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Rapid dephosphorylation of p107 following UV irradiation

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In response to UV irradiation, mouse NIH3T3 fibroblasts transiently arrest predominantly in the G1 phase of the cell cycle. Here, we investigate the role of the retinoblastoma-related pocket proteins in this biological process. We report here that UV induces an increase in p107/E2F complexes, shown previously to be repressors of E2F-dependent transcriptional activity. Several lines of evidence indicate that the increase of p107/E2F complexes following UV irradiation is a consequence of rapid dephosphorylation of p107. First, UV-mediated p107 dephosphorylation could be abolished by pretreatment of NIH3T3 fibroblasts with the serine/threonine phosphatase inhibitors calyculin A and okadaic acid. Second, alteration of protein phosphatase 2A holoenzyme composition by over-expression of specific B subunits interfered with UV-mediated dephosphorylation of p107. Consistent with this, p107 could be dephosphorylated *in vitro* with PP2A. Moreover, dephosphorylation of p107 was shown to be independent of the activity of p53 and p21, as it occurred also in UV-treated p53-null as well as p21-null mouse fibroblasts. We observed a close correlation between the UV dosages required for G1 cell cycle arrest and p107 dephosphorylation. Our data suggest a model in which UV radiation-induced cell cycle arrest depends, at least in part, on the induction of a PP2A-like phosphatase that acts on p107.

Keywords: E2F; p107; G1 arrest; PP2A; UV response

Introduction

The transcription factor E2F regulates the expression of genes essential for cell cycle progression (Beijersbergen and Bernards, 1996; Lam and La Thangue, 1994; Nevins, 1992). This is manifested through binding to specific sequences located within the promoters of cell-cycle regulated genes. The E2F transcription factor exists physiologically as heterodimers, consisting of a member of the E2F family of proteins and a DP protein (Lam and La Thangue, 1994). To date, five E2F proteins (E2F1–5) and at least three distinct DPs have been characterized (Hijmans *et al*., 1995; Lam and La Thangue, 1994; Sardet *et al*., 1995; Wu *et al*., 1995; Zhang and Chellappan, 1995). The E2F and DP proteins interact synergistically in binding to DNA as well as activating transcription of target genes (Lam and Thangue, 1994). E2F activity is negatively regulated by a family of 'pocket proteins', which includes the tumour suppressor protein pRB and structurally related proteins, p107 and p130 (Weinberg, 1995). All three pocket proteins have been shown to be able to repress E2F activity (Flemington *et al*., 1993; Johnson, 1995; Vairo *et al*., 1995; Zamanian and La Thangue, 1993), however it also appears that each E2F protein associates preferentially with a particular pocket protein family member *in vivo*. Thus, E2F 1, 2 and 3 interact specifically *in vivo* with pRB (Lees *et al*., 1993), E2F4 binds preferentially to p107 and p130, and E2F5 to p130 only (Beijersbergen *et al*., 1994; Ginsberg *et al*., 1994; Hijmans *et al*., 1995; Sardet *et al*., 1995).

The pocket proteins are phosphoproteins whose phosphorylation states are regulated in a cell cycle dependent manner (Beijersbergen *et al*., 1995; Buchkovich *et al*., 1989; Chen *et al*., 1989; DeCaprio *et al*., 1989; Mayol *et al*., 1995). pRB is hypophosphorylated in early G1 and becomes hyperphosphorylated in late G1 prior to entry of cells into S phase of the cell cycle. The consequence of this pRB phosphorylation is dissociation from E2F and relief of repression of E2F regulated genes (Ewen *et al*., 1993; Kato *et al*., 1993; Qian *et al*., 1992). In G0, p107 is present in a hypophosphorylated form, but at low levels. Expression of p107 increases dramatically in late G1, when the majority of the p107 protein becomes hyperphosphorylated. In S phase, hypophosphorylated p107 reappears which can associate with E2F4 and repress E2F-dependent S phase transcription (Beijersbergen *et al*., 1995). Hypophosphorylated forms of p130 are found in G0 and the early G1 stage of the cell-cycle. Upon entering mid-G1, the hypophosphorylated forms of p130 are converted to a unique hyperphosphorylated form and this form of p130 persists for the rest of the cell-cycle (Mayol *et al*., 1995). Phosphorylation of p130 at mid-G1 seems to play an important part in relieving E2F-mediated repression of G1/S phase genes (Johnson, 1995). These pocket proteins are the targets of positive cell cycle regulators such as cyclins and their dependent kinases (cdks). Accumulating evidence has shown that cyclin/dependent kinases associate with and phosphorylate these pocket proteins in a cell cycle dependent manner (Sherr, 1996; Weinberg, 1995).

It has been demonstrated that overexpression of pRB can induce growth arrest in G1 in certain susceptible cells (Ewen *et al*., 1993; Hinds *et al*., 1992; Zhu *et al*., 1993). The exact mechanism involved is unclear, but it is closely related to the ability of pRB to interact with E2F (Chellappan *et al*., 1991; Hiebert, 1993). Like pRB, overexpression of p107 and p130 also results in growth arrest of some cell lines, and can at least in part be explained by their interaction with E2F (Beijersbergen *et al*., 1995; Vairo *et al*., 1995; Zhu *et al*., 1993, 1995a). Overexpression of E2F4, the cellular target of both p107 and p130, can overcome the G1

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arrest imposed by either pocket protein (Beijersbergen *et al*., 1994, 1995; Vairo *et al*., 1995). It has also been shown that the pRB and p107 induced growth arrest can be over-ridden through phosphorylation of pRB and p107 by cyclins and cdks. While both the D-type cyclins and cyclin E contribute to pRB phosphorylation *in vivo* and can overcome a pRB induced cell cycle block, the cyclin D1/cdk4 complex (but not the cyclin E/cdk2 complex) can phosphorylate p107 *in vivo* and alleviate growth suppression by p107 (Beijersbergen *et al*., 1994, 1995; Ewen *et al*., 1993; Hatakeyama *et al*., 1994; Hinds *et al*., 1992).

Serine/threonine protein phosphatases play an important part in transducing intracellular signals and can be classified into at least four subgroups including protein phosphatase type-1 (PP1), -2A (PP2A), -2B (PP2B), and -2C (PP2C) (Wera and Hemmings, 1995). Besides kinases, protein phosphatases have also been implicated in regulating the phosphorylation state of pocket proteins. For example, a type-1 serine/threonine protein phosphatase (PP1) has been suggested to mediate the dephosphorylation of pRB during mitosis (Durfee *et al*., 1993; Nelson *et al*., 1997; Nelson and Ludlow, 1997). Moreover, anti-cancer drugs have been shown to activate a pRB specific phosphatase, most likely to be PP1 related, which induces pRb dephosphorylation and G1 cell cycle arrest (Dou *et al*., 1995). Protein phosphatase 2A (PP2A) is another group of serine/threonine protein phosphatases, which have been implicated in a number of biological processes, including cell cycle regulation, DNA synthesis and viral transformation (Mayer-Jaekel and Hemmings, 1994). The PP2A holoenzymes exist as heterotrimers consisting of a 65-kDa structural A subunit, a 36-kDa catalytic C subunit, and a variable regulatory B subunit. A high level of diversity exists within the PP2A B subunits, which are thought to modulate phosphatase activity, substrate specificity and subcellular localization/translocation of the heterotrimeric enzyme complex.

