The Cellular Distribution and Kinase Activity of the Cdk Family Member Pctaire1 in the Adult Mouse Brain and Testis Suggest Functions in Differentiation[1]

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Abstract

Pctaire1, a member of the family of cyclin-dependent kinases, has been shown to be particularly abundantly expressed in differentiated tissues such as testis and brain. However, very little is known about the cellular and subcellular distribution and function of Pctaire1 protein(s), which is the focus of this study. We show that *Pctaire1* encoded two major proteins of M_r ~62,000 and ~68,000, found predominantly in testis and brain. Within these two tissues, Pctaire1 was most abundant in the cytoplasm of terminally differentiated cells, notably, the pyramidal neurons in brain and elongated spermatids in testis. Immunoprecipitation experiments further showed that a kinase activity toward myelin basic protein was associated with Pctaire1 in the adult testis and brain and that its activity was potentially regulated through association with regulatory partner(s). These results suggest that Pctaire1 kinase might have an important role in differentiated cells such as postmitotic neurons and spermatogenic cells.

Introduction

Cdks[3] are serine/threonine kinases that have been shown to be key players in the control of cell cycle progression. Their activity is regulated by interaction with specific subunits known as cyclins and also by phosphorylation by other protein kinases and dephosphorylation by phosphatases (reviewed in Ref. 1). Cdks were first identified in yeast (2) and then in all eukaryotes examined to date (reviewed in Ref. 3). Whereas in yeast, cell cycle control is achieved by a single Cdk, $p34^{cdc2}$ (in *Saccharomyces pombe*) or $p34^{CDC28}$ (in *Saccharomyces cerevisiae*), in higher eukaryotes, multiple genes appear to function. These various Cdks possess high homology in their amino acid sequence, and the PSTAIRE motif, where binding of the cyclin occurs, is particularly highly conserved in Cdks across species.

Over the last few years, several proteins have been cloned that are clearly related to Cdks based on sequence homology, but their cyclin partner(s) remain unidentified and/or their role in the cell cycle control is not known. These Cdk-related kinases are usually named according to their amino acid sequence, which corresponds to the conserved PSTAIRE domain (4–9). Some of these Cdk-related kinases, such as Pitslre and Cdk5, have been shown to play important roles in events that are not directly related to mitosis, including apoptosis or neuronal differentiation (10–13). However, for most of the other Cdk-related kinases, very little is known.

Pctaire is a Cdk-related gene that was cloned from human (4), mouse (9), and lower organisms such as *Trypanosoma brucei* (14) and *Dictyostelium* (15), based on its similarity to *Cdc2*. Three human genes, *PCTAIRE1*, *PCTAIRE2*, and *PCTAIRE3* (4), have been isolated, whereas in mouse, two *Pctaire* genes, corresponding to *PCTAIRE1* and *PCTAIRE3*, have been identified (9). Analysis at the mRNA level has shown that *Pctaire1* is most abundantly expressed in highly differentiated tissues (4, 9). However, little is known about the proteins encoded by *Pctaire1*, and its potential kinase activity has not been demonstrated. Here, we have examined the expression of Pctaire1 proteins in different murine tissues and have determined its distribution at the cellular level as well. We showed that Pctaire1 proteins are present at high levels in the testis and the brain and that, in both tissues, Pctaire1 protein expression is abundant in the cytoplasm of terminally differentiated cells, elongated spermatids in the testis, and postmitotic neurons in the brain. We also demonstrated a kinase activity for Pctaire1 that was only detectable in testis and brain, suggesting a role for Pctaire1 in the process of differentiation in these tissues.

Results

Distribution of Pctaire1 in Mouse Tissues. The general distribution of *Pctaire1* mRNA distribution in various tissues and cell lines has been reported (4, 9, 16), but the cellular and subcellular distribution of Pctaire1 proteins has, so far, not been evaluated. We, therefore, performed immunoblot analysis of mouse embryos and adult tissues to examine the pattern of expression of Pctaire1 proteins. As shown in Fig. 1*A*, two bands of M_r~62,000 and ~68,000 were readily

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; RT-PCR, reverse transcription-PCR; MBP, myelin basic protein; HA, hemagglutinin; ODF, outer dense fibers; TBS, Tris-buffered saline; Ab, antibody.

