2003). Briefly, cells (approximately 50% confluent) were treated with 2 mM thymidine in complete medium for 16 h. The cells were released back into the cell cycle by washing with Hanks buffered saline solution (HBSS, Invitrogen) and were incubated for an additional 8 h in complete medium in the absence of thymidine. Cells were treated a second time with 2 mM thymidine in complete medium for 16 h, which resulted in an average of 85–90% of them synchronized at the G_1/S-phase boundary of the cell cycle (Dangi *et al*. 2003). Synchronized cells were washed with HBSS, were released back into the cell cycle and were harvested at various times after release with or without the indicated treatments. Protein lysates were collected from synchronized cells after two washes with cold phosphate buffered saline (PBS, pH 7.2; Invitrogen) by scraping with 300 µl of cold tissue lysis buffer (20 mM Tris-base, pH 7.4, 137 mM NaCl, 2 mM ethylenediamenetetraacetic acid (EDTA), 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine). The lysates were centrifuged at 20 000 (Xg) to remove insoluble material, were diluted with an equal volume of 2X sodium dodecyl sulphate (SDS)-sample buffer, and the proteins were separated on SDS-polyacrylamide gel electrophoresis for immunoblot analysis.

Immunofluorescence and mitotic index assays

HeLa or HCT116 cells were grown on round coverslips (No. 1, 18 mm; VWR, West Chester, PA) in 6-cm tissue culture plates and then were synchronized at the G_1/S boundary as described in the previous section. Cells were released back into the cell cycle for 7 h, which corresponded to G_2 phase, and were treated with PMA or EGF. In some cases, 20–30 min prior to PMA or EGF treatment, cells were pre-treated in the presence or absence of Bis or U0126. Immunofluorescence was performed as previously described (Cha & Shapiro 2001). Briefly, at varying times after release, coverslips were fixed with 4% paraformaldehyde diluted in PBS, from a 16% stock (Electron Microscopy Sciences, Hatfield, PA) for 5 min and were then permeabilized with 0.1% triton X-100 for 3 min. Alternatively, cells were fixed in cold methanol (−20 °C) for 10 min prior to staining. Following blocking with bovine serum albumin, cells were incubated with antibodies against γ-tubulin or p62 nucleoporin followed by fluoroscein or Texas red-conjugated secondary antibodies. Cellular DNA was stained with 4′, 6-diamidino-2-phenylindole (DAPI, 0.2 µg/ml in PBS) for 5 min. Mitotic chromosomes were identified, based on their characteristic condensed structure, using a Nikon E800 fluorescence microscope, and photographs were taken using a Hamamatsu CCD camera (ORCA-ER-285; Biovision Technologies, Exton, PA, USA). Images of mitotic cells, which included cells in prophase, pro-metaphase, metaphase, anaphase and telophase, were processed using IPLAB software (Scanalytics, Inc. Fairfax, VA). Mitotic cells were expressed as a fraction of total cells counted to determine the mitotic index (MI). Cells containing multiple nuclei (two or more) were counted and expressed as a percentage of the total cell number. Centrosomes were identified by γ-tubulin staining and cells containing

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greater that two centrosomes were expressed as a percentage of the total cell number. Approximately 250–300 cells were counted for each condition.

Flow cytometry

Synchronized cells were trypsinized, washed with cold PBS, fixed with cold (–20 °C) 70% ethanol and stored at 4 °C overnight. Cells were then incubated with 100 μg/mL propidium iodide (Sigma) dissolved in 0.2 M Tris pH 7.5, 20 mM EDTA, 1 mg/ml RNase A (Sigma) for 1 h at room temperature and were then diluted with an equal volume of PBS. DNA content was measured by flow cytometry (FACScan Analyser; Becton Dickinson, Franklin Lakes, NJ) and was analysed using the Sync Wizard Model, ModFit LT software (Becton Dickinson). Fifteen thousand cells were counted under each condition. To determine G_1, S, and G_2/M-phase cell populations, the settings for 2 N and 4 N DNA content peaks were obtained within each experiment using the G_1/S arrested cells (2N) as the reference and applied to all samples within a given experiment.

RESULTS

PKC mediates PMA-induced G_2 arrest

Protein kinase C proteins are linked to the regulation of the ERK pathway and cell cycle progression. Using PMA as a potent activator of PKC, we first addressed the requirement for PKC and ERK signalling in mediating PMA-induced cell cycle delay during G_2 phase. HeLa cells synchronized at the G_1/S phase boundary were released back into the cell cycle, pre-treated in the absence or presence of the PKC inhibitor, Bis during G_2 phase, and were then stimulated with or without PMA. Cells harvested at various times after release were analysed by flow cytometry for cell-cycle progression and ERK activation. Fluorescence-activated cell sorter (FACS) analysis of DNA content showed that untreated cells enter G_2/M phase after 7 h release from G_1/S, exit mitosis, and return to G_1 phase 13 h after release from the initial G_1/S-phase block (Fig. 1a). In contrast, PMA-treated cells remain primarily in G_2/M phase up to 13 h after release from G_1/S block (Fig. 1a). The PMA-induced arrest in G_2/M phase was reversed by the PKC inhibitor, Bis I (Fig. 1a). Delays in G_2/M-phase progression were also observed in non-transformed telomerase immortalized human retinal pigment epithelial (hTERT-RPE) cells (Fig. 1b) or HEK293 cells (data not shown) treated with PMA during G_2 phase.

In agreement with previous studies (Roberts *et al*. 2002), basal ERK activity was elevated in G_2/M phase and PMA treatment caused sustained ERK activation for up to 6 h (Fig. 2a). Bis had little effect on basal ERK activity but it did inhibit PMA-induced ERK activity (Fig. 2a). In control cells, cyclin B1 expression showed a typical transient expression as cells progressed through G_2 phase (7 and 7.3 h after G_1/S release), mitosis (9 h), and back into G_1 phase (11 and 13 h). In contrast, cyclin B1 expression in PMA-treated cells was sustained for up to 13 h and supported the FACS data, indicating that these cells were arrested in the G_2 or M phase (Fig. 2a). However, pre-treatment with Bis before PMA restored the transient expression of cyclin B1 indicate that PKC activity is required for maintaining PMA-induced G_2/M-phase arrest (Fig. 2a).

