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Lack of cyclin D–Cdk complexes in Rb-negative cells correlates with high levels of p16$^{INK4/MTS1}$ tumour suppressor gene product

David Parry, Stewart Bates, David J.Mann[1] and Gordon Peters[2]

Molecular Oncology and [1]Gene Regulation Laboratories, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

[2]Corresponding author

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D-type cyclins, in association with the cyclin-dependent kinases Cdk4 or Cdk6, regulate events in the G_1 phase of the cell cycle and may contribute to the phosphorylation of the retinoblastoma gene product (Rb). However, in cells in which the function of Rb has been compromised, either by naturally arising mutations or through binding to proteins encoded by DNA tumour viruses, Cdk4 and Cdk6 are not associated with D cyclins. Instead, both kinases form binary complexes with a stable 16 kDa protein (p16) encoded by the putative tumour suppressor gene *INK4/MTS1* on human chromosome 9p21. Here we show an inverse correlation between Rb status and the expression of p16. Since Rb-negative cells express high levels of p16, we suggest that in these cells p16 competes with D cyclins for binding to Cdk4 and Cdk6 and prevents formation of active complexes. In line with these predictions, DNA tumour virus oncoproteins do not disrupt cyclin D1–Cdk4 complexes in cells lacking p16.

Key words: cdk/cell cycle/cyclin D/retinoblastoma protein

Introduction

The cell division cycle proceeds in a temporally ordered series of events that involves both positive and negative regulators. Among the positive effectors of cell division are a family of protein kinases, known collectively as the cyclin-dependent kinases (Cdks), that rely for their activity on association with members of the cyclin family (Norbury and Nurse, 1992; Pines, 1993; Sherr, 1993). The classical cyclins undergo periodic accumulation and destruction in phase with the cell cycle, thus providing a mechanism whereby the activities of the various cyclin–kinase complexes are regulated throughout the cycle. For example, the cyclin E–Cdk2, cyclin A–Cdk2 and cyclin B–cdc2 complexes appear to act sequentially in the G_1/S, S/G_2 and G_2/M phases respectively (Motokura and Arnold, 1993; Pines, 1993; Sherr, 1993). The activity of the kinases is in turn regulated by phosphorylation and dephosphorylation, providing a further tier of control (Norbury and Nurse, 1992; Solomon, 1993).

Among the negative regulators are the product of the retinoblastoma gene, Rb, and the related p107 and p130 proteins (reviewed in Dyson and Harlow, 1992; Ewen, 1994). *RB1* is the archetypal tumour suppressor gene in that loss of function mutations contribute to both familial and sporadic forms of cancer (Weinberg, 1992; Goodrich and Lee, 1993). Various strands of evidence suggest that, in normal cells, Rb must be inactivated in order for the cells to leave the G_1 phase and begin DNA synthesis. The evidence also suggests that it is the unphosphorylated or hypophosphorylated forms of Rb that are functional and that the restraining influence of Rb is alleviated by phosphorylation (Goodrich and Lee, 1993; Ewen, 1994). Central to this argument is the observation that proteins encoded by DNA tumour viruses, such as the large T-antigen (T-Ag) of SV40, the E1A protein of certain adenovirus serotypes and the E7 protein of the oncogenic sub-types of human papilloma virus (HPV), bind preferentially to under-phosphorylated Rb (Dyson and Harlow, 1992; Weinberg, 1992; Goodrich and Lee, 1993; Ewen, 1994). In so doing, they presumably promote progression into S phase by preventing Rb from performing its normal functions. One of these functions appears to be an interaction with transcription factors of the E2F family which are needed for the expression of key genes involved in DNA synthesis (Hollingsworth *et al.*, 1993).

Many of the known phosphorylation sites on Rb conform to the consensus for cyclin–Cdk targets, providing a potential link between the positive and negative effectors of cell cycle control (Taya *et al.*, 1989; Lees *et al.*, 1991; Lin *et al.*, 1991; Hollingsworth *et al.*, 1993). Although there may be additional specificity that is not yet appreciated, the phosphorylation and inactivation of Rb might be accomplished by successive waves of cyclin–Cdk complexes. The earliest of these would be the D cyclins and indeed cyclins D1–D3 in conjunction with their major catalytic partners Cdk4 and Cdk6 are able to phosphorylate Rb *in vitro* (Ewen *et al.*, 1993; Kato *et al.*, 1993; Serrano *et al.*, 1993; Meyerson and Harlow, 1994). Analogous complexes immunoprecipitated from mammalian cells also have Rb kinase activity (Matsushime *et al.*, 1994; Meyerson and Harlow, 1994), but in this respect, the D cyclin–kinases appear much less effective than for example cyclin E–Cdk2.

An obvious prediction of this connection between cyclin-dependent kinases and Rb would be that the deregulated expression of a cyclin or Cdk could be oncogenic, since it would lead to constitutive phosphorylation of Rb. Although there are sporadic reports of amplification of cyclin E and Cdk4 (Keyomarsi and Pardee, 1993; Khatib *et al.*, 1993; Leach *et al.*, 1993), the most compelling data pertain to the D cyclins and suggest that cyclin D1 functions as a classical oncogene. Thus, the expression of cyclin D1 can be activated by chromosomal translocation, DNA amplification or retroviral integration in different tumour systems (reviewed in Fantl *et al.*, 1993; Motokura

and Arnold, 1993; Bates and Peters, 1994) and the oncogenic properties of the gene have been demonstrated by DNA transfection (Jiang *et al.*, 1993a; Hinds *et al.*, 1994; Lovec *et al.*, 1994) and in transgenic models (Bodrug *et al.*, 1994; Wang *et al.*, 1994). Since the D cyclins also bear the LXCXE amino acid sequence motif shared by DNA tumour virus proteins that bind directly to Rb (Dowdy *et al.*, 1993), they provide an intriguing example of oncogene−tumour suppressor gene interaction in a wholly cellular context.