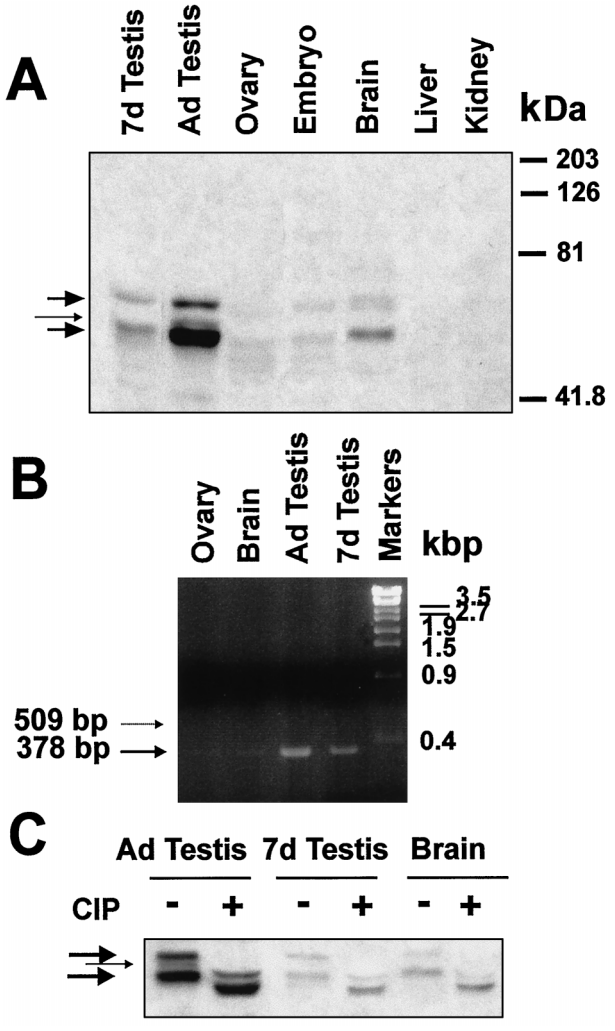

Fig. 1. Pctaire1 expression in mouse tissues. *A*, immunoblot analysis of Pctaire1 in mouse tissues. Protein extracts (75 μg) from murine tissues were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-Pctaire1 Ab (Santa Cruz Biotechnology). *Arrows*, positions of Pctaire1 proteins. The sizes of the protein markers are indicated on the *right*. *B*, RT-PCR analysis of *Pctaire1* mRNA in mouse tissues. For each reaction, 50 ng of reverse transcribed mRNA were used for RT-PCR as described in "Materials and Methods." The RT-PCR products were separated on a 2.5% agarose gel, stained with ethidium bromide, and photographed. The sizes of the DNA markers are indicated on the *right*. *C*, protein lysates from testis or brain (40 μg) were incubated in the presence (*Lanes* +) or absence (*Lanes* −) of calf intestine phosphatase (40 units) for 30 min at 37°C. Samples were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with anti-Pctaire1 Ab as described in "Materials and Methods." *Arrows*, positions of Pctaire1 proteins.

detected in the testis and, to a lesser extent, in the brain. Very low levels of Pctaire1 doublet were detected in all other tissues tested. These results correlated with the data obtained at the RNA level, with high expression in testis and brain and low basal levels in other tissues. The molecular weights of the proteins detected by immunoblot were comparable to the estimated sizes of the *in vitro* translated Pctaire1 polypeptides, M_r 60,000 and 65,000 (9).

In a recent study, Gao *et al.* (17) reported the presence of a *Pctaire1* mRNA in rat neonatal brain and in PC12 cells that contained an additional exon of 131 bp near the 5′ end. This mRNA would encode for a protein with a different NH_2-terminal end but identical amino acid residues downstream of the splicing site (at amino acid 68). As shown in Fig. 1*A*, we did not detect protein migrating at M_r 52,000, the predicted size of the protein encoded by the alternative transcript, in any of the murine tissues tested. Furthermore, no evidence for the presence of this transcript was obtained by RT-PCR using mRNA from several murine tissues, including brain (Fig. 1*B*). Fig. 1*B* depicts the products obtained from RT-PCR using primers spanning the NH_2-terminal region. For each tissue tested, a single band of 378 bp was found, corresponding to the Pctaire1 transcript lacking the alternatively spliced exon.

We did, however, detect an additional polypeptide migrating at M_r ~64,000 in the adult testis (Fig. 1*A*). As discussed above, this Pctaire1 isoform would not appear to be encoded by the alternative transcript. We, therefore, tested whether it could result from posttranslational modifications. As shown in Fig. 1*C*, the M_r 64,000 band is no longer detected after treatment of the adult testis lysate with calf intestine phosphatase, suggesting that this isoform resulted from differential phosphorylation. In all the tissues tested, however, the Pctaire1 doublet (M_r 62,000 and 68,000) remained, although migrating faster (at M_r ~60,000 and 62,000), indicating that Pctaire proteins are highly phosphorylated *in vivo*.

Expression of Pctaire1 Proteins in the Testis. Previous work in our laboratory had shown that *Pctaire1* transcript levels were high in the testis and were strikingly abundant in postmeiotic germ cells (16). Cellular localization of Pctaire1 proteins was, therefore, monitored by immunostaining of testis sections with anti-Pctaire1 antibody. Low levels of Pctaire1 were localized to all cell types of the immature testis (data not shown).

Low levels of Pctaire1 immunostaining were also present in the various cells in the adult testis but the intensity and the localization of the signal varied among the stages of the seminiferous epithelium. Fig. 2 depicts several of the 12 stages constituting the cycle of the seminiferous epithelium (18, 19) of an adult testis. There was low-level staining of Pctaire1 in round spermatids. More robust signal began to appear in early stages of spermatid elongation (Fig. 2*F*) and increased in intensity as elongating spermatids matured (Fig. 2, *A*–C). The most abundant expression of Pctaire1 protein corresponded to the late stages of spermiogenesis [stages 9–16 according to Oakberg (18)], when the nucleus and the cytoplasm of the spermatids undergo tremendous morphogenetic changes. At the subcellular level, Pctaire1 signal was restricted to the cytoplasm of the spermatids at all stages studied. At the terminal stages of spermiogenesis, spermatozoa extrude most of the cytoplasm as residual bodies just before being released into the lumen of the tubule. Pctaire1 staining was consistently detected in the resulting residual bodies, whereas no signal could be seen in the spermatozoa (Fig. 2, *D* and *E*). This pattern of expression correlates with the data obtained previously in the testis at the mRNA level (16), suggesting that translational regulation is not involved, and further establishes the subcellular localization of Pctaire1 protein.

