



Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein

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The proteins of the retinoblastoma family are potent inhibitors of cell cycle progression. It is well documented that their growth-inhibitory activity can be abolished by phosphorylation on serine and threonine residues by cyclin dependent kinases. In contrast, very little is known about the dephosphorylation of retinoblastoma-family proteins. We report here the isolation, by virtue of its ability to associate with p107, of a novel Protein Phosphatase 2A (PP2A) regulatory subunit, named PR59. PR59 shares sequence homology with a known regulatory subunit of PP2A, PR72, but differs from PR72 in its expression pattern and its functional properties. We show that PR59 co-immunoprecipitates with the PP2A catalytic subunit, indicating that PR59 is a genuine component of PP2A holo-enzymes. *In vivo*, PR59 associates specifically with p107, but not with pRb. Elevated expression of PR59 results in dephosphorylation of p107, but not of pRb, and inhibits cell proliferation by causing cells to accumulate in G1. These data support a model in which the distinct PP2A regulatory subunits act to target the PP2A catalytic subunit to specific substrates and suggest a role for PP2A in regulation of p107.

Keywords: cell cycle; p107; protein phosphatase 2A; Retinoblastoma protein

Introduction

Orderly progression through the cell division cycle requires the coordinate expression of groups of genes that are responsible for the various biochemical processes that take place during cell division. The E2F transcription factor family plays an important part in cell cycle-regulated gene expression as it activates a number of genes that are required for DNA synthesis (Beijersbergen and Bernards, 1996; Bernards, 1997). The E2F transcription factor, in turn, is regulated by the retinoblastoma family of growth-inhibitory proteins. This family consists of three members: the retinoblastoma protein (pRb) and the related p107 and p130 (Hannon *et al.*, 1993; Li *et al.*, 1993b; Zhu *et al.*, 1993; Weinberg, 1995). Collectively, these proteins are known as the ‘pocket’ proteins, as they have a domain, named the pocket, with which they can bind and inactivate several cellular proteins. The ability of the pocket proteins to bind proteins such as E2F is abolished through cell cycle-

regulated phosphorylation by cyclin-dependent kinases (cdks). In the G1 stage of the cell cycle the retinoblastoma protein is hypophosphorylated. At the G1- to S phase transition, pRb receives additional phosphates and phosphorylation increases even further as cells progress through S and G2. Not only pRb, but also p107 can be phosphorylated and thereby inactivated as a growth suppressor by the cyclin D1/cdk4 kinase complex. In addition, pRb, but not p107, is thought to be a substrate for cyclin A/cdk2 and cyclin E/cdk2 (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Beijersbergen *et al.*, 1995). It is somewhat enigmatic that even though p107 and p130 do not appear to be phosphorylated by cyclin A/cdk2 and cyclin E/cdk2, both pocket proteins can form stable complexes with cyclin A/cdk2 and E/cdk2 through a domain within the pocket called the spacer (Lees *et al.*, 1992; Shirodkar *et al.*, 1992; Li *et al.*, 1993a; Zhu *et al.*, 1995).

In contrast, little is known about the way in which phosphates are removed from pocket proteins during the cell cycle. The retinoblastoma protein has been shown to interact directly with the protein phosphatase 1 (PP1) catalytic subunit during the M phase of the cell cycle, when pRb is known to be dephosphorylated (Durfee *et al.*, 1993; Nelson and Ludlow, 1997). Moreover, a high molecular weight form of PP1 found in mitotic cell extracts was shown to be able to dephosphorylate pRb *in vitro* (Nelson *et al.*, 1997). Also, pRb immunoprecipitates from cells which have accumulated DNA damage as a result of exposure to anti cancer drugs contain a phosphatase activity that can dephosphorylate pRb *in vitro* and it is likely that PP1 is involved in this dephosphorylation of pRb in response to DNA damage (Dou *et al.*, 1995).

Besides PP1, other major cellular phosphatases exist that could potentially contribute to dephosphorylation of cell cycle control proteins. For example, Protein Phosphatase 2A (PP2A) is a major intracellular serine/threonine phosphatase that, apart from being involved in cell cycle regulation, plays a role in diverse cellular processes such as DNA-replication, intermediary metabolism, transcription and signal transduction (Hubbard and Cohen, 1993; Mumby and Walter, 1993; Virshup *et al.*, 1993; Mayer-Jackel and Hemmings, 1994). PP2A consists of a catalytic subunit (PP2Ac) (Cohen *et al.*, 1990) and a structural 65 kDa subunit (PR65) (Hemmings *et al.*, 1990). These two proteins form a core dimer (Hendrix *et al.*, 1993b) to which various other regulatory ‘B’ subunits associate. To date, three families of genes encoding regulatory B subunits have been cloned. The most prevalent isoform, called PR55, B55, or simply B subunit, is encoded by at least three related genes (Mayer *et al.*,

1991; Pallas *et al.*, 1992). Five genes have been cloned that fall into one closely related gene family of PR61 subunits (also called B56, or B' subunit) (McCright and Virshup, 1995; Tehrani *et al.*, 1996; Zolnierowicz *et al.*, 1996). One gene that gives rise to two differentially spliced mRNAs encodes two B-subunits, PR72 (also called B'') and PR130 (Hendrix *et al.*, 1993a). It is widely believed that the multitude of B subunits plays a role in regulation of phosphatase activity, control of substrate specificity and targeting to certain cellular compartments of the PP2A core dimer. That substrate specificity is indeed conferred by the subunit composition of PP2A is demonstrated by the finding that PR72-containing PP2A holo-enzyme purified from rabbit skeletal muscle *in vitro* preferentially dephosphorylates SV40 large T antigen phosphorylated by Casein Kinase I, whereas the PP2A core dimer, or PR55 containing holo-enzyme, preferentially dephosphorylates SV40 large T antigen on a site phosphorylated by recombinant cdc2 kinase (Cegielska *et al.*, 1994). The diversity of activities of the PP2A holo-enzyme is thus reflected in a diversity of possible trimeric PP2A complexes.

The notion that PP2A plays a role in cell cycle regulation is supported by several observations. First, okadaic acid, a strong inhibitor of PP2A, is a tumor promoting agent (Mumby and Walter, 1993). Furthermore, PP2A is a target of transforming proteins of several DNA tumor viruses (Sontag *et al.*, 1993). SV40 small t antigen and polyoma virus small t and middle T antigens, which have a helper function in transformation, can replace certain B subunits in PP2A complexes. As a result, SV40 small t antigen inhibits the PP2A phosphatase activity towards signal transduction kinases MEK and ERK (Sontag *et al.*, 1993). That there is specificity in this interference with the activity of PP2A holo-enzymes is illustrated by the finding that the variable subunit in PP2A trimeric complexes purified from bovine heart is not replaced by SV40 small t (Kamibayashi and Mumby, 1995). Finally, a complex of HIV-I encoded proteins, NCp7 and vpr, is implicated in direct activation of PP2A and subsequent cell cycle arrest at the G2 to M transition (Tung *et al.*, 1997).