The tumour suppressor protein p53 and its downstream effector, $p21^{WAF1/CIP1/SDI1}$, are key mediators of the G1 arrest induced by DNA damage (Brugarolas *et al*., 1995). This G1 growth arrest appears to involve both post-translational stabilization of p53 and activation of its transactivational activity, which in turn induces the expression of the p21 cyclin/cdk inhibitor (Hartwell and Kastan, 1994). Besides DNA damage, these DNA damaging agents also trigger an 'acute' reaction in mammalian cells called the 'eukaryotic UV response' (Devary *et al*., 1991; Ronai *et al*., 1990), equivalent to the bacterial 'SOS' response (Walker, 1985). In mammalian cells, this response involves activation of various cellular proteins that have a role in DNA repair and synthesis, transcription and regulation of the cell cycle (Herrlich *et al*., 1992; Holbrook and Fornace, 1991). The exact function of this response is not well established, but it is believed to be essential for maintaining genomic integrity and consequent long term survival of the living organism. The source of the signal for the UV response is not known but it has been demonstrated that it is unlikely to be DNA damage itself (Devary *et al*., 1992, 1993).

In the present study, we investigated the possible role of pocket proteins in mediating the response to UV. Our findings demonstrate that p107 is rapidly dephosphorylated in response to UV and there is a close correlation between p107 dephosphorylation and UV-induced cell cycle arrest. This UV-induced response does not require the function of the tumour suppressor p53 or p21, but appears to be mediated through activation of a PP2A-related phosphatase.

Results

Changes in E2F DNA-binding activity in UV-irradiated fibroblasts

In response to UV irradiation, mouse NIH3T3 fibroblasts transiently arrest predominantly at the G1 phase of the cell cycle (Figure 4b). To investigate the possible role of E2F and pocket proteins in mediating the response to UV, we performed DNA mobility-shift experiments on extracts from NIH3T3 cells to examine the E2F DNA binding activity after UV-treatment. Figure 1a shows that UV irradiation causes a rapid change in distribution of 'free' E2F to slower mobility complexes known to contain the pRB family of proteins. The amount of slower migrating E2F complexes increased within 2 h, and this shift of free E2F to pocket protein bound complexes was sustained for 24 h. Total levels of E2F binding activity gradually started to decline after 12 h. Subsequent super-shift experiments using pRB, p107 and p130-specific antibodies revealed that the slower migrating complexes contain predominantly p107 as well as relatively low levels of p130 (Figure 1b), but not pRB (data not shown). This is consistent with previous findings that p107 is the major pocket protein present in cycling NIH3T3 cells (Lam *et al*., 1994; Lam and Watson, 1993). The kinetics for the increase in levels of p107/E2F or p130/E2F complexes are similar. We subsequently focused our studies on the regulation of pocket protein p107/E2F complexes in response to UV because our present results and previous reports from other groups have shown that p130 is only a very minor component of E2F complexes in cycling cells and exists only in cells arrested in G0 or cells recently exited from G0 (Moberg *et al*., 1996; Smith *et al*., 1996).

Expression of p107 in response to UV irradiation

To ask how UV irradiation of NIH3T3 cells leads to a transient increase in p107/E2F complexes, p107 protein was analysed in these cell extracts by Western blotting (Figure 2a). Analysis of cycling NIH3T3 cells revealed at least two forms of p107 with different mobility. Upon UV-treatment, the slower migrating form(s) disappeared rapidly with a reciprocal increase in the faster migrating (lower) form within 2 h. Previous experiments have shown that dephosphorylation causes p107 to migrate faster on SDS gels (Beijersbergen *et al*., 1995) and it is likely that these different mobility species represent distinct phosphorylated forms of p107. There is a decline in p107 expression 12 h after UV treatment, which can be attributed to the fact that p107 is a cell cycle- and E2F-regulated gene, and is down-regulated in cell cycle arrested cells (Zhu *et al*., 1995b). This downregulation of E2F expression, does not however result in a change in the ratio of 'free'

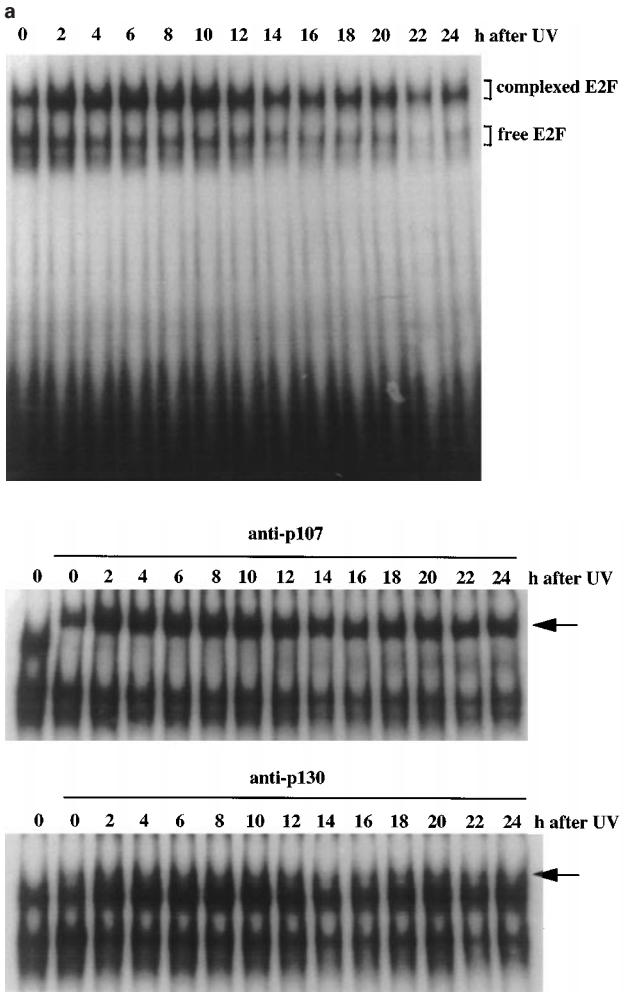

Figure 1 Electrophoretic mobility shift analysis of E2F DNA-binding complexes after UV irradiation. (**a**) NIH3T3 whole cell extracts were harvested at indicated times after UV-treatment with 50 J/m^2 and used for mobility shift experiments with a ^{32}P-labeled oligonucleotide containing an E2F site. The positions of the 'free' and complexed E2F DNA-binding activities are indicated. (**b**) Assay for the presence of p107 and p130 in E2F DNA-binding complexes. E2F DNA-binding activities detected after UV treatment were assayed for the presence of p107 and p130 using specific antibodies. Positions of the super-shift complexes are indicated

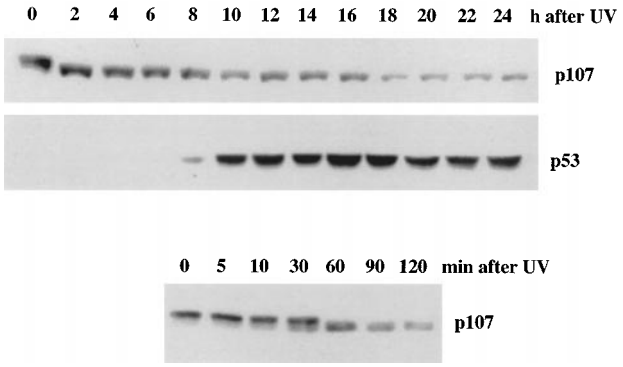

Figure 2 Western blot analysis of p107 and p53 expression after UV treatment. Expression of p107 and p53 after UV irradiation. Extracts prepared from NIH3T3 cells after UV treatment (see Figure 1) were separated on SDS polyacrylamide gels. Following transfer onto Immobilon-P membrane, proteins were detected by Western blotting using antiserum against p107 (C-18) and p53 (CM5). Expression of p107 during the first 2 h post-UV treatment. Cell extracts were prepared from NIH3T3 cells at 0, 5, 10, 30, 60, 90 and 120 min after irradiation with 50 J/m^2 of UV and subjected to Western blotting with anti-p107 antiserum (C-18)