To determine whether the PMA-induced cell cycle delay was occurring in the G_2 or the M phase, the MI was evaluated under various conditions. As shown in Fig. 2b, the MI of PMA-treated cells at 9 and 11 h after G_1/S release was greatly reduced compared to untreated control cells. By 13 h after release, the MI of PMA-treated cells began to increase, indicating a more than 4 h delay in mitotic entry (Fig. 2b). The delay in mitotic entry was reversed by pre-treatment with Bis (Fig. 2b), supporting the requirement for PKC in mediating PMA-induced G_2-phase arrest.

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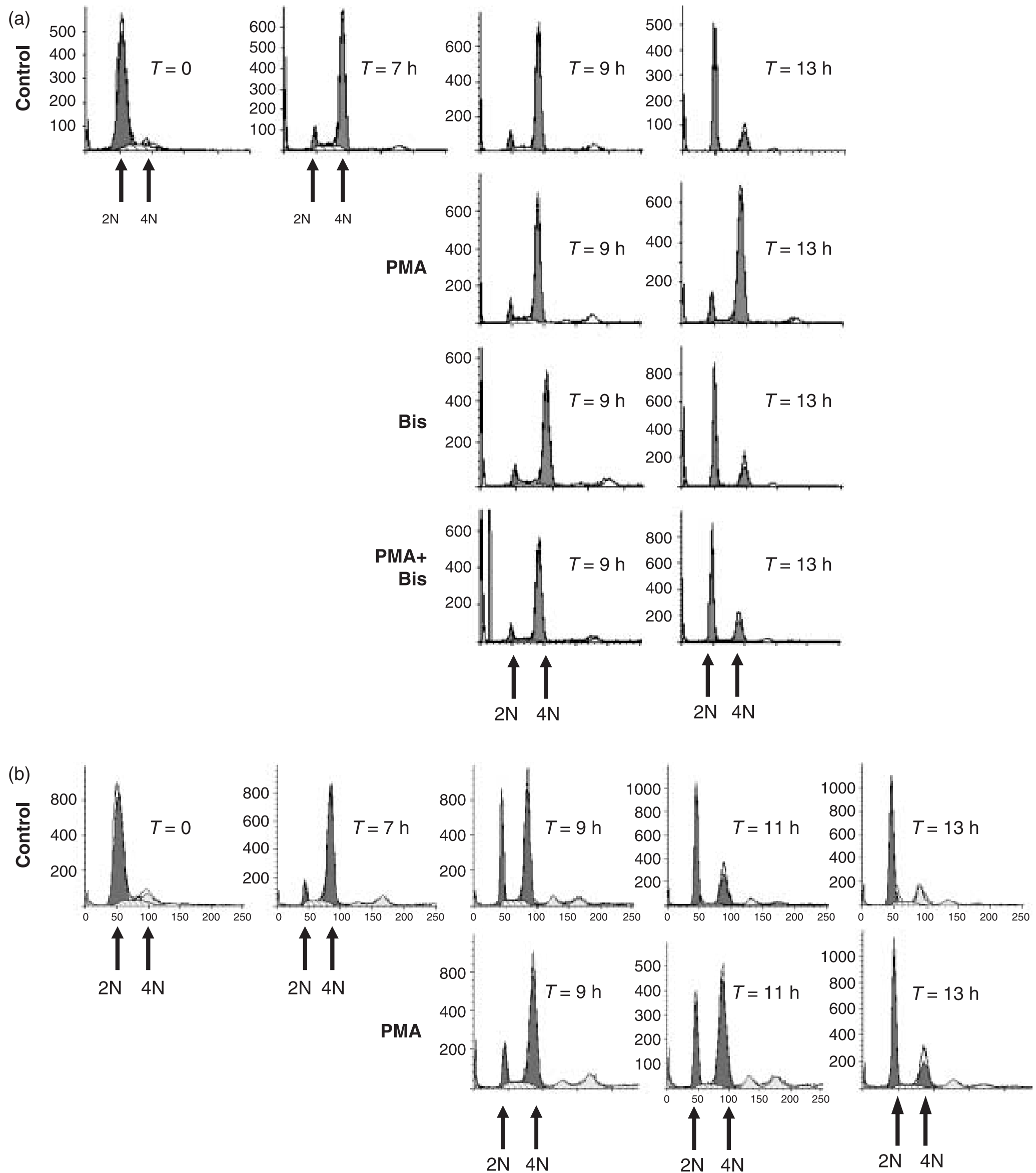

Figure 1. Fluorescence-activated cell sorting (FACS) analysis of PMA-mediated G_2-phase arrest. HeLa cells were synchronized at G_1/S boundary and released back into the cell cycle. (a) Cells were left untreated (control) or treated with PMA (0.1 μM) at 7 h after G_1/S release, with the bisindolylmaleimide (Bis, 1 μM) at 6.5 h after G_1/S release, or with PMA and Bis together. (b) FACS analysis of synchronized hTERT cells treated in the absence (control) or presence of PMA at 7 h after G_1/S-phase release. Cells were harvested at the times indicated after release from G_1/S and the number of cells (Y axis) containing 2N or 4N DNA content (X axis) were determined by FACS.

ERK mediates PMA-induced G_2-arrest

We next examined the requirement for ERK signalling in mediating PMA-induced G_2-phase arrest. G_2-phase cells generated as described in Fig. 1 were pre-treated in the absence or presence of the MEK1/2 inhibitor, U0126, followed by PMA stimulation. Similar to inhibition of

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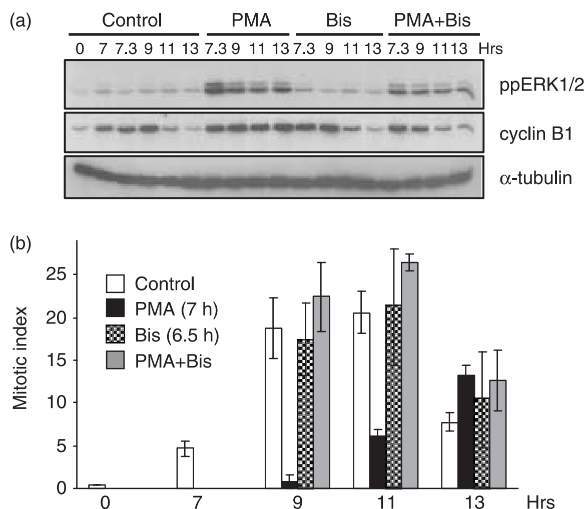