A new class of cell cycle regulator has recently been identified that impinges on these processes. These are the inhibitors of cyclin-dependent kinase activity, such as $p16^{INK4}$ (Serrano *et al.*, 1993), $p15^{INK4B}$ (Hannon and Beach, 1994), $p21^{CIP1/WAF1/SDI1}$ (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a; Noda *et al.*, 1994) and $p27^{KIP1}$ (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). Since the loss of these inhibitors would lead to an increase in kinase activity and promote cell cycle progression, they are attractive candidates for tumour suppressor genes. Indeed, recent reports have indicated that the p16 gene, located on chromosome 9p21, is frequently deleted or mutated in tumour cell lines (Kamb *et al.*, 1994; Nobori *et al.*, 1994), although its importance in primary tumours remains open to debate (Cairns *et al.*, 1994; Caldas *et al.*, 1994; Mori *et al.*, 1994; Spruck *et al.*, 1994). The p16 protein was originally identified and the cDNA cloned through binding to Cdk4 (Serrano *et al.*, 1993). Here we show that p16 also binds and inhibits the kinase activity of Cdk6, implying that its role as an inhibitor is specific for the D cyclins. In cells that lack functional Rb, the D cyclins are not found associated with either Cdk4 or Cdk6 (Xiong *et al.*, 1993b; Bates *et al.*, 1994b; Tam *et al.*, 1994). We now provide a likely explanation, since p16 is expressed at high levels in these cells and sequesters the majority of Cdk4 and Cdk6. As the expression of p16 is inversely correlated with the presence of functional Rb, these data suggest that p16 is part of a feedback loop that regulates Rb. They also imply a common mechanism of tumorigenesis in which loss of p16, over-expression of cyclin D1 and loss of Rb are functionally analogous.

Results

Association of p16 and Cdks in cell lysates

When comparing the interactions between the three D-type cyclins and members of the Cdk family in a variety of tumour cells, we were unable to detect cyclin D−Cdk complexes in cells in which the function of Rb had been compromised (Bates *et al.*, 1994b). For example, in the Rb-positive bladder carcinoma cell line T24, immunoprecipitates of labelled Cdk6 contained proteins of 36 and 34 kDa which we have identified as cyclins D1 and D3 respectively, whereas these proteins were not detectable in equivalent immunoprecipitates from the Rb-negative bladder cell line 5637 (Figure 1a). The same holds true for a number of breast and cervical carcinoma lines and can be reproduced by the transformation of cells with DNA tumour viruses that interfere with the function of Rb (Xiong *et al.*, 1993a; Bates *et al.*, 1994b; Tam *et al.*, 1994). More significantly, in 5637 cells and other Rb-negative cell lines, Cdk6 immunoprecipitates contained a 16 kDa protein that was not observed in corresponding analyses of T24 cells (Figure 1a).

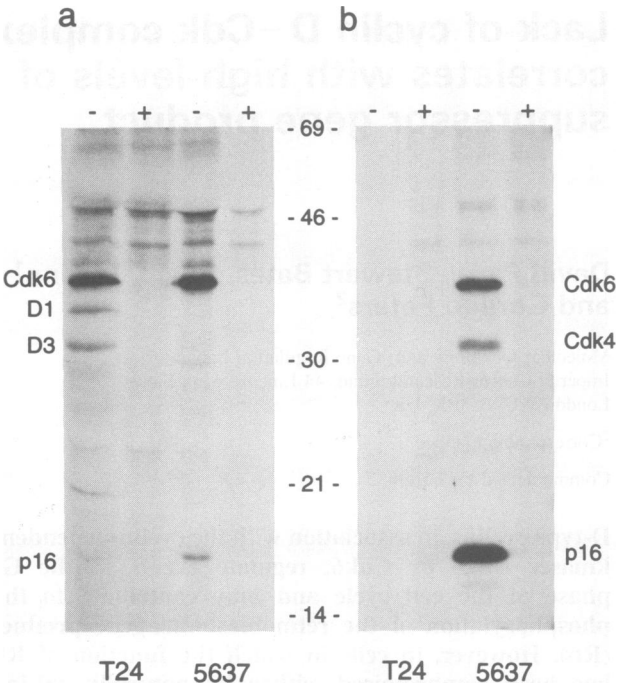

Fig. 1. Association of p16 with Cdk4 and Cdk6 in pRb-negative cells. The bladder carcinoma cell lines T24 and 5637 were metabolically labelled with [^{35}S]methionine for 30 min and cell lysates were immunoprecipitated with (**a**) polyclonal antiserum against Cdk6 or (**b**) polyclonal antiserum against p16. In each case, the antiserum was pre-incubated with and without an excess of the peptide antigen (+/−). The labelled proteins were fractionated by SDS−PAGE in a 12% gel and visualized by fluorography. The positions of p16, cyclin D1, cyclin D3, Cdk4 and Cdk6 are as indicated.

A p16 protein was previously described by Xiong and co-workers in SV40-transformed human fibroblasts (Xiong *et al.*, 1993b) and subsequently cloned in a two hybrid screen for proteins that interact with Cdk4 (Serrano *et al.*, 1993). We prepared a polyclonal antiserum against the C-terminal peptide of the published human $p16^{INK4}$ sequence (Serrano *et al.*, 1993). This antiserum specifically precipitated a 16 kDa protein from lysates of metabolically labelled 5637 cells, but there was no detectable equivalent in T24 cells (Figure 1b). Two prominent proteins of 33 and 38 kDa were also present in the immune complexes from 5637 cells and partial proteolytic mapping (not shown) indicated that these co-precipitating proteins correspond to Cdk4 and Cdk6 respectively.

Correlation of p16 levels and Rb status

To extend these observations, we surveyed the p16 levels in the panel of cell lines in which we had previously investigated cyclin D−Cdk interactions. Immunoblotting with a C-terminal antibody revealed susbtantial levels of p16 in a range of cell lines in which Rb has been functionally compromised and which lack cyclin D−Cdk complexes (Figure 2a). In contrast, little or no p16 could be detected in a series of tumour cell lines that express functional Rb. The faint signal observed in some of the latter cells is likely to be a cross-reacting protein, because the MCF-7 line, for example, is known to have a homozygous deletion of the p16 locus (Kamb *et al.*, 1994).