E2F *versus* p107 bound E2F complexes, as the total amount of E2F complexes also declined after 12 h (Figure 1a). The presence of p107/E2F complexes in unirradiated cycling cells is accounted for by the presence of hypophosphorylated forms of p107 in G1 phase of cycling cells (Beijersbergen *et al*., 1995). The fact that the initial increase in level of the hypophosphorylated form of p107 correlates well with an increase in E2F/p107 complexes detected in gel shift experiment (Figure 2a and b) argues that UV-irradiation leads to an increase in the hypophosphorylated form of p107 able to associate with E2F. Notably, there is a decline in p107 expression 12 h after UV treatment, which mirrors a similar down-regulation of p107 containing E2F complexes (Figure 1a). This is probably due to the fact that p107 is a cell cycle- and E2F-regulated gene, and is down-regulated in cell cycle arrested cells. This downregulation of p107/E2F complexes is accompanied by a corresponding increase in p130/E2F complexes, which could not be supershifted by specific antibodies against p107 (Figure 1b, upper panel). This continued growth arrest after 10–12 h could be a result of p53 induction and subsequent p21 activation, which represses cyclin-dependent kinases that mediate p130 and other pocket protein phosphorylation. Moreover, the role of p130 in mediating growth arrest also seems to be more significant with higher UV doses. We have also studied the E2F activity following UV irradiation using NIH3T3 cell lines harbouring E2F-dependent promoters (i.e. 3XE2F and B-*myb* promoters) (Lam *et al*., 1994; Lam and Watson, 1993) and detected that there is a downregulation of E2F-dependent transcriptional activity following UV (data not shown), which is associated with the accumulation of p107/E2F complexes and the hypophosphorylated forms of p107. Though p107 seems to be more sensitive to UV, we do not exclude a role for p130, especially to maintain the cell in an arrested state when the expression of p107 is downregulated.

The tumour suppressor p53 and its downstream target, the cdk inhibitor p21, have both been shown to be important for DNA damage-induced cell cycle arrest (Hartwell and Kastan, 1994). A possible mechanism for the p107 mobility shift, therefore, is that DNA damage induces accumulation of p53 and subsequently p21, resulting in the inhibition of cdk-mediated p107 phosphorylation. To examine this possibility, the expression level of p53 was examined in parallel with p107 (Figure 2a). The results showed that the change in migration rate of p107 occurred well before the accumulation of p53. To explore further the relationship between these different forms of the p107 protein and their kinetics after UV irradiation, we repeated the previous experiment over a shorter time course (Figure 2b). The result showed that the upper form(s) took between 30 min and 1 h to disappear completely, well before any increase in p53 was apparent. Moreover, it is evident that the increase in the lower form paralleled the disappearance of the

upper form, suggesting that the upper form(s) of p107 converts to the lower form in response to UV. It is unlikely that the p107 dephosphorylation is mediated through the p53 and p21 pathway because of the relative short time taken for this to occur, suggesting that a more direct dephosphorylation event is involved. Consistent with this hypothesis is the observation that Western blot analysis failed to detect any induction of p21 (data not shown).

The UV induced dephosphorylation of p107 is independent of p53 and p21

To investigate in more detail whether the dephosphorylation of p107 in response to UV is mediated through the p53 and p21 pathway, we irradiated p53 null and p21 null mouse embryo fibroblasts and examined the phosphorylation state of p107 using Western blotting after 90 min (Figure 3). As in NIH3T3 cells, p107 in both p53 null and p21 null fibroblasts was found to shift to a faster migrating form after UV irradiation (Figure 3). This observation confirms and extends previous results that the UV-induced dephosphorylation of p107 is independent of the action of the p53 tumour suppressor. It is notable that there is a relatively greater amount of dephosphorylated form of p107 in the normal cycling $p21^{-/-}$ cells. This in contrast to what is expected from the role of p21 as an inhibitor of cyclin-dependent kinase activity which in turn is responsible for p107 phosphorylation.

Dephosphorylation of p107 by UV correlates with G1 arrest

To assess the relationship between this UV-triggered p107 dephosphorylation and the G1 cell cycle arrest, we assessed the effect of different doses of UV on the phosphorylation status of p107 and on cell cycle arrest. Cycling NIH3T3 cells were irradiated with various doses of UV and the phosphorylation status of p107 was analysed by Western blotting after 90 min (Figure 4a). The serum starved NIH3T3 cells were included as a control. For comparison, the phosphorylation status of p130 was also examined. Duplicate samples were processed for analysis of cell cycle distribution using FACS analysis (Figure 4b). The Western blotting results demonstrated that p107 dephosphorylation was induced by UV doses as low as 5 J/m^2 but the dephosphorylation of p130 only occurred when the cells were treated with UV dosage of 50 J/m^2 or higher. It also appeared that the phosphorylation status of p107 and p130 differs between NIH3T3 arrested by UV irradiation and that by serum starvation, implying that this UV triggered G1 arrest is different from the cell cycle arrest induced by serum deprivation. To determine the critical dosage of UV required to induce p107 dephosphorylation, we performed a similar experiment but with lower doses of UV (Figure 4a). The results showed that 4 J/m^2 is the minimal dosage of UV required to trigger p107 dephosphorylation. Significantly, this UV dosage corresponds with that required to trigger G1 cell cycle arrest (Figure 4b), suggesting that the UV-induced p107 dephosphorylation is functionally related to this response. Taken together, these findings demonstrate that the p107 is

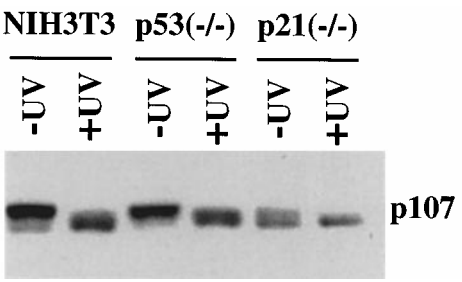

Figure 3 Western blot analysis of p107 expression in NIH3T3, $p53^{(-/-)}$ and $p21^{(-/-)}$ mouse fibroblasts in response to UV. Cycling NIH3T3, $p53^{(-/-)}$ and $p21^{(-/-)}$ mouse fibroblasts were exposed to 50 J/m^2 of UV or mock-irradiation and harvested 2 h post-irradiation. Cell lysates were Western blotted with anti-p107 antiserum (C-18)

