The CDC2-related kinase PITALRE is the catalytic subunit of active multimeric protein complexes

Judit GARRIGA, Xavier MAYOL and Xavier GRAÑA*

Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, 3307 North Broad St., Philadelphia, PA19140, U.S.A.

PITALRE is a human protein kinase identified by means of its partial sequence identity to the cell division cycle regulatory kinase CDC2. Immunopurified PITALRE protein complexes exhibit an *in vitro* kinase activity that phosphorylates the retinoblastoma protein, suggesting that PITALRE catalyses this phosphorylation reaction. However, the presence of other kinases in the immunopurified complex could not be ruled out. In the present work, an inactive mutant of the PITALRE kinase has been used to demonstrate that PITALRE is the catalytic subunit responsible for the PITALRE-complex-associated kinase activity. Ectopic overexpression of PITALRE did not increase the total PITALRE kinase activity in the cell, suggesting that PITALRE is regulated by limiting cellular factor(s). Characterization of the PITALRE-containing protein complexes indicated that most of the cellular PITALRE protein exists as a subunit in at least two different active multimeric complexes. Although monomeric PITALRE is also active *in vitro*, PITALRE present in multimeric complexes exhibits several-fold higher activity than monomeric PITALRE. In addition, overexpression of PITALRE demonstrated the existence of two new associated proteins of approx. 48 and 98 kDa. Altogether these results suggest that, in contrast to the situation with cyclin-dependent kinases, monomeric PITALRE is active, and that association with other proteins modulates its activity and/or its ability to recognize substrates *in vivo*.

INTRODUCTION

Protein kinases play a critical role in the regulation of numerous cellular processes [1]. They are traditionally classified as either tyrosine or serine/threonine protein kinases, although protein kinases with the ability to phosphorylate both types of residues have also been described [2,3]. A subgroup of serine/threonine protein kinases, of which the cell division cycle regulatory kinase CDC2 is a prototype, is known to control critical points of the cell cycle. Several kinases have been ascribed to this subgroup by means of partial primary sequence identity to CDC2. However, a role in the cell cycle has been demonstrated in only a few cases [4–7]. Cyclin-dependent kinases (CDKs) are those CDC2-related kinases that bind a regulatory subunit called cyclin to form active holoenzymes. These cyclin/CDK holoenzymes are regulated by phosphorylation/dephosphorylation of specific conserved residues on the CDK catalytic subunit, as well as by binding to a family of inhibitors called CKIs [8,9]. CDKs are proteins of approx. 30 kDa that contain essentially the catalytic domain of a protein kinase. Interestingly, one of these CDKs, originally found to associate with D-type cyclins and hence named CDK5, has been shown to associate with a brain-specific regulatory subunit called p35 which has no identity to cyclins. p35 is essential for CDK5 activation, and this holoenzyme is a candidate for the neurofilament kinase and the tau kinase [10].

PITALRE is a 43 kDa protein that was identified in a search for CDC2-related kinases [6]. PITALRE shares 38–43 % identity with CDKs and contains the hallmarks that are conserved in all known protein kinases [2]. Immunopurified PITALRE protein complexes possess a kinase activity that phosphorylates the retinoblastoma protein (pRB) and the myelin basic protein *in vitro*, and this activity seems not to be cell-cycle-regulated. However, since PITALRE protein complexes contain other unidentified cellular proteins of 80, 95 and 155 kDa [6], it is conceivable that one or more of these proteins is, indeed, a protein kinase, and therefore partially or totally responsible for the PITALRE-associated kinase activity.

In the present study, we have ectopically expressed a kinase-deficient point-mutated version of PITALRE to study its associated kinase activity. In addition, we have also characterized PITALRE protein complexes and identified two new cellular proteins that associate with PITALRE. Our findings indicate that PITALRE kinase activity does not require the association of additional cellular protein(s). However, association with other cellular proteins results in increased kinase activity, suggesting that PITALRE-associated proteins regulate its activity and/or facilitate its substrate recognition. Taken together, our results suggest that the mechanisms of activation of PITALRE are distinguishable from those of other CDC2-related kinases.

EXPERIMENTAL

Cell culture

Human embryonal kidney transformed 293 cells were cultured at 37 °C and 5 % CO_2 in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum (Summit). Cells at 60–80 % confluence were labelled with 0.2 mCi of [^{35}S]methionine (Dupont) in 2 ml of medium without methionine at 37 °C for 4–6 h (unless otherwise indicated). Protein extracts were prepared by lysing the cells in 50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 % Triton X-100, 0.1 mM Na_3VO_4, 1 mM PMSF and 10 μg/ml leupeptin (lysis buffer). Flow cytometric analysis was performed as previously described [11].

Transfections were performed essentially as previously described [12]. Briefly, confluent cultures of 293 cells were split 1:10 and transfected 24 h later with calcium phosphate precipitates of 10 μg (stable transfection) or 20 μg (transient transfection) of the corresponding plasmid DNA for each 100 mm dish. After 16 h,

Abbreviations used: CDK, cyclin-dependent kinase; pRB, retinoblastoma protein; GST, glutathione S-transferase; CMV, cytomegalovirus; PITALRE-HA, PITALRE with an added haemagglutinin peptide (HA) tag; PITALRE-CT, C-terminal peptide of PITALRE.

