Regulation of the CDK-related protein kinase PCTAIRE-1 and its possible role in neurite outgrowth in Neuro-2A cells

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Accepted 12 June 2002
Journal of Cell Science 115, 3479-3490 (2002) © The Company of Biologists Ltd

Summary

PCTAIRE-1 is a CDK-related protein kinase found in terminally differentiated cells in brain and testis, and in many immortalised and transformed cell lines. Bacterially expressed PCTAIRE is completely inactive as a protein kinase, but is a very good substrate for protein kinase A (PKA), which phosphorylates a total of four sites in the N-terminus of PCTAIRE-1. Phosphorylation of one of these sites, Ser119, generates a 14-3-3 binding site, which is functional in vitro as well as in vivo. Mutation of another PKA site, Ser153, to an alanine residue generated an activated kinase in transfected mammalian cells. This activity was comparable to that of CDK5 activated by a bacterially expressed, truncated version of p35^{nck}, p21. Gel filtration analysis of a brain extract suggested that monomeric PCTAIRE-1 was the active species, implying that PCTAIRE-1 may not be a true CDK, in that it does not require a partner (cyclin-like) subunit for kinase activity. Finally, we found that various forms of PCTAIRE-1 transfected into neuroblastoma cell lines could either promote or inhibit neurite outgrowth, suggesting a potential role for the PCTAIRE-1 gene product in the control of neurite outgrowth.

Key words: PCTAIRE-1, CDK-related, Protein kinase A, 14-3-3, Neurite outgrowth

Introduction

The cell cycle is an ordered process that is controlled mainly by a subset of protein kinases, the cyclin-dependent protein kinases or CDKs. Initially, Cdc2 (now CDK1), in association with a variety of different cyclin partners was thought to regulate progression through the individual phase transitions of the cell cycle. Soon afterwards, however, a larger set of CDKs and related kinases was discovered in humans (Meyerson et al., 1992). There is clear evidence that CDK2, CDK4 and CDK6 also play important roles in cell cycle regulation (Ekholm and Reed, 2000). CDK7 was found to activate CDK1, as well as CDK2 and CDK4, but it also plays a role in transcriptional activation forming a complex with transcription factor IIH, which phosphorylates the C-terminal domain of RNA polymerase II (Matsuoka et al., 1994; Poon et al., 1993; Roy et al., 1994). CDK8 forms a complex with cyclin C, and represses transcription via negatively regulating CDK7/cyclin H (Akoulitchev et al., 2000; Tassan et al., 1995). CDK9 in a complex with cyclin T forms pTEFb, a human transcription elongation factor, which also phosphorylates the C-terminal domain of RNA polymerase II, and was found to be required for HIV-1 Tat transactivation in vitro and in vivo (Gold et al., 1998; Wei et al., 1998). However, CDK5 does not play a role in proliferating cells; it is expressed and is active in terminally differentiated cells. Nevertheless, it does form a complex with a cyclin-like molecule, and is therefore a real CDK (Lew et al., 1994; Nikolic et al., 1996; Ohshima et al., 1996; Tsai et al., 1993). Others, like the PITSLRE kinases (Lahti et al., 1995b), are cleaved in cells undergoing apoptosis (Lahti et al., 1995a) but no cyclin partner has been identified yet.

Messenger RNAs encoding the 45-58 kDa PCTAIRE family of protein kinase subunits have been identified in frogs, mice and humans, and consist of two or three isoforms. The mRNAs for PCTAIRE-2 and 3 are found almost exclusively in the brain, whereas PCTAIRE-1 is located in both brain and testis of adult mice and rats (Hirose et al., 1997; Rhee and Wolgemuth, 1995). Unlike CDK1, which has only 10 residues before the Walker A motif, the PCTAIRE family have 127-198 residues in N-terminal extensions (Meyerson et al., 1992; Okuda et al., 1992). No cyclin partner has been identified for any of the PCTAIREs, and these extensions may play the role of an attached cyclin for these kinases, although they do not display obvious homology to known cyclin sequences. However, interacting proteins have been identified in 2-hybrid screens; Sladeczek and co-authors isolated 14-3-3 proteins and the annexin II-interacting protein p11 in a screen using the full-length mouse PCTAIRE-1 cDNA as a bait for a mouse brain cDNA library (Sladeczek et al., 1997). Although the interaction of PCTAIRE-1 with the 14-3-3 proteins was strong enough to allow the affinity purification of PCTAIRE-1 (Le Bouffant et al., 1998), no functional

significance could be ascribed in the absence of any clues about the function of PCTAIRE-1 itself. In another 2-hybrid screen, which used the isolated N-terminal region of rat PCTAIRE-2, a protein was pulled out with tudor repeats that showed some sequence homology to AKAP proteins (Hirose et al., 2000). This protein, called Trap, interacted in vitro with PCTAIRE-1 and -2, but not with PCTAIRE-3. Trap co-localized with PCTAIRE-2 in the brain (Hirose et al., 2000), but the kinase activity of PCTAIRE-2 was not influenced by Trap (Hirose et al., 2000). Thus, the PCTAIREs do form complexes with a number of other proteins, but none of them seem to act as a PCTAIRE-specific cyclin.

Although expression of the PCTAIREs is restricted to the brain and testis, our attention was aroused by the finding that high levels of expression occurred in a wide range of transformed and immortalised cell lines of epithelial origin (Charrasse et al., 1999; Hirose et al., 1997; Rhee and Wolgemuth, 1995). There is great uncertainty about the function of the PCTAIREs, some authors suggesting a role in the cell cycle (Charrasse et al., 1999), others in the differentiation of neurons or testicular cells (Besset et al., 1999; Rhee and Wolgemuth, 1995). Equally, the natural substrate for this family of protein kinases is not known, but some investigators found that PCTAIRE-1, and PCTAIRE-2 could phosphorylate histone H1 (Hirose et al., 1997; Le Bouffant et al., 1998), whereas others only detected MBP phosphorylation (Besset et al., 1999; Rhee and Wolgemuth, 1995). The levels of kinase activity, compared to CDK1 or protein kinase A (PKA), are extremely low. This makes it difficult to be sure that the observed kinase activity is due to PCTAIRE, rather than traces of contaminating protein kinases. At least one of the antibodies used in studies of PCTAIRE recognises a completely unrelated protein (Le Bouffant et al., 1998).

We originally came across PCTAIRE in an early screen for CDK homologues in *X. laevis* that revealed two frog homologues of PCTAIRE (R. Y. C. Poon, Cyclin-dependent kinase family and its regulation, PhD. thesis, University of Cambridge, 1993). The protein was only detectable after the start of neurogenesis, and had no detectable kinase activity when expressed in bacteria. We discovered the overexpression of PCTAIRE-1 in cell lines, and decided to revisit the subject, in case there might be a connection with cellular transformation. This paper presents our finding about the mode of activation and function of PCTAIRE-1 in cells. We present evidence that PCTAIRE-1 is regulated by PKA and may play a role in the control of neurite outgrowth.

Materials and Methods

PCTAIRE-1 constructs

The human PCTAIRE-1 cDNA was isolated from HEK 293 cells in a RT-PCR using the primers (5′PCT1 CGATCGCCATGGATCGGAT-GAAGAAGATCA and 3′PCT1 GGGCCTCGGTCTTAGGTCAT-GAACTCGGT). The PCTAIRE-1 ORF was confirmed by sequencing. For bacterial expression, a double c-myc tag was fused to the C-terminus of PCTAIRE-1, followed by six histidine residues. This construct was inserted into pET 21d using *Nco*I/*Hin*dIII sites. For mammalian expression, the PCTAIRE-1 ORF was fused upstream of YFP by ligating the *Nco*I/*Bsp*HI digested ORF into the *Nco*I site of pEF YFP. Site-directed mutagenesis was performed by PCR (Ausubel et al., 1999).

Expression of PCTAIRE-1 in *E. coli* and phosphorylation by brain extracts

The PCTAIRE-1 constructs in pET 21d were transformed into BL21(DE3), and the proteins were expressed and purified using nickel agarose according to standard methods (Crowe et al., 1994; Hochuli et al., 1987).