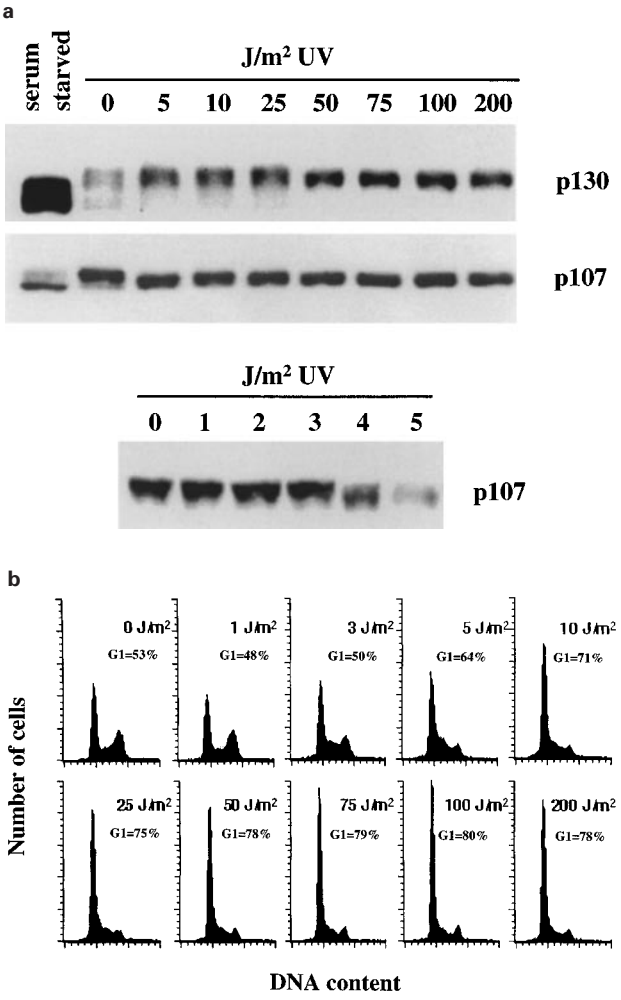

Figure 4 Dose-dependent effects of UV irradiation on dephosphorylation of p107 and p130, and proliferation of NIH3T3 cells. (**a**) Dose-dependent dephosphorylation of p107 and p130. Exponentially growing NIH3T3 cells were irradiated with various doses of UV as indicated and harvested 90 min post-irradiation. The whole cell extracts prepared were used for Western blotting with anti-p107 antiserum (C-18) and anti-p130 antiserum (C-20). (**b**) Dose-dependence of UV-induced cell cycle arrest. Cycling NIH3T3 cells were irradiated with different doses of UV as indicated and harvested at 12 h after UV treatment for FACS analysis. The cell cycle distribution was expressed as 'number of cells' against 'DNA content' and the percentage of cells in G1 phase of the cell cycle was also indicated

more sensitive than p130 to dephosphorylation induced by UV and suggest further that p107 has a role in the UV-induced repression of E2F dependent transcription and the subsequent G1 cell cycle arrest.

Phosphatase inhibitors arrest dephosphorylation of p107 and block the increase in p107/E2F complex formation induced by UV

To test whether the change in p107 mobility was due to induction of a UV-responsive phosphatase, we tested whether serine/threonine specific phosphatase inhibitors would block the effect of UV on p107. NIH3T3 cells were pre-treated with the phosphatase inhibitors calyculin A or okadaic acid for 15 min and then subjected to a UV dosage of 50 J/m^2. Cell extracts collected after 90 min were then Western blotted with p107 specific antibody. After a series of titrations, we found that a very low concentration of calyculin A (5 nM) was enough to prevent the change in p107 mobility in response to UV irradiation. A relatively higher concentration of okadaic acid, 500 nM, was needed to prevent the p107 mobility shift (Figure 5a). These observations strongly suggest that the rapid change in mobility of p107 in response to UV was the result of a dephosphorylation event mediated by a UV-responsive phosphatase. Next, we tested whether inhibition of p107 dephosphorylation by phosphatase inhibitors affected the composition of E2F/p107 complexes following UV treatment. Using the band-shift assay, it was found that concentrations of phosphatase inhibitors sufficient to abolish p107 dephosphorylation also prevented the increase in p107/E2F DNA binding activity associated with UV. At higher inhibitor concentrations, we observed a decrease in p107/E2F complexes as well as an increase of 'free' E2F DNA binding activity, when compared to the unirradiated control (Figure 5b).

To extend the previous observations and provide further evidence that the p107 dephosphorylation in response to UV was mediated by a phosphatase, the p107 dephosphorylation was studied in cell free conditions. Whole cell extracts prepared from NIH3T3 cells immediately after irradiation with 50 J/m^2 of UV were incubated for 90 min either at 4°C or at 37°C in the absence or presence of phosphatase inhibitors (Figure 5c). The results show that incubation of the irradiated NIH3T3 extracts at 37°C induced dephosphorylation of p107, reproducing the dephosphorylation detected in UV-irradiated cells. Similarly, both calyculin A and okadaic acid prevented the dephosphorylation of p107 (Figure 5c). This also implies that the ability of calyculin A and okadaic acid to block dephosphorylation of p107 following UV irradiation is not a result of their physiological effects on the cell cycle. Moreover, p107 dephosphorylation did not occur at 4°C, indicating that the dephosphorylation reaction is temperature dependent. Significantly, p107 dephosphorylation in cell free lysates could be blocked by lower concentrations okadaic acid (10 nM) than were used in previous *in vivo* experiments. This concentration of okadaic acid will specifically inhibit PP2A, but not PP1 (Cohen *et al*., 1989; Honkanen *et al*., 1994). This suggests that the p107 phosphatase that is induced by UV is PP2A or PP2A-related.

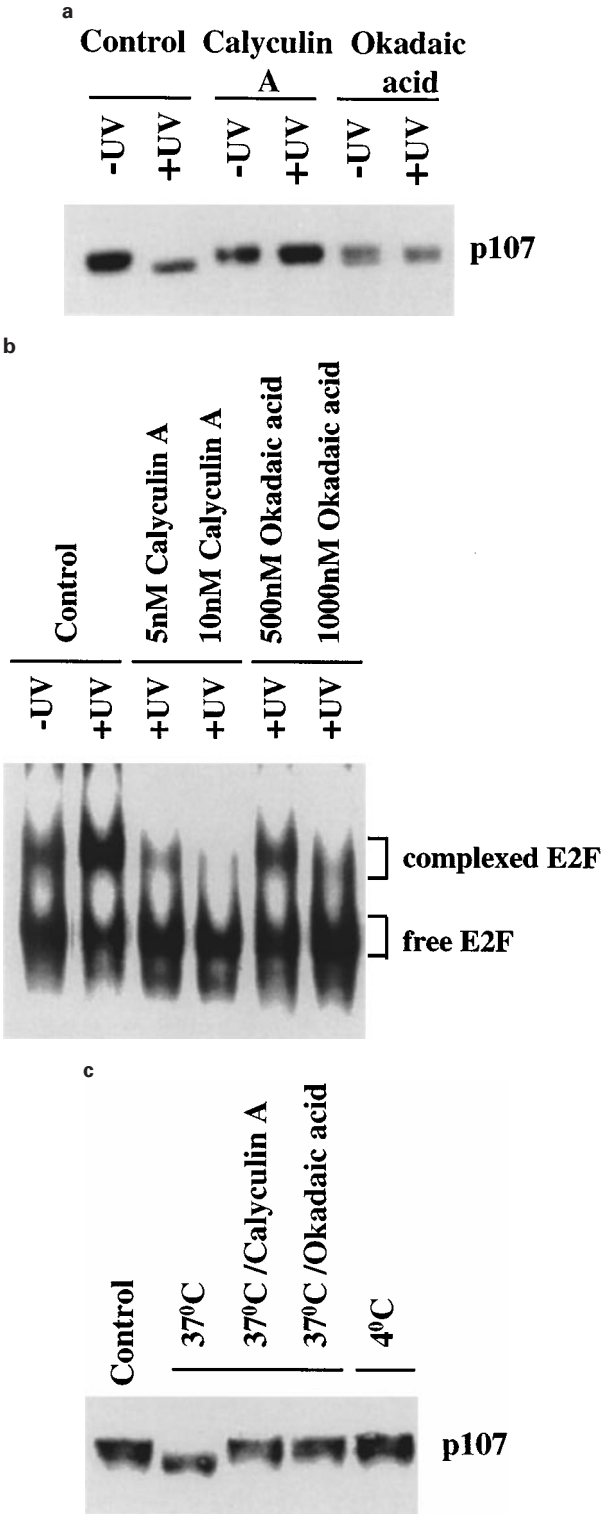

Figure 5 Effects of phosphatase inhibitors on p107 expression and E2F-binding after UV treatment. (**a**) NIH3T3 cells were treated with 5 nM of calyculin A or 500 nM okadaic acid before exposing to 50 J/m^2 of UV. Cells were harvested 2 h post-irradiation and whole cell extracts prepared were used for Western blotting with anti-p107 antiserum (C-18). (**b**) Mobility shift experiments using a ^{32}P-labelled E2F site containing oligonucleotide as probe were performed with cell extracts prepared as in (**a**) either in the absence or presence of the indicated amounts of phosphatase inhibitors. (**c**) Whole cell extracts were prepared from NIH3T3 cells harvested immediately after irradiation with 50 J/m^2 of UV. The cell extracts were incubated either at 4°C or at 37°C with or without phosphatase inhibitors (i.e. 5 nM calyculin A and 10 nM okadaic acid respectively) for 90 min before being analysed by Western blotting with anti-p107 antiserum (C-18)