* To whom correspondence should be addressed.

cells were washed twice with PBS and incubated with fresh medium. In the stable transfections, the process of selection was started 24 h after removal of the DNA precipitates by culturing the cells with medium containing G418 (500 μg/ml). After 3 weeks, individual drug-resistant clones were randomly picked and expanded by maintaining them in medium containing 250 μg/ml G418. In the transient transfection procedures, the cells were harvested 48 h after removal of the DNA precipitates.

Protein assays

Immunoprecipitations and Western blotting experiments were performed as previously described [6,11]. Kinase assays with immunoprecipitated complexes were performed at 30 °C for 20 min in 20 mM Hepes-Na (pH 7.4), 10 mM magnesium acetate, 1 mM dithiothreitol and 20 μM ATP [$(2–4) \times 10^4$ c.p.m./pmol] in the absence or presence of 0.3–0.5 μg of glutathione S-transferase (GST)–pRB fusion protein in a total volume of 35 μl. The reaction was terminated by the addition of 35 μl of 2 × Laemmli sample buffer. GST–pRB (amino acids 379–792) was obtained as previously described [12,13]. The *in vitro* translated proteins PITALRE and PITALRE-HA [PITALRE with an added haemagglutinin peptide (HA) tag; see below] were produced using the plasmids pBS-PITALRE and pBS-PITALRE-HA respectively as templates and the TNT rabbit reticulocyte lysate system (Promega) as described previously [7]. Protein determinations were performed by using the Bio-Rad protein assay.

Antibodies

Affinity-purified anti-PITALRE antibodies have been described [6]. Affinity-purified anti-PITALRE-CT (a polyclonal antibody directed to the C-terminal region of PITALRE) was from Santa Cruz Biotechnology, and a monoclonal antibody to HA (anti-HA; 12CA5) was from Boerhinger Mannheim. The PITALRE-CT peptide CSTNQSRNPATTNQTEFERVF (residues 353–372), the HA-epitope peptide YPYDVPDYA, and the GST–PITALRE fusion protein were used in specific competition assays.

Plasmids

The HA tag was added to PITALRE cDNA by PCR using the 5′ primer 5′-AATGTGCTTATCACTCG-3′ and the two 3′ nested primers 5′-ACTAGCATAATCAGGAACATCA-TAGAAGACGCGCTCAAACTCCGTCTGGTTGGT-3′ and 5′-CCGTCTAGACTAGAGACTAGCATAATCAGGAAC-3′ (the *Xba*I restriction site is underlined). The PCR product was digested with *Xba*I and *Stu*I. The digested products were gel-purified and subcloned into pBS-PITALRE [6] previously digested with the same enzymes, yielding pBS-PITALRE-HA. pBS-PITALRE-HA was then digested with *Xba*I and partially digested with *Hin*dIII, and the full-length coding sequence was subcloned into the pRc/cytomegalovirus (CMV) vector (Invitrogen). PITALRE$^{(D \rightarrow N)}$-HA was obtained by site-directed mutagenesis using the Altered Sites II *in vitro* mutagenesis system (Promega). The PITALRE cDNA [6] was subcloned into the *Eco*RI site of pALTER-EX1. We obtained single-strand DNA from this plasmid and annealed it to the mutagenic primer 5′-AAGCTGGCAAACTTTGGGCTG-3′, where A replaces a G resulting in a change of the encoded residue to Asn from Asp^{167}. All modified cDNAs were verified by DNA sequencing [14]. The resulting mutated cDNA was digested with *Nco*I–*Stu*I and, after gel purification, inserted into pBS-PITALRE-HA (see above) lacking the wild-type *Nco*I–*Stu*I cDNA fragment, yielding pBS-PITALRE$^{(D \rightarrow N)}$-HA. The point-mutated cDNA PITALRE$^{(D \rightarrow N)}$-HA was then subcloned into pRc/CMV (Invitrogen) as described for PITALRE-HA.

FPLC analysis

Cell lysates were fractionated by Superose 12 column FPLC gel filtration. Samples of 2 mg of protein extract in lysis buffer were injected at a flow rate of 0.5 ml/min. Fractions of 0.25–1.00 ml were collected (as indicated) and the protein concentration was monitored by absorbance at 280 nm. Identical absorbance profiles and Coomassie Blue staining patterns were obtained in all runs. Aliquots of 50 μl of molecular mass standards (Bio-Rad) containing thyroglobulin (670 kDa; fractions 8/8a); β-γ-globulin (158 kDa; fractions 12/12a); ovalbumin (44 kDa; fractions 14/14a); myoglobin (17 kDa; fractions 15/15a) and vitamin B12 (1.35 kDa; fractions 20/20a) were used to estimate the molecular masses of the proteins and protein complexes contained in each fraction.