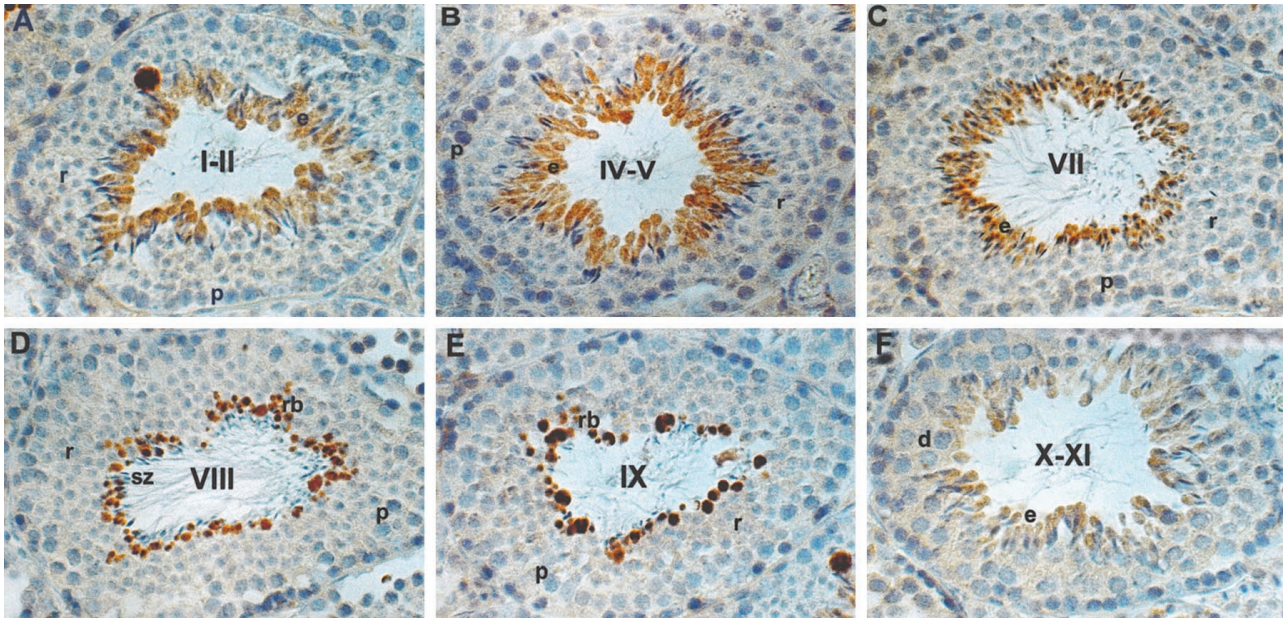

Fig. 2. Immunostaining of Pctaire1 in the adult testis. Paraffin-embedded sections of the testis were immunostained with anti-Pctaire1 antibody and counterstained with hematoxylin, which stains the nucleus blue. Pictures were taken at low magnification (×10). *Roman numerals*, the spermatogenic stages of the seminiferous epithelium (18, 19). *d*, diplotene spermatocytes; *e*, elongating spermatids; *p*, pachytene spermatocytes; *r*, round spermatids; *rb*, residual bodies; *sz*, spermatozoa.

Expression of Pctaire1 in Mouse Brain. Northern blot analysis had shown high levels of *Pctaire1* mRNA in the brain (4, 9); here, we extend these results to the protein level by immunoblot analysis. The brain was also the only tissue other than testis in which Pctaire1 proteins were readily detectable. However, nothing is known about *Pctaire1*'s cellular distribution within this complex tissue at either the mRNA or protein levels. *In situ* hybridization analysis performed on brain sections indicated that *Pctaire1* transcripts were ubiquitously expressed throughout the brain (Fig. 3). Strikingly high levels were observed in the hippocampus, the dentate gyrus, the mammillary bodies (which are part of the hypothalamus but receive their major input from the hippocampal formation), and the amygdala (a group of nuclei also associated with the function of the limbic system, which includes all of the above-mentioned structures).

Immunostaining of "free-floating" sections of the brain with anti-Pctaire1 antibodies allowed us to study the cellular and subcellular localization of Pctaire1 proteins in the brain. Pctaire1 protein staining correlated with the pattern of expression obtained for the mRNA by *in situ* hybridization analysis. In the sagittal section of the brain shown in Fig. 4*A*, a strong expression of Pctaire1 was detected in the layer II of the pyriform cortex, the hippocampal formation, and the cerebellum. The staining seen on the edges of the section was not specific because it was also detected when sections were incubated with rabbit IgG as the primary antibody (Fig. 4*F*). Examination of the cerebellum at high magnification showed that Pctaire1 expression was most abundant in the layer of the Purkinje cells (Fig. 4*B*). In coronal sections of the hippocampal area (comprised of the hippocampus itself and the dentate gyrus), Pctaire1 expression was very high in specific layers, namely, the pyramidal layer of the hippocampus, especially in the CA3 field and, to a lesser extent, in the granular layer of the dentate gyrus (Fig. 4*C*). These two layers are known to contain the cell bodies of the pyramidal neurons and the granule cells, respectively, whereas the molecular layer contains dendrites and axons. Strong immunostaining was also detected in the habenulae, which consists mainly of nuclei. In the neocortex, Pctaire1 levels were high in layer II–III and layers V and VI, where the proportion of pyramidal neurons is particularly high; these layers are believed to be analogous to the pyramidal layer of the hippocampal formation (Ref. 20; Fig. 4*D*). Therefore, it appears that Pctaire1 proteins are highly expressed in postmitotic neurons. Furthermore, as shown in the higher magnification of the pyriform cortex (Fig. 4*E*), the localization of the proteins was restricted to the cytoplasm of the cell bodies and the proximal processes, but no immunostaining was detected in the axons.