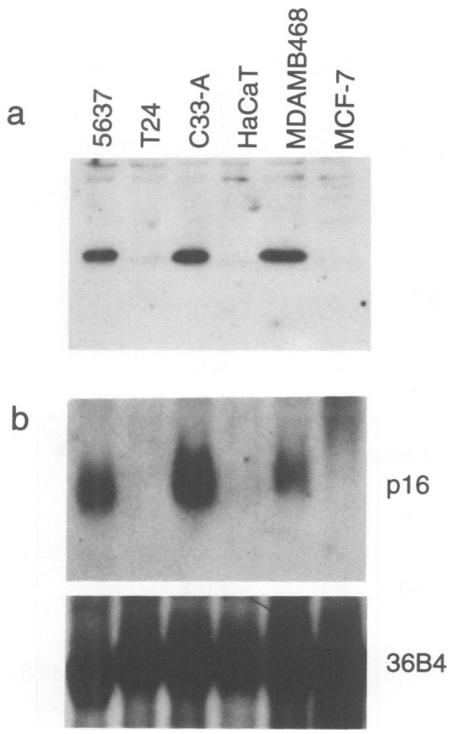

Fig. 2. Correlation of p16 levels with Rb status. (**a**) Lysates prepared from equivalent numbers of the indicated cells were fractionated by SDS–PAGE in a 12% gel and immunoblotted with a polyclonal antiserum against the C-terminal peptide of p16. Immune complexes were detected with ^{125}I-labelled protein A and autoradiography. (**b**) Samples (10 μg) of total RNA from the indicated cell lines were fractionated by electrophoresis in a formaldehyde–agarose gel and transferred to membrane. The filter was then hybridized to a probe representing the first exon of the p16 gene (Kamb *et al.*, 1994). The loading in each lane was monitored by the signal for 36B4, corresponding to the ribosomal protein PO (Laborda, 1991).

Since the inability to detect p16 protein in a particular cell line could reflect mutations in the coding domain, lack of expression or complete absence of the gene, we also examined the expression of p16 RNA in these cell lines. In the examples shown in Figure 2b, we used a probe specific for the first exon of the *MTS1* genomic locus to exclude the possibility of cross-reaction with the adjacent *MTS2* sequences (Kamb *et al.*, 1994). There was a striking correlation between the expression of p16 RNA and the status of Rb in these cell lines. For example, the Rb-negative cells 5637, MDA-MB468 and C33-A all contained relatively high levels of the ~1.6 kb transcript, whereas the matching Rb-positive cells T24, MCF-7 and HaCaT showed no or barely detectable levels of this RNA. This correlation applied to all of the tumour cell lines listed in Table I. As controls for these analyses, we also examined the primary human fibroblast strains MRC5 and WI38. Both expressed readily detectable but relatively low levels of p16 RNA and protein (data not shown), but these levels were dramatically increased in VA13 cells, an SV40-transformed derivative of WI38 (Xiong *et al.*, 1993b). Thus, in cell lines in which the p16 locus was grossly intact, there was an inverse relationship between the function of Rb and the expression of p16 RNA (Table I). If p16 interferes with the association of Cdk4 and Cdk6 with their regulatory cyclins, the elevated expression of

Table I. Correlation of p16 expression and Rb status in a series of primary, transformed and tumour cell lines

Cell line	Cell type	Rb	p16 RNA	p16 DNA
T24	bladder	+	+/−	+
5637	bladder	−	+++	+
HaCaT	keratinocyte	+	+/−	+
C33-A	cervical	−	+++	ND
CaSKI	cervical	HPV	+++	+
SiHa	cervical	HPV	+++	+
MCF-7	breast	+	−	−
MDA-MB468	breast	−	+++	+
BT549	breast	−	+++	ND
MTSV1-7	breast	SV40	+++	+
MOLT-4	T-cell	+	−	−
J6	T-cell	+	−	−
MRC-5	fibroblast	+	+	+
WI38	fibroblast	+	+	+
VA13	fibroblast	SV40	+++	+

The panel of cells surveyed here and in our previous report (Bates *et al.*, 1994b) are listed together with their tissues of origin. The status of Rb in each cell was determined either by immunoblotting or by reference to the literature (Bates *et al.*, 1994b). RNA and DNA analyses were performed as described in Materials and methods and examples are included in Figures 2 and 8.

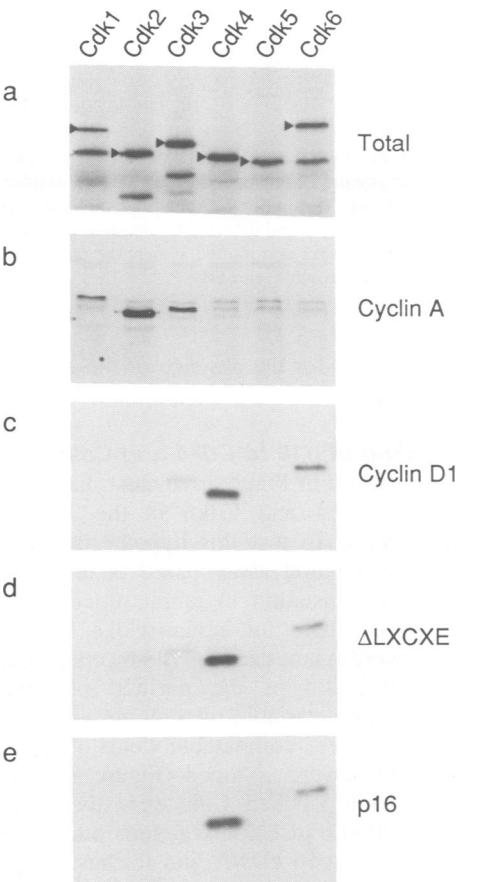

Fig. 3. *In vitro* binding of p16 and Cdks. The six known human Cdks were radiolabelled by *in vitro* translation (**a**) and mixed with similar reactions containing unlabelled cyclin A (**b**), cyclin D1 (**c**), an LXCXE mutant of cyclin D1 (**d**) or p16 (**e**). The samples were either analysed directly (a) or after immunoprecipitation with antisera against the cyclins or p16. The proteins were then fractionated by SDS–PAGE in a 10% gel and the labelled Cdks detected by autoradiography.

