Loss of Rb overrides the requirement for *ERK* activity for cell proliferation

Giovanna M. D'Abaco, Steven Hooper, Hugh Paterson and Christopher J. Marshall*

Cancer Research UK Centre for Cell and Molecular Biology, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK

*Author for correspondence (e-mail: chrism@icr.ac.uk)

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Summary

The Ras GTPase is a critical transducer of mitogenic signals ultimately leading to inactivation of the retinoblastoma (Rb) protein, but the molecular basis underlying Ras-dependent control of cell cycle kinetics remains to a great extent unknown. In an effort to further elucidate the role of Ras activation in cell cycle control, we have studied the role of the downstream Mek-ERK pathway in facilitating exit from the quiescent G0 state and passage through the G1/S transition. We have adopted a genetic approach in combination with U0126, an inhibitor of Mek activation to study the role of Mek in cell cycle progression. Here we report that whereas wild-type (Wt) mouse embryo fibroblasts (MEFs) depend on ERK activation to enter the cell cycle, Rb-deficient ($Rb^{-/-}$) MEFs have a reduced requirement for ERK signalling. Indeed in the presence of U0126 we found that Rb-null MEFs can exit G0, make the G1/S transition and proliferate. Analysis of Rb-deficient tumour cell lines also revealed a reduced requirement for ERK signalling in asynchronous growth. We discuss the molecular mechanism that may underlie this escape from MAP kinase signalling.

Key words: Ras, MAP kinase, ERK, Retinoblastoma

Introduction

Ras proteins function as a molecular switch cycling between the inactive GDP and active GTP bound state. In the GTP bound state Ras binds to and leads to the activation of downstream effectors. A number of potential effectors of Ras have been described including the Raf family of protein kinases, the Ral-GDS family of guanine nucleotide exchange factors, PI 3-kinase (Marshall, 1996) and Phospholipase C-epsilon (Kelley et al., 2001; Lopez et al., 2001; Song et al., 2001). Since many of these studies are based on over-expression studies, which of these molecules are true effectors remains to be determined. However genetic evidence from C. elegans and D. melanogaster model systems strongly supports a role for Raf and thereby the ERK-MAP kinase pathway as a downstream effector of Ras (Dickson and Hafen, 1994; Marshall, 1996).

In mammalian cells, entry into the cell cycle is controlled at the G1/S boundary (reviewed in Sherr, 1996). The requirement for Ras in cell cycle regulation and proliferation is now well established (Cai et al., 1990; Feig and Cooper, 1988; Mulcahy et al., 1985; Stacey et al., 1991; Dobrowolski et al., 1994; Aktas et al., 1997). These studies also show that Ras activity is required at multiple points in cell cycle entry. Following growth factor stimulation of quiescent non-proliferating cells, Ras activity is required for cells to exit G0 and pass through the G1/S transition of the cell cycle (Dobrowolski et al., 1994; Feig and Cooper, 1988; Mulcahy et al., 1985). In contrast to growth factor stimulation of quiescent cells, studies in exponentially growing cells indicate that Ras is required in the G2 phase of the preceding cycle and not during G1 (Hitomi and Stacey, 2001).

The key event that regulates the G1/S transition is inactivation of the retinoblastoma (Rb) protein via phosphorylation mediated by cyclin dependent kinases. In its hypo-phosphorylated state Rb is bound to the E2F transcription factor (reviewed in Weinberg, 1995). This represses transcription of target genes that are required for progression through S phase of the cell cycle. Phosphorylation of Rb leads to its inactivation and de-repression of E2F facilitating DNA replication. Inactivation of Rb by phosphorylation is mediated by the sequential activation of the cyclin dependent kinases (cdks) (Sherr, 1996; Weinberg, 1995). The D-type cyclins which bind cdk4 or cdk6 and cyclin E and cyclin A which bind cdk2 are implicated in this process (Sherr, 1996; Weinberg, 1995).

Many studies link cyclin D1 expression to Ras signalling. The use of inducible Ha-Ras expression constructs has demonstrated that overexpression of Ha-Ras in the rat epithelial cell line IEC (Filmus et al., 1994) and BalbC-3T3 cells (Winston et al., 1996) leads to the induction of cyclin D1 expression. Conversely expression of dominant negative Ras mutants has been demonstrated to inhibit expression of cyclin D1 following serum stimulation (Aktas et al., 1997). An acceleration of G1 progression and shorter cell doubling times in asynchronously growing Ha-Ras transformed NIH3T3 cells has been shown to correlate with overexpression of cyclin D1 (Albanese et al., 1995; Liu et al., 1995). These reports make a clear link between Ras expression and the induction of cyclin D1 expression in cell cycle control. Activation of ERK-MAP kinase appears to be a key event in mediating Ras-dependent regulation of cyclin D1 expression as well as cdk complex

formation (Lavoie et al., 1996; Weber et al., 1997; Cheng et al., 1999). PI 3-kinase and Ral-GEF have also been postulated to regulate cyclin D1 expression and stability (Diehl et al., 1997; Muise-Helmericks et al., 1998; Gille and Downward, 1999; Henry et al., 2000).

Levels of the cdk inhibitor $p27^{Kip1}$ are high in quiescent cells. Re-entry into the cell cycle is accompanied by a decline in the levels of $p27^{Kip1}$ facilitating activation of cyclin E/cdk2 complexes (Sherr and Roberts, 1999). Expression of oncogenic Ras had also been shown to regulate the levels of $p27^{Kip1}$ (Aktas et al., 1997; Takuwa and Takuwa, 1997). Studies indicate that regulation of $p27^{Kip1}$ expression by the Raf-ERK pathway and PI 3-kinase is mediated by regulating the rate of translation and protein stability (Aktas et al., 1997; Kerkhoff and Rapp, 1997; Takuwa and Takuwa, 1997). A further contribution to the relief of $p27^{Kip1}$inhibition of cdk2 complexes is made by the Mek-ERK pathway facilitating cyclin D1/cdk4 complex formation which results in sequestration of $p27^{Kip1}$ onto this complex and allows activation of cdk2 complexes (Cheng et al., 1998).

Microinjection studies using the neutralising Y13-259 antibody (Furth et al., 1982) against Ras show that the requirement for Ras signalling to promote cell cycle entry is altered in $Rb^{-/-}$ cells (Mittnacht et al., 1997; Peeper et al., 1997). Interestingly these experiments defined a difference between cells that are asynchronously growing and cells that have been rendered quiescent and then treated with growth factors to stimulate cell cycle entry. Regardless of whether they express Rb or are Rb null, cells require Ras activation in order to leave G0. In contrast microinjection of the Y13-259 antibody into asynchronously growing cells revealed that in comparison to wild-type MEFs, Rb-null MEFs have a reduced requirement for Ras activity (Mittnacht et al., 1997; Peeper et al., 1997). This reveals two interesting points. Ras activity is critical for both exit from G0 and for cells to make the G1/S transition. However with the loss of Rb, cells are released from the requirement for Ras for the G1/S transition. The observation that proliferation of asynchronously growing $Rb^{-/-}$ MEFs is much less affected by blocking Ras function raises the question whether $Rb^{-/-}$ cells have a reduced requirement for Ras dependent signalling pathways such as the ERK-MAP kinase signalling pathway. We find that $Rb^{-/-}$ cells no longer depend on ERK signalling for re-entry into the cell cycle from G0,or for asynchronous growth. Consistent with this phenotype is the observation that the main role of ERK-MAP kinase pathway is to regulate cyclin D1 expression and associated cdk activity rather than the regulation of cyclin E and cyclin A expression.

Materials and Methods

Cell culture

Mouse embryo fibroblasts (MEFs) were prepared from embryos at 12.5 days post-coitus (dpc) as described by D'Abaco and Olson (D'Abaco and Olson, 2000). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and all experiments were conducted using early passage (<6) MEFs. For BrdU experiments cells were seeded at 2500 cells/well on 13 mm coverslips in DMEM supplemented with 10% FCS. The following day, cells were washed once in DMEM supplemented with 0.5% FCS and then maintained in this medium for 24-48 hours. Subsequently cells were treated with inhibitor(s) for 60 minutes then stimulated with 10% (v/v) serum. U0126 (Promega) was used at 20 μM, with DMSO as the vehicle control. LY294002 (Biomol Research Laboratories) was used at 20 μM with ethanol as the vehicle control For growth curve experiments cells were plated at a density of 10,000/plate in 5 cm^2 plates in DMEM containing 10% FCS. Following an overnight incubation cells were treated with inhibitor(s) as described above.