RESULTS AND DISCUSSION

Analysis of 293 cells ectopically expressing HA-tagged wild-type PITALRE

Human 293 cells were transfected by using a cDNA encoding an HA-tagged version of PITALRE under the control of a CMV promoter (see the Experimental section). Transfected cells were selected in G418-containing culture medium and resistant colonies were isolated. No morphological differences were observed between 293 cells and the 293/pCMV and 293/pCMV-PITALRE-HA clones. Flow cytometric analysis of exponentially growing parental and stably transfected cell lines showed similar cell cycle distributions (~ G1, 61%; S, 22%; G2/M, 17%). Characterization of the resistant clones by Western blotting indicated that 11 out of 15 clones expressed detectable levels of PITALRE-HA. Figure 1(A) shows the presence of a specific band corresponding to PITALRE-HA recognized by an anti-HA monoclonal antibody in lysates of four representative clones (293/pCMV-PITALRE-HA#9, #13, #14 and #15), compared with the absence of this band in lysates of two pCMV clones (#1 and #5) and 293 cells. A similar filter immunoblotted with a polyclonal antibody directed to the C-terminal region of PITALRE (anti-PITALRE-CT) recognized both the endogenous PITALRE in all lysates and PITALRE-HA in clones #9, #13, #14 and #15 (Figure 1B). The slower migration of PITALRE-HA is due to the HA tag, and both PITALRE and PITALRE-HA co-migrate precisely with their respective *in vitro* translated products (results not shown).

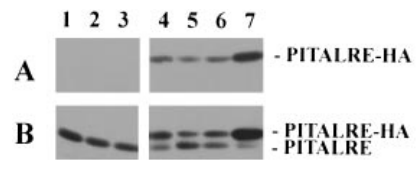

Figure 1 Analysis of 293 cells ectopically expressing HA-tagged wild-type PITALRE

293 cells (lane 1), 293/pCMV clones #1 and #5 (lanes 2 and 3 respectively) and 293/pCMV-PITALRE-HA clones #9, #13, #14 and #15 (lanes 4–7 respectively) were lysed as described in the Experimental section, and 40 μg samples of whole protein extracts were loaded on SDS/10%-PAGE and transferred to poly(vinylidene difluoride). (**A**) Cell lysates immunoblotted with anti-HA monoclonal antibody; (**B**) the same lysates immunoblotted with anti-PITALRE-CT. PITALRE-HA and PITALRE proteins are indicated on the right.

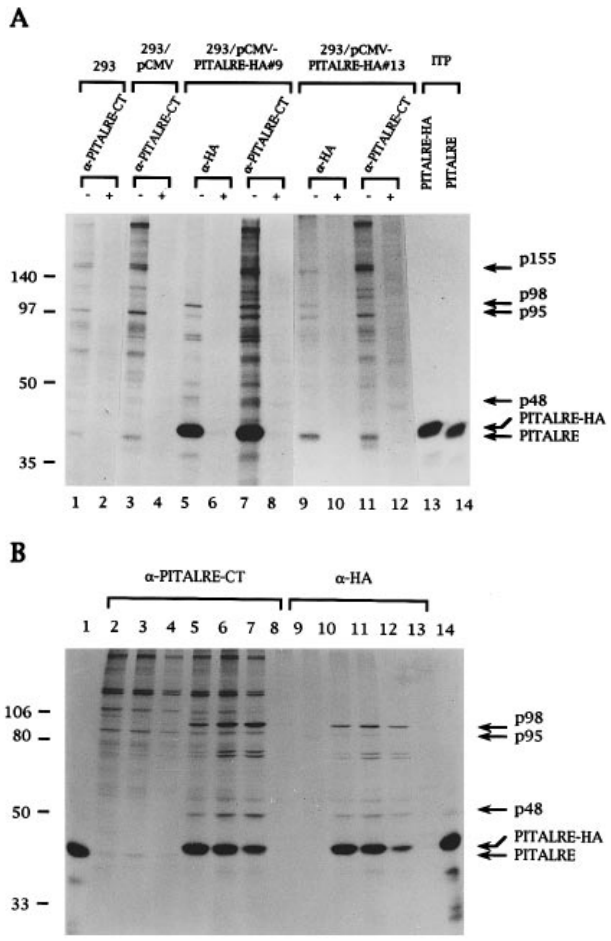

Figure 2 Comparison of PITALRE- and PITALRE-HA-associated proteins

(**A**) 293 cells or the indicated 293 stable clones were metabolically labelled as described in the Experimental section. Equivalent amounts of radioactivity (c.p.m.) of labelled proteins were immunoprecipitated with anti-PITALRE-CT (α-PITALRE-CT) and α-HA antibodies. Aliquots of 10 μg of the specific peptides were added for the control immunoprecipitations (presence and absence of peptide indicated by + and − respectively). *In vitro* translated PITALRE-HA and PITALRE were loaded as markers (indicated by ITP). (**B**) 293 cells (lanes 2–4 and 9) and the stable clone 293/pCMV-PITALRE-HA#15 (lanes 5–8 and 10–13) were metabolically labelled for different periods of time (lanes 2, 5 and 10, 5 h; lanes 3, 6, 8, 9, 11 and 13, 10 h; lanes 4, 7 and 12, 20 h). Equivalent amounts of radioactivity (c.p.m.) of labelled proteins were immunoprecipitated with α-PITALRE-CT and α-HA. The controls of the immunoprecipitations were performed by competition with the corresponding peptides, PITALRE-CT (lane 8) or HA (lane 13). *In vitro* translated PITALRE (lane 1) and PITALRE-HA (lane 14) were used as markers. Specific immunoprecipitated proteins are indicated on the right. Molecular mass markers, indicated on the left, are given in kDa.