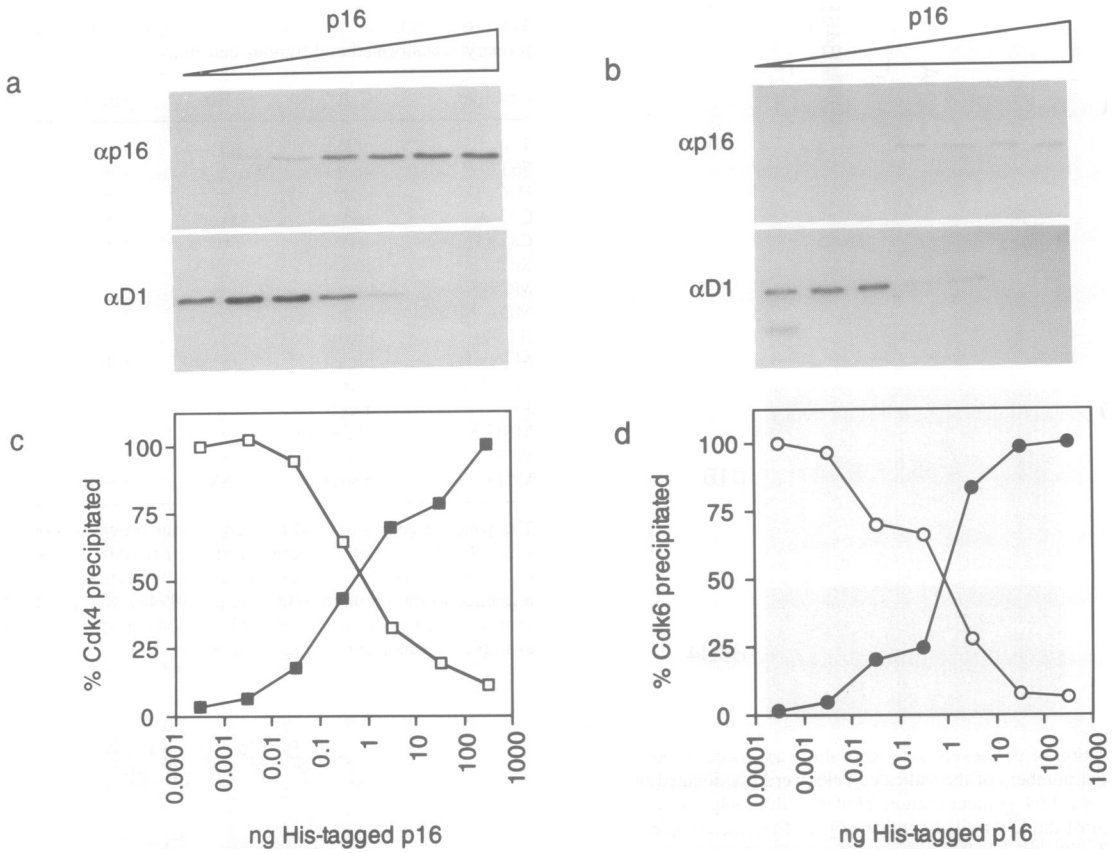

Fig. 4. Competititon between p16 and cyclin D1 for binding to Cdk4 and Cdk6. Serial dilutions of bacterially expressed histidine-tagged p16 were added to a fixed amount of unlabelled cyclin D1 synthesised by *in vitro* translation. Labelled Cdk4 (**a**) or Cdk6 (**b**) was then added and the mixtures incubated at 30°C for 30 min before immunoprecipitation with either p16 or cyclin D1 antiserum as indicated. The products were fractionated by SDS−PAGE in a 10% gel and detected by autoradiography. The labelled Cdk4 (**c**) and Cdk6 (**d**) in each track was quantitated using a Molecular Dynamics PhosphorImager and plotted against the amount of p16 added to the reactions. Closed symbols refer to precipitation via p16; open symbols to precipitation via cyclin D1.

p16 could account for the absence of these complexes in Rb-negative cells.

Direct binding of p16 to Cdk4 and Cdk6

The data presented in Figure 1 suggest that p16 interacts directly with Cdk4 and Cdk6 in the absence of an associated cyclin. To test this hypothesis, we exploited an *in vitro* association assay based on expressing each component by translation in rabbit reticulocyte lysates. The coding sequences for human Cdks 1–6 (Meyerson *et al.*, 1992) were synthesized *in vitro* by coupled transcription and translation of the purified plasmid DNAs. Allowing for their variable methionine content (ranging from 4 to 8 residues), comparable yields of the respective labelled proteins were obtained (Figure 3a). Equivalent amounts of each labelled Cdk were then mixed with similar unlabelled translation reactions programmed with either a cyclin or p16 cDNA and the resultant mixtures were subjected to immunoprecipitation using antisera specific for the unlabelled product. In this system, human cyclin A bound significantly to Cdks 1–3, but not to Cdks 4–6 (Figure 3b). Cyclins D1−D3, on the other hand, were each found to associate preferentially with Cdk4 and Cdk6 (Figure 3c and additional data not shown), although there was clearly a low level of binding to the other kinases tested. This is consistent with published findings based on immunoprecipitation of whole cell lysates (Matsushime *et al.*, 1992; Xiong *et al.*, 1992; Bates *et al.*, 1994a). As we had been unable to detect an association between cyclin D1 and Cdk4 or Cdk6 in Rb-negative cells (Figure 1), it was of interest to confirm that the direct binding of these components in the *in vitro* assay was not dependent on an interaction with Rb that might be present in the reticulocyte lysate. A mutant of cyclin D1 was therefore generated in which the putative Rb binding motif, LXCXE, was deleted. However, this five amino acid deletion had no effect on the association of cyclin D1 with either Cdk4 or Cdk6 (Figure 3d).