Cell cycle analysis

To monitor proliferation, following treatment with inhibitor(s) cells were pulsed with bromodeoxyuridine (BrdU) (Amersham Pharmacia Biotech) at the time of serum stimulation for 12-16 hours. Cells were fixed and stained for immunofluorescence as follows. Cells were rinsed once in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 minutes at room temperature. After rinsing twice in PBS cells were permeabilized in 0.2% Triton X-100/0.1 mM glycine for 30 minutes at room temperature. Cells were rinsed several times in PBS and then blocked by incubation in 5% FCS in PBS (PBS/FCS) for 30 minutes at room temperature. The cells were then treated with RNase free DNase I (Roche) at 1-2 units/μl for 60 minutes at 37°C. After washing three times in PBS/FCS cells were incubated with anti-BrdU rat monoclonal antibody (diluted to 5 μg/mL in PBS/BSA) for 1 hour at room temperature. After washing cells three times in PBS/BSA cells were incubated with fluorescent secondary antibody (Jackson Immunoresearch Laboratories) to detect incorporated BrdU and DAPI (Sigma) to detect total nuclei in each field. In addition a Texas-Red-X-conjugated phalloidin stain (Molecular Probes) was used to detect cells. Secondary antibodies and phalloidin were diluted at 1:200, DAPI was diluted to a working concentration of 1 μg/ml in PBS/FCS. Cells were then incubated for 1 hour at room temperature in a light-proof container. After rinsing three times in PBS and once in water, coverslips were mounted onto glass slides by inverting onto 5-10 μl of moviol mountant containing 0.1% para-phenylenediamine as an antiquenching agent. Stained preparations were examined with a Bio-rad MRC confocal imaging system in conjuction with a Nikon Diaphot epifluorescence microscope.

Antibodies

An ERK2 rabbit polyclonal antibody generated against a C-terminal ERK2 peptide (no. 122) was used for immunoprecipitation experiments (Leevers and Marshall, 1992). A phospho-specific antibody to ERK1 and 2 (clone MAP-YT) was purchased from Sigma. The antibody that detects the hypo and hyper phosphorylated forms of the Retinoblastoma protein (14001A), was purchased from PharMingen. Anti-cyclin D1 (72-13G), cyclin E (M20) and cyclin A (H-432) antibodies were purchased from Santa Cruz Biotechnology. An anti-$p27^{Kip1}$ (clone 57) antibody was purchased from Transduction Laboratories.

Preparation of cell extracts

Cells were washed twice in ice-cold PBS and lysed in ELB buffer (50 mM NaCl, 100 mM HEPES pH 7.0, 10 mM EDTA and 0.2% Triton-X-100) containing 20 mM β-glycerol phosphate, 20 mM sodium fluoride, 10 mM sodium vanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Cell debris was cleared by centrifugation at 18,000 ***g*** for 10 minutes. Protein concentrations were determined using the BCA protein assay reagent (Pierce).

Western blots

Immunoblotting was performed using 25-30 μg of cleared lysate. For detection of the Rb protein 250 μg of cleared lysate was loaded. Prior

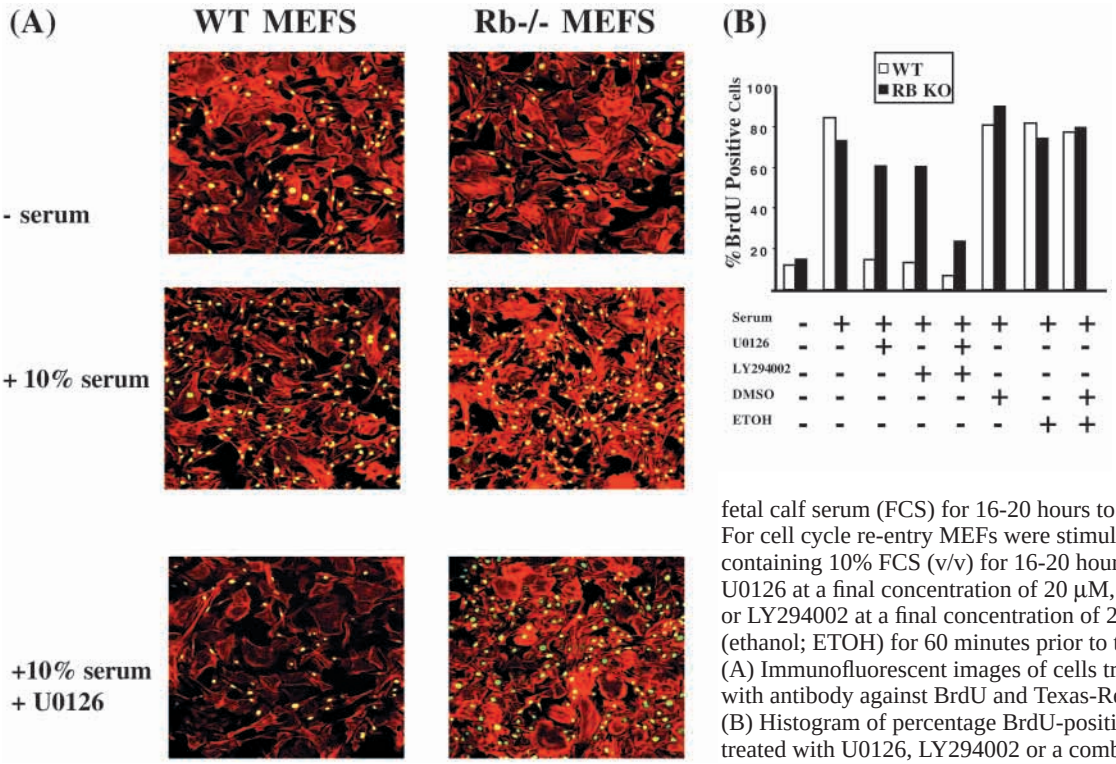

Fig. 1. Rb null but not wild-type MEFs enter the cell cycle in the presence of the Mek inhibitor U0126. Primary MEFs were plated at a 2500 cells/13 mm coverslip. The following day cells were placed in medium containing 0.5% (v/v) fetal calf serum (FCS) for 16-20 hours to render the cells quiescent. For cell cycle re-entry MEFs were stimulated with medium containing 10% FCS (v/v) for 16-20 hours. Cells were treated with U0126 at a final concentration of 20 μM, or vehicle control (DMSO) or LY294002 at a final concentration of 20 μM, or vehicle control (ethanol; ETOH) for 60 minutes prior to the addition of serum. (A) Immunofluorescent images of cells treated with U0126 stained with antibody against BrdU and Texas-Red-X-phalloidin. (B) Histogram of percentage BrdU-positive cells that have been treated with U0126, LY294002 or a combination.

to loading Laemmli buffer was added to each sample, followed by heating to 100°C for 3-5 minutes. Samples were resolved by electrophoresis through denaturing SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) (Millipore) membrane for western blotting. Membranes were probed with an appropriate dilution of primary antibody for 1-2 hours at room temperature followed by incubation with a horseradish peroxidase (HRP) conjugated secondary antibody (Bio-Rad) for 45 minutes at room temperature. Proteins were visualised using the enhanced chemiluminescence (ECL) reagent (Amersham).