Figure 2. PMA-induced G_2-phase arrest requires PKC activity. Cells synchronized at the G_1/S boundary were released back into the cell cycle for 6.5 h and pre-treated with or without Bis (1 μM). At 7 h post-G_1/S release, cells were treated in the absence or presence of PMA (0.1 μM) and harvested at the times indicated. (a) Immunoblot analysis of active ERK1/2 (ppERK1/2, top panel) and cyclin B1 (middle panel) as the marker of G_2/M progression. The expression of α-tubulin (lower panel) is shown for a protein loading control. (b) The MI at various times after G_1/S release in controls (open bars), PMA-treated (black bars), Bis-treated (checkered bars), or Bis- and PMA-treated (grey bars) cells. Data represent the mean and standard error from three independent experiments.

PKC, inhibition of ERK activation by U0126 also reversed most of the PMA-induced G_2-arrest (Fig. 3). As expected, U0126 blocked PMA-induced ERK activation (Fig. 4a) and reversed the PMA-induced G_2-phase arrest as indicated by MI measurements (Fig. 4b). In addition, U0126 treatment also enhanced the MI at 13 h post-G_1/S-phase release (Fig. 4b) and did not reverse elevated cyclin B1 expression (Fig. 4a). These findings indicated a delay in mitotic progression and are consistent with our previous studies that have demonstrated a requirement for ERK during metaphase to anaphase transitions (Roberts *et al.* 2002). Thus, these data indicate that PMA-induced G_2-phase arrest requires PKC activation of the ERK pathway.

Activation of ERK during G_2 phase by EGF delays M-phase progression

We next tested whether activating the ERK pathway by EGF during G_2 also affected G_2/M progression. Cells synchronized in the G_2 phase were pre-treated with or without U0126 and then were treated in the presence or absence of EGF. FACS analysis indicated that EGF mediated an approximately 2 h delay in G_2/M progression compared to controls (Fig. 5, 11 h time point) and that this delay was reversed by pre-treatment with U0126 (Fig. 5). As expected, EGF activation of ERK was inhibited by U0126 (Fig. 6a). Cyclin B1 degradation and mitotic entry as determined by MI were also delayed by approximately 2 h in cells treated with EGF in the G_2 phase and this was reversed by the presence of U0126 (Fig. 6a,b). As in Fig. 5b, U0126-treated cells showed an elevated MI at the 13-h time point (Fig. 6b), indicative of a delay in metaphase to anaphase transitions (Roberts *et al.* 2002). These data provide further support that the level of ERK activity during G_2 phase regulates mitotic entry and progression.

An ERK activation threshold determines M-phase progression

Our data suggest that PMA or EGF-mediated activation of the ERK pathway during the G_2 phase delays mitotic entry. We next determined whether an ERK activity threshold was needed

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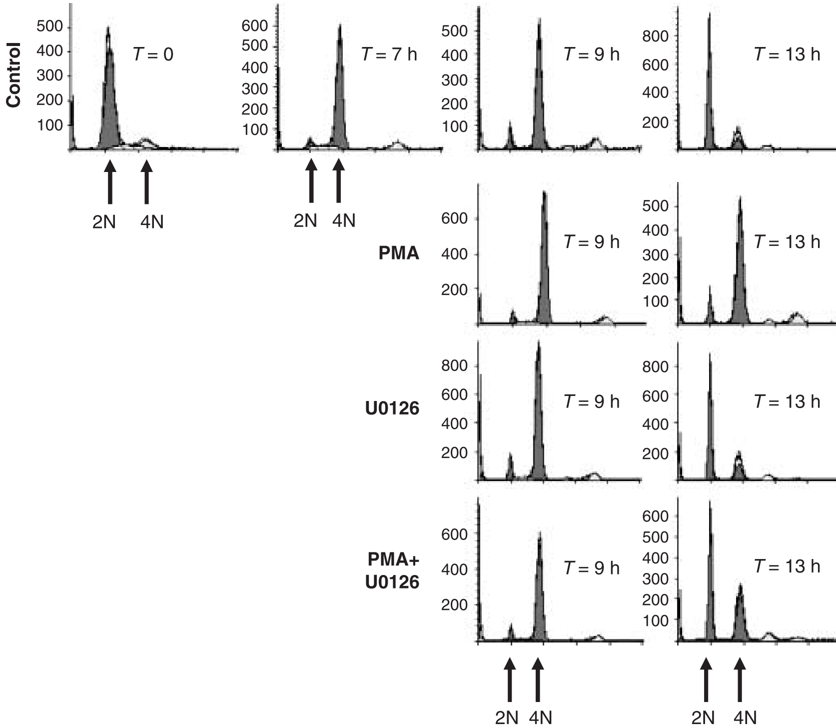

Figure 3. FACS analysis of ERK dependency in mediating PMA-induced G_2-phase arrest. HeLa cells were synchronized at the G_1/S boundary and released back into the cell cycle. Cells were left untreated (control), treated with PMA (0.1 μM) at 7 h after release, treated with U0126 (10 μM) at 6.5 h after G_1/S release or treated with PMA and U0126 together. Cells were harvested at the times indicated after release from G_1/S and the number of cells (Y axis) containing 2N or 4N DNA content (X axis) were determined by FACS.

to be reached before a delay in G_2/M-phase progression could be observed. To test this, cells synchronized at the G_1/S boundary were released into G_2 phase and were treated with varying doses of EGF or PMA. Treatment with 0.1 ng/ml of EGF caused a transient activation of the ERK pathway but had little effect on cyclin B1 expression (Fig. 7a). Increasing the EGF dose further enhanced the magnitude and duration of ERK activity and the expression of cyclin B1 at 11 h after G_1/S release as compared to untreated controls (Fig. 7a). As another measure of cell cycle progression, the expression of the Cdk inhibitor *p21^{CIP1}* was measured. Whereas the lowest dose of EGF that caused ERK activation had little effect on *p21^{CIP1}*, higher EGF doses increased *p21^{CIP1}* expression in a manner that correlated with ERK activity and cyclin B1 expression (Fig. 7a).