To analyse whether PITALRE-HA interacts with the same cellular proteins as endogenous PITALRE, immunoprecipitations from lysates of [^{35}S]methionine metabolically labelled 293, 293/pCMV and 293/pCMV-PITALRE-HA#9 and #13 cell lines were performed by using anti-HA and anti-PITALRE-CT antibodies. Figure 2(A) shows that PITALRE and PITALRE-HA interact specifically with similar sets of cellular proteins (compare lanes 3, 7 and 11 with lanes 5 and 9; and see below). The PITALRE-associated protein p95 is specifically present in the immunocomplexes of anti-PITALRE-CT and anti-HA in all cell lines [6] (Figure 2). However, the PITALRE-associated protein p155 is not easily detectable by using anti-HA antibodies, which suggests that the PITALRE domain required for this interaction is modified by the presence of the HA-epitope tag. A new protein of $\sim$ 98 kDa is detected in all clones expressing PITALRE-HA (Figure 2A, lanes 5, 7, 9 and 11; Figure 2B, lanes 5–7 and 10–12). Finally, another protein of $\sim$ 48 kDa is also detected in clones expressing high levels of ectopic PITALRE (Figure 2A, lanes 5 and 7; Figure 2B, lanes 5–7, 10–12). Detection of these two previously unidentified proteins may be attributable to the increased amount of PITALRE in the transfected clones, since the abundance of these two proteins appeared to increase concomitantly with the levels of the PITALRE-HA protein in the different clones (Figure 2A, lanes 5 and 9; Figure 2B, lanes 4 and 5). This suggests that these proteins are not limiting in the cell and that they can only be detected associated with PITALRE when the PITALRE protein is highly expressed.

Differences in the levels of PITALRE-HA protein were observed by either Western blot or immunoprecipitation analysis of PITALRE-HA stable clones (Figures 1, 2A and 2B, and results not shown). A decrease in the endogenous level of PITALRE in clones expressing PITALRE-HA was also detected (Figures 1 and 2, and results not shown). 293 and 293/pCMV-PITALRE-HA#15 cells metabolically labelled with [^{35}S]methionine for 5, 10 and 20 h were immunoprecipitated with anti-HA and anti-PITALRE-CT antibodies, showing similar patterns of associated proteins (Figure 2B; see below). The level of PITALRE-HA in 293/pCMV-PITALRE-HA#15 cells was about four times the level of endogenous PITALRE in 293 cells, as determined by Western blotting. Interestingly, the endogenous PITALRE is almost undetectable in this clone (Figures 1 and 2B; see below). Increasing the duration of labelling with [^{35}S]-methionine did not result in any changes in the compositions of PITALRE and PITALRE-HA protein complexes.

Ectopic expression of PITALRE-HA does not increase the total cellular PITALRE-associated kinase activity

We analysed the kinase activity associated with PITALRE immunoprecipitates from 293/pCMV and 293/pCMV-PITALRE-HA cells in the absence of exogenous substrate by using anti-HA, anti-PITALRE-CT and affinity-purified anti-PITALRE antibodies. Anti-HA monoclonal antibodies, as expected, did not immunoprecipitate any kinase activity from 293/pCMV cells (Figure 3A). However, the three antibodies immunoprecipitated similar kinase activities from 293/pCMV-PITALRE-HA#15 cells (Figure 3A). To ascertain whether the total cellular PITALRE-associated kinase activity was increased in 293 cells ectopically expressing PITALRE-HA, the kinase activity of different clones compared with that in 293 and 293/pCMV cells was measured by using GST–pRB as an exogenous substrate [6]. Anti-HA immunoprecipitates from the PITALRE-HA clones contained variable levels of kinase activity (Figure 3B and 3C, lanes 3–6), since different levels of PITALRE-HA are expressed in these stably transfected cell lines (Figures 1, 2A and 2B). Indeed, PITALRE-HA protein levels in clone 293/pCMV-PITALRE-HA#15 are approx. 4 times the levels of endogenous PITALRE in 293 cells (Figure 1). As expected, no kinase activity was detected in immunoprecipitates from 293 and two 293/pCMV cell lines (Figure 3B and 3C, lanes 1 and 2). However, anti-PITALRE-CT immunoprecipitates from all the cell lines, including 293 cells and all 293/pCMV and 293/pCMV-PITALRE-HA transfectants, contained similar levels of kinase activity (Figure 3B and 3C, lanes 7–12, and results not shown). Therefore, although increasing levels of exogenous PITALRE-HA were correlated with the levels of PITALRE-HA-associated kinase activity, the total cellular PITALRE-associated kinase