***In Vitro* Kinase Activity of Pctaire1.** Previous analysis of the deduced amino acid sequence of Pctaire1 had shown the presence of domains conserved among the Cdks, including the kinase subdomains as well as the three residues (Thr-14, Tyr-15, and Thr-161) that are involved in Cdc2 regulation (4, 9). Pctaire1 also contains the motif KLADFGLAR, known to be essential in the phosphotransfer reaction (21). Pctaire1 is, therefore, a putative functional kinase. However, to the best of our knowledge, Pctaire1 kinase activity has not been reported in either cultured cells or in tissues. We tested the kinase activity of Pctaire1 proteins immunoprecipitated from testis toward several substrates, including histone H1, neurofilament proteins, MBP, and p56Rb. Of these substrates, only myelin basic protein was found to be significantly phos-

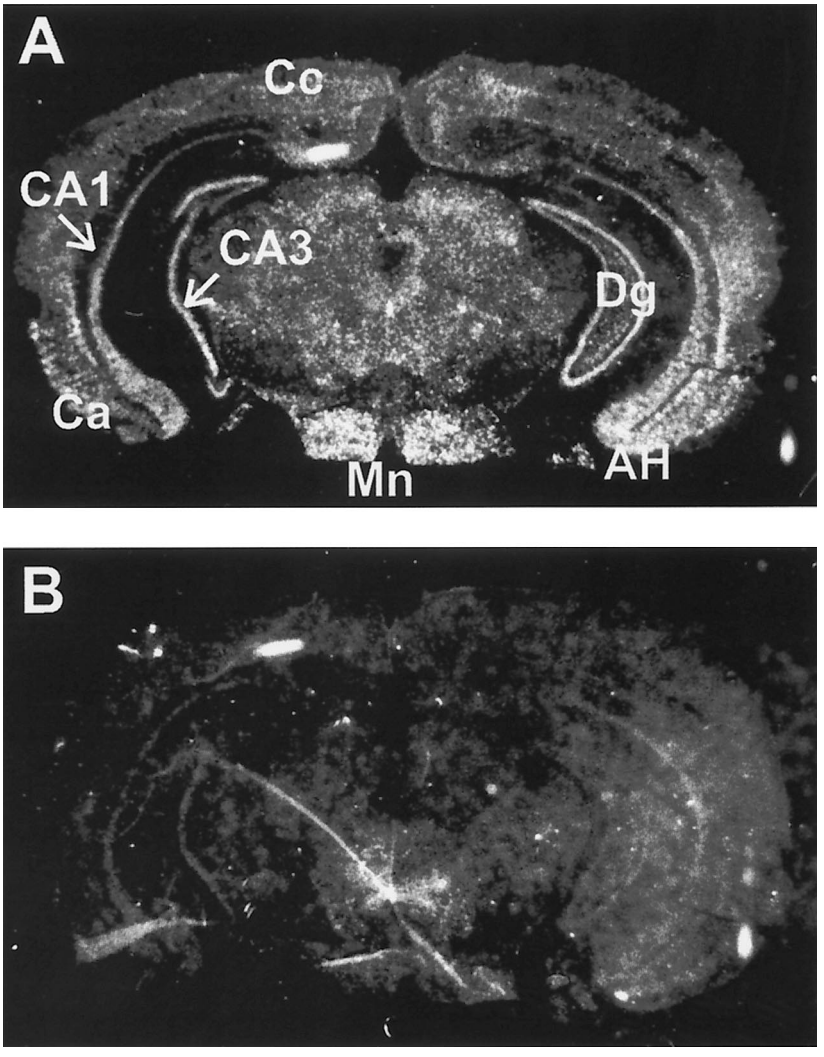

Fig. 3. Expression of *Pctaire1* transcripts in the adult mouse brain by *in situ* hybridization. Ten-μm coronal sections of adult brain were hybridized with antisense (*A*) or sense (*B*) ^{35}S-labeled *Pctaire1* riboprobe. Exposure time was 1 week. *AH*, amygdalohippocampal area; *Ca*, cortical amygdala nuclei; *CA*1 and *CA*3, CA1 and CA3 fields of the hippocampus; *Cc*, cerebral cortex; *Dg*, dentae gyrus; *Mn*, mammillary nuclei. Magnification, $\times$6.

phorylated by Pctaire1 kinase (data not shown). Fig. 5 depicts a representative kinase assay performed on several murine tissues. A robust activity toward myelin basic protein was detected in the adult testis and in the brain, to a lesser extent, but no significant kinase activity was observed in the other tissues tested, including the immature testis. This latter finding was surprising since our previous results had shown that Pctaire1 proteins were relatively abundant in the 7-day-old testis (see Fig. 1).