ERK2 assay

Cells were lysed in lysis buffer as described above. For each sample 50 μg of cleared lysate was immuopprecipitated with the 122 antibody coupled to protein-A-Sepharose beads for 90 minutes at 4°C. Immuno-complexes were washed twice in lysis buffer and twice in kinase buffer (30 mM Tris pH 8.0, 20 mM $MgCl_2$ and 2 mM $MnCl_2$) containing 10 mM β-glycerol phosphate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Kinase reactions were conducted in a final volume of 30 μl kinase buffer containing 10 μM ATP (Sigma), 5 μCi [γ-^{32}P]ATP Amersham) and 0.25 mg of myelin basic protein (Sigma) as the substrate per reaction. After a 30 minute incubation at 30°C reactions were stopped by the addition Laemmli buffer followed by heating to 100°C for 3-5 minutes. Proteins were then transferred to PVDF membrane and ERK2 activity was detected by PhosphoImager (Molecular Dymamics).

Cell-cycle-associated kinase assays

Cells were lysed in lysis buffer as described above. For each sample 500 μg of cleared lysate was immuoprecipitated with antibodies coupled to protein-A-Sepharose beads for 90 minutes at 4°C. Immuno-complexes were washed twice in lysis buffer and twice in kinase buffer (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$) containing 10 mM β-glycerol phosphate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM protein kinase inhibitor (Sigma). For cyclin D1/cdk4 associated kinase assays a GST-fusion construct encoding a C-terminal fragment (amino acids 763-928) of Rb (Zarkowska and Mittnacht, 1997) was used at 0.5 μg per reaction. For cyclin E/cdk2 kinase assays histone H1 was used as the substrate (a gift from G. Goodwin, ICR) at 2 μg per reaction. Kinase reactions were conducted in a final volume of 25 μl kinase buffer containing 50 μM ATP and 5 μCi [γ-^{32}P] ATP. After a 15 minute incubation at 30°C reactions were stopped by the addtion of SDS-PAGE sample buffer. Proteins were then transferred to PVDF membrane and kinase activity was detected by PhosphoImager.

Results

MAP kinase signalling is uncoupled from the G1/S transition in Rb-null cells

The Ras-neutralising antibody Y13-259 (Furth et al., 1982) has been shown to have little effect on proliferation when microinjected into asynchronously growing Rb-null cells suggesting that these cells have a reduced requirement for Ras signalling (Mittnacht et al., 1997; Peeper et al., 1997). It is well established that activation of ERK-MAPK pathway is Ras-dependent (Marshall, 1995). Therefore we compared the ability of serum growth factors to drive quiescent Wt and $Rb^{-/-}$ MEFs into S-phase in the presence of U0126, an inhibitor of Mek activation (Favata et al., 1998). Following serum starvation for 20-24 hours cells were rendered quiescent as judged by their failure to incorporate BrdU (Fig. 1A,B). Serum stimulation for 16-20 hours resulted in efficient cell cycle entry and DNA replication (Fig. 1A,B) whereas treatment of Wt MEFs with U0126 blocked cell cycle entry. Treatment of $Rb^{-/-}$ MEFs with U0126 did not result in a block of cell cycle entry (Fig. 1A,B). Similar results were obtained with PD098059

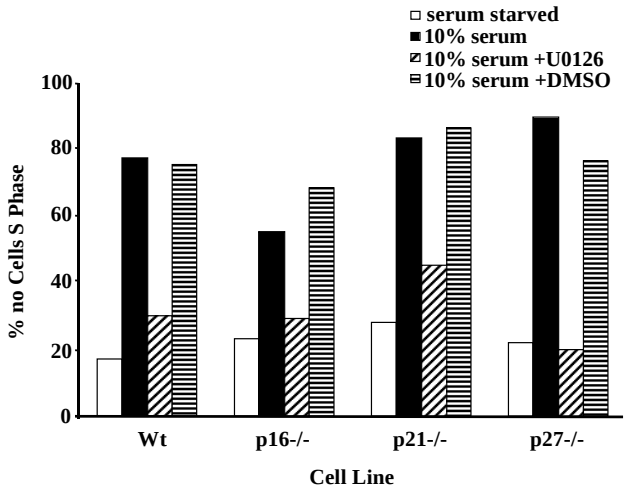

Fig. 2. Loss of expression of CDKIs does not result in escape from requirements for MAPK activation. MEFs derived from $p21^{Waf1/Cip1}$, $p27^{Kip1}$ or $p16^{Ink4}$-knockout mice were plated at a density of 2.5×10^4/ml. The following day the cells were placed in medium containing 0.5% (v/v) FCS for 24-48 hours. The cells were then treated with U0126 (20 μM final concentration) or vehicle control (DMSO) 60 minutes and then stimulated with 10% (v/v) FCS for 16-20 hours followed by immunofluorescence.

(Dudley et al., 1995), another inhibitor of Mek activation (data not shown). As $Rb^{-/-}$ MEFs appear to have a reduced requirement for ERK signalling we went on to study the role of PI 3-kinase which has also been shown to be an effector of oncogenic Ras (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1997; Rodriguez-Viciana et al., 1996; Kodaki et al., 1994). Analysis of proliferation in the presence of LY294002, an inhibitor of PI 3-kinase activation (Davies et al., 2000) revealed that unlike the Wt cells the $Rb^{-/-}$ MEFs are able to enter the cell cycle (Fig. 1B). However, treatment with the combination of inhibitors results in a block in cell cycle entry in both cell lines (Fig. 1B).

Since the requirement for ERK activation for cell proliferation was abrogated in $Rb^{-/-}$ cells, we investigated whether cells lacking other negative regulators of cell cycle entry had altered signalling requirements. Fig. 2 shows that MEFs that do not express the cyclin dependent kinase inhibitor(s) (CDKi) $p21^{Waf1/Cip1}$, $p27^{Kip1}$ or $p16^{Ink4}$ all required ERK activation for cell cycle entry. Thus it is only the loss of Rb that alleviates the requirement for ERK signalling.

Regulation of ERK activity is comparable in Wt and Rb-deficient MEFs

Lee et al. have shown that $Rb^{-/-}$ cells have elevated levels of Ras-GTP during the G1 phase of the cell cycle (Lee et al., 1999). In addition, Alessi et al. have shown that PD098059 does not block ERK activation at high levels of growth factor stimulation (Alessi et al., 1995). Higher levels of active Ras in $Rb^{-/-}$ cells might result in higher levels of ERK activation that cannot be blocked by U0126. Hence the inability of U0126 to block cell cycle entry in $Rb^{-/-}$ cells could be a consequence of higher levels of ERK activation following growth factor stimulation. We therefore investigated whether ERK activity was different in $Rb^{-/-}$ compared with Wt MEFs. Analysis of ERK activation (Fig. 3) by in vitro kinase assays (Fig. 3a,c) and western blotting for (active) phospho-ERK (Fig. 3b,d) revealed that the basal level of ERK activation was the same in quiescent $Rb^{-/-}$ and Wt MEFs and that it was stimulated to the same degree with similar duration in both $Rb^{-/-}$ and Wt MEFs. Furthermore, Fig. 3 shows that U0126 inhibited ERK activation to a similar degree in both cell lines. Thus the failure of U0126 to block cell proliferation of $Rb^{-/-}$ cells is a consequence of a reduced requirement for ERK signalling rather than the inability of U0126 to block ERK activation in these cells.