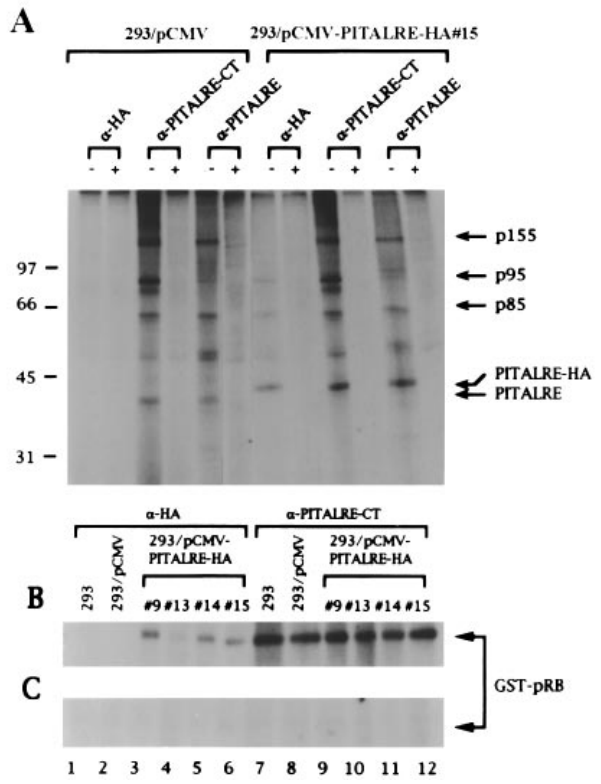

Figure 3 Overexpression of PITALRE does not increase the total PITALRE-associated kinase activity in the cell

Protein extracts from parental 293 cells or from the indicated 293 stable transfectants were immunoprecipitated with the indicated antibodies. (**A**) Kinase assays were performed on immunoprecipitates obtained with anti-HA (α-HA), α-PITALRE-CT and α-PITALRE in the absence of exogenous substrate. α-HA and α-PITALRE-CT immunoprecipitations were performed in the absence (−) or in the presence (+) of the corresponding peptides. α-PITALRE antibodies were pre-cleared with GST–PITALRE gluthatione beads for the control immunoprecipitations (+). (**B**) PITALRE immunocomplexes were assayed for their ability to phosphorylate the exogenous substrate GST–pRB: lanes 1–6, α-HA immunoprecipitates; lanes 7–12, α-PITALRE-CT immunoprecipitates. Cell lines: 293 (lanes 1 and 7), 293/pCMV (lanes 2 and 8), 293/pCMV-PITALRE-HA#9, #13, #14 and 15# (lanes 3 and 9, 4 and 10, 5 and 11, and 6 and 12 respectively). (**C**) As in (**B**), but antibodies were blocked with the corresponding specific peptides (see the Experimental section). Specific phosphorylated proteins are indicated on the right. Molecular mass markers, indicated on the left, are given in kDa.

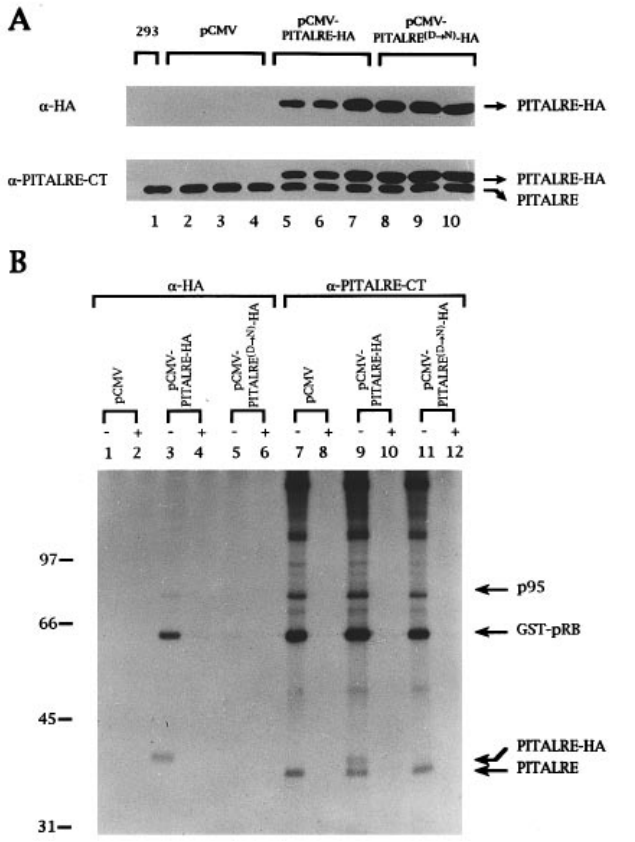

Figure 4 Transient expression of PITALRE-HA and PITALRE$^{(D\rightarrow N)}$-HA in 293 cells

293 cells were transiently transfected with different pCMV constructs (indicated at the top, lanes 2–10) as described in the Experimental section. (**A**) Western blot analysis of parental 293 cells and 293 cells transiently transfected with pCMV (lanes 2–4), pCMV-PITALRE-HA (lanes 5–7) and pCMV-PITALRE$^{(D\rightarrow N)}$-HA (lanes 8–10) by using anti-HA (α-HA) or anti-PITALRE-CT antibodies (indicated on the left). Relevant proteins are indicated on the right. (**B**) GST–pRB kinase assays were performed using anti-HA and anti-PITALRE-CT immunoprecipitates from the indicated cell extracts. Protein extracts were from pCMV (lanes 1, 2, 7 and 8), pCMV-PITALRE-HA (lanes 3, 4, 9 and 10) and pCMV-PITALRE$^{(D\rightarrow N)}$-HA (lanes 5, 6, 11 and 12) transiently transfected cells. Portions of 10 μg of the corresponding peptides were used in the competition assays (+). Specific phosphorylated proteins are indicated on the right. Molecular mass markers, indicated on the left, are given in kDa.