Similarly, the low doses of PMA (0.01 nM) activated ERK but had little effect on cyclin B1 expression as compared to controls (Fig. 7b). When PMA concentrations were increased by 10-fold or more, maximal ERK activation was observed and a corresponding increase in cyclin B1 and *p21^{CIP1}* expression consistent with G_2-phase arrest (Fig. 7b). Thus, the level of ERK activity during G_2 phase appears to regulate cell cycle progression through regulation of *p21^{CIP1}* expression.

ERK regulates *p21^{CIP1}* expression during G_2 phase

Several studies support a role for the CIP/KIP family of Cdk inhibitors in regulating cell cycle progression during G_2 phase in response to various stress conditions (Niculescu *et al.* 1998;

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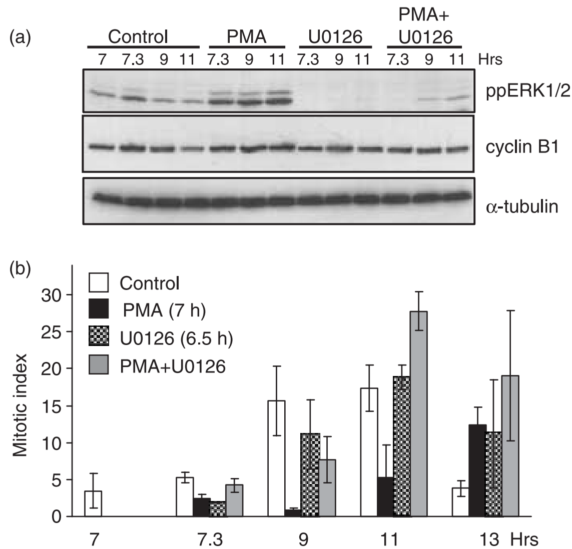

Figure 4. PMA-induced G_2-phase arrest requires ERK activity. Cells synchronized at the G_1/S boundary were released back into the cell cycle and treated in the presence of absence of PMA (0.1 μM, 7 h after G_1/S release), U0126 (10 μM, 6.5 h after G_1/S release), or PMA and U0126 together. (a) Immunoblot analysis of ppERK1/2 (top panel), cyclin B1 (middle panel), and α-tubulin (lower panel) as a protein loading control. (b) The MI at varying times after G_1/S release in untreated (white bars), PMA-treated (black bars), U0126-treated (checkered bars), or PMA- and U0126-treated cells (grey bars). Data represent the mean and standard error from three independent experiments.

Coqueret 2003; Di Gennaro *et al.* 2003). Thus, the requirement for PKC and ERK proteins in regulating the expression of *p21*^{CIP1} was examined in G_2-phase cells treated with PMA. Increased *p21*^{CIP1} expression was observed within 2 h of PMA treatment (9 h after G_1/S release) and continued to increase after 4 and 6 h treatment with PMA (11 and 13 h after G_1/S release, respectively) (Fig. 8a,b). The increased *p21*^{CIP1} expression corresponded to the accumulation of G_2/M-phase cells analysed previously by FACS analysis (Figs 1a and 3) and the accumulation of cyclin B1 protein (Figs 2a and 4a). Inhibition of PKC or ERK activation during G_2 phase with Bis or U0126, respectively, inhibited PMA-induced *p21*^{CIP1} expression (Fig. 8a,b). These data suggest that elevated PKC and ERK activities delay G_2/M progression by increasing the expression of *p21*^{CIP1} during G_2 phase.

It was next investigated whether PMA-induced expression of *p21*^{CIP1} during G_2 phase was the result of increased protein synthesis or to chromosome stability. Synchronized cells were treated with cyclohexamide at 7.5 h after G_1/S release to block protein synthesis. After 30 min, cells were treated with PMA (8 h after G_1/S release) and harvested after an additional 1 or 3 h incubation (9 and 11 h post-G_1/S-phase release). As shown, cyclohexamide blocked basal and PMA-induced *p21*^{CIP1} expression at 9 and 11 h post-G_1/S release (Fig. 8c). In contrast, inhibition of the proteasome and protein degradation with MG115 had little effect on PMA-induced *p21*^{CIP1} expression (Fig. 8c). MG115 treatment in the absence of PMA caused a small increase in *p21*^{CIP1} expression, suggesting a role for the proteasome in *p21*^{CIP1} turnover during G_2 and M phase (Fig. 8c). These data indicate that one mechanism for PMA-mediated G_2-phase arrest is through ERK-mediated *de novo* synthesis of *p21*^{CIP1}.

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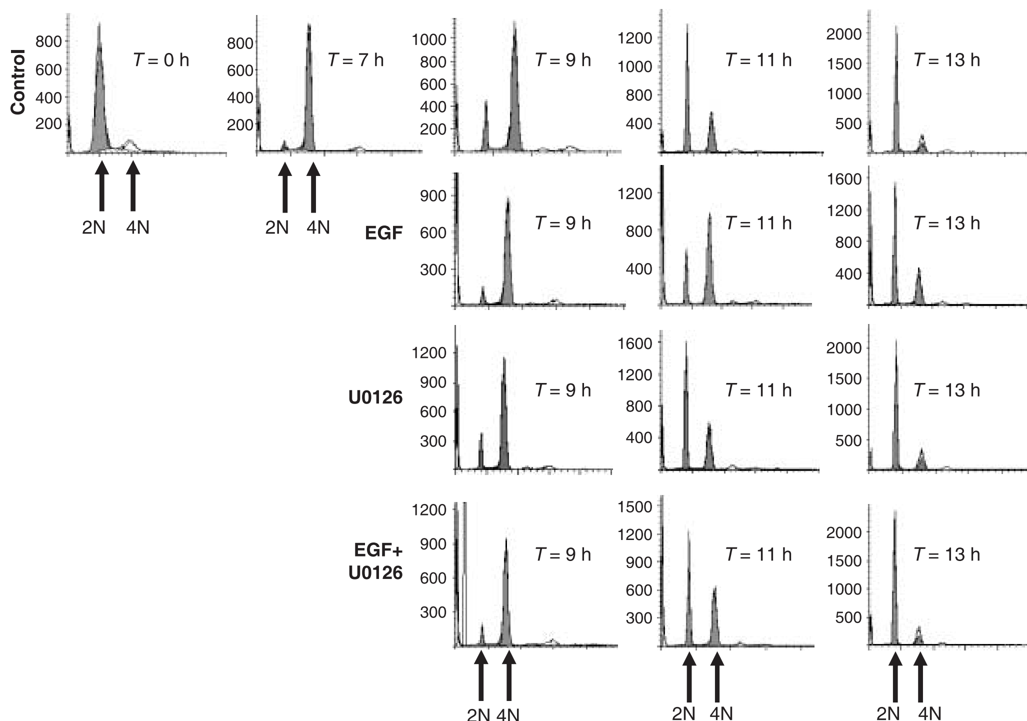