Lysates of brain or testis from mice were produced by homogenizing fresh tissue in an equal volume of lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin and leupeptin). Aliquots of these extracts containing 100 μg of total protein were incubated with bacterial extracts containing 1 μg of PCTAIRE-1 for 30 minutes at 30°C. The reaction was diluted with buffer A (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM DTT), and the recombinant PCTAIRE-1 recovered by affinity chromatography using

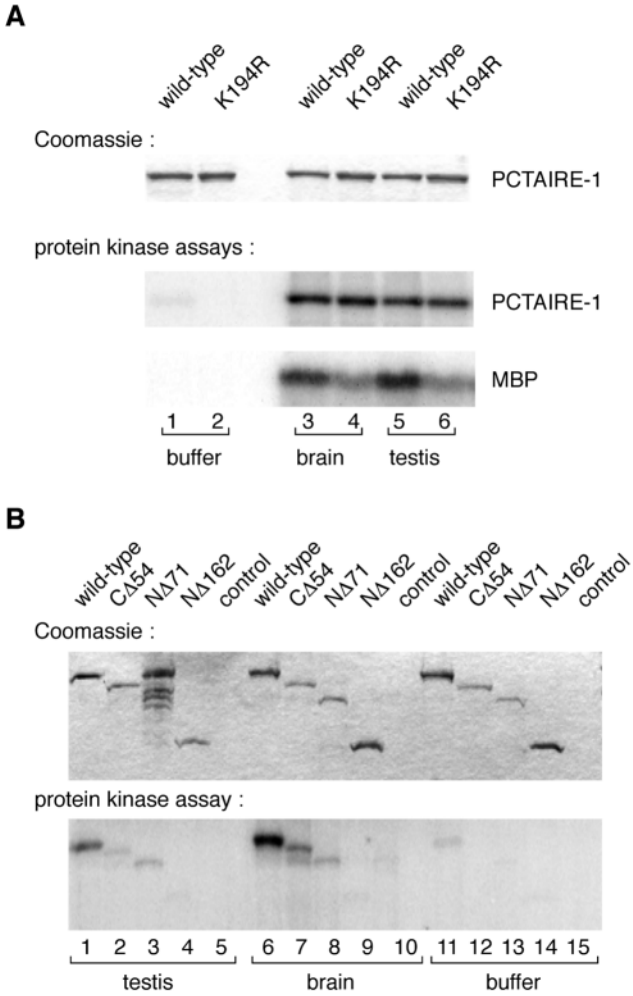

Fig. 1. Activation and phosphorylation of recombinant bacterially expressed PCTAIRE-1 in brain and testis extracts. (A) Bacterial lysates containing either wild-type or kinase-dead myc-tagged PCTAIRE-1 proteins were incubated in brain or testis extracts or buffer, and the bound material was assayed for protein kinase activity towards myelin basic protein (MBP). (top panel) Coomassie-blue-stained gel; (middle panel) autoradiograph at the position of PCTAIRE-1; (bottom panel) autoradiograph at the position of MBP. (B) Purified wild-type and truncated recombinant myc-tagged PCTAIRE-1 proteins were incubated in brain and testis extracts or buffer, immunoprecipitated, and incubated in the presence of [γ-^{32}P]ATP. (top panel) Coomassie-blue-stained gel; (bottom panel) autoradiograph at the position of PCTAIRE-1.

monoclonal antibody 9E10 covalently coupled to Affi-Prep beads (BioRad) (Schneider et al., 1982). The beads were washed twice with buffer A, twice with buffer A plus 500 mM NaCl, twice with buffer C (50 mM Tris pH 7.4, 15 mM $MgCl_2$) and assayed for kinase activity as described below.

To investigate the interaction of PCTAIRE-1 with GST-tagged 14-3-3 proteins [produced as described (Dubois et al., 1997)], the PCTAIRE-1 proteins were bound to 9E10 beads as described above. The beads were then incubated with 1 unit of purified PKA (Sigma) or buffer, washed twice with buffer A, incubated with purified GST-tagged 14-3-3 γ or ζ, washed again with buffer A, and the bound material was analysed by SDS-PAGE and immunoblotting using an antiserum against GST.

Cell lines, transfections and immunoprecipitations

For most experiments involving expression in mammalian cells, the cell line Neuro-2A (ATCC CCL-131) was used (Klebe and Ruddle, 1969). Transfections were carried out using $CaPO_4$ as described (Ausubel et al., 1999). Briefly, the precipitates were left on the cells for 16 hours, the medium was replaced, and the cells were left for 48 hours to express the transfected construct. Transfection efficiencies were generally around 30-50%. All other cell lines were transfected with Superfect (Gibco) according to the manufacturer's instructions.

For immunoprecipitations, the cells were extracted in lysis buffer, the insoluble material was removed by centrifugation, and the YFP-tagged PCTAIRE-1-constructs were immunoprecipitated using a rabbit serum against GFP covalently coupled to Affi-Prep protein A beads with dimethylpimelimidate (Schneider et al., 1982). The material bound to the beads was washed twice with buffer A, twice with buffer A plus 500 mM NaCl, and twice with buffer C.

Kinase assays

Proteins bound to beads were then assayed for kinase activity by incubating them in 10 μl of buffer C containing 1 mg/ml myelin basic protein (MBP), 20 μM ATP, 1 mM DTT and 1 μCi [γ-^{32}P]ATP for 30 minutes at 30°C.

Mass spectrometry analysis of phosphorylation sites

Recombinant PCTAIRE phosphorylated by PKA was loaded on a 12.5% SDS-PAGE to separate the phosphorylated protein from ATP. PCTAIRE was visualised on the gel by GelCODE (Pierce) staining and digested with trypsin as described (Aitken and Learmonth, 1997).

High performance liquid chromatography-electrospray mass spectrometry (LC-ESMS) was performed as follows. A modified Perkin Elmer/ABI 130A HPLC was coupled to a Micromass Platform electrospray mass spectrometer. Chromatography was performed with a Vydac C18 0.25×250 mm PEEK column (slurry packed in-house) at a flow rate of 10 ml/minute over a 0-60% B linear gradient in 60 minutes [buffer A is 0.12% formic acid, 98% water and 2% acetonitrile (ACN); buffer B is 10% formic acid, 15% water and 85% ACN] with in-line UV monitoring at 215 nm. The low flow rate was achieved by incorporating a valco tee stream splitter coupled to a microbore dummy column, just prior to the Rheodyne injection. For the identification of the phosphorylation sites, reverse phase chromatography was performed on a Beckman Gold HPLC using a 4.6×250 mm Merck superspher 100 RP18 end-capped column at 0.8 ml/min and a 1%/minute gradient [buffer A is 0.10% trifluoroacetic acid (TFA); buffer B is 0.10% TFA, 15% water and 85% ACN]. Fractions were dried then resuspended in 50% ACN, 0.10% formic acid and analysed by ESMS. The elution positions of the ^{32}P-labeled peptides were determined by Cerenkov counting and the phosphopeptide fractions were analysed by electrospray mass spectrometry as described (Dubois et al., 2001).

Results

Bacterially expressed PCTAIRE-1 is phosphorylated by brain and testis extracts

Although PCTAIRE-1 was expressed as a soluble protein in bacteria, it had no detectable activity in protein kinase assays with myelin basic protein (MBP) as substrate. A very faint autophosphorylation could be detected in the wild-type, but not the K194R (kinase dead) mutant (Fig. 1A, lanes 1,2). This lack of activity might be: (1) due to misfolding of the protein; (2) (by analogy with CDKs) because an activating cyclin-like subunit was missing; (3) because PCTAIRE-1 requires an activating phosphorylation on its T-loop; or (4) due to a combination of these defects. To test whether activators were present in mammalian cell extracts where PCTAIRE-1 is normally found, we mixed bacterially expressed PCTAIRE-1 with extracts prepared from mouse testis or brain. The recombinant PCTAIRE-1 protein was recovered using its c-myc tag, and the bound material was assayed with [γ-^{32}P]ATP and MBP as substrate (Charrasse et al., 1999). Fig. 1A shows that both the wild-type and the kinase-dead PCTAIRE-1 were strongly phosphorylated under these conditions, indicating the presence of a tightly-bound protein kinase in the immunoprecipitates. Nevertheless, the MBP was more

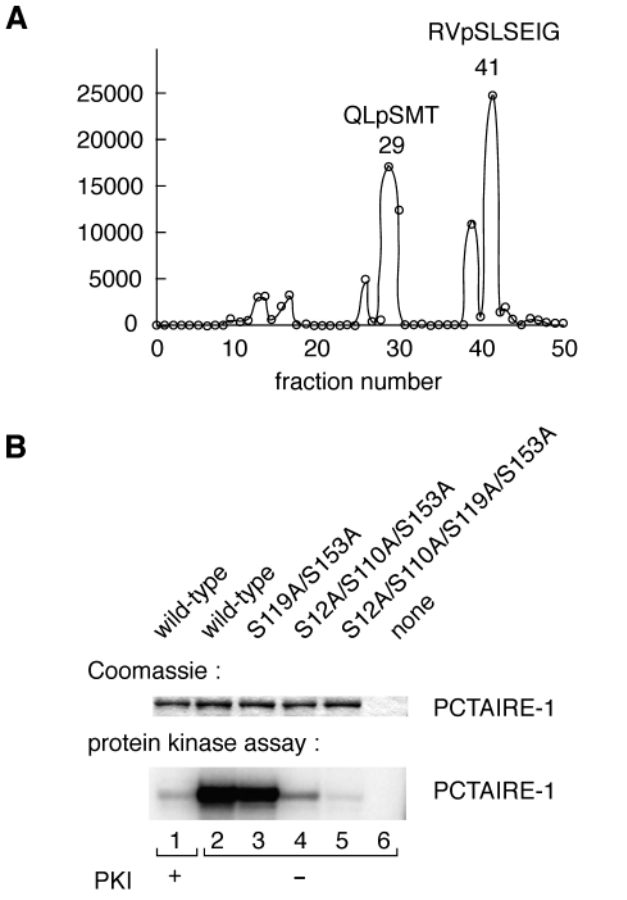

Fig. 2. PKA phosphorylates PCTAIRE-1. (A) Elution profile of labeled tryptic peptides of PCTAIRE-1, prepared as described in Materials and Methods. (B) Wild-type, or mutated recombinant PCTAIRE-1 proteins were tested as PKA substrates using 1 U of purified PKA. (top panel) Coomassie-blue-stained gel; (bottom panel) autoradiograph at the position of PCTAIRE-1.