Growth of Rb-null tumour cell lines is uncoupled from MAP kinase signalling

Since we observed a reduced requirement for ERK signalling in Rb-null MEFs, we were interested to investigate whether human tumour cell lines lacking Rb had a reduced requirement for ERK signalling. We compared tumour cell lines, which are deficient for Rb expression to cells lines of similar origin, which are wild-type for Rb. To facilitate these experiments we used two cell lines derived from glioma (SF295 Rb wild-type

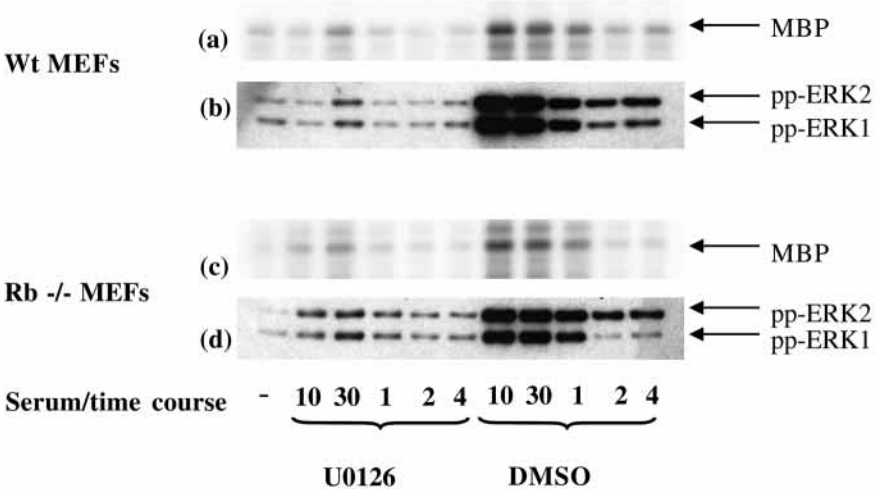

Fig. 3. ERK activation is similar in Wt and Rb-null cells and is sensitive to inhibition by U0126. Following overnight serum deprivation cells were treated with U0126 at a final concentration of 20 μM or vehicle control for 60 minutes. The cells were then stimulated with 10% serum for the indicated times. (a,c) Endogenous ERK activity was analysed by in vitro kinase assay. Equal amounts of cell lysates were subjected to immunoprecipitation followed by kinase assay using myelin basic protein (MBP) as the substrate. (b,d) Total cell lysates were analysed by western blotting for active phospho-ERK.

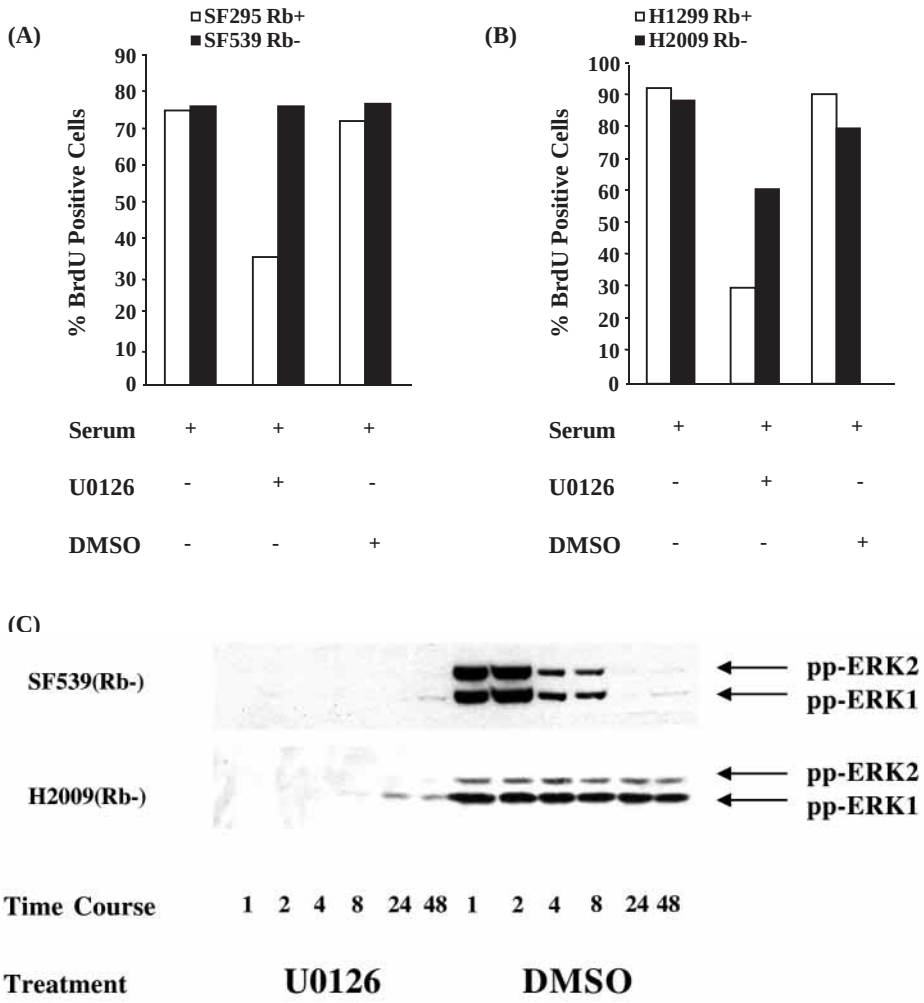

Fig. 4. ERK signalling is uncoupled from cell cycle progression in $Rb^{-/-}$ tumour cell lines. Asynchronously growing populations of tumour cells were plated at a density of 10^4/ml. The following day cells were treated with U0126 at a final concentration of 20 μM, or vehicle control (DMSO). Growth of cells was monitored by BrdU incorporation 48 hour post treatment followed by immunofluorescence. (A) Proliferation of tumour cell lines derived from gliomas treated with inhibitor(s): SF295 (Rb wild-type) and SF539 (Rb deficient). (B) Proliferation of tumour cell lines derived from non-small lung carcinomas treated with inhibitor(s): H1299 (Rb wild-type) and H2009 (Rb deficient). (C) Inhibition of ERK activation by U0126.

and SF539 Rb deficient) and two cell lines derived from non-small cell lung carcinoma (H1299 Rb wild-type and H2009 Rb deficient). Analysis of SF295 and H1299 (Rb wild-type) revealed that treatment with U0126 blocked proliferation of asynchronously growing cells (Fig. 4A and B, respectively). In contrast the Rb-deficient tumour cell lines, SF539 and H2009, continued to proliferate in the presence of U0126. Western blotting for phospho-ERK (Fig. 4C) showed that U0126 inhibited ERK activation in SF539 and H2009 (Fig. 4C). These data show that human tumour cell lines that lack Rb, like MEFs that are deficient for Rb expression, have a reduced requirement for ERK signalling.

Inactivation of retinoblastoma protein by phosphorylation is blocked by U0126 and LY294002 in Wt MEFs

The G1/S transition is facilitated by the inactivation of the retinoblastoma protein. At the molecular level, inactivation of pRb^{105} correlates with the hyper-phosphorylation of a number of Ser/Thr sites (Mittnacht, 1998; Weinberg, 1995) mediated by the G1 cyclin dependent kinases (cdks) (Sherr, 1996; Weinberg, 1995). Using a pan Rb antibody in a western blot we were able to probe for endogenous hypo (active) and hyper (inactive) forms of pRb as judged by altered electrophoretic mobility (Fig. 5). Upon serum deprivation only the active faster migrating (hypo-phosphorylated) form of pRb^{105} was detected (Fig. 5, lane 1) correlating with the quiescent state of the cells. On serum stimulation the inactive slower migrating (hyper-phosphorylated) form of retinoblastoma was also detected (Fig. 5, lane 2). In contrast, when cells were treated with U0126 prior to serum stimulation we did not detect the hyper-phosphorylated of Rb (Fig. 5, lanes 3). When the cells were treated with either LY294002 or the combination of both inhibitors we were also unable to detect the hyper-phosphorylated form of Rb (Fig. 5, lanes 4,5).

Levels of cyclin D1 but not cyclins E or A are regulated by ERK signalling

The G1/S boundary is marked by expression of cyclin D1 and

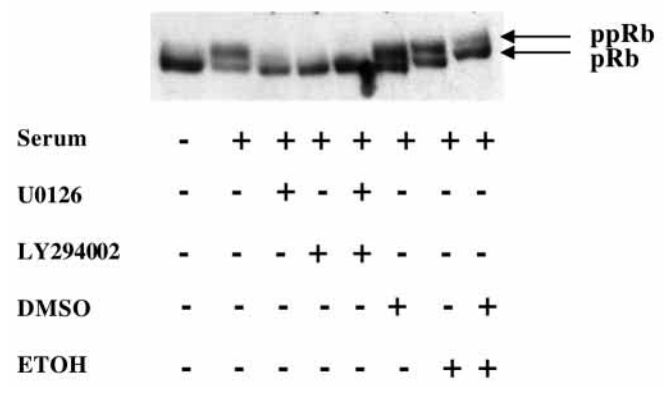

Fig. 5. Inactivation of the Rb protein is blocked by U0126 and LY294002 in Wt MEFs. After plating cells were placed in growth medium containing 0.5% FCS overnight, rendering the cells quiescent. Cells were then treated with U0126 at a final concentration of 20 μM or LY294002 at a final concentration of 20 μM or vehicle control for 60 minutes followed by stimulation with 10% (v/v) FCS. An equal amount of total cell lysate was then subjected to western blotting for Rb using a pan-antibody that detects both the hypo and hyper phosphorylated forms of the protein.