To gain more insight on the potential regulation of Pctaire1 kinase activity, we used Cos7 cells as an *in vitro* model. Cos7 cells have a low level of endogenous Pctaire1 protein and do not display any kinase activity in the *in vitro* assay (data not shown). These cells were transiently transfected with an HA-tagged Pctaire1 cDNA construct, and protein extracts were immunoprecipitated with anti-Pctaire1 antibodies and either used for *in vitro* kinase assay or immunoreacted with anti-HA antibody to assess the protein expression. As shown in Fig. 6, no kinase activity above the control (Cos7 cells transfected with vector alone) could be found in Pctaire1 cDNA-transfected Cos7 cells (Fig. 6, *top*). Testis extracts were run as a positive control for the kinase assay. As expected, a major band migrating at M_r 20,000 as well as a minor form of M_r ~16,000, corresponding to two isoforms of MBP were phosphorylated in the positive controls.

The absence of kinase activity in transfected cells was not due to a lack of Pctaire1 expression because immunoblots performed with HA antibodies showed the presence of two bands which migrated similarly to the Pctaire1 doublet found in the testis (Fig. 6, *bottom*). These findings show that, in transfected Cos7 cells as well as in the immature testis, the presence of substantial amounts of Pctaire1 protein is not sufficient to ensure the detection of kinase activity. This further suggested that an activation step might be necessary in order for Pctaire1 to be functional as a kinase.

To test whether the activation of Pctaire1 kinase activity is dependent upon association with one or several activating partner(s), Pctaire1 was immunoprecipitated from adult testis protein extracted in the presence of high salt concentrations. These conditions have been shown previously to disrupt protein-protein complexes (22). In high-salt concen-