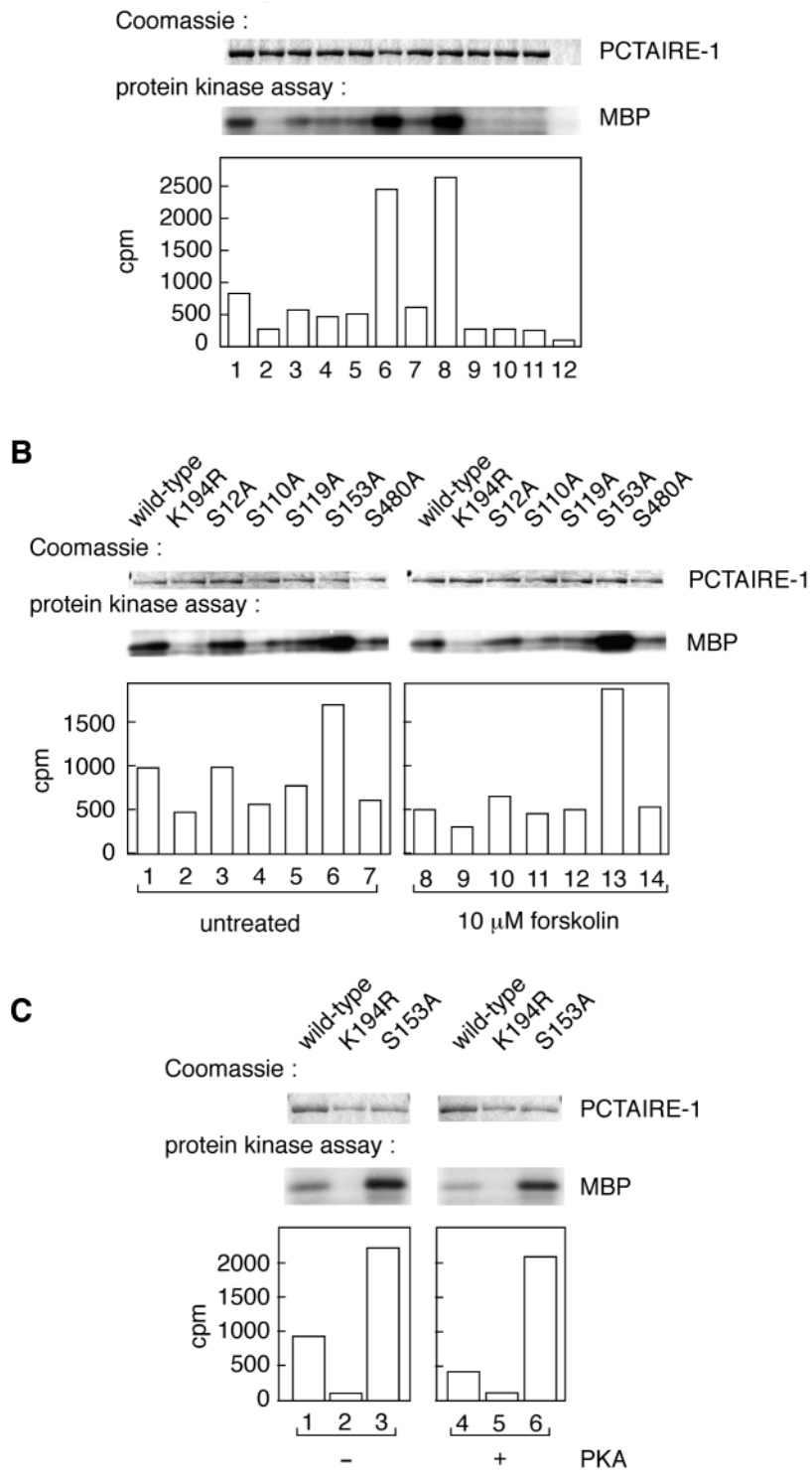

Fig. 3. Phosphorylation of S153 by PKA inhibits PCTAIRE-1 kinase activity. (A) Constructs encoding wild-type or mutated YFP-tagged PCTAIRE-1 were transfected into Neuro-2A cells. 48 hours after transfection, the cells were lysed, the YFP-tagged PCTAIRE-1 proteins immunoprecipitated using a rabbit serum against GFP, and the bound material was assayed for kinase activity towards MBP. (top panel) Coomassie-blue-stained gel; (middle panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the MBP bands. (B) Constructs encoding wild-type and YFP-tagged PCTAIRE-1 were transfected into Neuro-2A cells. 48 hours after transfection, the cells were treated with 10 μM forskolin to stimulate PKA for 2 hours, or left untreated. Cells were lysed, the YFP-tagged PCTAIRE-1 proteins were immunoprecipitated using a rabbit serum against GFP and assayed for kinase activity towards MBP. (top panel) Coomassie-blue-stained gel; (middle panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the MBP bands. (C) Constructs encoding wild-type and mutated YFP-tagged PCTAIRE-1 were transfected into Neuro-2A cells. 48 hours after transfection, the cells were lysed, the YFP-tagged PCTAIRE-1 proteins immunoprecipitated using a rabbit serum against GFP, and the bound material was incubated in the presence or absence of PKA, washed, and then assayed for kinase activity towards MBP. (top panel) Coomassie blue stained gel; (middle panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the MBP bands.

phosphorylated in reactions containing wild-type PCTAIRE-1 protein than in those containing the kinase-dead mutant, although significant background labelling was present. Apart from the recombinant PCTAIRE-1, no other protein was recovered in stoichiometric yield in the immune complexes (data not shown). Since the observed stimulation of PCTAIRE-1 kinase activity may be the result of an activating phosphorylation, we mapped the phosphorylation sites in PCTAIRE-1. Various truncated forms of the protein were therefore expressed in bacteria, and phosphorylated using brain and testis extracts as before. Fig. 1B shows that the construct lacking the N-terminal 162 residues was no longer a substrate in this assay, whereas CΔ54 and NΔ71 were still phosphorylated. This suggested that the phosphorylation sites, and perhaps the binding site for the tightly-bound contaminating protein kinase were to be found in the N-terminus of PCTAIRE-1 (Fig. 1B).

PCTAIRE-1 is phosphorylated by protein kinase A (PKA)

The recombinant PCTAIRE-1 phosphorylated by the tightly-bound kinase was analysed by mass spectrometry, which identified two serine residues, S12 (KRQLSM) and S153 (RRVSL) as the major sites of phosphorylation (Fig. 2A). Both sites conformed to the recognition sequence for phosphorylation by PKA, R-R/K-X_{1-2}-hydrophobic-S/T, -R-X_{1-2}-S/T-, or -R-R/K-X-S/T-hydrophobic, where hydrophobic stands mainly for I, L or V residues. Inspection of the N-terminus revealed two additional PKA sites, S110 (RKIST) and S119 (KRLSLP). We noted that the latter site might form part of a 14-3-3 binding motif (RLpSLP), consistent with the report that PCTAIRE-1 interacted with 14-3-3 proteins in a 2-hybrid screen (Sladeczek et al., 1997).

In order to test whether PCTAIRE-1 was a substrate for

PKA, we incubated bacterially expressed proteins with purified PKA. Mutants were prepared that had S119A/S153A, S12A/S110A/S153A or all four potential PKA phosphorylation sites replaced by alanines. Fig. 2B shows that wild-type PCTAIRE-1 was an excellent substrate for PKA, and addition of the PKA inhibitor, PKI, reduced the phosphorylation of PCTAIRE-1 to the background autophosphorylation level (lane 1). The quadruple mutant could no longer be phosphorylated (lane 5), but S12A/S110A/S153A was still weakly labelled, showing that PKA was able to phosphorylate PCTAIRE-1 on its putative 14-3-3 binding site, S119 (lane 4). However, it should be noted that S12 and S153 are much stronger phosphorylation sites under the conditions of this experiment, as found for the kinase derived from the extract. The N-terminus of PCTAIRE-1 has other potential phosphorylation sites, and we found evidence for the existence of additional contaminating protein kinase(s) when the protein was expressed in neuroblastoma cell lines. As this activity was unable to phosphorylate MBP, its origin was not explored further. However, we should note that the activity of these substoichiometric, tight-binding protein kinases was often comparable with that of PCTAIRE-1 itself, depending on the substrate used, and could give misleading results if misinterpreted.