In contrast to the relative promiscuity of the D cyclins, p16 was found to associate exclusively with Cdk4 and Cdk6 (Figure 3e). There was no evidence for an association between p16 and any of the other kinases or with any of the D cyclins, irrespective of which protein was labelled (Figure 3e and additional data not shown). We also tested the effect of adding increasing amounts of unlabelled p16 to mixtures of cyclin D1 and either Cdk4 or Cdk6. To this end, histidine-tagged p16 was expressed in bacteria and purified by affinity chromatography on chelating agarose. Serial dilutions of the protein were added to the *in vitro* binding reactions and the mixtures precipitated with either cyclin D1 or p16 antiserum. Precipitation through p16 showed that the amount of Cdk4 in the

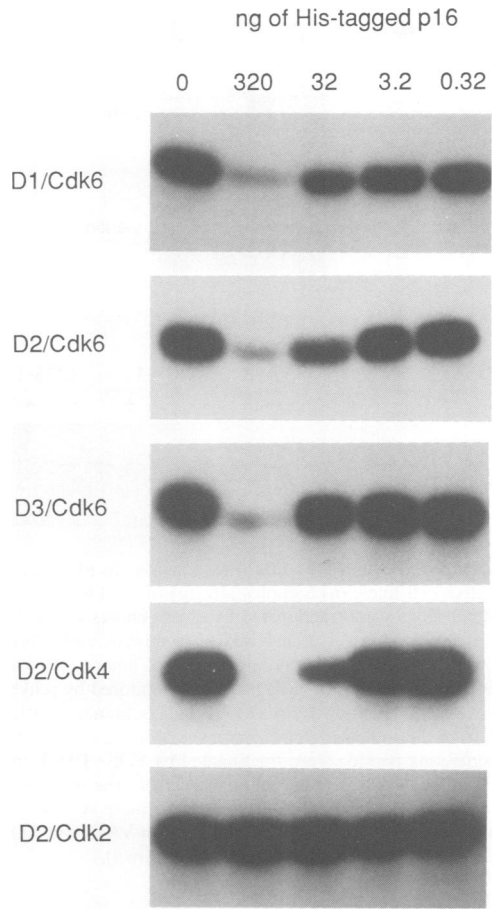

Fig. 5. Inhibition of cyclin D–Cdk6 kinase activity by p16. Extracts from baculovirus-infected Sf9 cells expressing the specified cyclin–Cdk combinations were pre-incubated with serial 10-fold dilutions of bacterially expressed p16 and then assayed for kinase activity towards GST–Rb as described (Kato *et al.*, 1993).

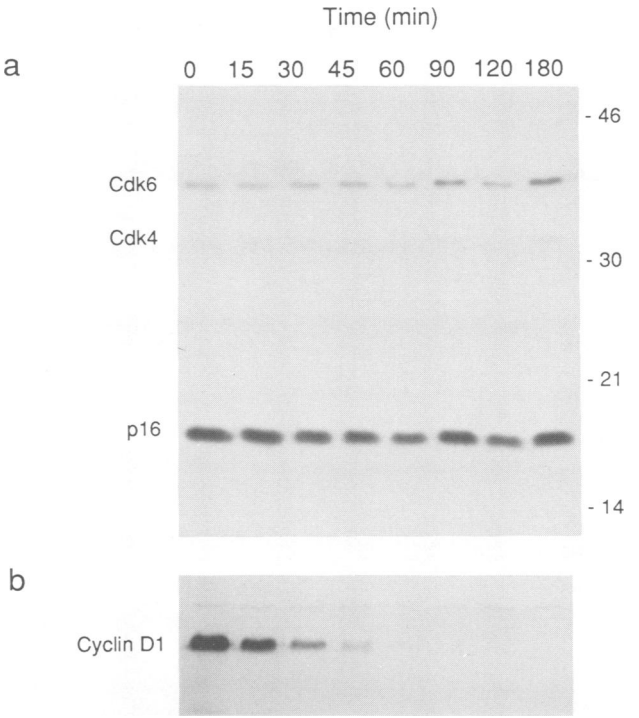

Fig. 6. Stability of p16, Cdk6 and cyclin D1 in Rb-negative cells. Logarithmically growing cultures of 5637 cells were pulse-labelled with [^{35}S]methionine and cysteine for 30 min and chased with medium containing unlabelled amino acids. At the indicated times, cell extracts were prepared and immunoprecipitated with either p16 antiserum (**a**) or cyclin D1 serum (**b**). The labelled proteins were fractionated by SDS–PAGE in a 12% gel, visualized by fluorography and the amounts of labelled p16, Cdk and cyclin D1 were quantitated by phosphorimaging.

immune complex was proportional to the amount of bacterially expressed p16 added (Figure 4a). Conversely, precipitation through cyclin D1 showed that the amount of Cdk4 associated with the cyclin decreased in proportion to the amount of added p16 (Figure 4a). Similar results were obtained with Cdk6 (Figure 4b). When the data were quantitated by phosphorimaging (Figure 4c and d), it was clear that the concentration of p16 required for half-maximal binding to Cdk4 and Cdk6 (~10 nM) was the same as the concentration that was required to compete half-maximally with cyclin D1 for binding to the kinases. This implies that p16 and cyclin D1 form independent binary complexes with Cdk4 and Cdk6.

Inhibition of Cdk4 and Cdk6 kinase activity by purified p16

To confirm that p16–Cdk6 complexes are inactive, we next asked whether the bacterially expressed p16 protein would inhibit the kinase activity associated with D cyclins and Cdk6. The most reliable assay for these kinases depends on co-expressing them with D cyclins in Sf9 insect cells, using baculovirus vectors. In this system, binary complexes of cyclins D1, D2 or D3 and either Cdk4 or Cdk6 are capable of phosphorylating a glutathione S-transferase (GST)–Rb fusion protein (Matsushime *et al.*, 1992; Ewen *et al.*, 1993; Kato *et al.*, 1993; Serrano *et al.*, 1993; Hannon and Beach, 1994; Meyerson and Harlow, 1994). Figure 5 confirms that the purified p16 protein inhibited the cyclin D2–Cdk4 complex as previously shown (Serrano *et al.*, 1993), but had no effect on cyclin D2–Cdk2 activity. As predicted, addition of p16 also inhibited the Rb kinase activity associated with complexes of Cdk6 and cyclins D1, D2 or D3. The low level of residual activity represents the endogenous phosphorylation observed using Sf9 cells infected with a virus expressing either a D cyclin or Cdk alone. Thus, p16 binds exclusively to Cdk4 and Cdk6 *in vivo* and *in vitro* and this binding correlates with inhibition of their kinase activity.