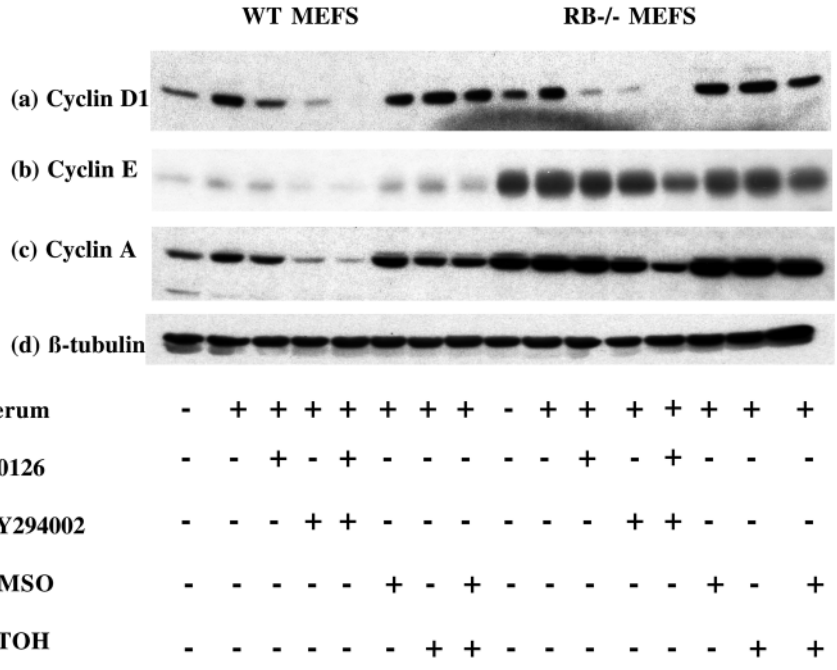

Fig. 6. Expression of cyclins is regulated differentially by ERK and PI 3-kinase signalling. Expression pattern of cyclins was assessed following serum starvation (–) or following 16 hour restimulation with 10% (v/v) FCS (+). For inhibitor experiments cells were first pre-treated with inhibitors for 60 minutes prior to serum stimulation. An equal amount of cell lysate was subjected to western blotting for (a) cyclin D1, (b) cyclin E expression and (c) cyclin A expression. (d) β-Tubluin was used as marker for loading control.

cdk4 activity (Sherr, 1996). Therefore we examined expression of cyclin D1 and cdk4 activity in MEFs treated with U0126 (Fig. 6a, Fig. 7A). Serum starved cells had low levels of cyclin D1 (Fig. 6a) that was induced upon serum stimulation (Fig. 6a). Treatment of cells with U0126 partially blocked induction of cyclin D1 expression in both the Wt and Rb-deficient MEFs. To determine whether this decrease in cyclin D1 expression affected cdk4 activity, cdk4 was immunoprecipitated from MEF cell lysates and assayed for in vitro kinase activity using a C-terminal fragment of Rb (amino acids 763-928) fused to GST as substrate (Zarkowska and Mittnacht, 1997). Serum starved MEFs had low cdk4 kinase activity (Fig. 7A), while serum stimulation lead to an increase in cdk4 activity (Fig. 7A). Treatment with U0126 resulted in a marked decrease in cdk4 kinase activity in both the Wt and $Rb^{-/-}$ MEFs (Fig. 7A). In agreement with previous studies, (Lavoie et al., 1996; Weber et al., 1997; Cheng et al., 1999), these results support the model that activation of ERK is required for cyclin D1 expression, cdk4 complex formation and kinase activity. We consistently observed that treatment with U0126 did not completely abrogate serum stimulated cyclin D1 expression or cdk4 activity although it did block entry into S-phase of WT cells (Fig. 1). Since the PI 3-kinase pathway has also been shown to regulate cyclin D1 expression at both the transcriptional (Gille and Downward, 1999), and postranslational level (Diehl et al., 1997; Diehl et al., 1998) we investigated how inhibition of PI 3-kinase affected cyclin D1 expression and cdk4 kinase activity. These experiments revealed that inhibition of PI 3-kinase with LY294002 lead to a partial block of cyclin D1 expression (Fig. 6a) and cdk4 activity (Fig. 7A). Interestingly inhibition of both the ERK and PI 3-kinase pathways by treatment with U0126 and LY294002 completely ablated serum stimulated cyclin D1 expression (Fig. 6a) and cdk4 activity (Fig. 7A). These results show that both the ERK and PI 3-kinase pathways contribute to cyclin D1 expression and that complete inhibition of cyclin D1 expression and associated cdk4 activity requires inhibition of ERK and PI 3-kinase signalling.

The G1-S phase of the cell cycle is regulated by cyclin E and cyclin A associated cdk2 activity (Sherr, 1996). We therefore investigated whether inhibition of ERK activation using U0126 would inhibit the expression of cyclin E and cyclin A (Fig. 6b,c). In Wt MEFs expression of cyclin E and A was unaffected by inhibition of the ERK pathway, whilst interestingly the level of cyclin E/cdk2 activity was low. In contrast inhibition of PI 3-kinase blocked expression of cyclin E and A (Fig. 6b,c) and activation of cyclin E/cdk2 activity in Wt MEFs. The lack of cdk4 and cdk2 activity that we observed in Wt MEFs upon treatment with either U0126 or LY294002 correlates with the absence of the inactive hyperphosphorylated form of Rb and the growth arrest that we observe. These data show that ERK signalling is required to regulate expression of cyclin D1 but does not impinge on the expression of cyclin E or cyclin A. This is in contrast to PI 3-kinase signalling which our studies show impinges on all 3 G1/S regulating cyclins and associated cyclin D1/cdk4 and cyclin E/cdk2 activity.

As previously described (Herrera et al., 1996), $Rb^{-/-}$ cells have elevated levels of cyclin E and cyclin A both in serum starved and serum stimulated conditions (Fig. 6b,c). As was observed in Wt MEFs, treatment with U0126 had no effect on the levels of cyclin E or cyclin A in $Rb^{-/-}$ cells (Fig. 7B,C). Stimulation of $Rb^{-/-}$ cells with serum induces a higher level of cyclin E/cdk2 activity in comparison to the Wt MEFs. Whilst treatment with U0126 had no effect on cyclin E expression in $Rb^{-/-}$ MEFs, analysis of cyclin E/cdk2 activity by immunoprecipitation of cyclin E from cell lysates indicated that treatment with U0126 had a small but consistent decrease in cyclin E associated kinase activity (Fig. 7B). However, the remaining kinase activity was greater than or equal to that observed in serum stimulated Wt MEFs in the absence of inhibitors. This high residual cyclin E/cdk2 activity in U0126-treated $Rb^{-/-}$ MEFs, coupled with the knowledge that $Rb^{-/-}$ cells do not require cyclin D1 associated kinase activity for cell cycle progression (Lukas et al., 1995) may explain why $Rb^{-/-}$ cells can proliferate when the ERK pathway is inhibited.

Analysis of PI 3-kinase signalling in $Rb^{-/-}$ MEFs revealed that unlike Wt cells inhibition of PI 3-kinase had no effect on cyclin E or cyclin A expression levels (Fig. 6b,c). The combination of U0126 and LY294002 lead to a small ihhibition in the level of both cyclin E and cyclin A. Interestingly, we did not observe complete inhibition in expression levels (Fig.