Although it is generally believed that the B subunits control the activity of the core PP2A dimer (Kamibayashi *et al.*, 1994; Mayer-Jaekel and Hemmings, 1994), very few substrates are known with which the regulatory B-subunits associate directly *in vivo* and that are subject to dephosphorylation by PP2A. An isoform of the B' (B56, PR56) PP2A regulatory subunits (B' α 1) has been shown to form stable complexes *in vivo* with a cellular protein, cyclin G, but it remains unclear whether this B subunit-bound cyclin actually serves as a substrate for PP2A (Okamoto *et al.*, 1996).

In this paper we describe the cloning of a new PP2A component, PR59, which shares sequence homology with a known regulatory B subunit. By showing that PR59 associates with p107 which results in a subsequent dephosphorylation of p107, our data for the first time provide experimental evidence in support of a model in which PP2A regulatory subunits function, at least in part, by specifically recruiting the catalytic subunit to distinct cellular substrates.

Results

Identification of clones that interact with p107

To identify proteins that interact with the retinoblastoma protein-related p107, we performed a yeast two-hybrid screen with the pocket domain of human p107 fused to the DNA binding domain of the yeast transcription factor GAL4 as previously described (Hijmans *et al.*, 1995). Partial sequence analysis of two positive clones indicated that they shared significant sequence homology with human PR72, a regulatory subunit of protein phosphatase 2A (PP2A) (Hendrix *et al.*, 1993a). Upon further sequence analysis, both clones (pp44 and pp31) were found to be derived from the same gene and contained an open reading frame of 603 nucleotides and 381 nucleotides, respectively. The partial mouse cDNA pp44 was then used as a probe to screen a mouse PCC4 teratocarcinoma cDNA library, which yielded a 1.9 kilo base pair cDNA with an open reading frame of 1692 base pairs (Figure 1a).

Two potential AUG initiator codons are present at the 5' end of the ORF in the PR59 cDNA: one at nucleotide -126, the other at nucleotide +1 of the cDNA sequence. Only the second AUG matches well the consensus start of translation as defined by Kozak (1992). There are no in-frame stop codons in the cDNA upstream of these putative AUG initiator codons. To ask which of the two possible AUG codons is used as a start of translation, we transcribed both the full length cDNA and a 5' truncated cDNA that lacks the first 189 base pairs (including the first AUG) *in vitro* into RNA and translated the RNAs in a rabbit reticulocyte lysate. Both the full length RNA and the 5' truncated RNA generated a protein of the same apparent molecular weight on SDS-PAGE (data not shown). This indicates that most likely the initiator codon at nt +1 is used as a start of translation. Following the nomenclature used for its homologue PR72 (Hendrix *et al.*, 1993a), we named this novel protein PR59.

PR59 is homologous to PR72

The amino acid comparison between PR59 and PR72 is shown in Figure 1b. PR59 shares 56% identity and 65% similarity with PR72. PR72 and PR130 are splice variants from the same gene, that differ in their N-terminal sequences. The homology between PR59 and PR72 extends into the diverged N-terminus of PR72, which suggests that PR59 is a homologue of PR72 rather than of PR130. The region of highest homology between PR59 and PR72 is evolutionary very conserved, as it is also found in *D. melanogaster* (DM25H1T), *T. Brucii* (W99281), *C. elegans* (U41014), *A. thaliana* (AB005239) and in *O. sativa* (RICC10055A), as determined by BLAST search of various databases (accession number of clone with highest homology given in brackets).

mRNA expression of PR59

To assess the expression of PR59, 2 μ g of poly(A)⁺ RNA from a panel of murine tissues was hybridized at high stringency with a probe derived from the PR59 cDNA. Figure 2 shows that there are multiple