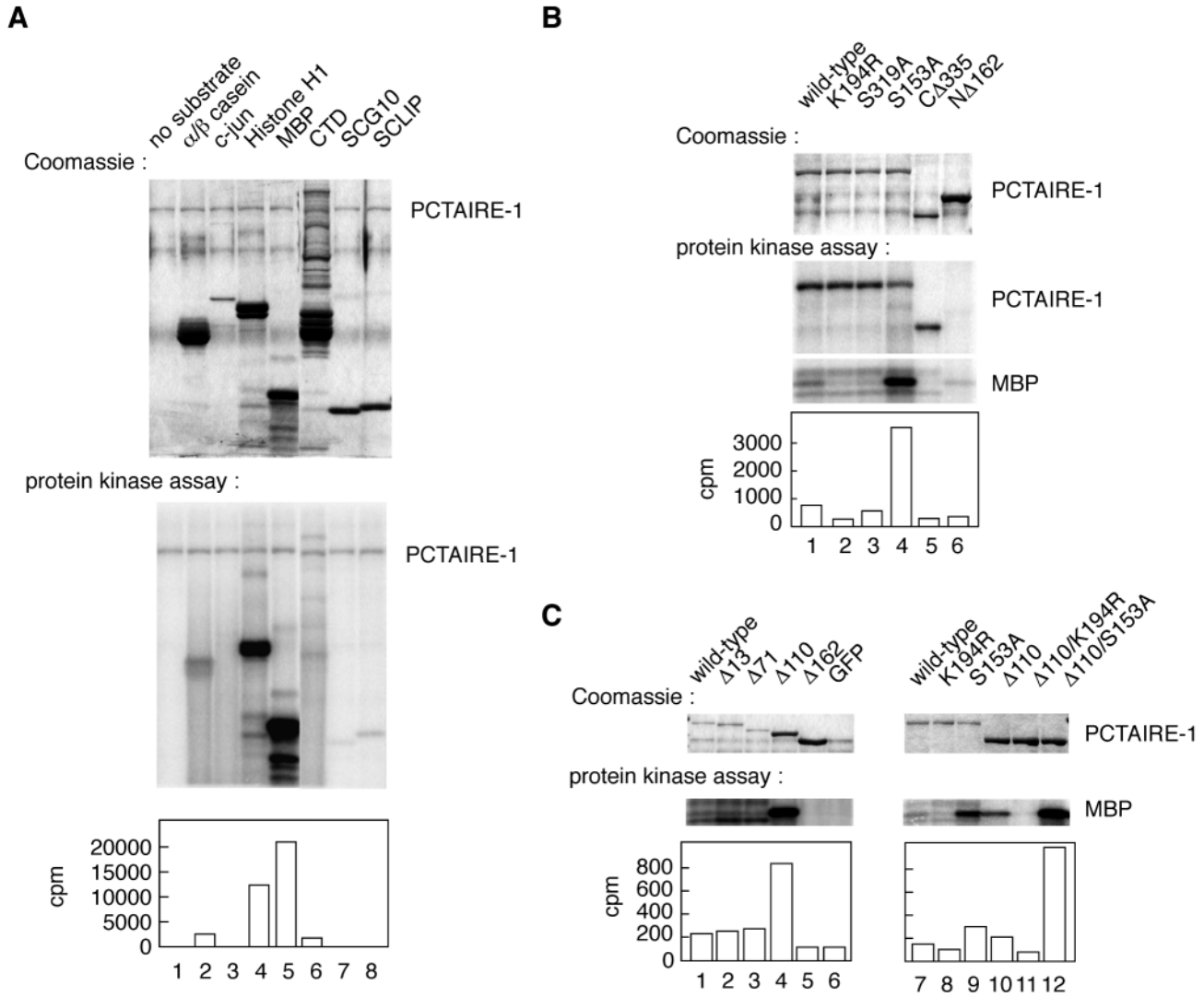

Fig. 4. PCTAIRE-1 kinase activity – substrates and structural requirements. (A) Neuro-2A cells were transfected with S153A mutated YFP-tagged PCTAIRE-1. 48 hours after transfection, the cells were lysed, the YFP-tagged PCTAIRE-1 proteins immunoprecipitated using a rabbit serum against GFP, and the bound material was assayed for kinase activity towards various substrates. (top panel) Coomassie-blue-stained gel; (middle panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the substrate bands. (B) Neuro-2A cells were transfected with constructs encoding wild-type, mutated and truncated YFP-tagged PCTAIRE-1. Two days after the transfection, the cells were lysed and the YFP-tagged PCTAIRE-1 proteins were immunoprecipitated using a rabbit serum against GFP, washed, and assayed for kinase activity towards MBP. (top panel) Coomassie-blue-stained gel; (second panel) autoradiograph at the position of PCTAIRE-1; (third panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the MBP bands. (C) Neuro-2A cells were transfected with constructs encoding N-terminally truncated, kinase-dead and S153A mutated YFP-tagged PCTAIRE-1. Two days after the transfection, the cells were lysed, and the YFP-tagged PCTAIRE-1 proteins were immunoprecipitated using a rabbit serum against GFP, washed, and assayed for kinase activity towards MBP. (top panel) Coomassie-blue-stained gel; (middle panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the MBP bands.

PKA phosphorylation of PCTAIRE-1 on S153 inhibits its kinase activity

Incubation of the bacterially expressed PCTAIRE-1 with PKA and ATP did not stimulate the MBP kinase activity of the PCTAIRE-1 (data not shown). To check whether PKA phosphorylation had an effect on the kinase activity of PCTAIRE-1, wild-type, kinase-dead, and PKA phosphorylation-site mutated forms of PCTAIRE-1 were introduced into Neuro-2A cells by transfection. Two days later, the cells were harvested, lysed, and the YFP-fused PCTAIRE-1 was immunoprecipitated with antibodies against GFP covalently coupled to Affi-prep protein A beads. The beads were then assayed for kinase activity towards MBP. Fig. 3A shows that wild-type PCTAIRE-1 was active in this assay, whereas the K194R and YFP-alone controls were not (compare lane 1 with 2 and 12). Three out of the four PKA