Figure 5. FACS analysis of ERK dependency in mediating EGF-induced delay in G_2/M progression. HeLa cells were synchronized at the G_1/S boundary and released back into the cell cycle. Cells were left untreated (control), treated with EGF (100 ng/mL) at 7 h after G_1/S release, treated with U0126 (10 μM) at 6.5 h after G_1/S release, or treated with EGF and U0126 together. Cells were harvested at the times indicated after release from G_1/S and the number of cells (Y axis) containing 2N or 4N DNA content (X axis) were determined by FACS.

p21^{CIP1} is required for PMA-induced G_2-phase arrest

To demonstrate that *p21^{CIP1}* expression is required for PMA-induced G_2-phase arrest, cell cycle progression was monitored in HCT116 colorectal carcinoma cells lacking *p21^{CIP1}*. FACS analysis showed that greater than 85% of both parental and *p21^{CIP1}* knockout cells synchronized at the G_1/S-phase boundary with 2N DNA content using excess thymidine (data not shown). Treatment with PMA at 6 h after release, when most cells have entered G_2/M-phase, caused a delay in mitotic progression as measured by FACS in the parental but not the cells lacking *p21^{CIP1}* (Fig. 9a,b). Examination of mitotic indices demonstrated that PMA induced a 2-h delay in mitotic entry in parental cells but had no effect on mitotic entry in cells lacking *p21^{CIP1}* (Fig. 9c,d). Although the data also show that cells lacking *p21^{CIP1}* progress through G_2/M-phase at different rates than the parental cells, these findings nevertheless indicate that *p21^{CIP1}* is required for PMA-induced G_2-phase arrest.

A role for ERK-mediated *p21^{CIP1}* expression during G_2 phase in regulating chromosome stability

The last set of experiments described here examined the consequence of ERK activation during G_2 phase and *p21^{CIP1}* expression, on hallmark characteristics of chromosome stability. Defects in mitosis and cytokinesis are manifested by cells that are binucleate and/or contain multiple

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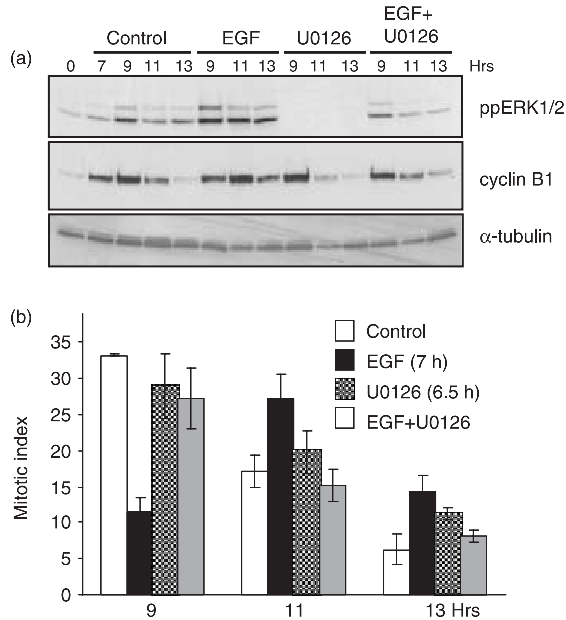

Figure 6. EGF-induced G_2-phase arrest requires ERK activity. Cells synchronized at the G_1/S boundary were released back into the cell cycle and treated with EGF (100 ng/ml, 7 h after G_1/S release), U0126 (10 µM, 6.5 h after G_1/S release), or both EGF and U0126. (a) Immunoblots show the levels of ppERK1/2 (top panel), cyclin B1 (middle panel) and α-tubulin (lower panel) as a protein loading control. (b) The MI at varying times after G_1/S release in untreated (white bars), EGF-treated (black bars), U0126-treated (checkered bars), or EGF- and U0126-treated cells (grey bars). Data represent the mean and standard error from three independent experiments.

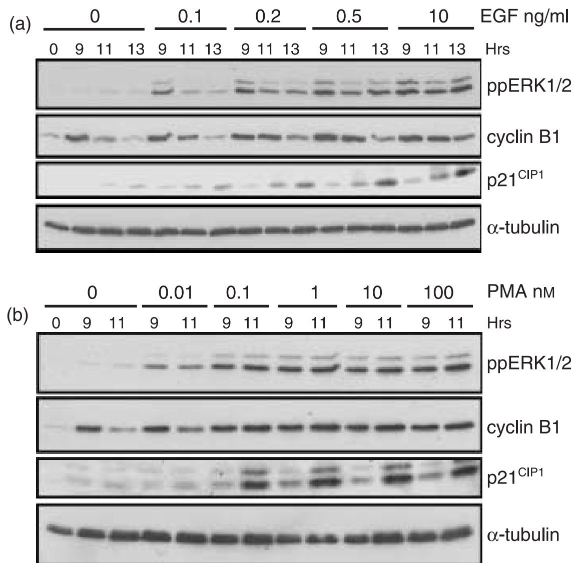

Figure 7. Cyclin B1 and *p21^{CIP1}* expression during G_2/M-phase correlate with ERK activity. Cells synchronized in G_1/S were released back into the cell cycle and treated with the indicated dose of EGF (a) or PMA (b). Immunoblot analysis shows the level of ppERK1/2 (top panel), cyclin B1, and *p21^{CIP1}* (middle panels) expression. The expression of α-tubulin (lower panel) is shown for a protein loading control.