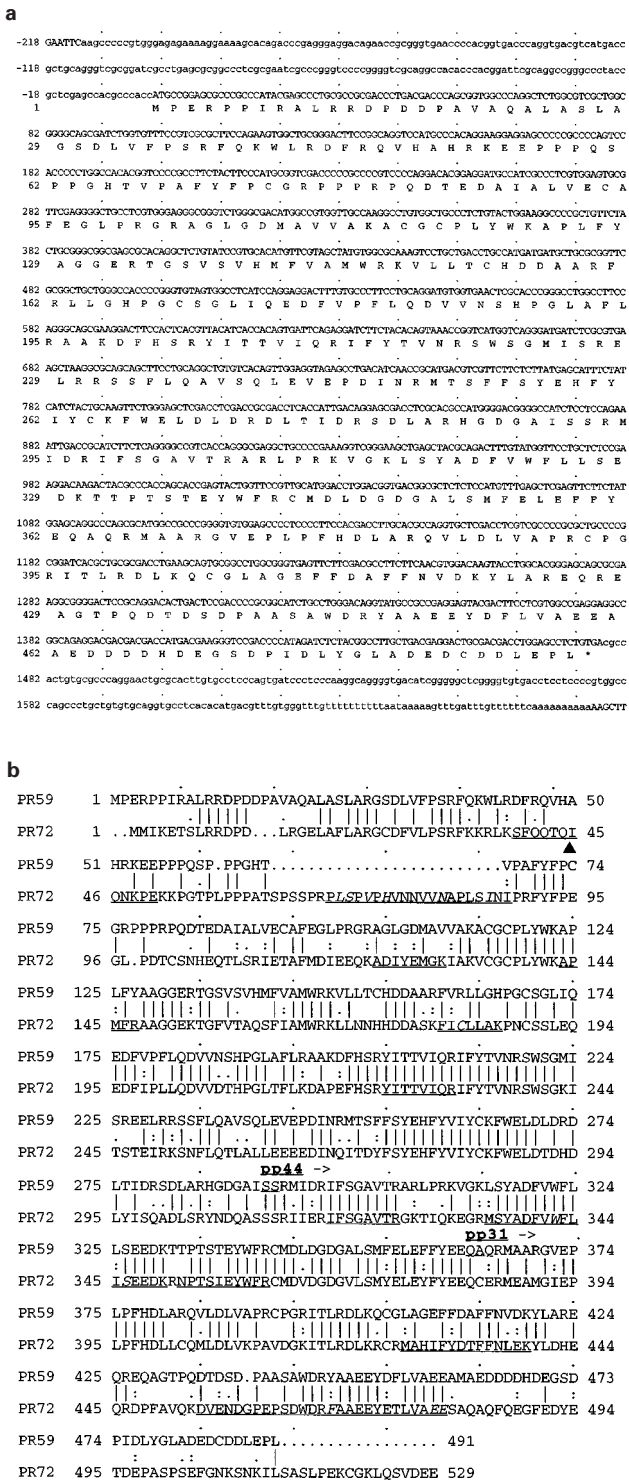


Figure 1 Nucleotide and predicted amino acid sequence, and alignment to human PR72 of the mouse cDNA encoding PR59. (a) The mouse PR59 cDNA sequence. The nucleotides are numbered starting with the first nucleotide of the initiator codon, and nucleotides extending in the 5'-non-coding region are designated with negative numbers. The predicted protein sequence starts at the second methionine in the ORF that extends from the 5' end of the clone to nt 1472. Nt -218 to -213 and 1575 to 1580 represent respectively *EcoRI* and *HindIII* restriction sites from the cloning vector. (b) The predicted PR59 protein was aligned to PR72 using the gap program in gcg (Genetics Computer Group, 1994). Underlined in PR59 are the amino acids at which the two clones start that were originally isolated in the two hybrid screen (pp44 and pp31). Underlined in PR72 are residues that are identical to rabbit PR72 peptide sequences (Hendrix *et al.*, 1993a), underlined in italics are amino acids that correspond to rabbit peptides, but are not identical. The triangle denotes the point of N-terminal divergence between PR72 and PR130

transcripts, of approximately 1.5, 2 and 8 kb, the relative abundance of which differs in the various tissues. The 2 kb transcript correlates well with the size of our 1.9 kb *PR59* cDNA clone, indicating that this clone is full-length or very close to being full length. The larger *PR59* transcripts on the Northern blot suggest that there may be PR130-like splice variants of *PR59*. The finding that this probe did not detect a signal in skeletal muscle indicates that this probe does not react with the related *PR72* as this gene is highly expressed in this tissue (Hendrix *et al.*, 1993a).

PR59 associates with PP2A activity

To determine whether *PR59* is a *bona fide* PP2A regulatory subunit that can associate with a functional PP2A catalytic subunit, we first generated several reagents. By repeated immunization of a rabbit with a GST-*PR59* fusion protein, a polyclonal antiserum was generated that specifically reacted with *PR59* in both immunoprecipitation and Western blotting experiments. This antibody did not cross react with *in vitro* translated *PR72* in immunoprecipitation experiments, nor with transfected *PR72* in Western blot experiments (data not shown). Furthermore, we made expression vectors in which the coding sequence of *PR59*, *PR65*, *PR72* or the PP2A catalytic subunit (PP2Ac) was fused to a 10 amino acid epitope (HA) that is recognized by the monoclonal antibody 12CA5. Using these reagents, we tested whether *PR59* associates with PP2A phosphatase activity *in vivo*. For this purpose, an immunoprecipitation-phosphatase assay was used in which PP2A catalytic subunit activity was measured through the release of $^{32}\text{PO}_4$

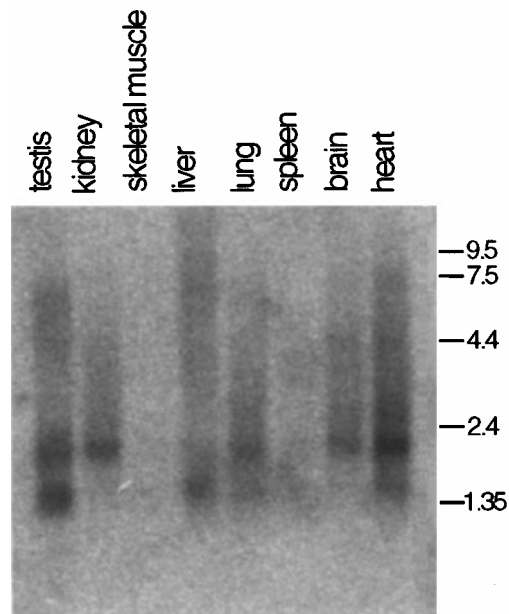


Figure 2 *PR59* mRNA expression in mouse tissues. A mouse tissue blot (Clontech) loaded with 2 μg of poly(A)⁺ RNA from the indicated tissues was hybridized with a ^{32}P labeled partial *PR59* cDNA. Hybridization and washing were performed as described in the manufacturers instructions. After a final wash at 65°C in 0.1 \times SSC the blot was exposed for 2 days on a phosphorimager screen

from a peptide containing a phosphorylated PP2A consensus site. U2-OS human osteosarcoma cells were transfected with the HA-tagged PR59 (HA-PR59), PR65 (HA-PR65), PR72 (HA-PR72) or PP2A catalytic subunit (HA-PP2Ac) expression vectors, and mild

detergent 12CA5 immunoprecipitates of transfected cells were tested for phosphatase activity in the assay described above. Figure 3a shows that, as expected, a 12CA5 immunoprecipitate of HA-tagged PP2Ac-transfected cells contained significant amounts of