activity remained constant. These results indicate that PITALRE protein levels up to four times the endogenous level of expression are insufficient to increase the total PITALRE kinase activity in the cell. Therefore limiting cellular factors must be required for modulation of PITALRE kinase activity and/or for substrate recognition (see below).

Transient transfection of the inactive point mutant PITALRE$^{(D\rightarrow N)}$-HA

PITALRE-associated kinase activity was previously identified by measuring the kinase activity present in PITALRE immunocomplexes in the presence or absence of exogenous substrate [6]. Since PITALRE immunoprecipitates contained at least three cellular associated proteins, we were not able to rule out the possibility that one of these proteins was a kinase. In such a case, the activity detected could be totally, or partially, due to the associated kinase. To demonstrate whether PITALRE is the catalytic subunit of these complexes and therefore responsible for their kinase activity, we constructed a point-mutated version of PITALRE by site-directed mutagenesis in which Asp^{167} was substituted by Asn (see the Experimental section). This residue is conserved among all known protein kinases [2] and has been mutated previously to produce dominant-negative forms of other members of the CDC2 family of protein kinases [15]. 293 cells were transiently transfected with pCMV, pCMV-PITALRE-HA or pCMV-PITALRE$^{(D\rightarrow N)}$-HA in triplicate (see the Experimental section). After transfection, cells were lysed and analysed by Western blotting. Figure 4(A) shows the levels of PITALRE-HA, PITALRE$^{(D\rightarrow N)}$-HA and endogenous PITALRE, indicating similar levels of wild-type and point-mutated PITALRE-HA proteins. Immunocomplex kinase assays were performed with the same protein extracts using either anti-HA or anti-PITALRE-CT with GST–pRB as an exogenous substrate. A kinase activity capable of phosphorylating GST–pRB was obtained from anti-HA immunoprecipitates of 293 cells expressing PITALRE-HA. Most significantly, the cells transfected with pCMV-PITALRE$^{(D\rightarrow N)}$-HA failed to show such a kinase activity, indi-

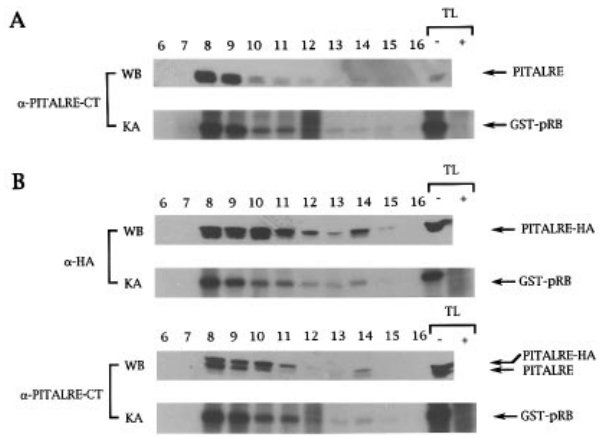

Figure 5 Comparison of gel-filtration fractionation of PITALRE cellular protein complexes compared with PITALRE-HA cellular protein complexes

Portions of 2 mg each of protein extracts from clones 293/pCMV#1 and 293/pCMV-PITALRE-HA#14 were applied to a Superose 12 column (fraction size 1 ml). Equal aliquots of fractions 6–16 (indicated above the lanes), as well as the input lysate (indicated by TL), were analysed by Western blotting (WB) and GST–pRB kinase assays (KA). In the kinase assays, 200 μg of the input lysate was immunoprecipitated in the absence (−) or presence (+) of 10 μg of the corresponding peptide. (**A**) Western blots and GST–pRB kinase assays of 293/pCMV#1 fractionated cell lysates were performed by using anti-PITALRE-CT (α-PITALRE-CT). (**B**) Western blots and GST–pRB kinase assays of 293/pCMV-PITALRE-HA#14 fractionated cell lysates were performed by using anti-HA and anti-PITALRE-CT antibodies (indicated on the left). Relevant proteins are indicated on the right.