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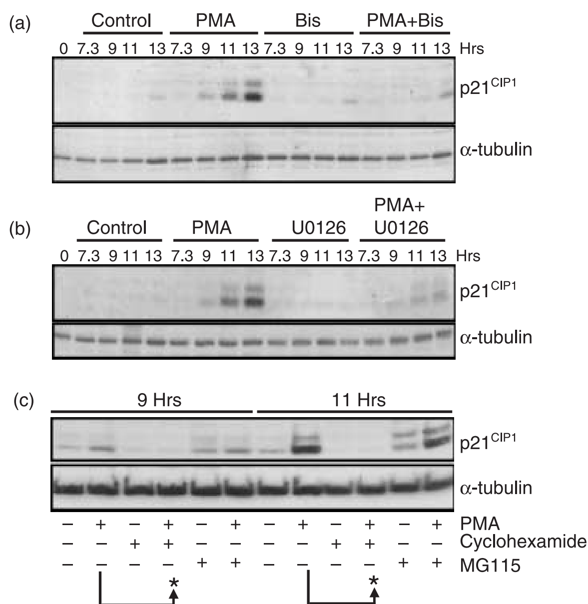

Figure 8. ***De novo*** **synthesis of** ***p21^{CIP1}*** **during G_2-phase requires PKC and ERK activity.** HeLa cells were synchronized at the G_1/S boundary, released, and harvested at the times indicated. (a) Immunoblot analysis of *p21^{CIP1}* expression in control cells or cells treated with PMA, Bis, or PMA plus Bis as described in Figs 1 and 2. (b) Immunoblot of *p21^{CIP1}* in control cells or cells treated with PMA, U0126, or PMA plus U0126 as described in Figs 3 and 4. (c) Immunoblot analysis of *p21^{CIP1}* in untreated or cells treated with PMA in the presence or absence of cyclohexamide (10 μM) or MG115 (10 μg/mL) at 7 h after G_1/S-phase release. The arrows and asterisks highlight the expression of *p21^{CIP1}* at 9 or 11 h after G_1/S release in cells treated with PMA minus or plus cyclohexamide or MG115. The expression of α-tubulin (lower panel) is shown for a protein loading control in a, b, and c.

centrosomes; both of which are indicators of chromosome instability (Saavedra *et al.* 1999; Fenech 2002), among the cells described in these experiments. HeLa cells synchronized in G_2 were treated with EGF in the presence or absence of U0126, and then were incubated for 17 h to allow completion of one cell cycle, and then were processed for immunofluorescence. Approximately 15% of the cells treated with EGF were bi-nucleate compared to approximately 3% in untreated cells (Fig. 10a,b). The increase in bi-nucleate cells caused by EGF could be prevented with U0126 treatment (Fig. 10a). This response was unique to ERK activation during the G_2 phase, as asynchronous cells treated with EGF showed only a slight increase in bi-nucleate cells as compared to G_2-phase synchronized cells treated with EGF (Fig. 10c). Similarly, G_2-phase cells treated with EGF also exhibited an increase in the number of cells containing greater than two centrosomes, which also was partially prevented with U0126 (Fig. 10d). These findings suggest that ERK activation during G_2 phase promotes cellular changes that are consistent with chromosome instability.

Finally, a function for *p21^{CIP1}* expression during G_2 phase in regulating the generation of bi-nucleate cells was examined in HCT116 parental and *p21^{CIP1}* knockout cells. Synchronized cells were treated during G_2 phase in the absence or presence of PMA. At times after PMA treatment, cells were fixed and stained with DAPI for nuclear analysis. Similar to EGF, PMA treatment increased the number of bi-nucleate cells in both parental and *p21^{CIP1}* knockout cells (Fig. 10e,f). However, in the absence of *p21^{CIP1}*, there were substantially more bi-nucleate cells in untreated or PMA-treated conditions, as compared to the parental cells (Fig. 10e,f). The ratio

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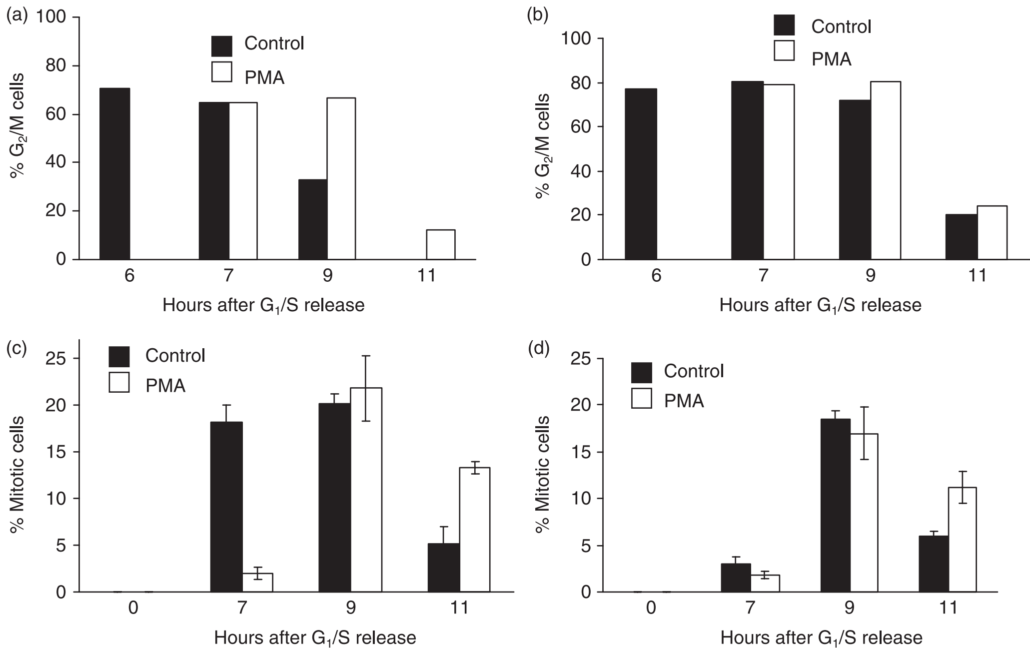

Figure 9. Expression of *p21^{CIP1}* is required for PMA-induced G_2-phase arrest. HCT116 parental (a and c) or *p21^{CIP1}* knockout cells (b and d) were synchronized at the G_1/S-phase boundary, treated in the absence (closed bars) or presence (open bars) of 0.1 μM PMA at 6 h after release, and harvested at the times indicated after G_1/S release for FACS analysis of G_2/M-phase cells (a and b) or mitotic indices (c and d).