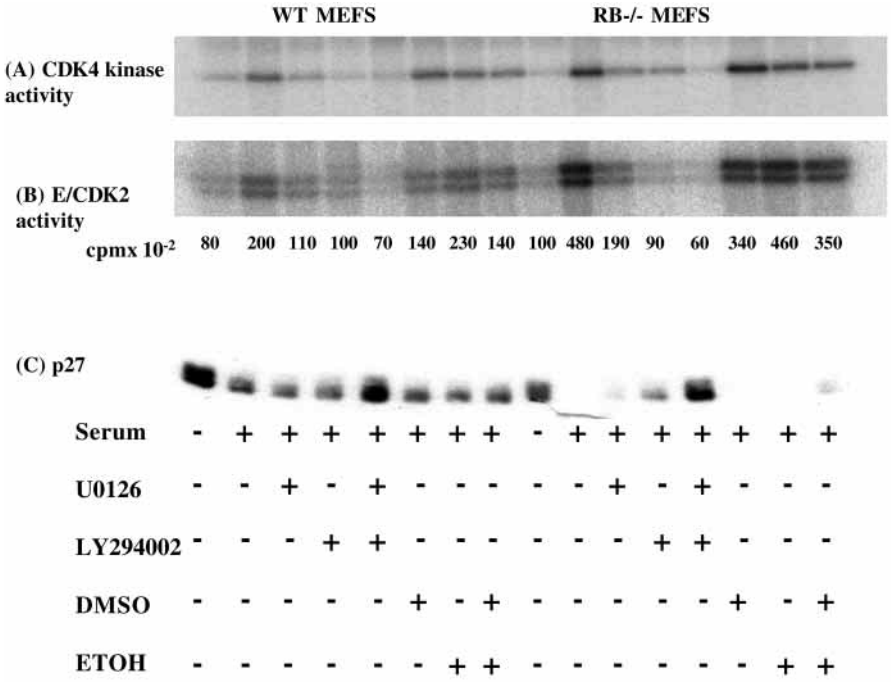

Fig. 7. Levels of $p27^{Kip1}$ are regulated by both MEK and PI 3-kinase pathways in Wt and Rb-deficient MEFs. Cyclin-dependent kinase activity was assessed following serum starvation (–) or following 16-20 hour restimulation with serum (+). For inhibitor experiments, cells were first pre-treated with inhibitors for 60 minutes prior to serum stimulation. Complexes were immunoprecipitated from 500 μg of cell lysates and cdk activity was assayed by in vitro kinase assay. (A) Endogenous cdk4 was immunoprecipitated and assayed using a C-terminal fragment of Rb as substrate. (B) Endogenous cyclin E was immunoprecipitated and assayed using Histone H1 as substrate. (C) Expression of $p27^{Kip1}$ was assessed by western blotting.

6b,c). Analysis of cyclin E/cdk2 activity revealed that despite a negligible effect on the levels of cyclin E, treatment with LY294002 and the combination of inhibitors lead to a marked inhibition of kinase activityin the $Rb^{-/-}$ MEFs (Fig. 7B).

Regulation of $p27^{Kip1}$ is controlled by ERK and PI 3-kinase signalling

The level of the expression of the cdk inhibitor $p27^{Kip1}$ is known to be important determinant for cell cycle entry at least in part as a regulator of cdk2 activity (Sherr and Roberts, 1999). In an effort to study the mechanism by which cdk2 activity is regulated as a consequence of ERK and PI 3-kinase signalling, we examined how inhibition of these pathways would affect $p27^{Kip1}$ expression levels. Consistent with previous studies (Slingerland and Pagano, 2000) removal of growth factors led to an accumulation of $p27^{Kip1}$ (Fig. 7C) that was downregulated upon serum restimulation in both Wt and Rb-deficient cells (Fig. 7C). Analysis of cells treated with either U0126 or LY294002 revealed that following serum stimulation, the levels of $p27^{Kip1}$ were still downregulated in both Wt and $Rb^{-/-}$ MEFs (Fig. 7C). However, in the presence of both U0126 and LY294002 serum stimulation was unable lead to the downregulation of $p27^{Kip1}$ in Wt and $Rb^{-/-}$ MEFs (Fig. 7C). These results show that either ERK or PI 3-kinase signalling can regulate the levels of $p27^{Kip1}$ suggesting that redundancy exists between these two signalling pathways for the regulation of $p27^{Kip1}$ levels.

Asynchronous proliferation of Wt MEFs but not $Rb^{-/-}$ MEFs is reduced by inhibition of MEK

We have shown that $Rb^{-/-}$ MEFs are capable of cell cycle entry in the presence of U0126 and LY294002 in a synchronised cell growth assay (Fig. 1). While we observe residual cyclin E/cdk2 activity in $Rb^{-/-}$ MEFs treated with U0126, treatment with LY294002 results in complete loss of kinase activity. Due to this surprising result we were interested to assess the capacity of $Rb^{-/-}$ cells to proliferate in a long term growth assay in the presence of these inhibitors (Fig. 8). As expected treatment of asynchronously growing Wt MEFs with U0126 retarded proliferation over a 4 day period (Fig. 8a). In contrast, $Rb^{-/-}$ MEFs were capable of sustained growth over a 4 day growth period in the presence of U0126 (Fig. 8a), which was comparable to the growth kinetics of untreated MEFs (Fig. 8a,b). Quiescent $Rb^{-/-}$ MEFs stimulated with serum in the presence of LY294002 entered DNA synthesis but after 24 hours entered apoptosis. Furthermore treatment with LY294002 led to inhibition of proliferation through apoptosis of asynchronous $Rb^{-/-}$ MEFs over a 4 day period (Fig. 8b)

Discussion

It is now well established that active Ras is required for mitogenic signalling in a variety of systems (Feig and Cooper, 1988; Mulcahy et al., 1985; Dobrowolski et al., 1994; Stacey et al., 1991). Recently the role of Ras in cell cycle progression has been more clearly defined (Mittnacht et al., 1997; Peeper et al., 1997). These studies showed that the requirement for Ras signalling is reduced in asynchrononously growing Rb-null MEFS (Mittnacht et al., 1997; Peeper et al., 1997). However Peeper et al., showed that Ras signalling is still required for quiescent Rb-null MEFs to exit G0 and enter G1 (Peeper et al., 1997). The observation that Ras signalling is still required for quiescent Rb-null MEFs to enter the cell cycle is consistent with the observation that these cells do not have a reduced serum requirement for proliferation (Herrera et al., 1996). These results imply that Ras signalling is required at two points: first, to leave G0 and second for the Rb-dependent G1-S transition. The observation that the requirement for Ras signalling is still required for Rb-null cells to leave G0 led us to investigate whether these cells require activation of the ERK-MAPK pathway for cell cycle entry. This pathway is a major downstream effector of Ras (Marshall, 1995) and has been strongly implicated in cell cycle entry particularly for the

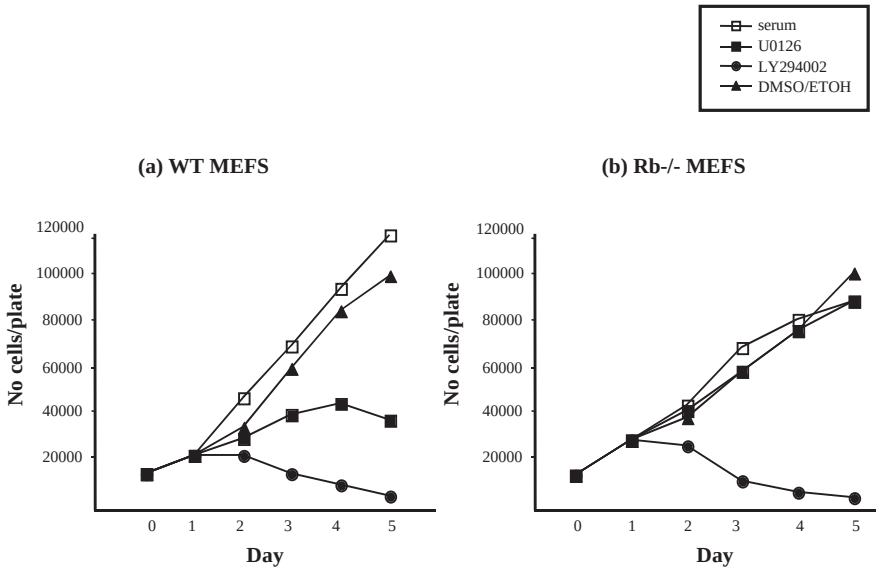

Fig. 8. Asynchronous proliferation of Wt MEFs but not $Rb^{-/-}$ MEFs is reduced by inhibition of MEK. Asynchronously growing populations of cells were plated at a density of 10^4/ml. The following day cells were counted (day 1) followed by treatment with U0126 (20 μM final concentration) or vehicle control (DMSO), or LY294002 (20 μM final concentration) or vehicle control (ETOH). Growth of cells was then monitored each day for four more days (day 2-5). On each day the cells' media was changed and retreated with inhibitor(s) or vehicle control.

induction of cyclin D1 expression (Cheng et al., 1998; Lavoie et al., 1996; Weber et al., 1997).