Stoichiometry of Cdk–p16 interactions in Rb-negative cells

The ability of p16 to compete with cyclins for binding to Cdk4 and Cdk6 *in vitro* was consistent with its suggested role in blocking the formation of these complexes in Rb-negative cells. However, for such an explanation to be tenable, it was important to consider the stability and stoichiometry of the various components in whole cells. A pulse-chase experiment was therefore performed to compare the half-lives of p16, Cdk4, Cdk6 and cyclin D1. We have previously reported that in Rb-negative cells, where cyclin D1 is not in a complex with Cdks, its half-life is of the order of 12 min (Bates *et al.*, 1994b). This

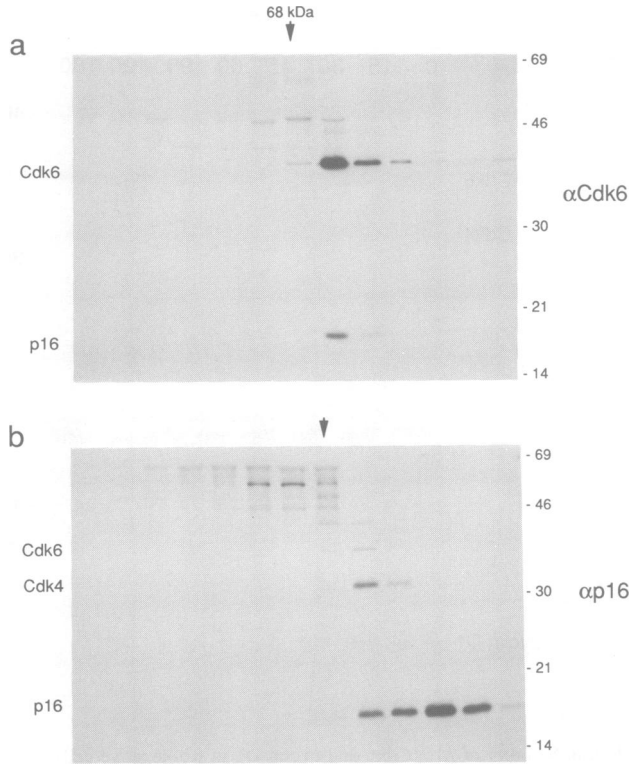

Fig. 7. Excess of p16 over Cdk6 in Rb-negative cells. Logarithmically growing 5637 cells were labelled for 4 h with [^{35}S]methionine and the resultant cell lysate was fractionated by centrifugation in a 15–30% glycerol gradient. Alternate samples were immunoprecipitated with antisera against (**a**) Cdk6 or (**b**) p16. The immune complexes were analysed by SDS–PAGE in a 12% gel and labelled proteins visualized by fluorography. Sedimentation was from right to left and the arrow shows the position of a size standard (BSA) analysed in parallel. The sizes and location of electrophoretic markers are shown on the right and the positions of Cdk4, Cdk6 and p16 are indicated on the left.

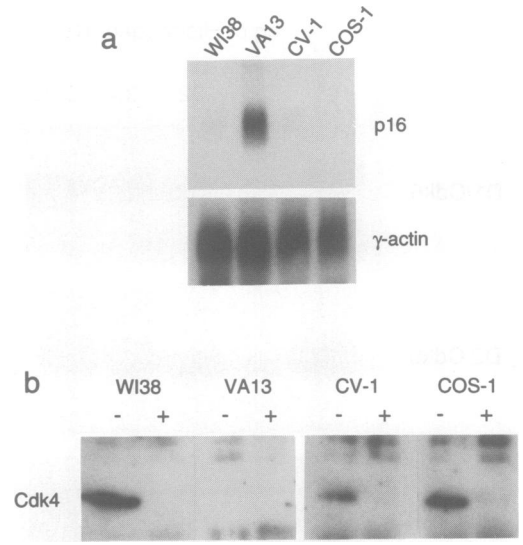

Fig. 8. Changes in cyclin D1–Cdk 4 associations in p16-positive and -negative cell lines. (**a**) Samples (10 μg) of total RNA from the indicated cell lines were fractionated by electrophoresis in a formaldehyde–agarose gel, transferred to membrane and hybridized with a probe representing the coding domain of human p16 (Serrano *et al.*, 1993). The loading in each lane was monitored by rehybridizing the filter with a probe for γ-actin. (**b**) Cyclin D1 immunoprecipitates prepared from the indicated cell lines in the absence (−) or presence (+) of competing peptide were fractionated by SDS–PAGE in a 10% gel and immunoblotted with an antisera to Cdk4. The immune complexes were visualized with ^{125}I-labelled protein A and autoradiography. The presence of SV40 T-Ag in VA13 and COS-1 cells was confirmed by immunoblotting (not shown).

is confirmed in Figure 6, which further shows that in the time taken for the label to be chased from cyclin D1, there was no discernible change in the labelling of p16 or the Cdks. Although this experiment did not provide an exact figure, the half-life for p16 is clearly in excess of 3 h.

To address the stoichiometry of the complexes in Rb-negative cells, a lysate from metabolically labelled 5637 cells was subjected to sedimentation in a glycerol gradient and the fractions were precipitated with antisera against Cdk6 or p16. Precipitation through the kinase indicated that almost all the labelled Cdk6 was present in an ~1:1 complex with p16, that sedimented at a position consistent with the sum of their molecular weights (Figure 7a). In the reciprocal experiment, precipitation through p16 revealed an excess of free p16 near the top of the gradient, with the remainder in more rapidly sedimenting complexes with Cdk4 and Cdk6 (Figure 7b). These data indicate that in Rb-negative cells, p16 is present in molar excess over the kinases and that virtually all of the Cdk6 (and presumably Cdk4) is sequestered in an inactive complex with p16.

Loss of cyclin D–Cdk interactions in Rb-deficient cells is p16 dependent

If the disruption of cyclin D–Cdk complexes in Rb-negative cells is attributable to an excess of p16, we would predict that viral oncoproteins that interfere with the function of Rb should have no effect on these complexes in cells that lack p16. To test this prediction, we compared the effects of SV40 T-Ag in two well-characterized pairs of cells, WI38/VA13 and CV-1/COS-1, that differ in their ability to express p16. As described in Table I, WI38 human diploid fibroblasts express low levels of p16 RNA, but these levels are markedly increased in the SV40-transformed derivative line VA13 (Figure 8a). In contrast, there is no up-regulation of p16 RNA in COS-1 cells, a T-Ag-transformed derivative of the CV-1 line of African green monkey kidney cells (Figure 8a). We have not established why p16 is absent from the latter cells, since at least one copy of the DNA is retained, but we have also failed to detect the protein by immunoblotting or by immunoprecipitation of metabolically labelled lysates (data not shown). Significantly, the presence or absence of SV40 T-Ag in COS-1 and CV-1 cells had no discernible effect on the co-precipitation of Cdk4 with cyclin D1, whereas T-Ag completely disrupted such interactions in VA13 cells (Figure 8b). Similarly, cyclin D–Cdk interactions were found to be unchanged upon expression of HPV E7 in p16-negative MCF-7 cells (Hickman *et al.*, 1994; data not shown). Thus, perturbation of Rb function in p16-negative cells had no discernible effect on cyclin D1–Cdk4 interactions.