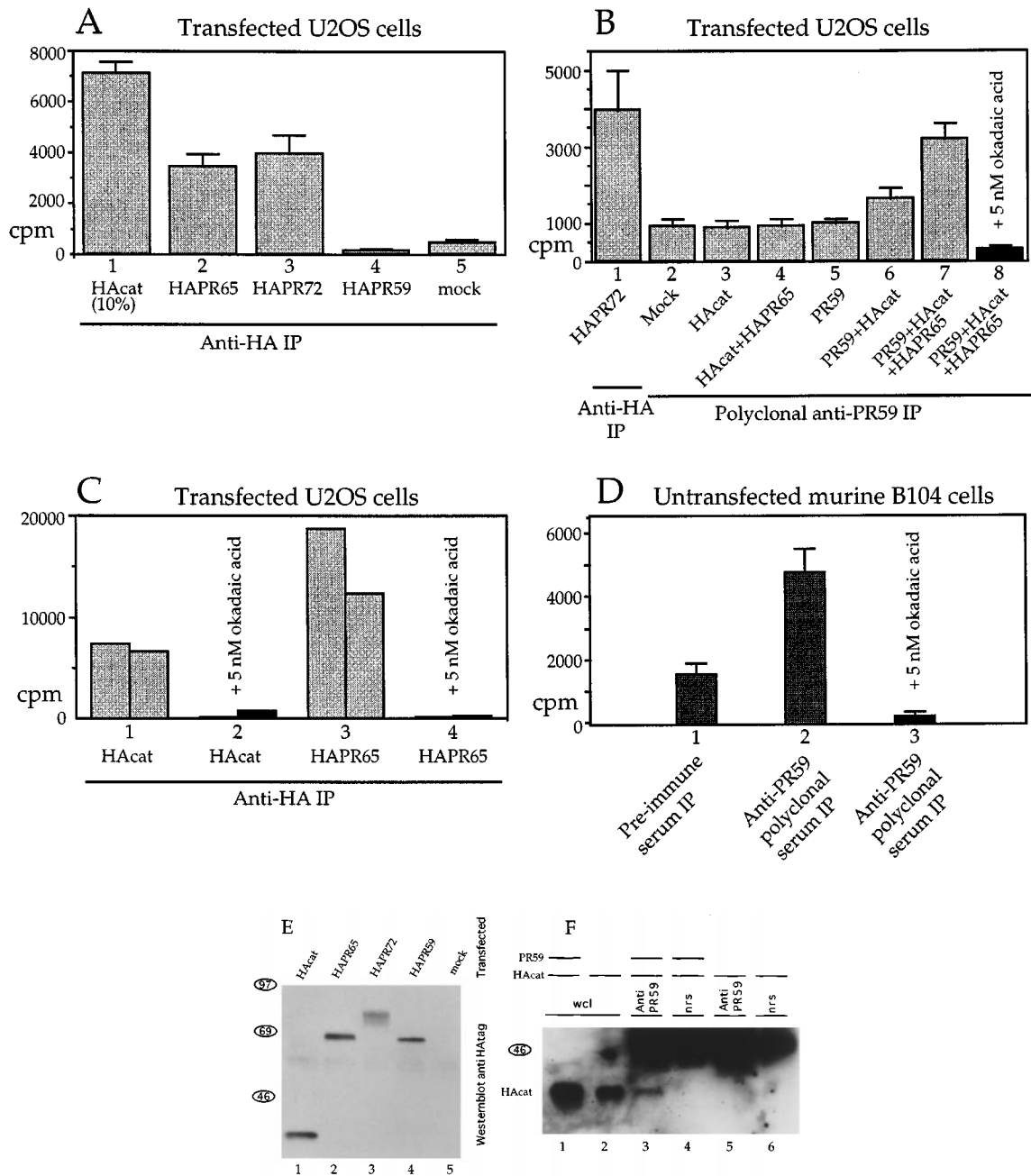


Figure 3 PR59 immunoprecipitates contain PP2A activity. (a) U2-OS cells were transfected with expression vectors directing the expression of the indicated proteins (HA-cat is HA-tagged PP2Ac) (lanes 1 and 2: 10 μ g expression vector, lanes 3 and 4, 20 μ g expression vector), lysed, and an anti-HA tag immunoprecipitate was assayed in triplicate for phosphatase activity measured as a release of 32 P $_4$ from a phosphorylated substrate peptide (vertical axis is released c.p.m. minus released c.p.m. from peptide incubated with assay buffer alone). The average with standard error bars is shown. All phosphatase assays are representative of at least three independent experiments. For the phosphatase assay with the HA-cat lysate, only 10% of the amount used in the other assays was used because of the high activity of this lysate (labeled: '10%'). (b) As in a, U2-OS cells were transfected (HA-PR72 and PR59 10 μ g, HA-cat and HA-PR65 5 μ g each), and indicated immunoprecipitates were assayed for phosphatase activity. (c) As in a, the indicated proteins were expressed in U2-OS and phosphatase activity was measured in duplicate immunoprecipitates, with or without addition of okadaic acid to 5 nM final concentration. (d) Untransfected rat neuroblastoma B104 cells were lysed, and triplicate immunoprecipitates with the indicated sera were assayed for phosphatase activity as in b. (e): 25% of the immunoprecipitate from (a) was Western blotted with an anti-HA tag antibody and protein A-linked peroxidase second antibody. Markers on the left indicate molecular weight markers in kDa. (f) U2-OS cells were transfected with expression vectors for PR59 and HA-cat (10 μ g and 5 μ g respectively) (lanes 1, 3, 4) or HA-cat alone (5 μ g) (lanes 2, 5, 6), lysed and the lysates were split in two and immunoprecipitated with anti-PR59 polyclonal rabbit serum or normal rabbit serum (nrs). Whole cell lysates (wcl, 3% of the amount used in the immunoprecipitations) and the immunoprecipitates were Western blotted with anti-HA tag antibody. Marker on the left indicates molecular weight in kDa. The strong signal at the top of the gel in lanes 3–6 is due to Ig heavy chains

phosphatase activity. Similarly, the 12CA5 immunoprecipitate from HA-PR65 and HA-PR72 transfected cells contained substantial amounts of phosphatase activity, suggesting that the endogenous PP2A catalytic subunit can be immunoprecipitated via a PP2A regulatory subunit in this assay. To confirm that the phosphatase activity seen in these assays was indeed PP2A activity, okadaic acid was added to several of the immunoprecipitates to a final concentration of 5 nM. This concentration of okadaic acid will specifically inhibit PP2A, but not PP1 (Cohen *et al.*, 1989; Honkanan *et al.*, 1994). Figure 3c shows that the phosphatase activity in immunoprecipitates of the HA-PR65 and the HA-PP2Ac-transfected cells could be inhibited by this concentration of okadaic acid, strongly suggesting that the phosphatase activity seen was indeed PP2A.

Although all HA-tagged proteins were expressed and immunoprecipitated at comparable levels, as determined by 12CA5 Western blot analysis of an aliquot of the immunoprecipitate used for the phosphatase assay (Figure 3e), no significant phosphatase activity was found in the HA-PR59 immunoprecipitate. In contrast, when U2-OS cells were transfected with expression vectors encoding non-tagged PR59 in combination with HA-PP2Ac and HA-PR65 subunits, and PR59 was immunoprecipitated with polyclonal PR59 antiserum, a significant amount of phosphatase activity could be detected in complex with PR59 (Figure 3b, lane 7). Similar to the results obtained with the HA-tagged PR59, no significant phosphatase activity was seen when a non-tagged version of PR59 was transfected alone (Figure 3b, lane 5). This suggests that PR59 competes poorly with endogenous B subunits for binding to the PP2Ac/PR65 core dimer. This latter result also excludes the possibility that the lack of phosphatase activity in the N-terminally tagged PR59 is the result of interference by the HA tag with PP2A complex assembly. Indeed, no functional differences were observed between PR59 or HA-tagged PR59 in our experiments.

Next, we asked whether endogenous PR59 can be found associated with endogenous PP2A catalytic subunit. Untransfected rat neuroblastoma B104 cells were immunoprecipitated with polyclonal rabbit PR59 serum and tested for phosphatase activity. Figure 3d shows that the PR59 immunoprecipitate contained significant phosphatase activity as compared to the control immunoprecipitate with pre-immune rabbit serum. Furthermore, the PR59-associated phosphatase activity could be completely inhibited with 5 nM okadaic acid (Figure 3d). Together, this suggests that endogenous PR59 is associated with PP2A activity. However, we cannot formally exclude the possibility that a PR59-like protein cross-reacts with this antiserum.