of PMA to unstimulated cell was also expressed using the basal levels of bi-nucleate cells in parental or *p21^{CIP1}* knockout cells. When five time points for the unstimulated conditions were averaged, the *p21^{CIP1}* knockout cells showed approximately 2.5-fold more bi-nucleate cells compared to the parental cells in the absence of PMA treatment. When this difference is taken into account, PMA still induced approximately threefold more bi-nucleate cells in *p21^{CIP1}* knockout cells compared to the parental cells at the 11-h time point (data not shown). However, at the 25- or 33-h time points, this analysis showed no difference in the PMA-induced bi-nucleate cells between the *p21^{CIP1}* knockout cells and the parental cells (data not shown). Thus, although the magnitude in number of bi-nucleate cells in *p21^{CIP1}* knockout cells treated with or without PMA are greater than the parental cells (Fig. 10e,f), the fold increase as a result of PMA is only observed during the first cell cycle. Nevertheless, these data support a role for *p21^{CIP1}* expression during G_2 phase in providing protection against a phenotype characteristic of chromosome instability.

DISCUSSION

Using synchronized cells, these studies demonstrate that activation of the ERK pathway during G_2 phase with PMA or EGF will initiate a rapid delay in G_2 to M-phase progression. The ERK-mediated delay during the G_2 phase appears to occur, in part, through the *de novo* synthesis of the cell-cycle inhibitor *p21^{CIP1}* in p53 defective cells. PMA or EGF dose–response assays

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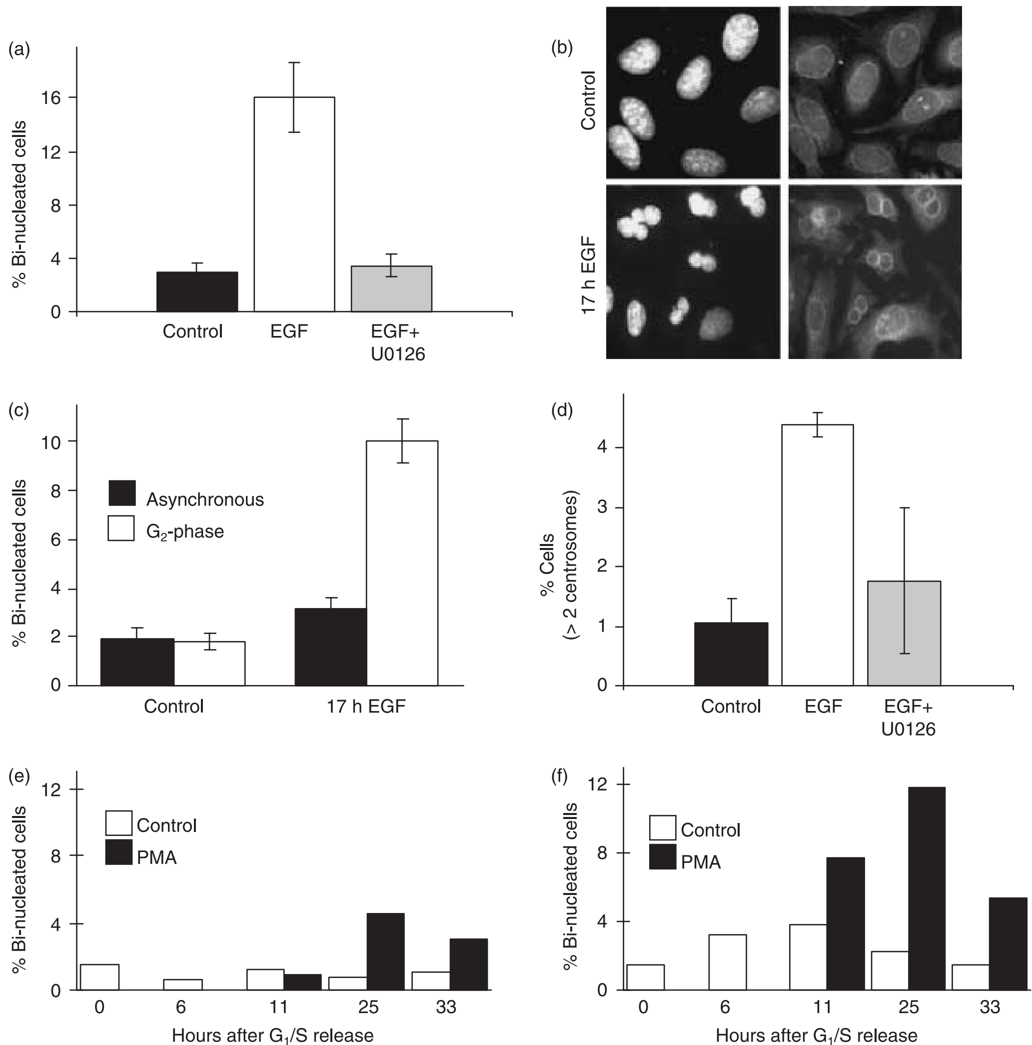

Figure 10. ERK activation during G_2-phase promotes hallmarks of chromosome instability; protective function of *p21*^{CIP1} expression. (a) Cells synchronized at the G_1/S-phase boundary were released for 7 h and treated with or without EGF (10 ng/mL) in the absence or presence of U0126 (10 μM). After 17 h, cells were immunostained for p62 nucleoporin as a marker of the nuclear envelope and counter-stained with DAPI. The graph shows the percentage of bi-nucleated cells under each condition. (b) Representative image of DAPI and p62 nucleoporin staining in control and EGF treated cells showing bi-nucleated cells. (c) Percentage of bi-nucleated cells in asynchronous or G_2-phase synchronized cells treated for 17 h in the absence or presence of EGF. (d) Percentage of cells containing greater than two centrosomes in control, EGF or EGF plus U0126 treated cells as described for (a). HCT116 parental (e) or *p21*^{CIP1} knockout cells (f) were synchronized at G_1/S phase, treated in the absence or presence of PMA at 6 h after release, and the percentage of bi-nucleated cells were determined at the times indicated.

indicated that some ERK activity above basal levels is tolerated during G_2 phase. However, once an ERK activity threshold was reached, then the delay in G_2-phase progression could be observed. A potential consequence of activated ERK during G_2 phase is an increased number of bi-nucleate cells that, in this case, are an indicator of defects in mitosis and cytokinesis and a characteristic of chromosome instability. Although a role for the ERK pathway during PMA or EGF-induced

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G_1 or G_2-phase arrest was implied previously (Kinzel *et al.* 1990; Kaszkin *et al.* 1991; Kosaka *et al.* 1996; Barboule *et al.* 1999), the current studies are to our knowledge, the first to show a link between ERK activity and the regulation of $p21^{CIP1}$ expression during G_2 phase.