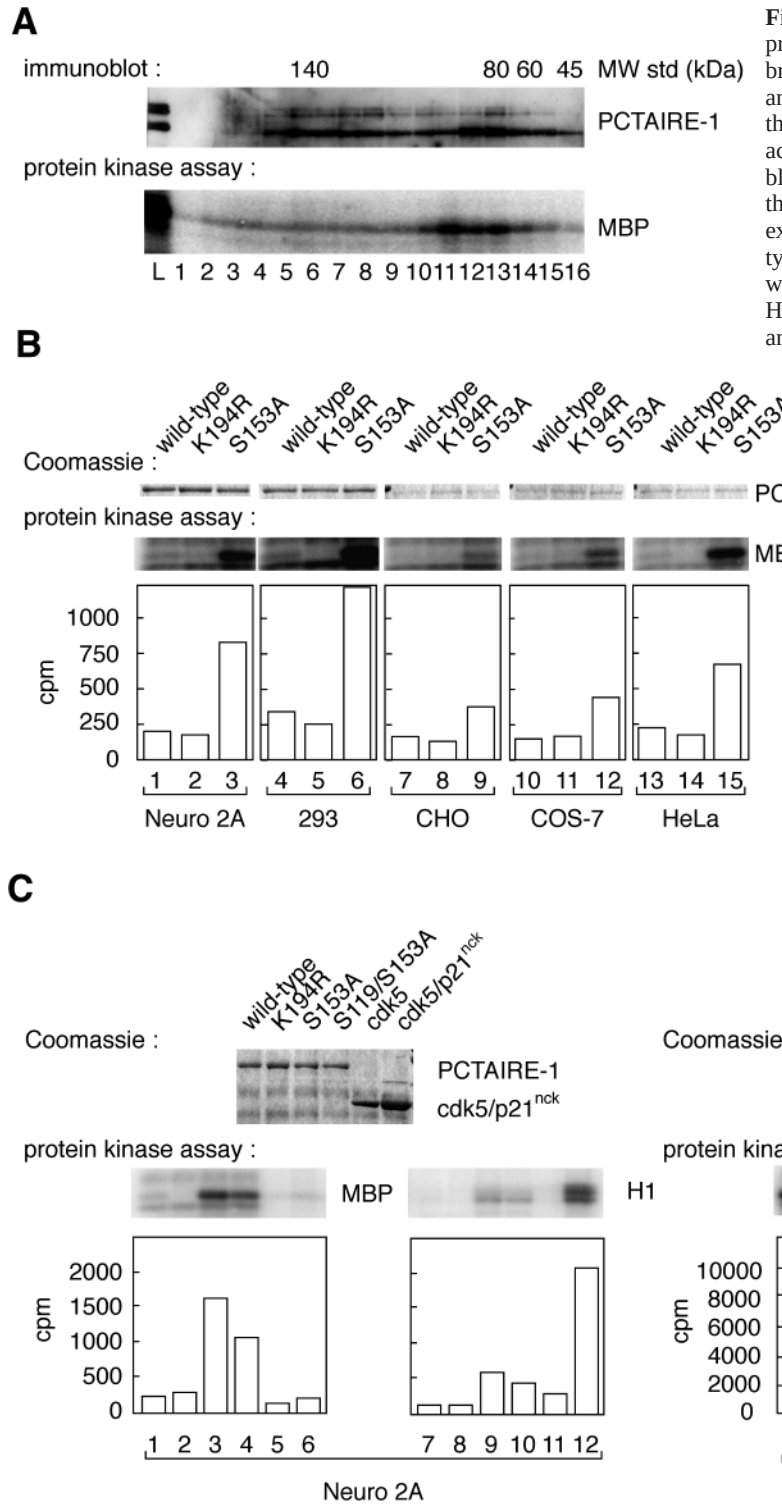

Fig. 5. Does PCTAIRE-1 require a brain-specific partner protein? (A) Gel filtration analysis of an extract from mouse brain. The lysate prepared from one mouse brain was applied on an AcA34 column, PCTAIRE-1 was immunoprecipitated from the individual fractions using mAb G6, and assayed for kinase activity. The reaction mix was then applied on an SDS-PAGE, blotted, and checked with a polyclonal PCTAIRE-1 antibody for the presence of PCTAIRE-1. Subsequently, the blot was exposed for autoradiography. (B) Constructs encoding wild-type, kinase-dead, or S153A mutated YFP-tagged PCTAIRE-1 were transfected into Neuro-2A, HEK 293, CHO, COS-7 and HeLa cells. Two days after the transfection, the cells were lysed, and the YFP-tagged PCTAIRE-1 proteins were immunoprecipitated using a rabbit serum against GFP, and assayed for kinase activity on MBP. (top panel) Coomassie-blue-stained gel; (middle panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the MBP bands. The S153A mutant PCTAIRE-1 protein kinase is active in a wide range of cell lines. (C) Constructs encoding wild-type, kinase-dead, S153A, S119A/S153A mutated YFP-tagged PCTAIRE-1 and YFP-tagged CDK5, were transfected into Neuro-2A and HEK 293 cells. Two days after the transfection, the cells were lysed, and the YFP-tagged proteins were immunoprecipitated using a rabbit serum against GFP, and assayed for kinase activity towards MBP. $p21^{nck}$ was added to reactions with CDK5 as indicated. (top panel) Coomassie-blue-stained gel; (middle panel) autoradiograph at the position of MBP; (bottom panel) radioactivity (cpm) in the MBP bands. Note that PCTAIRE-1 and CDK5/$p21^{nck}$ display comparable kinase activity.

phosphorylation site mutants had somewhat reduced activity (S12A, S110A, and S119A; lanes 3-5), but the S153A mutation increased the protein kinase activity of the immunoprecipitated PCTAIRE-1 by at least three-fold (lane 6). The increased activity of the S153A mutant can be accounted to PCTAIRE-1 kinase activity, as the S153A/K194R double mutant had no detectable protein kinase activity (lane 10).

These results suggested that phosphorylation of PCTAIRE-1 on S153 might regulate its kinase activity. To check this, wild-type YFP-PCTAIRE-1 was transfected into Neuro-2A cells. After 48 hours, the cells were treated with 10 μM forskolin, which should increase PKA activity in the cells. Wild-type, S12A, S110A, S119A and S480A mutants showed about half the activity from forskolin-treated cells compared with the untreated control (Fig. 3B, compare lanes 1,3,4,5 with 8,10,11,12), whereas the S153A mutant showed the same increased activity with or without the forskolin treatment (lanes 6,13).

As a further test of these effects, YFP-PCTAIRE-1 was recovered from transfected Neuro-2A cells and incubated with purified PKA. Fig. 3C shows that wild-type PCTAIRE-1 kinase activity was reduced by 50%, whereas the S153A mutant, which was about twice as active as the wild-type enzyme, was not significantly affected by incubation with PKA.

Characterisation of the PCTAIRE-1 protein kinase

Previous investigators have reported that either MBP (Le Bouffant et al., 1998) or histone H1 (Rhee and Wolgemuth, 1995) were substrates for PCTAIRE-1. To resolve this discrepancy, we compared MBP, histone H1, casein, the C-terminal domain of RNA polymerase II, c-jun and two neuronal forms of stathmin as substrates for the YFP-tagged S153A mutant form of PCTAIRE-1 isolated from Neuro-2A cell lysates. Only MBP and histone H1 were good substrates for this form of PCTAIRE-1 (Fig. 4A, and data not shown). In our hands, MBP was a better substrate for PCTAIRE-1 than histone H1 (Fig. 4A, lanes 4,5) because, as mentioned previously, contaminating protein kinases seemed to prefer histone H1 over MBP.

Given a reliable source of PCTAIRE-1 and a reliable assay, we investigated the role of other parts of the molecule for its activity: in particular, the N-terminal extension and the role of S319 in the so-called activation or T-loop domain (the equivalent of T160 in CDK2). We produced two truncated versions of PCTAIRE-1 as YFP fusion proteins: the isolated N-terminus, residues 1-161-YFP, and the catalytic domain comprising residues 162-491-YFP. Neuro-2A cells were transfected with plasmids encoding these mutants as well as with wild-type, S153A and K194R full-length PCTAIRE-1-YFP. Fig. 4B shows that the S153A mutant displayed at least fourfold elevated kinase activity compared with the wild-type enzyme (lane 4), while both K194R and S319A mutants were completely inactive (lanes 2,3). The N-terminus of PCTAIRE-1 displayed significant autophosphorylation despite lacking a functional kinase domain, which must be due to a contaminating protein kinase that bound to the immunoprecipitate (lane 5). By contrast, the C-terminal portion of PCTAIRE-1, which includes the catalytic domain, was almost completely devoid of MBP kinase activity, and displayed no autophosphorylation (lane 6).

We next produced a series of N-terminal truncations of PCTAIRE-1. The constructs were transfected into Neuro-2A cells, and assayed for MBP kinase activity. Similar activity was shown by full-length and truncated versions up to residue 71 (Fig. 4C, lanes 1-3), but a version starting at residue 111 showed enhanced activity towards MBP, although this version was expressed at higher levels than the longer constructs (lane 4). The version of PCTAIRE-1 lacking all 162 residues of the N-terminus was catalytically inactive (lane 5). Thus, a minimum of 52 residues of the N-terminus are required for kinase activity. This region is well-conserved amongst all three PCTAIRE genes and contains both the potential 14-3-3-binding motif, and the phosphorylation site at S153. Lane 12 shows that the NΔ106/S153A mutant was about four times more active against MBP than the NΔ106 form, confirming the importance of S153 as a potential regulator of kinase activity.

Does PCTAIRE-1 kinase require a partner subunit?

Preparations of the PCTAIRE-1 protein kinase never contained

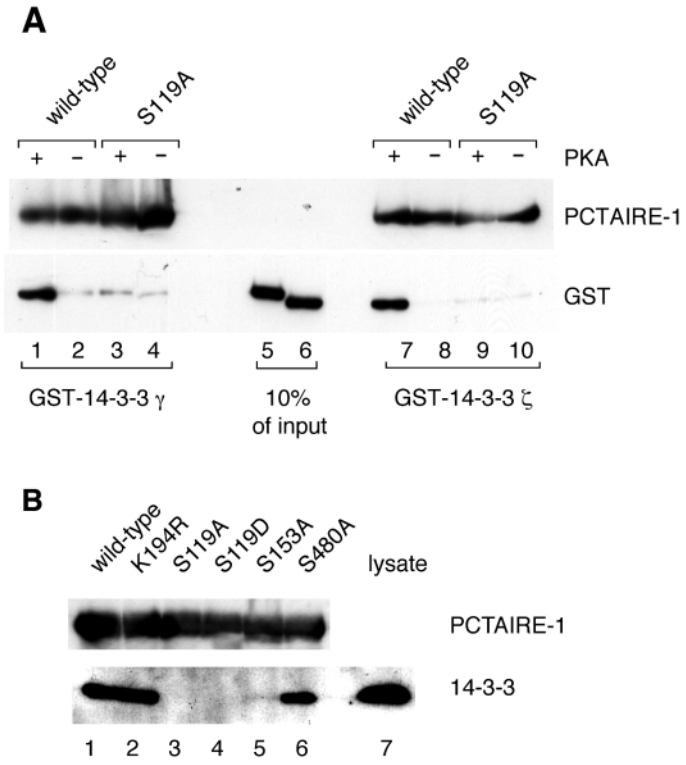

Fig. 6. Phosphorylation of S119 by PKA generates a 14-3-3 binding motif. (A) Wild-type and S119A mutated recombinant PCTAIRE-1 proteins were bound to 9E10 beads, incubated either in the presence or absence of 1U PKA, followed by incubation with GST-tagged 14-3-3 γ or ζ isoforms. The beads were washed, and the bound material was analysed by western blotting using antisera against PCTAIRE-1 (top panel) and GST (bottom panel). In lanes 5 and 6, 10% of the input of the respective 14-3-3 proteins were applied. (B) Constructs encoding wild-type or mutated YFP-tagged PCTAIRE-1 were transfected into Neuro-2A cells. Two days after the transfection, the cells were lysed, the YFP-tagged PCTAIRE-1 proteins were immunoprecipitated using a rabbit serum against GFP, and the bound material was checked for the presence of 14-3-3 proteins and PCTAIRE-1 by western blotting using antisera against 14-3-3-proteins (pan) and PCTAIRE-1, respectively.