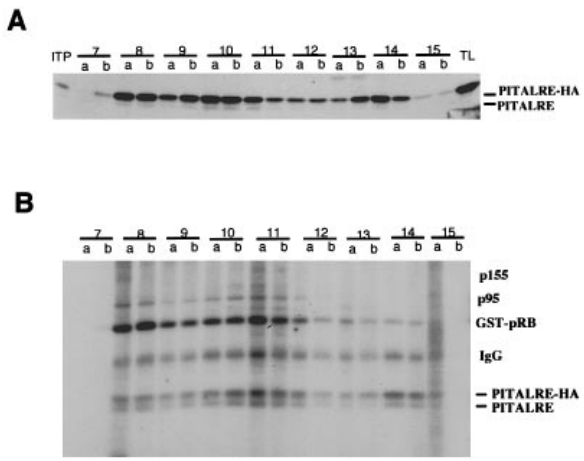

Figure 6 High-resolution chromatographic separation of PITALRE multimeric complexes and monomeric PITALRE

Portions of 2 mg of protein extracts from clone 293/pCMV-PITALRE-HA#15 were applied to a Superose 12 column as described in the legend to Figure 5 (fraction size of 0.5 ml). (**A**) Equal aliquots of fractions (indicated above the lanes) were resolved by SDS/PAGE and immunoblotted with anti-PITALRE-CT. A sample of 40 μg of the protein lysate loaded on to the column was also analysed by Western blotting (TL). (**B**) Kinase assays were performed on anti-PITALRE-CT immunoprecipitates of each fraction by using GST–pRB as an exogenous substrate. Phosphorylated proteins are indicated on the right. a and b denote the first and second half of each 1 ml fraction (so that the fraction numbers match those given in Figure 5).

cating that PITALRE is the catalytic subunit responsible of all of the pRB-phosphorylating activity detected in PITALRE immunocomplexes (Figure 4B, lanes 1–6). Identical assays performed by using anti-PITALRE-CT showed similar levels of GST–pRB phosphorylation (Figure 4B, lanes 7–12).

It has been shown previously that PITALRE is phosphorylated in this assay, indicating that PITALRE itself or another kinase present in the complex can use PITALRE as a substrate *in vitro* [6]. In this context, it is interesting to note that CDKs are positively or negatively regulated by phosphorylation of specific residues [9], and that some of these residues are conserved in the PITALRE protein [6]. Therefore it is conceivable that a kinase present in the complex having a different substrate specificity may be using PITALRE as a substrate, but not the exogenous substrate GST–pRB or the PITALRE-associated protein p95. Interestingly, regardless of the identity of the kinase responsible for this phosphorylation, it appears that the phosphorylation of PITALRE depends on the kinase activity of PITALRE, since PITALRE$^{(D \rightarrow N)}$-HA was not phosphorylated (Figure 4B, lanes 5 and 11). Therefore it is most likely that PITALRE shows autophosphorylation activity *in vitro*.

Characterization of PITALRE cellular protein complexes

To characterize PITALRE protein complexes, cellular lysates from the 293/pCMV and 293/pCMV-PITALRE-HA#14 clones were fractionated by gel filtration using a Superose 12 column connected to an FPLC system (1 ml fractions were collected). The presence of PITALRE in these fractions was analysed by Western blotting (protein standards were used for molecular mass estimations; see the Experimental section). As shown in Figure 5(A) (panel labelled WB), most endogenous PITALRE was present in high-molecular-mass complexes. Ectopic overexpression of PITALRE, however, allowed its detection in its monomeric form (Figure 5B, panels WB, lanes 14). To ascertain whether monomeric PITALRE is active, kinase assays were performed on PITALRE complexes immunoprecipitated from each fraction by using GST–pRB as an exogenous substrate. By comparing 293/pCMV and 293/pCMV-PITALRE-HA#14 protein lysates fractionated by gel filtration, we took advantage of the fact that overexpression of PITALRE-HA results in the detection of higher levels of monomeric PITALRE. As mentioned above, endogenous PITALRE was present mainly in high-molecular-mass fractions of 293/pCMV fractionated lysates, and this was correlated with phosphorylation of GST–pRB (Figure 5A). PITALRE kinase analysis of 293/pCMV-PITALRE-HA#14 fractionated lysates showed that, although phosphorylation of GST–pRB was primarily catalysed by the high-molecular-mass complexes, GST–pRB phosphorylation was also detected in fraction 14 (Figure 5B). These results indicate that the monomeric form of PITALRE is active and does not require association with other cellular factors for activity (see below).

To define precisely the complexes containing PITALRE, we performed additional FPLC gel-filtration assays of protein lysates of clone 293/pCMV-PITALRE-HA#15, collecting fractions of smaller size. We chose the 293/pCMV-PITALRE-HA#15 clone for these experiments because it expresses the highest levels of PITALRE-HA. Fractions were analysed by Western blotting and PITALRE kinase assays as described above. Figure 6(A) shows the presence of at least three peaks of PITALRE for a fraction size of 0.5 ml (identical results were obtained with fractions of 0.25 ml), as determined by Western blotting using anti-PITALRE-CT antibodies (note that lower levels of endogenous PITALRE were detected in clones expressing high levels of ectopic PITALRE-HA; see above). In particular, PITALRE was found in multimeric complexes of $\sim$ 670 kDa, in protein complexes of $>$ 158 kDa and also as a monomeric protein (Figure 6). Western blots performed with anti-HA

antibodies exhibited an identical distribution of PITALRE-HA (results not shown). The presence of PITALRE in different complexes is not surprising, since PITALRE is known to be associated with several proteins ([6]; the present work). Interestingly, the p95 protein that associates with and is phosphorylated by PITALRE was detected by a kinase assay in all high-molecular-mass complexes (Figure 6B). The GST–pRB exogenous substrate was phosphorylated preferentially by the multimeric PITALRE complexes, but also by monomeric PITALRE, although to a much lower extent (Figure 6B). However, autophosphorylation of PITALRE appears to be more consistent with the levels of PITALRE protein, regardless of its association with other proteins (Figure 6B), suggesting that PITALRE activity towards the GST–pRB exogenous substrate is positively regulated by proteins acting in the multimeric complexes (compare phosphorylation of PITALRE and GST–pRB in fractions containing either monomeric or complexed PITALRE; Figure 6B, lanes 8, 10 and 11 compared with lane 14).