To further investigate whether PR59 can associate with the PP2A catalytic subunit, cells were transfected with HA-PP2Ac and PR59 expression vectors. Lysates of transfected cells were immunoprecipitated with anti PR59 or control serum and analysed by Western blot for the presence of the catalytic subunit. Figure 3f shows that indeed PP2Ac is present in anti-PR59 immunoprecipitates from cells expressing PR59 and PP2Ac, but not in control immunoprecipitates, nor in anti-PR59 immunoprecipitates from cells not expressing exogenous PR59. Taken together, these data show

that the PR59 cDNA encodes a functional PP2A regulatory subunit with relatively low affinity for the PP2A core dimer.

PR59 binds preferentially to p107

To determine whether PR59 also interacts specifically with p107 in mammalian cells, we performed a co-immunoprecipitation experiment (Figure 4). U2-OS cells were co-transfected with an HA-PR59 expression vector and expression vectors for both human p107 and human pRb. As controls, expression vectors encoding PR59 without HA tag or HA-PR72 were co-transfected. Transfected cells were lysed in a non-ionic detergent buffer and lysates were immunoprecipitated with 12CA5 monoclonal antibody. Immunoprecipitated proteins were separated on a SDS-polyacrylamide gel and Western blotted with polyclonal antiserum C15 to detect pRb. The filter was subsequently stripped of antibody, and reprobed with polyclonal antiserum C18 to detect p107. 12CA5 Western blot showed that the HA-tagged proteins were immunoprecipitated efficiently and equally (data not shown). Figure 4 shows that p107 is present only in the HA-PR59 immunoprecipitate, but not in the control or HA-PR72 immunoprecipitates. No pRb was detected in the HA-PR59 immunoprecipitate, even though the expression of pRb and p107 was equivalent in the transfected cells. Consistent with these data, we found that in a yeast two hybrid assay PR59 interacted with p107 and not with pRb. Furthermore, in an *in vitro* GST pull down assay, PR59, but not PR72, associated specifically with GST-p107 (data not shown). Taken together, these data indicate that PR59 associates with p107, but not with pRb, in mammalian cells, whereas PR72 associates with neither of the two pocket proteins under these conditions.

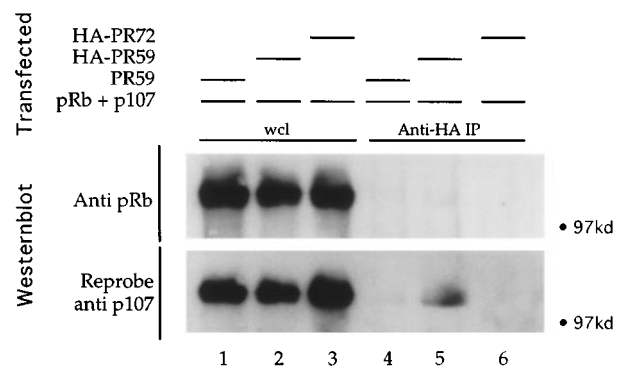


Figure 4 HA-PR59 associates preferentially with p107 in mammalian cells. U2-OS cells were transfected with the indicated expression vectors (10 μ g for PR59, HA-PR59 and HA-PR72, 5 μ g for pRb and p107). Three percent of the cell lysate was used to check expression of pRb or p107 (lanes 1, 2, 3). The remainder of the cell lysate was immunoprecipitated with anti-HA tag monoclonal 12CA5 and Western blotted (lanes 4, 5, 6). The presence of pRb in the immunoprecipitates was detected with a polyclonal rabbit antiserum against the C-terminal 15 amino acids of pRb (C15). After this, the blot was stripped of antibodies, and reprobed with a polyclonal antiserum against the C-terminal 18 amino acids of p107 (C18) to detect p107 in the immunoprecipitates. Both HA-tagged proteins were efficiently immunoprecipitated as determined using an anti-HA tag Western blot of the immunoprecipitates (data not shown)

p107 is dephosphorylated in vivo by PR59

Next, we determined whether association of PR59 to p107 also results in dephosphorylation of p107 *in vivo*. For this purpose, we again used U2-OS cells, which have high endogenous cyclin D1/cdk4 kinase activity towards pocket proteins and consequently hyperphosphorylate both endogenous and ectopically expressed p107 and pRb. U2-OS cells were transfected with expression vectors for HA-tagged p107 (to distinguish transfected from endogenous p107) and increasing amounts of PR59 expression vector (Figure 5). As a control, HA-p107 was co-transfected with a construct that directs the synthesis of a dominant negative form of cdk4 to generate hypophosphorylated p107. Figure 5 shows that expression of HA-p107 in U2-OS cells resulted in hyperphosphorylated p107 (lane 1), whereas co-expression of increasing amounts of PR59 resulted in increasing amounts of hypophosphorylated p107 (lanes 2, 3, 4; lane 5 shows the position in the gel of hypophosphorylated p107). HA-pRb is also hyperphosphorylated in U2-OS cells (lane 6), but no hypophosphorylated pRb appeared when PR59 was co-expressed with pRb (lanes 7, 8, 9), whereas co-expression of a dominant negative form of cdk4 did result in the appearance of hypophosphorylated pRb (lane 10). To establish that the difference seen here in dephosphorylation of p107 and pRb was not due to a difference in expression of PR59, the cell lysates were also analysed by Western blot with antiserum against PR59 (Figure 5, lower panel). These results show that co-expression of PR59 with p107 leads to an increase in the amount of hypophosphorylated p107, whereas overexpression of PR59 does not influence pRb phosphorylation status.

It may seem contradictory at first glance that expression of PR59 alone does not allow the detection of associated phosphatase activity (Figure 3), whereas it

is sufficient to cause dephosphorylation of p107 (Figure 5). The difference between these experiments is that the first experiment is an *in vitro* assay in which PR59 is subjected to immunoprecipitation, which may cause dissociation of labile trimeric phosphatase complexes. In the p107 dephosphorylation experiment however, PR59-complexes are assayed *in vivo* for activity towards a substrate.

Overexpression of PR59 results in inhibition of cell cycle progression

Expression of hypophosphorylated p107 inhibits progression through the cell cycle by causing cells to accumulate in G1 (Zhu *et al.*, 1993; Beijersbergen *et al.*, 1995). We therefore asked whether expression of PR59 caused an increase in the G1 population of transfected cells. To this end U2-OS cells, which contain p107 in an inactive, hyperphosphorylated form, were transfected with increasing amounts of PR59, or HA-tagged PR59 expression vector, and the cell cycle profile of the transfected cells was analysed by FACS. Expression of PR59 resulted in a marked increase in cells in G1 compared to control cells (Figure 6a–d).

To further investigate the effects of PR59 on cell proliferation, a colony formation assay was performed. Figure 6e shows that transfection of U2-OS cells with a puromycin-selectable marker and increasing amounts of PR59 expression vector, causes a marked reduction in the number of puromycin-resistant colonies. Taken together, these data indicate that elevated expression of PR59 causes a reduction of cell proliferation that results from an accumulation of cells in the G1 phase of the cell cycle.