Discussion

Cdk4 and Cdk6 form a distinct subset of the cyclin-dependent kinases based on sequence conservation (the

proteins are ~70% identical) and their exclusive associations with the D cyclins (Bates *et al.*, 1994a). Although the expression of Cdk6 may be less widespread than Cdk4, it is clear that many cell types contain significant amounts of both proteins (Bates *et al.*, 1994a,b; Meyerson and Harlow, 1994; Tam *et al.*, 1994). Similarly, most cell types we have examined express more than one of the D-type cyclins, resulting in multiple binary complexes within the same cell (Bates *et al.*, 1994b). In the light of these observations, we considered it essential to confirm that the p16 tumour suppressor gene product (Serrano *et al.*, 1993), widely reported as an exclusive inhibitor of Cdk4, would interact with and inhibit the related kinase Cdk6. The co-immunoprecipitation of p16 and Cdk6 from labelled cell lysates (Figures 1 and 7), the *in vitro* binding and competition data (Figures 3 and 4) and the kinase inhibition asssays (Figure 5) demonstrate convincingly that p16 acts equally as well on Cdk6 as on Cdk4. Similar findings have recently been reported by Hannon and Beach (1994).

Since p16 therefore inhibits all six of the possible complexes between the D cyclins and Cdk4 and Cdk6, it is interesting to consider whether the multiple complexes have similar functions or have as yet unrecognized substrate specificities. For example, all six complexes are capable of phosphorylating Rb when the components are expressed in insect cells. Although the target sites on Rb have not been accurately defined, there are indications that they correspond to sites that are phosphorylated *in vivo* (Kato *et al.*, 1993). However, the notion that phosphorylation by cyclin D–Cdk complexes alone can inactivate Rb seems untenable, since other cyclin–Cdk combinations are equally if not more potent Rb kinases *in vitro* and when immunoprecipitated from cycling cells (Figure 5; Lees *et al.*, 1991; Lin *et al.*, 1991; Ewen *et al.*, 1993; Kato *et al.*, 1993; Meyerson and Harlow, 1994). We therefore favour the idea that the D cyclin–kinases are only partly responsible for phosphorylating Rb, perhaps resulting in conformational changes that make the protein more accessible to other kinases. While p16 would inhibit this initial phosphorylation, the data presented in Figure 5 and elsewhere (Serrano *et al.*, 1993) suggest that it has no effect on complexes between Cdk2 and cyclins E, D2 or D3, all of which phosphorylate Rb in insect cell lysates.

In relation to tumorigenesis, the loss of p16 function observed in tumours and tumour cell lines (Cairns *et al.*, 1994; Caldas *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Nobori *et al.*, 1994; Spruck *et al.*, 1994) would presumably lead to increased or constitutive D cyclin activity and would have the same effect as the overexpression of cyclin D1. Although this has not been fully explored, the inverse correlation between cyclin D1 amplification and Rb status noted in oesophageal tumours (Jiang *et al.*, 1993b) and the inverse correlation between Rb and p16 status reported here (Figure 2 and Table I) lend credence to the idea that perturbations in D cyclins, p16 and Rb are part of a common pathway to tumorigenesis. For example, there may be no selective advantage to sustain a p16 mutation in an Rb-deficient cell and vice versa.

Significantly, Rb-deficient cell lines express much higher levels of p16 RNA and protein than is observed in primary fibroblasts, suggesting that Rb may negatively regulate p16 expression (Figure 2). A definitive answer will require delineation of the p16 promoter and experiments along these lines are in progress. Whatever the mechanism, the elevated levels of p16 observed in Rb-deficient cells provide a tenable explanation for the absence of cyclin D–Cdk complexes in these cells (Bates *et al.*, 1994b). As the D cyclins turn over much more rapidly than p16 (Figure 6), it is conceivable that they are progressively replaced by the excess of stable p16 (Figure 7), rather than being displaced from pre-existing complexes. This would also explain why cyclin D1 function in G_1 is dispensible in cells lacking Rb, since no active Cdk4 or Cdk6 complexes could form in the presence of p16 (Lukas *et al.*, 1994; Tam *et al.*, 1994). Conversely, disruption of Rb function in a p16-negative cell line has no effect on the cyclin D–Cdk complexes (Figure 8), providing clear evidence for a feedback loop in which Rb regulates the proteins that initiate its phosphorylation. What remains unclear is how the relative levels of p16, D cyclins and their Cdk partners are balanced in normally cycling cells.

Materials and methods

Antisera

We have previously described the preparation and characterization of antisera against synthetic peptides representing the C-terminal regions of cyclin D1, Cdk4 and Cdk6 (Bates *et al.*, 1994a). A similar procedure was used to generate antisera specific for p16, using CHARIDAA-EGPSDIPD as the immunizing peptide. An additional antiserum against the C-terminus of human p16 was generously provided by Dr Margaret Frame (CRC Beatson Insititute, Glasgow, UK).

Immunoprecipitation and immunoblotting of cell proteins

The origins of the cell lines used in this study and the relevant culture conditions have been reported in detail elsewhere (Gluzman, 1981; Xiong *et al.*, 1993; Bates *et al.*, 1994b; Hickman *et al.*, 1994). Cells were metabolically labelled with [^{35}S]methionine (60 μCi/ml) for 30 min to favour labelling of cyclins or for 4 h to label the kinases and p16 (Bates *et al.*, 1994a). For immunoprecipitation, cell extracts were prepared from ~10^7 cells by lysis in 1 ml ice-cold NP40 buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin). The lysates were centrifuged to remove debris and 900 μl of the supernatant mixed with 5 μl of antiserum on ice for 60 min. Where appropriate, the anti-peptide sera were pre-incubated for 60 min at 37°C in the presence or absence of cognate peptide as described (Bates *et al.*, 1994a). Immune complexes were collected on protein A–Sepharose beads (Pierce) and washed four times in 1 ml of NP40 lysis buffer and once in 50 mM Tris–HCl, pH 7.5. The beads were resuspended in 20 μl of 2× sample buffer and boiled for 5 min.