Dephosphorylation of p107 following UV is not restricted to mouse fibroblasts and is mediated via the activation of a PP2A-related phosphatase

To determine whether the UV-induced dephosphorylation of p107 is a more universal feature of mammalian cells, we irradiated the human osteosarcoma cell line U2-OS, the rat neuroblastoma line B104 (Ciment and de Vellis, 1978) and the mouse neuroblastoma cell line N115 (Bachrach, 1975), with 100 J/m^2 UV and followed the phosphorylation status of p107. The Western blotting results (Figure 6) indicate that p107 in both cell lines underwent rapid dephosphorylation after UV treatment, suggesting that the UV-induced p107 dephosphorylation is a common event in mammalian cells and not restricted to mouse fibroblasts.

Using the specific phosphatase inhibitor okadaic acid, we have demonstrated that the dephosphorylation of p107 following UV is likely to be mediated by a PP2A-related phosphatase. We have recently isolated a novel regulatory subunit of PP2A, named PR59, that interacts specifically with p107 and whose overexpression caused dephosphorylation of p107, but not of pRb (Voorhoeve *et al*., in press). It is highly possible therefore that PR59 targets the PP2A catalytic subunit to p107 to cause its dephosphorylation in response to certain extracellular stimuli. To ask whether PP2A holoenzyme subunit composition influenced the effect of UV irradiation on p107 dephosphorylation, we tested if a PP2A B subunit, PR72, which does not interact with p107 (data not shown), could sequester the catalytic PP2Ac and 65 kDa dimer from p107, thus preventing the dephos-phorylation of p107 following UV irradiation. For this purpose, U2-OS cells were co-transfected with HA-tagged p107 expression vector and a vector that directs the synthesis of the PP2A B subunit PR72. Since PR72 does not influence p107 phosphorylation status (Figure 7, lane 4) we asked whether saturation of the PP2A core dimer (consisting of catalytic PP2Ac and 65 kDa) with PR72 prevented the dephosphorylation of p107 following UV irradiation. As a control, the U2-OS cells were cotransfected with HA-tagged p107 only. The results (Figure 7) show that overexpression of PR72 prevents dephosphorylation of p107 following UV irradiation. In contrast, dephosphorylation of p107 was evident in U2-OS cells after UV in the control cells transfected with HA-p107 expression vector only. These results indicate that alteration of the PP2A holoenzyme composition abolishes the effect of UV on p107 and further implicate PP2A in mediating the dephosphorylation of p107 following UV irradiation.

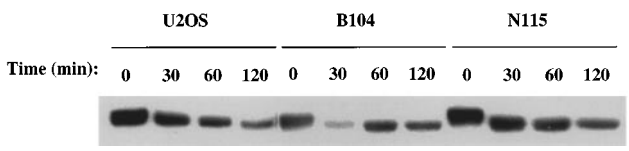

Figure 6 Rapid dephosphorylation of p107 in response to UV in mammalian cells. Murine neuroblastoma cells (B104 and N115) or human osteosarcoma cells (U2-OS) were treated with 100 J/m^2 UV, incubated for the indicated times and used for Western blotting analysis

p107 is a direct substrate for PP2A

We next set out to determine whether p107 is a direct substrate for PP2A using an *in vitro* dephosphorylation assay. U2-OS cells were transfected with either the HA-tagged 36 kDa catalytic C or the HA-tagged 65 kDa structural A subunit of PP2A. These respective PP2A subunits were then immunoprecipitated via the HA-tag from the transfected cells in a non-ionic detergent buffer to preserve protein–protein interactions. The substrate, p107, was immunoprecipitated from a U2-OS cell line stably expressing HA-tagged p107, which was predominantly in its hyperphosphorylated form. The hyperphosphorylated p107 was then incubated with the different PP2A subunit immunoprecipitates at either 4°C or 30°C and the phosphorylation status of p107 was analysed by Western blotting. The results (Figure 8) showed that p107 was completely dephosphorylated by the PP2A catalytic C subunit immunoprecipitate. Similarly, the PP2A structural A subunit immunoprecipitate dephosphorylated p107, but to a slightly lesser extent. This is probably due to the fact that the endogenous catalytic C subunit is limiting in these cells. However, no sign of p107 dephosphorylation was detected when incubated with precipitates from the mock transfected cells. This result indicates that p107 can

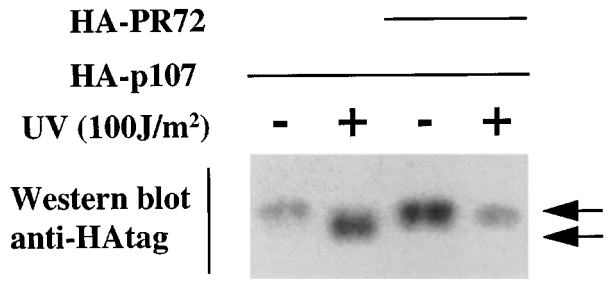

Figure 7 Overexpression of a PP2A regulatory subunit inhibits UV-induced dephosphorylation of p107. U2-OS cells transfected with the expression plasmids for the indicated proteins were mock-irradiated or irradiated with UV as described in Figure 6. After 60 min the cells were lysed and the phosphorylation status of the transfected p107 was analysed by Western blotting. Equal expression of HA-PR72 was verified by Western blot (data not shown) Arrows indicate hyper- (above) and hypo- (below) phosphorylated p107

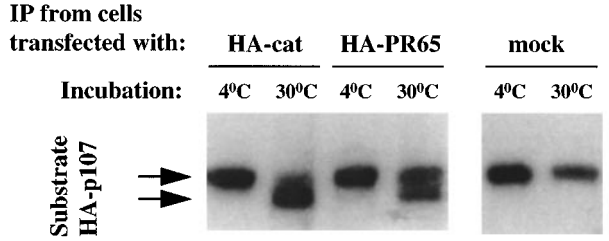

Figure 8 p107 is dephosphorylated *in vitro* by PP2A. Hyperphosphorylated HA-tagged p107 immunoprecipitated from U2-OS cells was mixed with immunoprecipitates against either HA-tagged PP2A catalytic subunit, or HA-tagged PR65 or with an immunoprecipitate from mock-transfected cells. The samples were divided into two halves and each half was incubated at either 4°C or 30°C for 3 h. The phosphorylation status of the HA tagged p107 was again analysed by Western blotting. Arrows as in Figure 7

indeed be dephosphorylated by PP2A and is consistent with our other experiments which also indicate that p107 is dephosphorylated by a PP2A-related protein phosphatase after UV treatment.