Discussion

In this paper we describe a new Protein Phosphatase type 2A (PP2A) regulatory subunit, named PR59, which was cloned by virtue of its interaction with p107, a member of the retinoblastoma family of growth-inhibitory proteins. Several lines of evidence indicate that PR59 is a genuine PP2A regulatory subunit. First, PR59 is structurally highly related to a pair of human PP2A regulatory subunits, PR72 and PR130 (Figure 1b). Second, PR59 immunoprecipitates contain associated PP2A phosphatase activity. Third, in co-transfection experiments, PR59 co-immunoprecipitates with the PP2A catalytic subunit. Based on these observations, we conclude that PR59 is a new and functional PP2A subunit.

The structural similarity between PR59 and PR72 would predict that these proteins are functionally related. However, our data reveal significant differences between these proteins. For instance, the PR59 mRNA is expressed in kidney, heart, but not in skeletal muscle, whereas PR72 is expressed both in skeletal muscle and in heart (Hendrix *et al.*, 1993a). The divergence between PR59 and PR72 is most pronounced in the carboxyl terminus of PR59 (Figure 1b), the region that is required for interaction with p107. That PR72 differs in function from PR59 is supported by our finding that PR59, but not PR72, associates specifically with p107 *in vitro* and *in vivo*. Consistent with this difference only overexpression of PR59, but

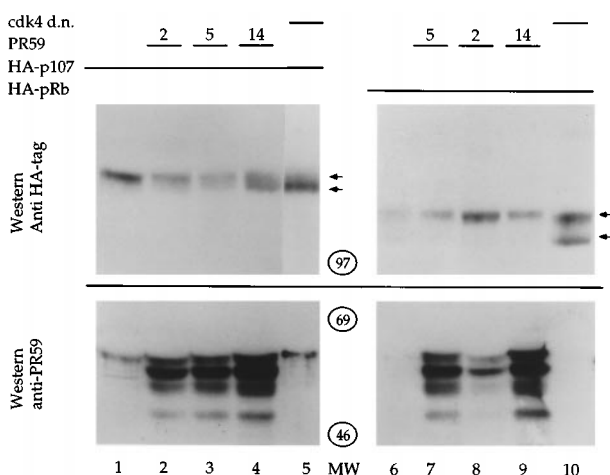


Figure 5 PR59 targets p107 for dephosphorylation *in vivo*. U2-OS cells were transfected with HA-p107 and HA-pRb expression vectors (3 and 6 μ g respectively), and expression vectors for PR59 (lanes 2, 3, 4, 7, 8, 9 with indicated amounts in μ g) or cdk4 d.n. (10 μ g) were co-transfected. Whole cell lysates were separated on a SDS-PAGE gel (7% PAA for HA-p107 and HA-pRb, 10% PAA for PR59) and Western blotted with the indicated antisera. MW indicates molecular weight in kDa, arrows indicate the positions of hypophosphorylated (lower arrows) and hyperphosphorylated (upper arrows) p107 and pRb

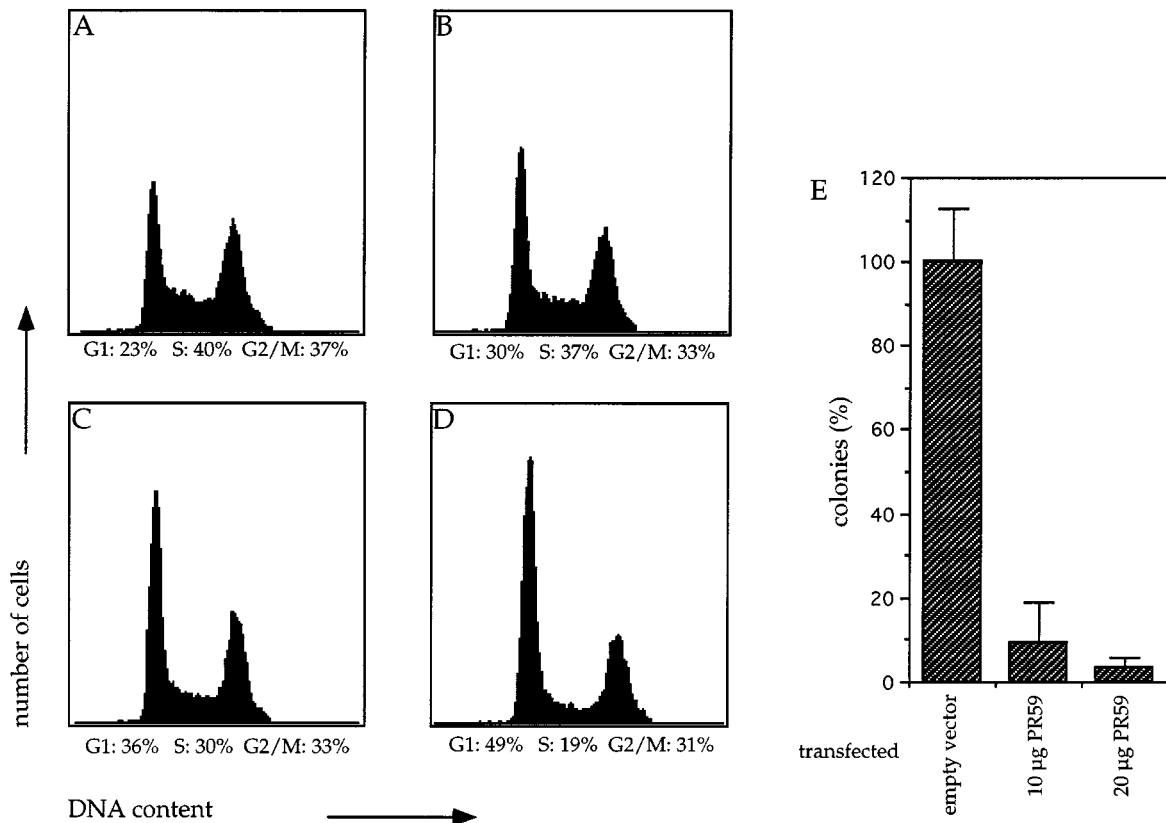


Figure 6 Inhibition of cell proliferation by PR59. U2-OS cells were transfected with 20 µg of empty vector (a), 10 µg (b) or 20 µg (c) of PR59 expression vector, or 20 µg HA-tagged PR59 expression vector (d), together with 2 µg CD20 expression vector. Forty-eight hours after transfection, cells were stained for DNA content and CD20 expression, and the cell cycle profile of CD20 positive cells was measured. The percentages of cells in G1, S, or G2/M phase is given below the panels. (e): U2-OS cells were transfected in triplicate with increasing amounts of PR59 expression vector or control empty vector, together with 0.5 µg of puromycin resistance vector. Forty-eight hours after transfection, the cells were placed under puromycin selection, and after 12 days colonies were counted. The number of colonies and the standard deviation is given as a percentage of colonies with the control vector alone

not of PR72, results in accumulation of dephosphorylated p107 (Figure 5 and data not shown). Thus it is quite likely that the members of the PR72-related family of PP2A subunits differ in their ability to associate with target proteins.