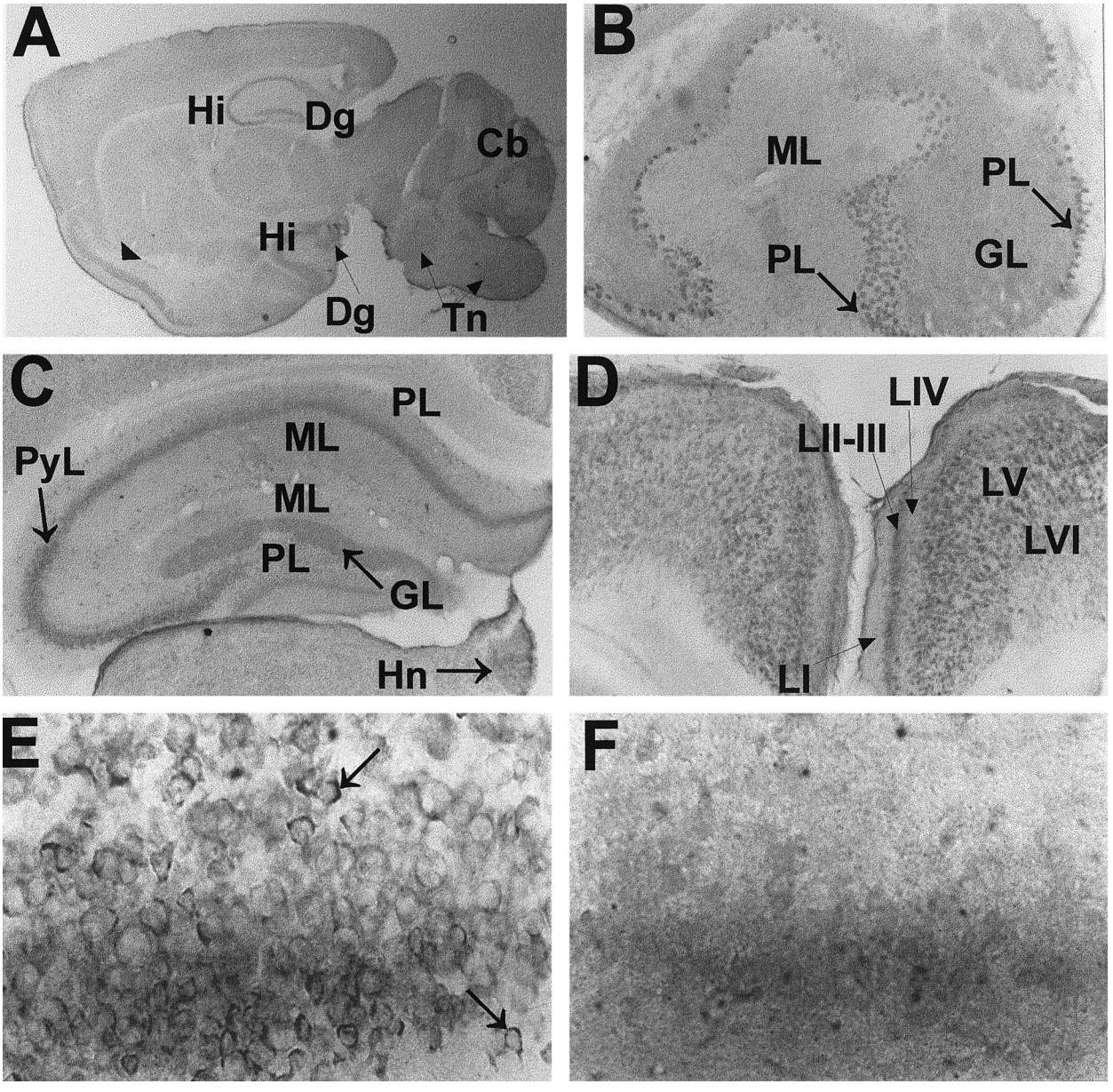

Fig. 4. Cellular localization of Pctaire1 proteins in the adult mouse brain. Fifty-μm sagittal (*A*, *B*, *E*, and *F*) or coronal (*C* and *D*) sections were immunostained with an anti-Pctaire1 antibody (*A–E*) or rabbit IgG (*F*). *B–E*, high-magnification photographs of the cerebellum (×10), the hippocampus (×10), the cortex (×10), and the pyriform cortex respectively (×40). *A*, *Cb*, cerebellum; *Dg*, dentae gyrus; *Hi*, hippocampus; and *Tn*, trigeminal nuclei. Magnification, ×6. *B*, *GL*, granular layer; *ML*, molecular layer; and *PL*, Purkinje layer. *C*, *GL*, granular layer; *Hn*, habenulae nuclei; *ML*, molecular layer; *PL*, polymorphic layer; and *PyL*, pyramidal layer. *D*, *LI*, layer 1 of the cortex; *LII*, layer 2 of the cortex; *LIII*, layer 3 of the cortex; *LIV*, layer 4 of the cortex; *LV*, layer 5 of the cortex; and *LVI*, layer 6 of the cortex. *E*; high magnification (×40) of the pyriform cortex (*arrowhead* in *A*) showing the localization of Pctaire1 signal in the perinuclear cytoplasm of the neurons (*arrows*). *F*, negative control using purified rabbit IgG as a primary antibody. High magnification (×40) of the pyriform cortex.

tration conditions, the kinase activity of Pctaire1 immunocomplexes was greatly reduced, compared with the level observed under low-salt conditions (150 mM; Fig. 7). The kinase activity in the high-salt assay was not significantly different from the negative control (high-salt testicular protein extracts immunoprecipitated with rabbit IgG). Pctaire1 doublet proteins were readily immunoprecipitated in both cases, as shown in Fig. 6, *bottom*. These results suggest that Pctaire1 kinase activity is dependent upon association with regulatory partner(s) that can be released under high-salt conditions.

Discussion

Pctaire1 is a Cdk-related kinase that has been identified in a variety of organisms. The predicted amino acid sequence of Pctaire1 revealed highly conserved homology throughout the entire protein in mammals. Most of the studies of *Pctaire1*

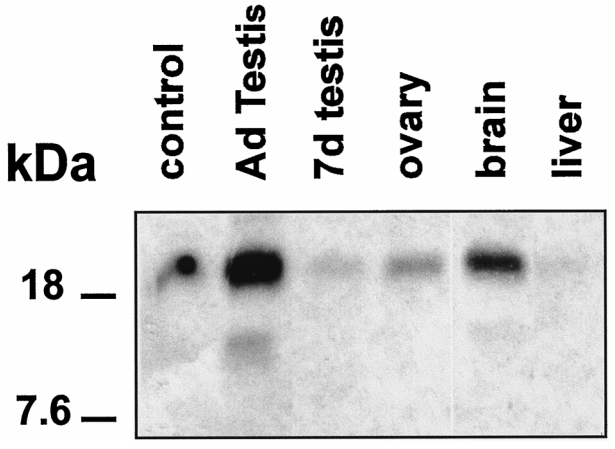

Fig. 5. *In vitro* Pctaire1 kinase activity of murine tissues. Protein extracts (500 μg) were incubated with anti-Pctaire1 antibodies or rabbit IgG (adult testis extracts used as a negative control). The immunocomplexes were assayed for *in vitro* kinase activity toward MBP, as described in "Materials and Methods." Autoradiographic exposure was overnight. Molecular weight standards are indicated on the *left*.

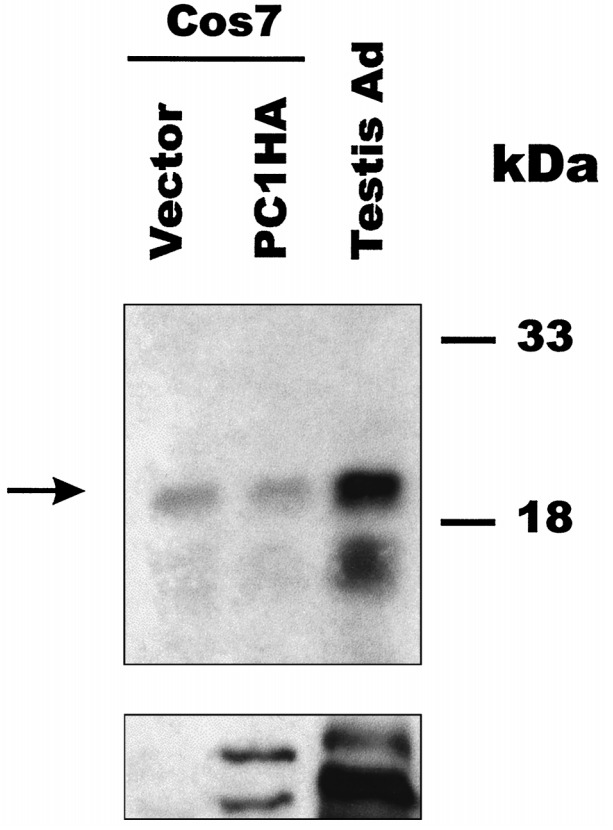

Fig. 6. *In vitro* kinase activity of Pctaire1 constructs expressed in Cos7 cells. Cos7 cells were transiently transfected with either pCDNA3 (vector) or pcDNA/Pctaire1HA (*PC1HA*) constructs. Protein extracts (400 μg), from Cos7 cells or testis, were incubated with anti-Pctaire1 antibodies. Immunocomplexes were processed for kinase activity toward MBP as described in "Materials and Methods" (*top*). *Bottom*, immunoprecipitation performed in parallel and immunoblotted with either HA (Cos7 cells) or anti-Pctaire1 antibodies (testis). Molecular weight standards are indicated on the *right*.

have focused on expression at the level of mRNA, so little is known about the cellular localization and the function of the protein.