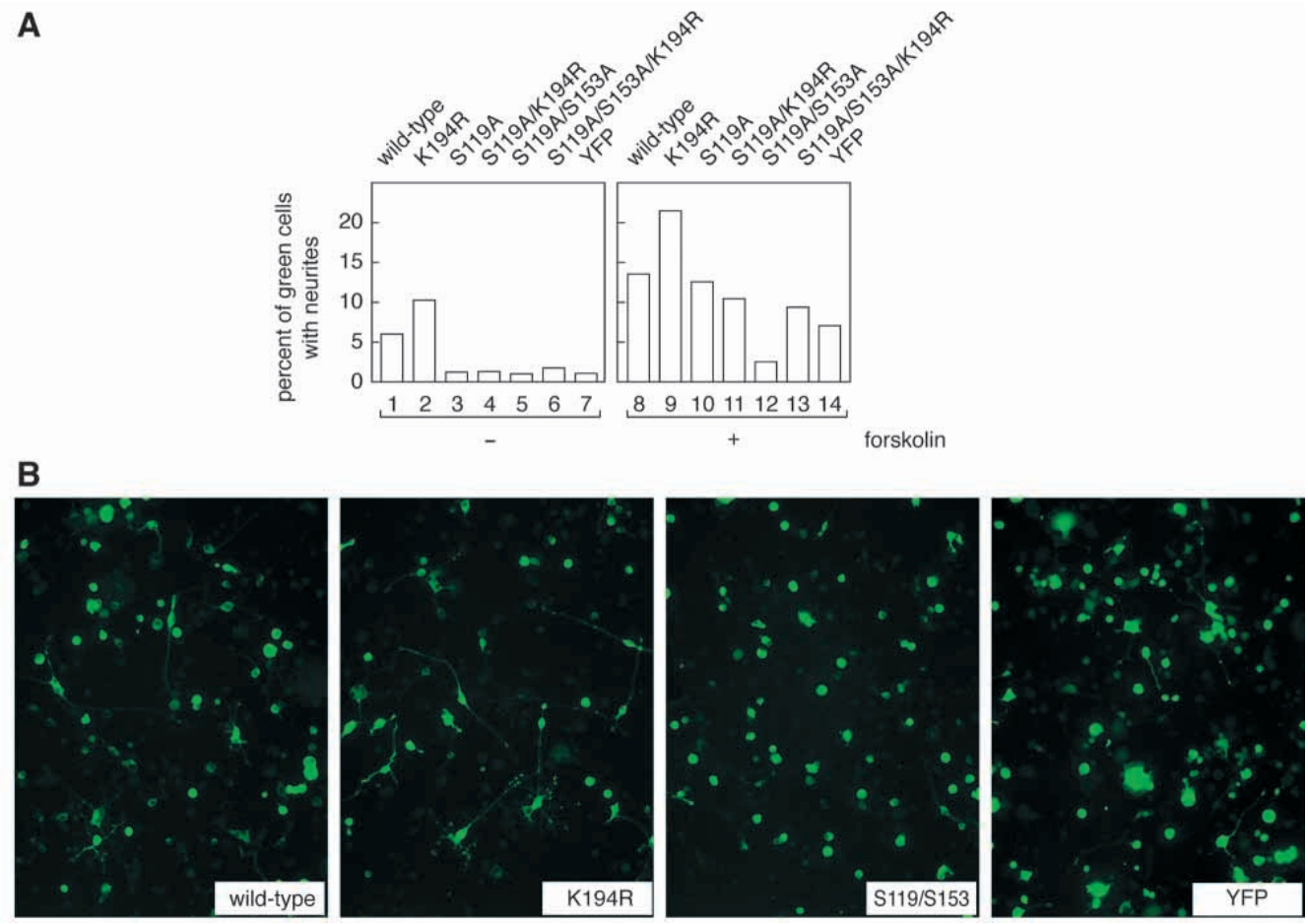

Fig. 7. Morphological effects of PCTAIRE-1-YFP constructs transfected into Neuro-2A cells suggest a role of PCTAIRE-1 in the prevention of neurite outgrowth. (A) Indicated constructs were transfected into Neuro-2A cells. Two days after transfection, the individual wells were scored for green cells that had neurites longer than twice the length of the cell body. The cells were then treated with 10 μM forskolin, left for 24 hours, and scored again. 5-10 fields were counted totalling 500-1000 transfected cells. The experiment was repeated twice with similar results. (B) Images of cells transfected with YFP, or wild-type, kinase-dead, or the double mutant S119/S153-YFP-PCTAIRE-1 constructs, showing the effects on neurite outgrowth.

significant amounts of other polypeptides, suggesting that the N-terminus might be acting as an intrinsic cyclin. By contrast, the kinase activity of PCTAIRE-1 was so low that it was possible that only very low levels of a heterologous activating subunit were present in the cells we were using as a source of the activity. To measure the size of the PCTAIRE-1 kinase activity we investigated the elution profile of endogenous PCTAIRE-1 in brain extracts by gel filtration. The PCTAIRE-1 protein was immunoprecipitated from the individual fractions using a monoclonal antibody specific for the N-terminus of PCTAIRE-1, G6, and the bound material was assayed for kinase activity. The reaction mix was then applied to an SDS-PAGE, and immunoblotted for PCTAIRE-1 using a polyclonal antibody against the C-terminus. Fig. 5A shows the elution pattern of PCTAIRE-1 on an AcA34 column, which suggested that some of the PCTAIRE-1 protein was present in high molecular weight complexes, but the active form of the enzyme eluted in the position expected for the monomeric protein.

An alternative way to test whether the PCTAIRE-1 protein kinase required a specific partner was to examine its activity in a variety of non-neuronal cell lines. Indeed, Besset et al. found that PCTAIRE-1 expressed in COS-7 cells lacked detectable activity, which they ascribed, by analogy with CDK5, to the absence of an activating partner subunit (Besset et al., 1999). Wild-type, K194R or the S153A mutants were introduced by transfection into a variety of cell lines, and tested for MBP kinase activity. Fig. 5B shows that in many cell lines, the wild-type protein was essentially inactive as a protein. However, the S153A mutant displayed significant kinase activity in all the cell lines we tested, suggesting that the lack of PCTAIRE-1 kinase activity was due to inactivation by PKA rather than a lack of a partner subunit (Fig. 5B, lanes 3,6,9,12,15).

For a comparison with a related neuronal protein kinase that is known to require an activating subunit, YFP-tagged CDK5 was expressed in either Neuro-2A cells or HEK 293 cells. Fig. 5C shows that CDK5 was inactive unless its partner, in the form of bacterially expressed GST-p21^{nck}, was added to the kinase assays. PCTAIRE-1 preferentially phosphorylated MBP, whereas CDK5 preferred histone H1, and the kinase activity of the S153A mutant was comparable with that of the activated CDK5.

The RLpS^{119}LP motif is a 14-3-3-binding site in vitro and in vivo

There is one report about a potential regulatory subunit for PCTAIRE-1: Sladeczek and co-workers reported that the PCTAIRE-1 protein interacted with 14-3-3 proteins (Sladeczek et al., 1997). The motif surrounding S119 (RLS*LP) might present a phosphorylation-dependent 14-3-3 protein-binding site. The ability of PKA to phosphorylate PCTAIRE-1 on S119 enabled us to check whether this created a 14-3-3 protein-binding site. We bound wild-type recombinant PCTAIRE-1 or the S119A mutant to 9E10 beads, incubated an aliquot of each with PKA and ATP, and finally added recombinant GST-14-3-3 γ and ζ proteins. The beads were washed, and the bound material was analysed by SDS-PAGE followed by immunoblotting using antiserum against GST. Both 14-3-3 isoforms bound to the PCTAIRE-1 protein in a phosphorylation-dependent manner (Fig. 6A). To check whether PCTAIRE-1 bound endogenous 14-3-3 proteins in vivo, neuroblastoma cells were transfected with constructs encoding various YFP-tagged forms of wild-type or mutant PCTAIRE-1. Fig. 6B shows that anti-GFP immunoprecipitates from lysates of cells expressing wild-type or K194R PCTAIRE-1 contained 14-3-3 proteins, which were absent from the immunoprecipitates of cells expressing the S119A mutant (compare lanes 1 and 2 with lane 3). Replacing S119 by aspartic acid to mimic a phosphorylated residue did not restore the binding (lane 4). Surprisingly, mutation of S153 also inhibited binding of 14-3-3 proteins (lane 5). By contrast, mutation of a serine residue in a second putative 14-3-3 binding site, S480, did not significantly affect 14-3-3 binding (lane 6).