In mammalian cells, PR59 co-immunoprecipitated with p107, but not with pRb, indicating that PR59 can discriminate between p107 and pRb *in vivo* (Figure 4). Consistent with the association seen in immunoprecipitation, immunofluorescence staining experiments indicated that PR59 co-localizes with PP2Ac and p107 in nuclear dots. Importantly, no co-localization of PR59 was seen with pRb, which corroborates the immunoprecipitation data (data not shown). Together, these data provide strong evidence for the model that B subunits of PP2A act as targeting devices that recruit cellular substrates of PP2A to the PP2A catalytic subunit. This situation is reminiscent of the interaction of D type cyclins with pRb, in which the regulatory cyclin component can also target the catalytic cdk component to its substrate (Dowdy *et al.*, 1993; Ewen *et al.*, 1993). In most cases, such interactions between enzymes and substrates are unstable and transient in nature. Indeed, the specific interaction between PR59 and p107 has thusfar only been seen in transient transfection experiments. Our inability to detect

association in the absence of overexpression may reflect the transient nature of their interaction.

The finding that PR59 can interact both with p107 and with PP2Ac suggests that PP2Ac is targeted to p107 by PR59, resulting in the dephosphorylation of p107. Indeed, expression of PR59 with p107 resulted in the appearance of dephosphorylated p107. Importantly, elevated expression of PR59 results in an increase in G1 in transfected cells. Overexpression of PR59 in U2-OS cells also inhibited the outgrowth of puromycin resistant colonies (Figure 6). This suggests that the dephosphorylation of p107 by the PP2A/PR59 complexes restores the ability of p107 to inhibit G1 to S phase progression. However, we cannot exclude the possibility that PR59 acts on additional cellular targets to mediate its effects on cell cycle.

It is possible that the interaction between PR59 and p107 not only serves to recruit the PP2A catalytic subunit to p107, but also to additional proteins that are complexed to p107. For instance, serine phosphorylation of DP and E2F proteins can inhibit their DNA binding activities (Dynlacht *et al.*, 1994; Krek, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995) and DP-1 has been shown to be a target for PP2A *in vitro*, effectively restoring the E2F DNA binding capacity (Altioik *et al.*, 1997). Thus, targeting of PP2A to p107 could not only result in more p107/E2F complexes, but also in a

higher affinity for DNA through dephosphorylation of E2F/DP, and consequently higher repressor activity of these complexes.

pRb was not dephosphorylated by PR59 expression, which is in agreement with the observed lack of association between pRb and PR59. This suggests that interaction between the B-subunit and the substrate is necessary for effective dephosphorylation of the substrate. That pRb phosphorylation was not affected by PR59 expression argues against a non-specific effect of PR59 on cell cycle progression or inactivation by PR59 of cyclin/cdk complexes that phosphorylate both p107 and pRb. Instead, these data suggest strongly that association of PR59 to p107 results in specific dephosphorylation of p107 *in vivo*.

It is noteworthy that p107 and p130 on the one hand and pRb on the other appear to belong to distinct pocket protein subclasses based on several criteria. For instance, the E2F partners of pRb (E2F-1, 2 and 3) differ from the E2F partners of p107 and p130 (E2F-4 and 5) (Beijersbergen and Bernards, 1996; Bernards 1997). Also, p107 and p130, but not pRb have a spacer motif that can bind cyclins A and E. Finally, disruption of the *Rb* gene in mice predisposes to cancer, whereas homozygous loss of *p107* or *p130* alone does not (reviewed in Bernards, 1997). Our present data add another feature to this list of differences in that PR59 mediates dephosphorylation of p107 and not of pRb in mammalian cells.

It is currently unknown whether the PP1 activity which dephosphorylates pRb during anaphase is also active towards p107. Proteins containing 'LXCXE' motifs are often able to interact with the retinoblastoma-family proteins. Durfee *et al.* point out that PP1 contains two 'LXSXE' motifs, which are thought to contribute to the ability of PP1 to form a complex with pRb (Radulescu 1995). PP1 may in this way also be relevant for p107 dephosphorylation during mitosis. However, both PR59 and PR72 contain an LXSXE motif (amino acid 324–328 and amino acid 344–348 respectively), whereas PR59 binds only to p107, and not to pRb and PR72 does not interact with either pocket protein. This indicates that merely the possession of such a motif is insufficient for stable interaction between these regulatory subunits and the pocket proteins. Furthermore, binding of PR59 to p107 may be distinct from binding of E2F to p107 in that PR59 association to p107 is more transient in nature than the p107-E2F interaction. Consistent with this, the binding of PR59 to p107 does not appear to require an intact 'B pocket' (PMV and RB unpublished data), which further suggests that PR59 and p107 interaction is distinct from the stable LXCXE-motif-mediated interactions with the 'A + B pocket' of p107.

The preferential dephosphorylation of p107 by PR59 raises the question why only one class of pocket proteins is targeted to PP2A by PR59. As p107 and pRb are thought to have distinct cellular targets through their interaction with different E2Fs, a differential dephosphorylation of the pocket proteins may allow the control of distinct subsets of pocket protein target genes in response to different stimuli. We are currently investigating which stimuli trigger PR59-mediated dephosphorylation of p107.

Materials and methods

Yeast two-hybrid screen and cDNA library screen

Yeast strain Y190 (Harper *et al.*, 1993), containing 'bait' plasmid pPC97-p107, encoding the GAL4 DNA binding domain (DBD) fused to the pocket region of p107, was transformed with a day 14.5 CD1 mouse embryo library (Chevray and Natans, 1992) by the lithium acetate method (Schiestl and Gietz, 1989). Transformants were selected for growth on plates lacking histidine and supplemented with 25 mM 3-aminotriazole. His⁺ colonies were subsequently analysed for β -galactosidase activity as previously described (Chevray and Natans, 1992, Hijmans *et al.*, 1995). cDNA library plasmids derived from double positive yeast colonies were tested for bait specificity by re-transformation with different GAL4 DBD fusion plasmids: pPC97-p107, pPC97-bmi, and pPC97 without an insert.

A partial mouse PR59 cDNA clone pp44 (nucleotide 871–1429) was used to screen additional mouse cDNA libraries by DNA-DNA hybridization. The full length cDNA was obtained from a mouse PCC4 teratocarcinoma cell line cDNA library (Stratagene).