In this study, we report the expression pattern of Pctaire1 protein in murine tissues, especially testis and brain, in which the level of expression is the highest. Pctaire1 proteins were detected as a doublet of M_r ~62,000 and ~68,000. These molecular weights are in agreement with the sizes of the two polypeptides previously obtained by *in vitro* translation of Pctaire1 murine cDNA (9). In none of the tissues tested were we able to detect additional proteins or transcripts that might have resulted from an alternatively spliced exon, such as that described by Gao *et al.* (17) in rat PC12 cells and in neonatal brain. Such an alternative transcript could encode for a predicted Pctaire1 protein of M_r 52,000 with a different NH_2-terminal end containing unique potential phosphorylation sites. Such sites could provide a means to regulate Pctaire1 function in the tissue(s) and/or cells in which this isoform is potentially expressed.

It is of interest to note, however, that a third Pctaire1 isoform of M_r ~64,000 was consistently found in the adult testis. This protein is not encoded by the alternative transcript described by Gao *et al.* (17) but is rather due to a posttranslational phosphorylation of Pctaire1 because this band was no longer detected after treatment of the cell extracts with alkaline phosphatase. This experiment also showed that Pctaire1 proteins of M_r 62,000 and 68,000 are highly phosphorylated, as demonstrated by the clear shift in the protein migration after alkaline phosphatase treatment. We still detected a doublet after treatment with the enzyme, suggesting that these two Pctaire1 proteins might result from the initiation of translation from two different methionines.

Although Pctaire1 is expressed at low levels in nearly every tissue, the analysis of Pctaire1 protein distribution in the testis and brain showed an interesting pattern. In both tissues, strong immunostaining was found in terminally differentiated cells. In the testis, high levels of Pctaire1 protein were detected in the early to late elongating spermatids (stages 9–16 of spermiogenesis). During these stages, the male germ cells undergo dramatic morphological changes. The nucleus condenses, elongates, and moves toward the cell surface. Concomitantly, the axoneme forms, and the cytoplasm then stretches along the developing flagellum. The flagellum is mainly composed of the ODF, which surround the axoneme, and the fibrous sheath, which overlies the ODF in the principal piece of the flagellum (19). The multiple proteins composing these structures share the characteristic of being insoluble in ionic detergent and highly phosphorylated, reflecting their high content of serine residues (23–26). These fibrous sheath proteins are spread throughout the cytoplasm, and the ODF are stored in granulated bodies before being assembled in the flagellum (27). Very little is known about the mechanisms involved in the assembly of these proteins along the flagellum and how this process is regulated. Pctaire1 proteins are not detectable in the spermatozoa (16), but strong immunostaining is found in the cytoplasm of the spermatids at the late stages of sper-

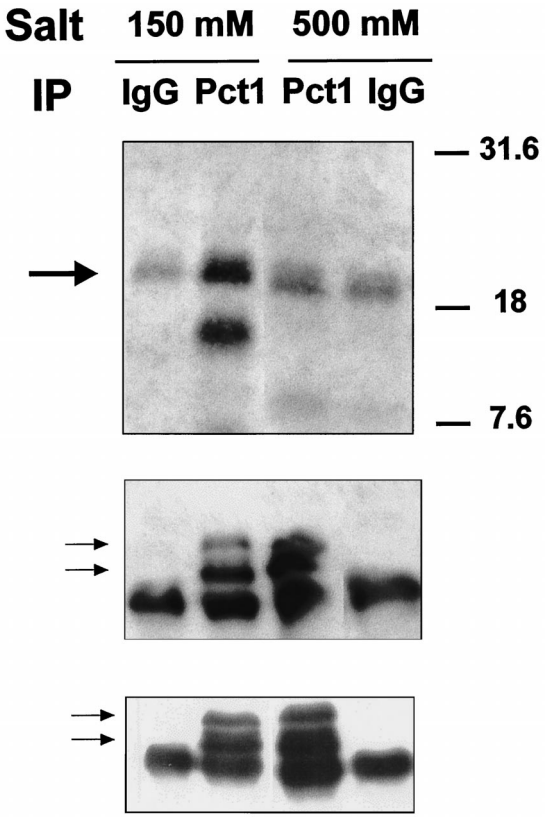

Fig. 7. Requirement of activating factor(s) for kinase activity of Pctaire1 immunocomplexes. Testicular proteins were extracted with either regular lysis buffer (150 mM NaCl) or with high-salt condition (500 mM) lysis buffer. Protein lysates were incubated either with rabbit IgG as a negative control or anti-Pctaire1 antibodies. Immunocomplexes were washed with the lysis buffer used for protein extraction and then processed for kinase activity assay, as described in "Materials and Methods" (*top*) or immunoblotted with anti-Pctaire1 antibody for detection of Pctaire1 proteins (*bottom*). Molecular weight standards are indicated on the *right*. *Arrows*, migration of Pctaire1 proteins in the *middle* and *bottom*. Because of the distorted migration obtained for the high-salt lysis buffer condition in the immunoblot analysis (*middle*), the experiment was repeated with the same extracts (*bottom*).

miogenesis (Fig. 2). Pctaire1 might not be directly involved in the assembly of the proteins constituting the flagellum because its distribution is uniform throughout the cytoplasm rather than associated specifically with the differentiating flagellum. However, the serine/threonine kinase activity of Pctaire1 might be required for the postmodification of these structural elements so that they can be recruited for assembly into the flagellum.