However, it is unlikely that binding of 14-3-3 proteins regulated the kinase activity of PCTAIRE-1 directly, given the results shown previously in Fig. 3A. Both S119A and S153A mutants lost the ability to bind 14-3-3 proteins, yet the former mutation slightly inhibited and the latter strongly stimulated the kinase activity of PCTAIRE-1 (Fig. 3A, compare lanes 5 and 6).

Effect of transfected PCTAIRE-1 constructs on neurite outgrowth of Neuro-2A cells

Despite the lack of an effect on kinase activity, binding of 14-3-3 proteins might still play a role in the regulation of PCTAIRE-1 function in cells. Since PCTAIRE-1 is found mainly in brain and testis, we used the neuroblastoma cell line Neuro-2A for these examinations. Neuro-2A cells were transfected with a variety of PCTAIRE-1-YFP constructs and the effects on the morphology or the cell cycle distribution of the cells were examined 2 days later. No change was caused in the cell cycle distribution, as determined by FACS analysis, by any of the transfected constructs (data not shown). However, when the cells were scored for neurite outgrowth (cells with neurite extensions longer than twice the size of the cell body were scored positive), we noticed that the K194R mutant induced neurite outgrowth in about 10 percent of the transfected cells (Fig. 7A, lane 2). A similar, but less pronounced effect could also be observed in cells transfected with wild-type PCTAIRE-1.

It is known that addition of forskolin to the medium tends to induce neurite outgrowth in a neuroblastoma cell line (Shea et al., 1992). Given the apparent link between PCTAIRE-1 and PKA, we checked the combined effects of forskolin and PCTAIRE-1 expression by adding the drug to cells 48 hours after transfection. After a further 16 hours of incubation, 5-20% of the transfected cells produced neurites, except for the cells transfected with the double mutant S119A/S153A, which were refractory to the neurite-inducing effect of forskolin (Fig. 7A, lane 12, and 7B). However, cells expressing the kinase-inactive S119A/S153A/K194R triple mutant were able to produce neurites under these conditions, suggesting that the inhibition of neurite outgrowth observed with the kinase active S119A/S153A form might be attributed to PCTAIRE-1 kinase activity (Fig. 7A, lanes 12,13).

In the Neuro-2A cells, PCTAIRE-1 is largely excluded from the nucleus, and presumably exerts its effects on neurite outgrowth as a cytoplasmic kinase [(Charrasse et al., 1999) (and our own observations)]. We produced constructs to target PCTAIRE-1 to the nucleus by adding the nuclear localisation signal of nucleoplasmin to the N-terminus of wild-type PCTAIRE-1 and the S119A/S153A mutant form. The

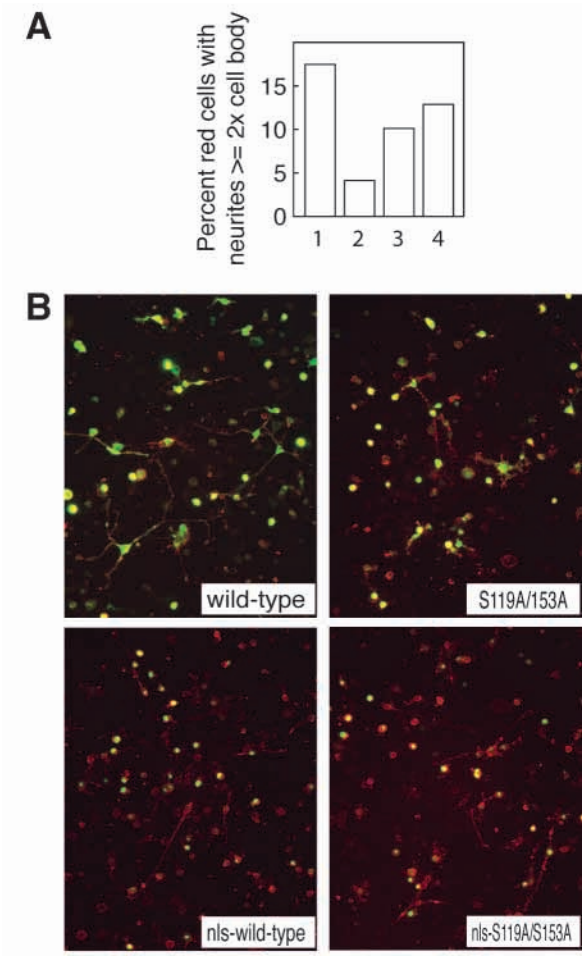

Fig. 8. Nuclear targeting of PCTAIRE-1 abolishes its effects on neurite outgrowth. (A) Indicated constructs were transfected into Neuro-2A cells together with a CD2 marker construct. Two days after transfection, the individual wells were treated with 10 μM forskolin and left for another 24 hours. The wells were incubated for 5 minutes using a Cy3 coupled CD2 antibody, and scored for green/red cells that had neurites longer than twice the length of the cell body. (B) Images of the transfected cells showing the effects on neurite outgrowth.

PCTAIRE-1 constructs were co-transfected with a membrane-localised form of CD2 (Lassus and Hibner, 1998), in order to be able to determine the morphology of the transfected cells. After 48 hours, the cells were treated for 12 hours with forskolin to induce PKA and neurite outgrowth. Fig. 8A,B shows that cells transfected with wild-type, non-nuclear PCTAIRE-1 readily produced extensions (lane 1), whereas the cells expressing the non-nuclear S119A/S153A mutant produced about 70% fewer outgrowths, which were also shorter (lane 2). When PCTAIRE-1 was targeted to the nucleus, however, the difference between wild-type and S119A/S153A mutant forms was abolished (Fig. 8A, lanes 3,4, and 8B), suggesting that the activated form of PCTAIRE-1 must be located in the cytoplasm to exert its inhibitory effect on neurite outgrowth.

Discussion

Despite a number of studies of the PCTAIRE family of protein kinases, very little is known of their function, substrate specificity or regulation. PCTAIRE-1 is expressed almost exclusively in the brain and the testis, where it is found in late stages of sperm development, after completion of the meiotic divisions. Thus, it is almost certainly not a cell cycle regulator despite its sequence homology with the CDKs. When expressed in bacteria, PCTAIRE-1 is inactive as a protein kinase. By analogy with the CDKs, this could reflect the lack of an activating, cyclin-like partner subunit and lack of phosphorylation on the activation loop at S319. When the bacterially expressed protein was incubated with lysates of either brain or testis, we found a weak activation of PCTAIRE-1-kinase activity towards MBP and a strong phosphorylation of PCTAIRE-1 itself. No binding partner was detected in these assays, however. The major phosphorylation sites were identified as S12 and S153. By analysing the context of the phosphorylated serine residues we could identify PKA as a potential kinase phosphorylating these residues. Phosphorylation of the bacterially expressed PCTAIRE-1 protein with PKA did not activate the kinase. However, mutation of S153 to alanine reproducibly gave a more active form of PCTAIRE-1 when expressed in eukaryotic cells, which may reflect a physiologically significant role for PKA in inhibiting PCTAIRE-1 activity. Nevertheless, PCTAIRE-1 seems to rely on an activating phosphorylation, since mutation of the T-loop residue S319 to alanine gave an inactive protein kinase (Fig. 4B, lane 3; Fig. 9 for a summary of all phosphorylation sites). However, we found it difficult to identify the S319 kinase, or other factors present in the cell lysates that were responsible for the activation, because even after activation, the bacterial PCTAIRE-1 displayed very low protein kinase activity. Moreover, we discovered more than one protein kinase that tended to bind to PCTAIRE-1, which could easily give misleading false positive results.

When PCTAIRE-1 was expressed in mammalian cell lines, it displayed higher kinase activity than was observed in bacteria, which allowed partial characterisation of its function. Thus, the conserved lysine 194 in the ATP-binding pocket was essential for activity. Truncation of the N-terminus beyond residue 106 also produced an inactive form of the kinase, suggesting that a minimal 50-residue N-terminal extension, which is highly conserved amongst all three PCTAIREs (Fig. 9), was required for PCTAIRE-1 activity. We suspect that this N-terminal sequence may provide the activation that cyclins normally provide to the rest of the CDK family. Neither we nor