Discussion

In the present study, we have examined the role of retinoblastoma-related proteins in mediating the cellular response to UV and investigated the molecular mechanism involved. Previously, we have shown that E2F activity is negatively regulated by binding of p107/E2F complexes to the E2F site in NIH3T3 cells (Lam *et al*., 1994; Lam and Watson, 1993). Our present data lead us to postulate that G1 arrest following UV in NIH3T3 is the result, at least in part, of the increased capacity of p107 to bind E2F. Consistent with our deduction that p107 is a mediator of the UV induced G1 arrest in these NIH3T3 cells are previous microinjection experiments demonstrating that p107 can prevent NIH3T3 fibroblasts from entering S phase (Zhu *et al*., 1995a). Moreover, it has been demonstrated that p107-mediated G1 cell cycle arrest could be rescued by co-expression of E2F-4 and DP1 (Beijersbergen *et al*., 1995), suggesting that one of the mechanisms whereby p107 arrests cell-cycle progression is by targeting E2F activity.

While there was an increase in p107/E2F complex DNA-binding activity immediately after UV treatment (Figure 1a), there was no significant increase in p107 protein expression (Figure 2a). Rather we observed a change in p107 electrophoretic mobility to a form reminiscent of the hypophosphorylated form found in NIH3T3 cells at G1 of the cell cycle (Beijersbergen *et al*., 1995). The appearance of the hypophosphorylated form of p107 after UV treatment is consistent with the well-accepted notion that this species is the active growth inhibitory form of p107. Our finding that this effect was blocked by specific serine/threonine phosphatase inhibitors strongly suggests that pre-existing p107 is rapidly dephosphorylated after UV treatment, increasing its binding to DNA and resulting in repression of E2F dependent transcription activity and cell cycle arrest.

Our data demonstrate a close correlation between p107 dephosphorylation and G1 cell cycle arrest following UV irradiation, while the finding that p130 dephosphorylation does not correlate directly with cell cycle arrest, and that p130 constitutes only a minor proportion of pocketproteins in cycling cells, suggests that p107 is more directly involved in the initial response to UV irradiation. This also implies that p107 is an important modulator of E2F activity in response to UV treatment, suggesting a more specific functional role for p107 in UV induced cell cycle arrest. We also show that the phosphorylation status of p107 and p130 in UV activated G1 cell cycle block is different from that in serum deprived quiescent (G0) cells. This observation indirectly demonstrates that the G1 cell cycle block induced by UV is different from that observed in quiescent (G0) cells, which has previously been demonstrated to be modulated by p130. These two separate cell cycle events could also be predominantly controlled by different upstream cell cycle regulators.

One of the best established mechanisms whereby DNA damage triggers cell cycle arrest is the p53-mediated induction of p21 expression. Increased p21 levels repress the activity of cyclin-dependent kinases, which are consequently unable to phosphorylate the pocket proteins to allow release of complexed E2F. It has also been shown that the induction of p21 expression can also be manifested through a p53-independent mechanism (Sheikh *et al*., 1994; Steinman *et al*., 1994). It is clear from our results with the p53 and p21 null fibroblasts, however, that the dephosphorylation of p107 does not require either p53 or p21 function. Moreover, the relatively short time required (between 30 and 60 min) for p107 to be completely dephosphorylated implies that this effect was mediated through a phosphatase rather than by blocking the phosphorylation of p107 by inhibition of cyclin-dependent kinases. Consistent with this conclusion, we found that phosphatase inhibitors can block the UV-induced dephosphorylation of p107 (Figure 3).

Intriguingly, pre-treating UV-irradiated cells with increased levels of phosphatase inhibitor led to a decrease in p107/E2F complex abundance compared to unirradiated controls (Figure 5b), suggesting that the phosphorylation state of p107 is tightly regulated during the normal cell cycle, being subject to a flux of phosphorylation and dephosphorylation. UV activates (or enhances) the phosphatase, which consequently drives the equilibrium towards p107 hypophosphorylation. This equilibrium can also be affected by specific phosphatase inhibitors which shift the equilibrium towards the hyperphosphorylated and non E2F-binding form of p107. Other recent experiments also suggest the existence of a p53-independent G1 checkpoint induced by DNA damaging agents, for example, it has also been reported that p21 null mouse embryo fibroblasts demonstrate a partial G1 growth arrest in response to γ-irradiation, suggesting that the p21 signal pathway might not be the only one responsible for growth suppression at G1 induced by DNA damage agent (Brugarolas *et al*., 1995; Deng *et al*., 1995). Our results also indicate that this UV induced cellular response might not be the direct consequence of DNA damage. This suggestion is based on the observation that it requires only a short time (between 30–60 min) for the majority of p107 to convert to its hypophosphorylated form and also a relatively low dosage (about 5 J/m^2) to elicit the p107 dephosphorylation and the consequent G1 cell cycle arrest in the majority of the irradiated cells. We therefore hypothesise that the rapid dephosphorylation of p107 in response to UV is a result of the acute 'mammalian UV response' to impose an immediate but transient G1 arrest to prevent replication of damaged DNA.

Three lines of evidence indicate that the UV-mediated dephosphorylation of p107 involves a PP2A-like phosphatase. First, okadaic acid, a potent inhibitor of PP2A but less efficient towards PP1 at the low concentrations used here, prevented the UV-induced dephosphorylation of p107. Second, we show here that PP2A catalytic subunit can dephosphorylate p107 *in vitro*, showing that p107 can in principle be a substrate for PP2A. Third, elevated expression of the PP2A regulatory B subunit PR72, which in itself has no effect on p107 phosphorylation status, blocked the

UV-induced dephosphorylation of p107. We speculate that ectopic PR72 expression alters the PP2A subunit composition in the cell and thus replaces the B subunit in the UV-activated PP2A complex, thereby preventing the dephosphorylation of p107. Together with the finding that okadaic acid can inhibit p107 dephosphorylation after UV irradiation, this implies that indeed PP2A, and not PP1 is involved in this UV-response. We have recently identified a PR72-related PP2A regulatory B subunit (named PR59, Voorhoeve *et al*., submitted) that interacts with p107 and mediates its dephosphorylation by PP2A after overexpression. We are currently investigating whether this particular B subunit is solely responsible for the UV mediated dephosphorylation of p107.

It is notable that p107 is also dephosphorylated by a PP2A related phosphatase activated (or enhanced) by the cell lysing process (data not shown). The phosphatase activity involved might be the same or related to the one induced by UV. Nevertheless, this observation also highlights one of the possible mechanisms by which UV could activate the p107 specific phosphatase through changing the subcellular localization of its subunits. Indeed, the regulatory B subunits of PP2A have been shown to encode the targeting information that directs the phosphatase heterotrimer to distinct intracellular locations (Sontag *et al*., 1995). It is possible that the cell lysing process destroys the subcellular compartmentalization and therefore bring together p107 and the activated subunits of the UV inducible phosphatase. This idea is supported by the rapid kinetics of p107 dephosphorylation in response to UV.

It has been reported that anti-cancer drugs can cause dephosphorylation of pRB, and subsequent G1 arrest and apoptosis (Dou *et al*., 1995). Interestingly, this dephosphorylation process is mediated by a serine/threonine phosphatase and is also independent of the p53 signaling pathway. However, it is unlikely that this pRB specific phosphatase could be identical to the one which dephosphorylates p107 in response to UV, as it has been shown that the phosphatase responsible for dephosphorylation of pRB is a type-1 protein phosphatase, while our results indicate that the p107-specific phosphatase is PP2A related.