Plasmids

pPC97-p107 was generated by cloning a cDNA fragment that specifies the pocket region of p107 (amino acids 240–816) in frame with the GAL4 DBD (amino acids 1–147) of pPC97 (Chevray and Natans, 1992). GST-PR59 was constructed by cloning the ORF of the PR59 cDNA with the insert from the *ApaI* site at nucleotide –26 relative to the initiator ATG codon in frame in the *SmaI* site of pGEX-2TK.

pMV-HAtag was created by cloning an oligonucleotide that specifies a consensus AUG start codon, followed by the 10 amino acid influenza virus haemagglutinin epitope tag from the *XhoI* to *SalI* sites of pBSK⁺. The PR59 cDNA was then cloned from the *ApaI* site 26 bases upstream of the putative initiator codon in frame in pMV-HAtag, introducing 17 amino acids between the HA tag and the first PR59 methionine. HA-PR59 was then subcloned into a cytomegalovirus promoter-containing expression vector (pCMV). HA-tagged PP2A catalytic subunit was created by cloning human catalytic subunit (Stone *et al.*, 1988) from nucleotide 58–1022 in pMV-HAtag, introducing 19 additional amino acids between the HA tag and the first methionine. HA-PR65 was created by cloning the human PR65 α cDNA (Hemmings *et al.*, 1990), starting from amino acid 3, in pMV-HAtag. HA-PR72 was created by PCR using as 5' primer 5'-TCGCGTTCGACGATGATGATCAAGGAAACATC-3' and T7 as 3' primer on the full length human PR72 cDNA in pBSK (Hendrix *et al.*, 1993a). The PCR product was cloned into the *SalI* site of pMV-HAtag. HA tagged PP2Ac and PR72 were then subcloned into pCMV. HA tagged PR65 was subcloned into the mammalian expression vector pRC/CMV (Invitrogen). pCMV-pRb, pCMV-p107, pCMV-HAp107 and expression vectors for Cyclin D1, cdk4 and cdk4 dominant negative have been described previously (Hinds *et al.*, 1992; van den Heuvel and Harlow, 1993; Zhu *et al.*, 1993; Beijersbergen *et al.*, 1994). pECE-HApRb was a gift from K Alevizopoulos.

Cell lines and transfections

U2-OS and B104 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Transfections were performed by the calcium phosphate precipitation method (Van der Eb and Graham, 1980). Total DNA transfected was adjusted to 20 μ g with empty vector.

Northern blot analysis

For PR59 expression analysis, a mouse tissue blot was obtained from Clontech and probed with a ^{32}P -labeled partial mouse PR59 cDNA (nucleotides 760–1075) according to the suppliers instructions. After washing at 65°C in $0.1 \times \text{SSC}$, the blot was exposed to a phosphor-imager screen.

Immunological reagents

To generate a polyclonal antiserum against PR59, GST-PR59 (see plasmids) was made in *Escherichia coli* and purified using glutathione Sepharose beads (Pharmacia). The fusion protein (200 μg) in complete Freund's adjuvant was injected in a rabbit. Subsequent booster injections (200 μg of protein in incomplete Freund's adjuvant) were given at three-weekly intervals. After six rounds of immunization, polyclonal serum was obtained. Before the first immunization, pre-immune serum was obtained from the rabbit. 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) were obtained from Santa Cruz.

Immunoprecipitation–phosphatase–assay

PP2A catalytic subunit activity in immunoprecipitates was measured as release of $^{32}\text{PO}_4$ from a peptide phosphorylated with protein kinase A and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Favre *et al.*, 1994). Immunoprecipitations from transfected U2-OS or untransfected B104 cells were performed as described previously (Beijersbergen *et al.*, 1994). In short, the cells were scraped 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. Insoluble material was removed by centrifugation and the supernatant was then rocked with 25 μl of a pre-formed complex of antibody 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).

For the *in vitro* phosphatase assay, 50 μl phosphatase assay buffer with 125 nM $[\text{P}^{32}]\text{phosphorylated peptide}$ and, if applicable, 10 nM okadaic acid was added, and rocked at 30°C for 3 h. After this, 350 μl stop mix (80 mM NaPP_i, 1.6 mM NaH_2PO_4 , 0.9 M HCl, 3.2% w/v active charcoal) was added to the reaction, tubes were vortexed for 10 min, spun down, and 300 μl of the supernatant was counted. The amount of released $^{32}\text{PO}_4$ never exceeded 25% of the input counts. The peptide Leu-Arg-Arg-Ala-Ser-Val-Ala (Kemptide Val⁶-Ala⁷, Bachem) was phosphorylated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by the catalytic subunit of protein kinase A as described previously (Favre *et al.*, 1994). Subsequently, the peptide was separated from un-incorporated $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ on a

Dowex 1 (Fluka AG) column with 30% acetic acid, freeze dried twice, and aliquoted at 25 mM in 50 mM Tris-HCl pH 7.5, 0.1 mM EDTA.

Immunoprecipitations and Western blotting

Transfected cells were immunoprecipitated as described previously (Beijersbergen *et al.*, 1994). The immunoprecipitate or the total cell lysate were separated on a SDS 10% or 7% polyacrylamide gel and transferred to nitrocellulose. The membrane was blocked in PBS containing 0.05% Tween-20 and 5% dried non-fat milk for 1 h at room temperature, and incubated for 16 h at 4°C with the indicated antibodies in PBS with Tween and 1% milk. Anti HAtag (12CA5) culture supernatant was used 1:500 diluted, anti-p107 (C18), anti-pRb (C15) and polyclonal anti-PR59 were used 1:4000. The membrane was then washed in PBS with Tween and 1% milk and the antibody was detected using horseradish peroxidase-linked goat anti mouse IgG (Biorad), peroxidase-linked goat anti rabbit (Biosource Ltd.) or peroxidase-linked protein A (Amersham Life Sciences) and enhanced chemiluminescence (Amersham).

FACS analysis and colony formation assays

FACS analysis of transfected U2-OS cells was performed as described previously (Beijersbergen *et al.*, 1995). For colony formation assays, 0.5 μg pBABE puro vector was co-transfected with 20 μg DNA. Forty-eight hours after transfection, cells were split 1:20, and cultured for 12 days in medium containing 2.5 $\mu\text{g}/\text{ml}$ puromycin. Then the colonies were fixed, stained with Coomassie-blue and counted.

Sequencing and alignments

Deletion clones were made of the largest clone with a nested deletion kit (Pharmacia) and sequenced using T7 sequenase (Pharmacia) or by terminator cycle sequencing (Applied biosystems). Both cDNA strands were completely sequenced.

Sequence alignments were performed with the University of Wisconsin GCG package (Genetics Computer Group, 1994) or using the BLAST algorithm (Altschul *et al.*, 1990) to search Genbank.

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