The function of the ERK pathway in modulating G_2 and M-phase transitions remains largely unknown. Recent studies using pharmacological inhibitors, catalytically inactive mutants of ERK pathway proteins, and RNA interference suggests that a basal ERK activity is important for G_2 and M-phase progression in several transformed and non-transformed cell lines (Abbott & Holt 1999; Wright *et al.* 1999; Hayne *et al.* 2000; Roberts *et al.* 2002; Liu *et al.* 2004). In addition, activation of the ERK pathway caused by over-expression of constitutively active Ras, Raf-1, or MEK1 mutants can induce a p53-dependent cell cycle arrest and senescence in non-transformed cells (Lin *et al.* 1998; Zhu *et al.* 1998; Ferbeyre *et al.* 2002). Moreover, over-expression of the Mos kinase and subsequent ERK activation caused a p53-dependent cell-cycle arrest characterized by an increased number of bi-nucleate cells (Fukasawa & Vande Woude 1997). Thus, although some basal level of ERK activity is needed during G_2/M phase, ERK activation above basal levels may also be detrimental at this time of the cell cycle.

Our studies used primarily p53 defective cells, which have suggested that G_2-phase arrest mediated by ERK activation and $p21^{CIP1}$ induction is regulated through p53-independent mechanisms. This is in agreement with others that used conditional activation of Raf kinase and ERK to cause a G_1-phase arrest in mouse fibroblasts through p53-independent induction of $p21^{CIP1}$ (Woods *et al.* 1997). Thus, p53-independent mechanisms for cell cycle control as previously suggested (Taylor & Stark 2001) and p53-dependent mechanisms appear to function in ERK-mediated G_1 or G_2-phase arrest.

One role for G_2-phase arrest as a result of ERK activation may be to protect cells from signals that may interfere with mitotic transitions. A major function for the ERK pathway is to mediate transcription events in response to extracellular signals. However, the structural constraints imposed by condensed chromosomes result in transcription inhibition in mitotic cells. Thus, elevated ERK activity throughout the cell during G_2 phase might be sensed as an inappropriate activation of transcription events that would detract from the high energy requirements needed for the dynamic structural changes that occur during mitosis. To compensate, our findings suggest that ERK may negatively regulate the cell cycle by imposing a G_2-phase checkpoint through induction of $p21^{CIP1}$ until ERK activity returns to levels that are compatible with proper mitotic transitions.

Increased expression of $p21^{CIP1}$, which is found in some cancer cells, may act to protect cells against apoptosis-inducing agents (Gartel & Radhakrishnan 2005). Similarly, activation of the ERK pathway in cancer cells may be involved in the development of drug resistance (Davis *et al.* 2003). ERK-induced G_2-phase arrest following treatment with genotoxic agents, such as doxorubicin, or microtubule-interfering drugs, such as paclitaxel, may provide a protective mechanism that prevents cells with extensive DNA damage from entering mitosis and generating additional damage that would initiate an apoptotic response. Thus, G_2-phase arrest may allow the cells to repair DNA damage induced by chemotherapeutic drugs. As such, efforts towards simultaneous inhibition of ERK or other signalling pathways may be a more effective approach to treating drug-resistant cancer cells. For example, simultaneous inhibition of the PKC, Chk1, and the ERK pathway with UCN-01 and MEK1/2 inhibitors has been shown to be effective in killing leukaemia cells that harbour the BCR-Abl fusion protein and are resistant to the anticancer drug ST1571, also known as Gleevec (Yu *et al.* 2002).

The role of other MAP kinase signalling pathways in regulating cell cycle progression through G_2 phase and mitosis may also play a role during genotoxic or other stress conditions. Activation of p38 MAP kinase was reported to mediate G_2-phase arrest in response to genotoxic

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or osmotic stress (Bulavin *et al*. 2001; Dmitrieva *et al*. 2002; Hirose *et al*. 2003). Similarly, conditional activation of MEKK3, a kinase that mediates inflammatory responses, induces G_2-phase arrest through a mechanism that is partially dependent on p38 MAP kinase activity (Ellinger-Ziegelbauer *et al*. 1999; Garner *et al*. 2002). Several MAP kinases may mediate cell cycle arrest in the context of breast cancer cell lines treated with the chemotherapeutic drug paclitaxel, which causes G_2-phase arrest and apoptosis in a manner that requires ERK and p38 MAP kinase activities but not a functional p53 protein (Bacus *et al*. 2001). However, in our studies, we have found no evidence that p38 MAP kinase is activated in G_2 or M-phase cells treated with or without PMA (data not shown). This agrees with others who report that PMA is a potent activator of ERK but not p38 MAP kinase (Morton *et al*. 2004).

During DNA damage or microtubule disruption, the c-Jun N-terminal kinase (JNK) pathway may also promote G_2-phase arrest and susceptibility to undergo apoptosis through a mechanism involving inhibition of cdc2 and the anti-apoptotic protein Bcl-2 (Yamamoto *et al*. 1999; Goss *et al*. 2003). Similar to ERK, a basal level of JNK activity may regulate proper progression through G_2 and M phase of the cell cycle. Recent studies suggest that inhibition of JNK activity with relatively non-specific chemical inhibitors or by genetic knockout of MKK7, a JNK activator, causes a delay in G_2 to M-phase progression (Mingo-Sion *et al*. 2004; Wada *et al*. 2004). Although we have found no evidence that JNK proteins are activated in untreated or PMA-treated synchronized HeLa cells during G_2 phase (data not shown), the three major MAP kinase family members may co-ordinate cell cycle progression during G_2 phase in a manner that may be dependent on cell type and extracellular signal.

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