The PITALRE-associated proteins may modulate PITALRE activity and/or its ability to recognize substrates, suggesting the possibility that a cyclin-like protein or an analogous regulatory polypeptide interacts with PITALRE. Two kinds of CDC2-related kinase regulatory subunits have been described hereto: cyclins [8] and the CDK5-associated p35 [10]. Cyclins associate specifically with CDKs to form active holoenzymes that are generally activated at specific stages of the cell division cycle. It has also been proposed that cyclins may confer substrate specificity [9]. In contrast, p35 is a protein that is preferentially expressed in post-mitotic neuronal cells and which binds to and activates CDK5, indicating a function for this holoenzyme outside the cell cycle [10]. Other CDC2-related kinases such as PITSLRE [16] and PCTAIRE(s) [17,18] contain other domains in addition to the catalytic core, suggesting that a regulatory subunit may not be required for their kinase activity. Moreover, other serine/threonine kinases of molecular mass similar to that of the PITALRE kinase, such as mitogen-activated protein kinases, are known to be active as monomers [19].

PITALRE appears to be active as a monomer, although most PITALRE is complexed with other proteins even when it is several-fold overexpressed, and the PITALRE pRB-phosphorylating activity is much greater with the multimeric complexes. This suggests that association of PITALRE with other cellular proteins may modulate its activity. In this context, it is important to note that PITALRE overexpression does not result in increased total cellular PITALRE activity, indicating that cellular factors are limiting for PITALRE activity. It is, therefore, conceivable that specific PITALRE-associated proteins are rate-limiting for PITALRE and therefore unable to modulate the activity and/or to confer specific substrate recognition to the overexpresed PITALRE. However, additional points of control may exist, since monomeric PITALRE is active. In this context, the PITALRE-associated protein p95 is a good candidate for a regulatory subunit, since it has been found to co-purify with PITALRE in all the cell lines and tissues tested so far ([6], and results not shown). Interestingly, p95 levels in PITALRE immunoprecipitates do not increase with PITALRE overexpression (Figures 2A and 2B), which is in agreement with a rate-limiting function for p95 on the kinase activity of PITALRE. In contrast, the 48 and 98 kDa proteins identified in PITALRE immunocomplexes from 293 cells overexpressing PITALRE-HA do not appear to be limiting, since their detection is dependent on the overexpression of PITALRE and their levels increase concomitantly with the increase in PITALRE expression (Figures 2A and 2B). The identification and characterization of PITALRE-associated proteins will be critical to our understanding of PITALRE regulation and will provide new clues regarding the cellular function of PITALRE.

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REFERENCES

1 Krebs, E. G. (1994) Trends Biochem. Sci. **19**, 439–440
2 Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) Science **241**, 42–52
3 Lindberg, R. A., Quinn, A. M. and Hunter, T. (1992) Trends Biochem. Sci. **17**, 114–119
4 Pines, J. (1993) Trends Biochem. Sci. **18**, 195–197
5 Brambilla, R. and Draetta, G. (1994) Oncogene **9**, 3037–3041
6 Graña, X., De Luca, A., Sang, N., Fu, Y., Claudio, P. P., Rosenblatt, J., Morgan, D. O. and Giordano, A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3834–3838
7 Graña, X., Claudio, P. P., De Luca, A., Sang, N. and Giordano, A. (1994) Oncogene **9**, 2097–2103
8 Graña, X. and Reddy, E. P. (1995) Oncogene **11**, 211–219
9 Morgan, D. O. (1995) Nature (London) **374**, 131–134
10 Lew, J. and Wang, J. H. (1995) Trends Biochem. Sci. **20**, 33–37
11 Mayol, X., Garriga, J. and Graña, X. (1995) Oncogene **11**, 801–808
12 Ausubel, F. M., Brent, R., Kington, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.) (1988) Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York
13 Kaelin, W. G., Pallas, D. C., DeCaprio, J. A., Kaye, F. J. and Livingston, D. M. (1991) Cell **64**, 521–532
14 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
15 van den Heuvel, S. and Harlow, E. (1993) Science **262**, 2050–2054
16 Bunnell, B. A., Heath, L. S., Adams, D. E, Lathi, J. M. and Kidd, V. J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 7467–7471
17 Meyerson, M., Enders, G. H., Wu, C.-L., Su, L.-K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L.-H. (1992) EMBO J. **11**, 2909–2917
18 Okuda, T., Cleveland, J. L. and Downing, J. R. (1992) Oncogene **7**, 2249–2258
19 Pelech, S. L. and Sanghera, J. S. (1992) Science **257**, 1355–1356