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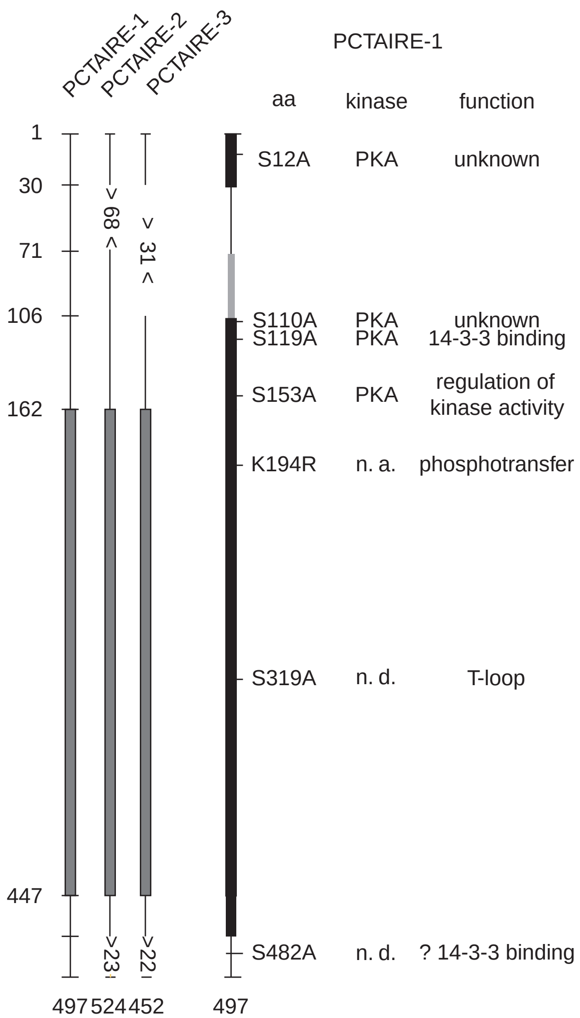

B

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PCT-1  103 NHPPRKISTEDINKRLSLPADIRLPEGYLEKLTLNSPIFDKPLSRRLRRVSLSEIGFGKLETYIK 167
           :  |: :   |:   |:| |:: ||       :  :|   : : | |  ::|    :      :
 Difo      DGAPHCVP-RDLS-WLDLEANMCLPGAAGLDSADGAP---HCVPRDLSWLDLEANMLPGAAG-LE
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Fig. 9. Schematic representation of PCTAIRE-1 compared with PCTAIRE-2 and -3. (A) On the left-hand side of the sketch the three known PCTAIREs are aligned with their catalytic kinase domain represented as a grey box. On the right hand side, PCTAIRE-1 is depicted again with regions of high homology between all three genes in a black box, and those between PCTAIRE-1 and -2 in a grey box. Additionally, the ATP-binding site and the T-loop residue, as well as the residues phosphorylated by PKA, are shown along with their function for PCTAIRE-1. (B) Alignment of the PCTAIRE-1 region surrounding the residues involved in 14-3-3 binding, S119 and S153, with a dimeric 14-3-3 peptide inhibitor [difopein (Masters and Fu, 2001)]. The position of S119 and S153 are marked with asterisks. Identical residues are connected with a bar, and related residues with a colon.

others have ever been able to identify an activating binding partner, and size exclusion chromatography by gel filtration indicated that monomeric PCTAIRE-1 was active as a protein kinase, although (1) its partner might be small, and (2) perhaps, if a partner were to be found, the activity of PCTAIRE-1 as a protein kinase might be very much enhanced. However, we found that the activity we were able to detect was comparable with that of CDK5 when associated with its partner.

Two other sites, S110 and S119 were also phosphorylated when the bacterially expressed protein was incubated in the extracts, and when purified PKA was added. Phosphorylation of S119 generated a 14-3-3 binding site that is functional in vitro when using purified proteins, as well as when the protein is expressed in cells. However, as shown in Fig. 6B, lane 5, mutation of S153 to alanine also compromised the 14-3-3 binding capability of PCTAIRE-1, suggesting an interaction of the two sites. In at least some cases, for full functionality the dimer of 14-3-3 is essential (reviewed by Tzivion and Avruch, 2002; Aitken, 2002) and this may also lead to 14-3-3 isoform preference due to the interaction of residues further away from the immediate phosphoserine (or -threonine) ligand-binding site. The binding to a dimer of 14-3-3 through two sites on a protein that might be individually of relatively low affinity could greatly increase target protein specificity of recognition. For example, a double peptide containing a repeat of a mode 1 phosphopeptide (Yaffe et al., 1997) linked by a hexa-repeat of 6-aminohexanoic acid has been shown to bind across a 14-3-3 dimer and span the binding grooves, which enhances binding by more than 30-fold because of cooperative binding. In addition, the R18 (non-phosphorylated peptide motif linked by an 11-mer peptide) has also been shown to bind very efficiently to the groove in 14-3-3 (Masters and Fu, 2001).

There is a lot of flexibility in these loops and so the exact length may not be important, but it is interesting to note that the spacing between the phosphorylated serines in PCTAIRE-1 (residues S119 and S153) is similar to that in the above double peptides (Fig. 9B). This hypothesis is also supported by the recent elucidation of the arylamine N-acetyl transferase (AANAT) structure in complex with 14-3-3, where two molecules of AANAT bind to a dimeric 14-3-3 (Ganguly et al., 2001) resulting in enhanced enzymic activity. It may also be noteworthy that both the PCTAIRE-1 phosphoserines are in LSL and VSL sequences, which resemble the high affinity non-phospho-interaction motif DALDL in ExoS (Hallberg, 2002) and WLDL in peptide R18 (Masters and Fu, 2001).

What is the function of PCTAIRE-1 in the brain? And is its expression in immortalized cell lines of any significance? In contrast to CDK5, which is expressed in many cell lines without its activating partner and is therefore inactive, we believe (as argued above) that PCTAIRE-1 is potentially active. Although Charrasse et al. suggested a cell-cycle-dependent regulation of PCTAIRE-1 kinase activity (Charrasse et al., 1999), we were unable to detect any significant differences in the cell cycle distribution attributable to either wild-type or mutant PCTAIRE-1, using a variety of cell lines expressing wild-type or kinase-dead PCTAIRE-1. We also tested the effects of expression of the PKA-site mutants, but again there was no detectable effect on the cell cycle. The related PITSLRE protein kinases were shown to be activated during the process of apoptosis (Lahti et al., 1995a). When 293 cell lines expressing various forms of PCTAIRE-1 were deprived of serum, they underwent apoptosis irrespective of the PCTAIRE levels and there was no evidence to support a role of PCTAIRE-1 in the induction or execution of apoptosis. The protein kinase activity of PCTAIRE did not change upon induction of apoptosis (data not shown).

We next examined the effects of manipulating PCTAIRE levels in neuronal cells, which are the natural source of PCTAIRE. The induction of neurite outgrowth in neuronal cell lines is normally preceded by an exit of the cells from the cell cycle. When Neuro-2A cells were transfected with cDNAs encoding either wild-type, kinase-dead or activated forms of PCTAIRE, there was no effect on the FACS profile of the cell cycle distribution; that is, cells were neither inhibited from cell cycle exit, nor did any form of PCTAIRE promote such exit. By contrast, reproducible effects on neurite outgrowth were observed, although their interpretation is complex. In Neuro-2A cells, addition of forskolin to the medium tends to promote neurite outgrowth, presumably as a result of protein kinase A activation. This stimulation was almost completely abolished by introduction of an activated form of PCTAIRE-1 that contains mutations of S119 and S153 to alanine. S119 lies in a 14-3-3-binding site, and its mutation to alanine seriously compromised the ability of PCTAIRE-1 to bind 14-3-3 proteins. We also showed that PKA could phosphorylate S119 and thereby generate a strong 14-3-3 protein-binding site. PKA also phosphorylated S153, which decreased the kinase activity of PCTAIRE-1. Thus, the kinase activity of the doubly mutant S119A/S153A form of PCTAIRE-1 should be unaffected by cAMP levels in the cell. Whether the inhibition of neurite outgrowth by this form of PCTAIRE is significant or not is difficult to say. We also observed a reproducible stimulation of neurite outgrowth by forms of PCTAIRE-1 that contained active 14-3-3-binding sites and, significantly, kinase dead forms of PCTAIRE-1 were somewhat more active than the wild-type in this respect. The simplest interpretation of the effects of PCTAIRE-1 on Neuro-2A cells would be that sequestration of 14-3-3 proteins tends to promote neurite outgrowth, whereas active PCTAIRE-1 tends to inhibit it. One way to think about this would be to propose that these cells contain a repressor of neurite outgrowth that is active when bound to 14-3-3 proteins. Raising PCTAIRE levels would sequester some of the 14-3-3 proteins, and reduce the activity of this repressor. But PCTAIRE kinase activity must also act, either to activate the repressor independently of the effects on 14-3-3 proteins or to inhibit the (implied) activator of neurite outgrowth. Until more is understood about the underlying control of neurite outgrowth, however, these models only help us understand the rather complex data.

It is intriguing that PCTAIRE-1 is normally found in cells that contain stable microtubules, neurons and sperm. In the Neuro-2A cells expressing high levels of activated PCTAIRE, time-lapse movies of the cells suggested that they were ‘trying’ to build neurites, but were unable to stabilise the processes they put out, as though PCTAIRE-1 antagonised the formation of stable microtubules. At first sight, these are contradictory observations, but it may be that the presence of the correct level of PCTAIRE permits the remodelling of stable microtubules that probably occurs during their assembly. What is required, of course, is a mouse lacking PCTAIRE, but although we were able to generate (male) ES cells lacking PCTAIRE-1, they never showed germline transmission (data not shown). One

explanation for this failure could be that the enzyme is required for the formation of viable sperm, although other explanations are feasible.

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