Protein samples were fractionated by electrophoresis in 10 or 12% SDS–polyacrylamide gels according to standard protocols (Harlow and Lane, 1988). Coloured ^{14}C-labelled protein markers (Amersham) were included in each gel as molecular weight standards. For immunoblotting, the proteins were transferred to Immobilon P membranes (Millipore) under semi-dry conditions and detected with secondary antibodies as previously described (Goldfarb *et al.*, 1991). The polyclonal rabbit antisera were used at a 1:500 dilution in blocking buffer [25 mM Tris–HCl, pH 8.0, 0.14 M NaCl, 0.2% Tween, 3% bovine serum albumin (BSA)]. After a 1 h incubation at room temperature (or overnight at 4°C), immune complexes were detected using a 0.1 μCi/ml solution of ^{125}I-labelled protein A (Amersham) in blocking buffer for 60 min at room temperature. With [^{35}S]methionine-labelled samples, the gels were directly fixed for 30 min in acetic acid–methanol, treated with Enlightning (NEN) for 30 min and dried on 3MM paper. Autradiography was performed with Kodak XAR5 film at −70°C.

Purification of bacterially expressed p16

The p16 coding sequences were cloned into the pRSET-A vector (Invitrogen) for expression in bacteria. The recombinant proteins were

expressed in *Escherichia coli* BL21(DE3)pLysS and recovered from insoluble inclusion bodies by solubilization in 6 M urea and affinity chromatography on chelating Sepharose (Pharmacia), using the buffers and conditions recommended by the supplier. The purified proteins were dialysed to remove urea and concentrated by ultrafiltration. The protein concentration (160 μg/ml) was determined using the Pierce BCA protein assay kit and its purity assessed by SDS–PAGE and staining with PAGE Blue-83. Histidine-tagged p16 migrated with an apparent molecular weight of 20–21 kDa.

Construction of mutant cyclin D1

A mutant form of human cyclin D1 was constructed by using a synthetic oligonucleotide spanning the N-terminal 16 amino acids but lacking the codons for the LXCXE motif. This oligonucleotide was used as the 5′ primer for amplification of cyclin D1 coding sequence from a wild-type cDNA clone using the polymerase chain reaction. The 3′ primer spanned the translational stop codon and unique restriction sites were introduced at each end to facilitate cloning into Bluescript KS^+.

In vitro binding assays

The coding sequences for human Cdks 1–6 were transferred into the Bluescript KS^+ plasmid vector to permit transcription with T7 RNA polymerase. [^{35}S]Methionine-labelled proteins were then synthesized by coupled transcription and translation of plasmid DNA using the TNT expression system (Promega). Similar conditions were used to express unlabelled cyclin A, cyclin D1 and p16. Samples (2 μl) of each reaction were mixed and incubated at 30°C for 30 min. In some experiments, 2 μl of bacterially expressed p16 (and 10-fold serial dilutions in 25 mM Tris–HCl, pH 8.0, 0.14 M NaCl) were added to the reaction mixtures prior to incubation. The volume was adjusted to 1 ml using ice-cold 500 mM NaCl, 1% NP40, 3% BSA and the mixture centrifuged at 14 000 r.p.m. for 10 min at 4°C. The supernatant (950 μl) was then precipitated with 5 μl of antiserum against the unlabelled component and the immune complexes collected with protein A–Sepharose. The labelled proteins were then analysed by SDS–PAGE in 10 or 12% gels and visualized by autoradiography.

Glycerol gradient centrifugation

Metabolically labelled 5637 cells were lysed in a minimal volume (200 μl) of lysis buffer without scraping, centrifuged briefly in a microfuge to remove cellular debris and the supernatant was loaded directly onto a 15–30% v/v glycerol gradient prepared in lysis buffer without NP40 (Zhang *et al.*, 1993). The gradient (total volume 12.5 ml) was centrifuged at 40 000 r.p.m. for 36 h in a Beckman SW40.1 rotor at 4°C. The gradient fractions (300 μl) were diluted to 1.0 ml in lysis buffer (adjusted to a final NP40 concentration of 1%) and immunoprecipitated as described above. Separate fractions were precipitated via p16 and Cdk6 and analysed by SDS–PAGE. BSA loaded on a parallel gradient was detected by SDS–PAGE and staining with PAGE Blue-83 dye in acetic acid–methanol.

RNA and DNA analyses

Total RNA was prepared from ~10^8 asynchronously growing cells by extraction with RNAzol (Cinna/Biotecx Laboratories) and ethanol precipitation. The RNA was quantitated by its absorbance at 260 nm and 10 μg samples were denatured and fractionated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. After transfer to Hybond N+ membranes, the RNAs were hybridized to a genomic DNA fragment containing the first exon of the p16 gene, ^{32}P-labelled by random priming. Hybridization was performed at 65°C in a buffer containing 0.4 M sodium phosphate, pH 7, 7% SDS, 15% formamide and 1 mg/ml BSA. Filters were washed in 40 mM sodium phosphate, 1% SDS at 65°C and exposed to Kodak XAR5 film. Southern blot analysis on cell line DNA was performed as described (Fantl *et al.*, 1990).

Kinase inhibition assays

Sf9 insect cells were co-infected with the appropriate recombinant baculoviruses and whole cell extracts prepared 48 h post-infection as described by Kato *et al.* (1993). Extracts (5 μl) were pre-incubated with an equal volume of histidine-tagged p16 (serial 10-fold dilutions) for 10 min at 30°C. Phosphorylation reactions (40 μl final) were performed in 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA and incubated with 500 ng GST–Rb bound to glutathione–Sepharose beads, 100 μM ATP and 5 μCi [γ-^{32}P]ATP. After 5 min at 30°C, reactions were terminated by the addition of 500 μl ice-cold NETN (20 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40). The glutathione–Sepharose beads were recovered by centrifugation, boiled in dissociation buffer and analysed by SDS–PAGE in a 10% gel.

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