When quiescent serum starved Wt MEFs were treated with the Mek inhibitor U0126, serum restimulation did not lead to S-phase entry. In contrast Rb-null MEFS were able to exit the G0 state, enter the cell cycle and proliferate when Mek activity is inhibited. This inability of U0126 to block cell cycle entry in Rb-null cells is not a consequence of the inhibitor failing to block Mek1/2 and thereby ERK1/2 activation in these cells because ERK1/2 activation was similarly inhibited in both Wt and Rb-null cells. U0126 and PD098059, another Mek inhibitor, have been shown to also block Mek5 in the ERK5/BMK1 pathway (Kamakura et al., 1999). Since the ERK5/BMK1 pathway has been implicated in cell cycle control (Kato et al., 1998) these results could imply that loss of Rb may also abrogate the requirement for ERK5 activation. However we have been unable to reproducibly measure ERK5 kinase activity and therefore do not know whether it is activated in the cell system we have used. These results show that while Rb-null cells still require Ras signalling to exit G0 they do not require ERK1/2 activation. The role of the ERK1/2 pathway is to bring about the inactivation of Rb and the G1-S transition rather than the Ras dependent step in G0 exit. While oncogenes have long been known to affect cell signalling pathways, these results show for the first time that loss of a tumour suppressor gene can alter the requirement for the MAP kinase signalling pathway. However the requirement for ERK signalling was unaffected in MEFs null for $p21^{Waf1/Cip1}$, $p27^{Kip1}$ or $p16^{Ink4}$ which are also tumour suppressor genes linked to cell cycle control. Thus it is only the loss of Rb that alleviates the requirement for ERK signalling.

We were interested to try and understand the mechanism that abrogates the requirement for ERK signalling in Rb-null cells. These cells do not depend on cyclin D1/cdk activity cdk (Lukas et al., 1995) arguing that they no longer depend on ERK signalling for cyclin D1/cdk activity. However Rb-null cells are known to require growth factor stimulation so they might require ERK signalling for other cell cycle events (Herrera et al., 1996). Although U0126 treatment reduced cyclin E/CDK2 activity by approximately 50% in $Rb^{-/-}$ MEFS (without affecting cyclin E expression), the serum stimulated level of E/CDK2 activity after U0126 treatment in $Rb^{-/-}$ MEFs was comparable to that in serum-stimulated untreated Wt MEFs. This suggests that this level of E/CDK2 activity is sufficient for proliferation. Rb-null human tumour cell lines – unlike their Rb Wt counterparts – also proliferated in the presence of U0126 despite complete inhibition of ERK activation. We conclude that Rb-null cells do not depend on ERK signalling for proliferation because the requirement for cyclin D dependent kinase activity is abrogated by loss of Rb and cells have elevated cyclin E/Cdk2 activity. This increased activity probably results from deregulated cyclin E transcription caused by the de-repression of E2F that is associated with loss of Rb (Herrera et al., 1996).

Like the ERK pathway PI 3-kinase signalling has been shown to be required for quiescent cells to enter the cell cycle (Gille and Downward, 1999; Jones et al., 1999; Jones and Kazlauskas, 2001). Furthermore PDGF dependent PI 3-kinase signalling is known to occur at G0/G1 and mid to late G1, interestingly however entry into S-phase depends on PI 3-kinase activity during the mid to late phase of G1 (Jones et al., 1999; Jones and Kazlauskas, 2001). Therefore we were interested to investigate the effect of inhibition of PI 3-kinase signalling on cell cycle progression in Rb-null cells. In contrast to Wt MEFS, treatment of quiescent Rb-null cells with serum in the presence of the PI 3-kinase inhibitor LY294002 did not inhibit S-phase entry. However unlike inhibition of the ERK pathway, cells could not proliferate long term when PI 3-kinase was inhibited and died through apoptosis. At the molecular level treatment with LY294002 partially blocked induction of cyclin D1 expression, cdk4 activity but in contrast to U0126 also blocked expression of cyclin E, A and cyclin E/cdk2 activity. Thus, cyclin D1 expression is responsive to both ERK and PI 3-kinase dependent signalling, but cyclin E and cyclin A only requires PI 3-kinase signalling. Together these data are consistent with a role for PI 3-kinase in regulating the G1/S transtion that is overcome in the absence of the Rb check-point. However Rb-null cells are still dependent on PI 3-kinase signalling for survival.

Downregulation of the CDKI $p27^{Kip1}$ is a key element for

the movement of cells out of G0 and into S-phase (Malek et al., 2001; Slingerland and Pagano, 2000). Our data show that in both Wt and Rb-null cells $p27^{Kip1}$ degradation occurs after serum stimulation in the presence of either U0126 to block ERK signalling or LY294002 to block PI 3-kinase signalling but not in the presence of both inhibitors. Similar results were found with human tumour cell lines (data not shown). These results demonstrate that there is redundancy between these signalling pathways for regulation of $p27^{Kip1}$. Cell cycle analysis of MEFS revealed that in the presence of both inhibitors, S-phase entry is inhibited regardless of Rb status. Our data predicts that it is the block of $p27^{Kip1}$ degradation that occurs when both pathways are inhibited that underlies the block in cell cycle entry that we observe. The residual levels of cyclin D1 and CDK4 activity that persist after treatment with either U0126 or LY294002 suggest that these low levels of the cyclin D1/CDK4 complex may be important to sequester $p27^{Kip1}$ and permit cell cycle entry in $Rb^{-/-}$ cells (Cheng et al., 1998). The mechanism which underlies the accumulation of $p27^{Kip1}$ is still to be elucidated. However is has been shown that Ras signalling pathways can control expression of $p27^{Kip1}$ by regulating the rate of translation and protein stability (Aktas et al., 1997; Kerkhoff and Rapp, 1997; Takuwa and Takuwa, 1997).

Since the Ras protein is a critical transducer of proliferative signals, it is not surprising that a high percentage of some human cancers contain activating Ras gene mutations (Bos, 1989). Most oncogenic Ras containing tumours that have been examined contain Wt Rb (Horowitz et al., 1990; Kashii et al., 1994) and would be predicted to be sensitive to Mek inhibitors. However where Rb is lost for example in non-small-cell lung cancers our data predict that inhibition of the Mek pathway will not have an anti-proliferative effect. Thus effective therapeutic use of signal transduction inhibitors may require knowledge of the genetic alterations to the cell cycle machinery for different tumours.

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References

Aktas, H., Cai, H. and Cooper, G. M. (1997). Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. *Mol. Cell. Biol.* **17**, 3850-3857.

Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. and Pestell, R. G. (1995). Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J. Biol. Chem.* **270**, 23589-23597.

Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen- activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem.* **270**, 27489-27494.

Bos, J. L. (1989). ras oncogenes in human cancer: a review. *Cancer Res.* **49**, 4682-4689.

Cai, H., Szeberenyi, J. and Cooper, G. M. (1990). Effect of a dominant inhibitory Ha-ras mutation on mitogenic signal transduction in NIH 3T3 cells. *Mol. Cell. Biol.* **10**, 5314-5323.

Cheng, M., Sexl, V., Sherr, C. J. and Roussel, M. F. (1998). Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proc. Natl. Acad. Sci. USA* **95**, 1091-1096.

Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M. and Sherr, C. J. (1999). The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J.* **18**, 1571-1583.

D'Abaco, G. M. and Olson, M. F. (2000). Mouse embryo fibroblasts: a genetic model system for studying Rho- and Ras-dependent cell cycle progression. *Methods Enzymol.* **325**, 415-425.

Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95-105.

Dickson, B. and Hafen, E. (1994). Genetics of signal transduction in invertebrates. *Curr. Opin. Genet. Dev.* **4**, 64-70.

Diehl, J. A., Zindy, F. and Sherr, C. J. (1997). Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.* **11**, 957-972.

Diehl, J. A., Cheng, M., Roussel, M. F. and Sherr, C. J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12**, 3499-3511.

Dobrowolski, S., Harter, M. and Stacey, D. W. (1994). Cellular ras activity is required for passage through multiple points of the G0/G1 phase in BALB/c 3T3 cells. *Mol. Cell Biol.* **14**, 5441-5449.

Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **92**, 7686-7689.

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F. et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273**, 18623-18632.

Feig, L. A. and Cooper, G. M. (1988). Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol.* **8**, 3235-3243.

Filmus, J., Robles, A. I., Shi, W., Wong, M. J., Colombo, L. L. and Conti, C. J. (1994). Induction of cyclin D1 overexpression by activated ras. *Oncogene* **9**, 3627-3633.

Furth, M. E., Davis, L. J., Fleurdelys, B. and Scolnick, E. M. (1982). Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. *J. Virol.* **43**, 294-304.

Gille, H. and Downward, J. (1999). Multiple ras effector pathways contribute to G(1) cell cycle progression. *J. Biol. Chem.* **274**, 22033-22040.

Henry, D. O., Moskalenko, S. A., Kaur, K. J., Fu, M., Pestell, R. G., Camonis, J. H. and White, M. A. (2000). Ral GTPases contribute to regulation of cyclin D1 through activation of NF-kappaB. *Mol. Cell. Biol.* **20**, 8084-8092.

Herrera, R. E., Sah, V. P., Williams, B. O., Makela, T. P., Weinberg, R. A. and Jacks, T. (1996). Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. *Mol. Cell. Biol.* **16**, 2402-2407.

Hitomi, M. and Stacey, D. W. (2001). Ras-dependent cell cycle commitment during G2 phase. *FEBS Lett.* **490**, 123-131.

Horowitz, J. M., Park, S. H., Bogenmann, E., Cheng, J. C., Yandell, D. W., Kaye, F. J., Minna, J. D., Dryja, T. P. and Weinberg, R. A. (1990). Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. *Proc. Natl. Acad. Sci. USA* **87**, 2775-2779.

Jones, S. M. and Kazlauskas, A. (2001). Growth-factor-dependent mitogenesis requires two distinct phases of signalling. *Nat. Cell Biol.* **3**, 165-172.

Jones, S. M., Klinghoffer, R., Prestwich, G. D., Toker, A. and Kazlauskas, A. (1999). PDGF induces an early and a late wave of PI 3-kinase activity, and only the late wave is required for progression through G1. *Curr. Biol.* **9**, 512-521.

Kamakura, S., Moriguchi, T. and Nishida, E. (1999). Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. *J. Biol. Chem.* **274**, 26563-26571.

Kashii, T., Mizushima, Y., Monno, S., Nakagawa, K. and Kobayashi, M. (1994). Gene analysis of K-, H-ras, p53, and retinoblastoma susceptibility genes in human lung cancer cell lines by the polymerase chain reaction/single-strand conformation polymorphism method. *J. Cancer Res. Clin. Oncol.* **120**, 143-148.

Kato, Y., Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J. and Lee, J. D. (1998). Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. *Nature* **395**, 713-716.

Kelley, G. G., Reks, S. E., Ondrako, J. M. and Smrcka, A. V. (2001). Phospholipase C(epsilon): a novel Ras effector. *EMBO J.* **20**, 743-754.

Kerkhoff, E. and Rapp, U. R. (1997). Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. *Mol. Cell. Biol.* **17**, 2576-2586.

Kodaki, T., Woscholski, R., Hallberg, B., Rodriguez-Viciana, P., Downward, J. and Parker, P. J. (1994). The activation of phosphatidylinositol 3-kinase by Ras. *Curr. Biol.* **4**, 798-806.

Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R. and Pouyssegur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J. Biol. Chem.* **271**, 20608-20616.

Lee, K. Y., Ladha, M. H., McMahon, C. and Ewen, M. E. (1999). The retinoblastoma protein is linked to the activation of Ras. *Mol. Cell. Biol.* **19**, 7724-7732.

Leevers, S. J. and Marshall, C. J. (1992). Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. *EMBO J.* **11**, 569-574.

Liu, J. J., Chao, J. R., Jiang, M. C., Ng, S. Y., Yen, J. J. and Yang-Yen, H. F. (1995). Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. *Mol. Cell. Biol.* **15**, 3654-3663.

Lopez, I., Mak, E. C., Ding, J., Hamm, H. E. and Lomasney, J. W. (2001). A novel bifunctional phospholipase c that is regulated by Galpha 12 and stimulates the Ras/mitogen-activated protein kinase pathway. *J. Biol. Chem.* **276**, 2758-2765.

Lukas, J., Bartkova, J., Rohde, M., Strauss, M. and Bartek, J. (1995). Cyclin D1 is dispensable for G1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. *Mol. Cell. Biol.* **15**, 2600-2611.

Malek, N. P., Sundberg, H., McGrew, S., Nakayama, K., Kyriakidis, T. R. and Roberts, J. M. (2001). A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. *Nature* **413**, 323-327.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-185.

Marshall, C. J. (1996). Ras effectors. *Curr. Opin. Cell Biol.* **8**, 197-204.

Mittnacht, S. (1998). Control of pRB phosphorylation. *Curr. Opin. Genet. Dev.* **8**, 21-27.

Mittnacht, S., Paterson, H., Olson, M. F. and Marshall, C. J. (1997). Ras signalling is required for inactivation of the tumour suppressor pRb cell-cycle control protein. *Curr. Biol.* **7**, 219-221.

Muise-Helmericks, R. C., Grimes, H. L., Bellacosa, A., Malstrom, S. E., Tsichlis, P. N. and Rosen, N. (1998). Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. *J. Biol. Chem.* **273**, 29864-29872.

Mulcahy, L. S., Smith, M. R. and Stacey, D. W. (1985). Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. *Nature* **313**, 241-243.

Peeper, D. S., Upton, T. M., Ladha, M. H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J. A. and Ewen, M. E. (1997). Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. *Nature* **386**, 177-181.

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D. and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370**, 527-532.

Rodriguez-Viciana, P., Warne, P. H., Vanhaesebroeck, B., Waterfield, M. D. and Downward, J. (1996). Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.* **15**, 2442-2451.

Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A. and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89**, 457-467.

Sherr, C. J. (1996). Cancer cell cycles. *Science* **274**, 1672-1677.

Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501-1512.

Slingerland, J. and Pagano, M. (2000). Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J. Cell Physiol.* **183**, 10-17.

Song, C., Hu, C. D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohge, M., Wu, D., Satoh, T. and Kataoka, T. (2001). Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. *J. Biol. Chem.* **276**, 2752-2757.

Stacey, D. W., Roudebush, M., Day, R., Mosser, S. D., Gibbs, J. B. and Feig, L. A. (1991). Dominant inhibitory Ras mutants demonstrate the requirement for Ras activity in the action of tyrosine kinase oncogenes. *Oncogene* **6**, 2297-2304.

Takuwa, N. and Takuwa, Y. (1997). Ras activity late in G1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. *Mol. Cell. Biol.* **17**, 5348-5358.

Weber, J. D., Raben, D. M., Phillips, P. J. and Baldassare, J. J. (1997). Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem. J.* **326**, 61-68.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. *Cell* **81**, 323-330.

Winston, J. T., Coats, S. R., Wang, Y. Z. and Pledger, W. J. (1996). Regulation of the cell cycle machinery by oncogenic ras. *Oncogene* **12**, 127-134.

Zarkowska, T. and Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. *J. Biol. Chem.* **272**, 12738-12746.