From these results, we concluded that UV induces dephosphorylation of p107 via activation of a type-2A protein phosphatase and it is likely that this may be one mechanism by which UV induces cell cycle arrest. The identification of this UV sensitive PP2A related phosphatase is the focus of our current work.

Materials and methods

Tissue culture and UV treatment of cells

NIH3T3, p53-null (Donehower *et al*., 1992) and p21-null (Deng *et al*., 1995) fibroblasts as well as U2-OS, B104 and N115 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, glutamine and penicillin/streptomycin. For UV irradiation experiments, cells were grown to 60% confluence and the tissue culture medium changed before irradiation. Just before UV irradiation, the tissue culture medium was removed and cells were then exposed to a 50 J/m^2 dose, unless stated otherwise, of UV delivered by an XL-1500 UV crosslinker (Spectronics Corporation). After treatment, the original culture medium was returned to the irradiated cells for further incubation.

Plasmids

The expression vector pMV-HAtag was generated by inserting an oligonucleotide encoding a consensus AUG start codon followed by the 10 amino acid influenza virus haemagglutinin epitope from the *Xho*I to *Sal*I sites of the vector pBSK$^+$. HA tagged PP2Ac was created by cloning human catalytic subunit cDNA (Stone *et al*., 1988) from nucleotide 58–1022 into pMV-HAtag, introducing 19 additional amino acids between the HA tag and the first methionine. HA-PR65 was created by inserting the human PR65 cDNA (Hemmings *et al*., 1990), starting from amino acid 3, in pMV-HAtag. HA-PR72 was created by PCR using the primer 5′-TCGCGTCGACGATGATGAT-CAAGGAAACATC-3′ and T7 primer on the full-length human PR72 cDNA in pBSK (Hendrix *et al*., 1993). The PCR product was cloned into the *Sal*I site of pMV-HAtag. HA tagged PP2Ac and PR72 were then subcloned into pCMV (Beijersbergen *et al*., 1994), while HA tagged PR65 into the mammalian expression vector pRC/CMV (Invitrogen). The expression vector pCMV-HAp107 has been described previously (Beijersbergen *et al*., 1994).

Transfections and establishment of stable cell lines

Transfection of U2-OS were performed using the calcium phosphate co-precipitation method as described previously (van der Eb and Graham, 1980). For establishment of transfected cell lines, U2-OS cells were transfected with linearized pCMV-HAp107 by the calcium phosphate precipitation method. G418 resistant U2-OS cells were pooled and tested for their ability to produce HA tagged p107.

FACS analysis

Cell cycle analysis was performed by flow cytometry as described before (Lam and Watson, 1993). Cells were trypsinized, collected by centrifugation, washed with PBS and fixed in 35% ethanol/65% DMEM prior to DNA staining. Both floating and adherent cells were pooled for FACS analysis. Cells were stained with propidium iodide (40 mg/ml) in the presence of 100 mg/ml RNAse A (Sigma) and stored at room temperature for 30 min prior to scanning using a Becton Dickinson FACSort analyser. The cell cycle profile was analysed using the Cell Quest software.

Phosphatase inhibitor treatment of cultured cell lines

Calyculin A and okadaic acid (Sigma) were dissolved in PBS with 10% dimethyl sulfoxide at concentrations of 100 mM and 500 mM respectively before use. The cell cultures were incubated with the indicated amount of phosphatase inhibitor in culture medium for 15 min prior to the treatment with UV. Cells were harvested at 90 min after UV irradiation.

Gel retardation and super-shift assays

Whole cell extracts from fibroblasts were prepared as described previously (Bandara *et al*., 1994). Protein yield was quantified by Bradford analysis (Bio-Rad). E2F gel retardation assays were performed essentially as described (Lam and Watson, 1993) using a double stranded oligonucleotide containing the distal E2F binding site from the adenovirus type 5 E2a promoter (Bandara *et al*.,

1994). Twenty mg of whole cell extract was incubated with 1–2 ng of ^{32}P-labeled DNA probe in the presence of 2 mg of sonicated salmon sperm DNA and 200 ng of a comparable unlabeled double stranded oligonucleotide with mutated E2F site at 30°C for 15 min. The reactions were electrophoresed on 4% polyacrylamide gels in 0.33XTBE buffer. The gels were then dried and exposed to X-ray films. Supershift assays were performed by adding 1 ml of either SD15 p107 monoclonal antibody (a gift from N Dyson), XZ104 pRB monoclonal antibody (Hu *et al*., 1991), or a rabbit polyclonal antiserum against the C-terminus of p130 (kindly provided by A Giordano) to the gel shift reaction prior to the addition of probe.

Immunoprecipitation and in vitro *phosphatase assay*

Immunoprecipitations were performed as described previously (Beijersbergen *et al*., 1994). Briefly, the cells were collected in ELB (250 mM NaCl, 0.1% NP40, 50 mM HEPES pH 7.0, 5 mM EDTA) supplemented with protease inhibitors (Complete, Boehringer Mannheim and 1 mM phenylmethylsulfonylfluoride) and incubated on ice for 25 min. After clearing by centrifugation, the supernatant was then rocked with 25 μl of a pre-formed complex of monoclonal antibody 12CA5 coupled to protein A Sepharose beads. After 1 h, the beads were washed three times in protease inhibitor-supplemented ELB buffer and once in phosphatase wash buffer (250 mM NaCl, 50 mM HEPES pH 7.0). The beads were then resuspended in 50 μl phosphatase assay buffer (50 mM Tris pH 7.5, 0.1 mM EDTA, 0.9 mg/ml Bovine Serum Albumin (Sigma), 0.09% β-mercaptoethanol, 1 mM $MnCl_2$). The immunoprecipitates from the transfected cells were then mixed with 50 μl immunoprecipitate from the U2-OS cells stably expressing HA tagged p107 and incubated at 4°C or 30°C for 3 h.

Western blot analysis

Whole cell extracts or immunoprecipitates, prepared as above, were separated on 5% SDS–PAGE gels. Following electrophoresis, proteins were transferred to nitrocellulose, before incubation with the indicated antibodies. Monoclonal antibodies to p107 (SD9) and the 12CA5 haemagglutinin tag antibody were described previously (Field *et al*., 1988; Zhu *et al*., 1993). Polyclonal antibodies against p107 (C18) and pRb (C15) and p130 (C20) were obtained from Santa Cruz. Polyclonal antibodies against PP2Ac were a gift from BA Hemmings (FMI, Basel). For detection of mouse p53, rabbit polyclonal antibody CM-5 were used. The antibodies were detected using horseradish peroxidase-linked goat anti-mouse (Biorad) or anti-rabbit (Biosource Ltd) and visualized by the enhanced chemiluminescent (ECL) detection system (Amersham).

Abbreviations

HA, haemagglutinin; UV, ultraviolet radiation.

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References

Bachrach U. (1975). *Proc. Natl. Acad. Sci. USA,* **72,** 3087–3091.

Bandara LR, Lam EW-F, Sorensen TS, Zamanian M, Girling R and La Thangue NB. (1994). *EMBO J.,* **13,** 3104–3114.

Beijersbergen RL and Bernards R. (1996). *Biochim. Biophys. Acta,* **1287,** 103–120.

Beijersbergen RL, Carlee L, Kerkhoven RM and Bernards R. (1995). *Genes Dev.,* **9,** 1340–1353.

Beijersbergen RL, Kerkhoven RM, Zhu L, Carlee L, Voorhoeve PM and Bernards R. (1994). *Genes Dev.,* **8,** 2680–2690.

Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T and Hannon GJ. (1995). *Nature,* **377,** 552–557.

Buchkovich K, Duffy LA and Harlow E. (1989). *Cell,* **58,** 1097–1105.

Chellappan SP, Hiebert S, Mudryj M, Horowitz JM and Nevins JR. (1991). *Cell,* **65,** 1053–1061.

Chen PL, Scully P, Shew JY, Wang JY and Lee WH. (1989). *Cell,* **58,** 1193–1198.

Ciment G and de Vellis J. (1978). *Science,* **202,** 765–768.

Cohen P, Klumpp S and Schelling DL. (1989). *FEBS Lett.,* **250,** 596–600.

DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwnica-Worms H, Huang CM and Livingston DM. (1989). *Cell,* **58,** 1085–1095.

Deng C, Zhang P, Harper JW, Elledge SJ and Leder P. (1995). *Cell,* **82,** 675–684.

Devary Y, Gottlieb RA, Lau LF and Karin M. (1991). *Mol. Cell. Biol.,* **11,** 2804–2811.

Devary Y, Gottlieb RA, Smeal T and Karin M. (1992). *Cell,* **71,** 1081–1091.

Devary Y, Rosette C, DiDonato JA and Karin M. (1993). *Science,* **261,** 1442–1445.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery Jr CA, Butel JS and Bradley A. (1992). *Nature,* **356,** 215–221.

Dou QP, An B and Will PL. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 9019–9023.

Durfee T, Becherer K, Chen PL, Yeh SH, Yang Y, Kilburn AE, Leee WH and Elledge SJ. (1993). *Genes Dev.,* **7,** 555–569.

Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J and Livingston DM. (1993). *Cell,* **73,** 487–497.

Field J, Nikawa J, Broek D, MacDonald B, Rodgers L, Wilson IA, Lerner RA and Wigler M. (1988). *Mol. Cell. Biol.,* **8,** 2159–2165.

Lerner RA and Wigler M. (1988). *Mol. Cell. Biol.,* **8,** 2159–2165.

Flemington EK, Speck SH and Kaelin Jr WG. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 6914–6918.

Ginsberg D, Vairo G, Chittenden T, Xiao ZX, Xu G, Wydner KL, DeCaprio JA, Lawrence JB and Livingston DM. (1994). *Genes Dev.,* **8,** 2665–2679.

Hartwell LH and Kastan MB. (1994). *Science,* **266,** 1821–1828.

Hatakeyama M, Herrera RA, Makela T, Dowdy SF, Jacks T and Weinberg RA. (1994). *Cold Spring Harb. Symp. Quant. Biol.,* **59,** 1–10.

Hemmings BA, Adams-Pearson C, Maurer F, Muller P, Goris J, Merlevede W, Hofsteenge J and Stone SR. (1990). *Biochemistry,* **29,** 3166–3173.

Hendrix P, Mayer-Jackel RE, Cron P, Goris J, Hofsteenge J, Merlevede W and Hemmings BA. (1993). *J. Biol. Chem.,* **268,** 15267–15276.
Herrlich P, Ponta H and Rahmsdorf HJ. (1992). *Rev. Physiol. Biochem. Pharmacol.,* **119,** 187–223.
Hiebert SW. (1993). *Mol. Cell. Biol.,* **13** 3384–3391.
Hijmans EM, Voorhoeve PM, Beijersbergen RL, van't Veer LJ and Bernards R. (1995). *Mol. Cell. Biol.,* **15,** 3082–3089.
Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI and Weinberg RA. (1992). *Cell,* **70,** 993–1006.
Holbrook NJ and Fornace Jr AJ. (1991). *New Biol.,* **3,** 825–833.
Honkanen RE, Codispoti BA, Tse K, Boynton AL and Honkanan RE. (1994). *Toxicon,* **32,** 339–350.
Hu QJ, Bautista C, Edwards GM, Defeo-Jones D, Jones RE and Harlow E. (1991). *Mol. Cell. Biol.,* **11,** 5792–5799.
Johnson DG. (1995). *Oncogene,* **11,** 1685–1692.
Kato J, Matsushime H, Hiebert SW, Ewen ME and Sherr CJ. (1993). *Genes Dev.,* **7,** 331–342.
Lam EW and La Thangue NB. (1994). *Curr. Opin. Cell. Biol.,* **6,** 859–866.
Lam EW, Morris JD, Davies R, Crook T, Watson RJ and Vousden KH. (1994). *EMBO J.,* **13,** 871–878.
Lam EW and Watson RJ. (1993). *EMBO J.,* **12,** 2705–2713.
Lees JA, Saito M, Vidal M, Valentine M, Look T, Harlow E, Dyson N and Helin K. (1993). *Mol. Cell. Biol.,* **13,** 7813–7825.
Mayer-Jaekel RE and Hemmings BA. (1994). *Trends Cell. Biol.,* **4,** 287–291.
Mayol X, Garriga J and Grana X. (1995). *Oncogene,* **11,** 801–808.
Moberg K, Starz MA and Lees JA. (1996). *Mol. Cell. Biol.,* **16,** 1436–1449.
Nelson DA, Krucher NA and Ludlow JW. (1997). *J. Biol. Chem.,* **272,** 4528–4535.
Nelson DA and Ludlow JW. (1997). *Oncogene,* **14,** 2407–2415.
Nevins JR. (1992). *Science,* **258,** 424–429.
Qian Y, Luckey C, Horton L, Esser M and Templeton DJ. (1992). *Mol. Cell. Biol.,* **12,** 5363–5372.
Ronai ZA, Lambert ME and Weinstein IB. (1990). *Cell. Biol. Toxicol.,* **6,** 105–126.
Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, Chen A and Weinberg RA. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 2403–2407.
Sheikh MS, Li XS, Chen JC, Shao ZM, Ordonez JV and Fontana JA. (1994). *Oncogene,* **9,** 3407–3415.
Sherr CJ. (1996). *Science,* **274,** 1672–1677.
Smith EJ, Leone G, DeGregori J, Jakoi L and Nevins JR. (1996). *Mol. Cell. Biol.,* **16,** 6965–6976.
Sontag E, Nunbhakdi-Graig V, Bloom GS and Mumby MC. (1995). *J. Cell. Biol.,* **128,** 1131–1144.
Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA and el-Houseini ME. (1994). *Oncogene,* **9,** 3389–3396.
Stone SR, Mayer R, Wernet W, Maurer F, Hofsteenge J and Hemmings BA. (1988). *Nucleic Acids Res.,* **16,** 11365.
Vairo G, Livingston DM and Ginsberg D. (1995). *Genes Dev.,* **9,** 869–881.
van der Eb AJ and Graham FL. (1980). *Methods Enzymol.,* **65,** 826–839.
Walker GC. (1985). *Annu. Rev. Biochem.,* **54,** 425–457.
Weinberg RA. (1995). *Cell,* **81,** 323–330.
Wera S and Hemmings BA. (1995). *Biochem. J.,* **311,** 17–29.
Wu CL, Zukerberg LR, Ngwu C, Harlow E and Lees JA. (1995). *Mol. Cell. Biol.,* **15,** 2536–2546.
Zamanian M and La Thangue NB. (1993). *Mol. Biol. Cell,* **4,** 389–396.
Zhang Y and Chellappan SP. (1995). *Oncogene,* **10,** 2085–2093.
Zhu L, Enders G, Lees JA, Beijersbergen RL, Bernards R and Harlow E. (1995a). *EMBO J.,* **14,** 1904–1913.
Zhu L, van den Heuvel S, Helin K, Fattaey A, Ewen M, Livingston D, Dyson N and Harlow E. (1993). *Genes Dev.,* **7,** 1111–1125.
Zhu L, Zhu L, Xie E and Chang LS. (1995b). *Mol. Cell. Biol.,* **15,** 3552–3562.