As in the testis, Pctaire1 staining in the brain was detected at low levels quite ubiquitously, but strong signal was characteristic of pyramidal neurons in the cortex and the hippocampus and of Purkinje cells in the cerebellum. Interestingly, in these fully differentiated neurons, the staining of Pctaire1 proteins was found in the cytoplasm of the cell bodies and the proximal processes but not in the axonal tracts. This is in contrast to the expression pattern observed for Cdk5, another Cdk-related kinase. *In vivo* as well as *in vitro*, Cdk5 was found in the axons of neuronal cells (12, 28, 29), consistent with its suggested role in the phosphorylation of neurofilaments and, therefore, in axon and neurite outgrowth. The cytoskeletal elements that compose the axon are synthesized in the cell body, sorted, and then transported in either the dendrites or axons. These last two events involved a complex network of proteins that are modulated by phosphorylation (30, 31). Our results showed that Pctaire1 is a kinase. The fact that Pctaire1 signal is localized in the cell body but not in the axon suggests that this kinase could be involved in the sorting process of the cytoskeletal elements toward the axon and/or the dendrites rather than in the extension of the neuronal processes. To verify this hypothesis, it will be of great interest to identify the physiological substrate(s) and the potential regulatory partner(s) of Pctaire1.

Unlike other related serine-threonine kinases such as Pctaire2 (22) or Cdk5 (12), Pctaire1 does not efficiently phosphorylate histone H1 in our *in vitro* kinase assay. Among the different substrates tested, histone H1, p56 Rb protein, neurofilaments, and MBP, only MBP was found to be a potent substrate for Pctaire1 kinase activity. Cdk4 and Pitalre are two other Cdks that have been shown to phosphorylate MBP but not histone H1 (7, 32). Among the different tissues tested, significant Pctaire1 kinase activity was found only in the adult testis and to a lesser extent, in the brain. This difference in kinase activity toward MBP may reflect, in part, the difference in overall levels of Pctaire1 protein in the two tissues. Furthermore, the fact that most of the other tissues expressed only very low levels of Pctaire1 proteins compared to testis and brain could explain why no kinase activity was detected in these tissues.

However, it was surprising that we could not detect any kinase activity in protein extracts from 7-day-old testis in which immunoblot analysis showed that the levels of Pctaire1 protein were comparable to those observed in the brain. We, therefore, expressed an HA-tagged Pctaire1 cDNA construct in Cos7 cells and tested the proteins obtained in the *in vitro* kinase assay. Our results showed that, although transfected Cos7 cells encoded for a Pctaire1 protein doublet migrating at similar sizes as found in the testis and brain, no kinase activity could be found associated with Pctaire1 in these cells. This absence of kinase activity could be due, in part, to a lack of specific posttranslational modifications of Pctaire1 proteins in transfected Cos7 cells as the doublet migrated faster than that observed with the testis extracts. This pattern was also similar to the one obtained after treatment of testis extracts with alkaline phosphatase. However, the bands in the day 7 testis extracts migrated similarly to adult testis, suggesting that additional factors (possibly interacting proteins) may also be required for kinase activity.

In a recent study, Hirose *et al.* (22) showed that rat Pctaire2, a Cdk-related kinase displaying 63% homology with Pctaire1, exhibited a kinase activity toward histone H1. This activity was dependent on its association with regulatory partner(s) that were released under high-salt conditions. Similarly, we showed that the kinase activity associated with Pctaire1 in the adult testis is lost when protein extracts are prepared with a high-salt lysis buffer. We cannot rule out the possibility that these conditions are denaturing for Pctaire1

proteins. However, these results, together with the absence of kinase activity of Pctaire1 protein expressed in Cos7 cells, suggest that Pctaire1 may require one or several partner(s) to be active as a kinase. This hypothesis could explain the absence of detectable kinase activity in the 7-day-old testis, presumably due to a lack of activating partner(s) in the tissue at this stage of development.

Using a yeast two-hybrid system, Sladeczek *et al.* (27) recently showed that Pctaire1 interacts with 14-3-3 proteins. The analysis of the predicted amino acid sequence of Pctaire1 showed that it contains at least two RSXSXP motifs, which have been shown to be essential for binding to 14-3-3 proteins (33). The θ 14-3-3 isoform has recently been cloned in the mouse and shown to be highly expressed in the brain and testis (34). In the testis, θ 14-3-3 protein was especially abundant in the differentiating spermatids. Because 14-3-3 proteins have been shown to modulate the interaction between several kinases involved in signal transduction (35), it was of interest to investigate whether Pctaire1 interacted with this class of protein. However, our attempt to show an interaction between Pctaire1 and 14-3-3 protein by coimmunoprecipitation experiments in the testis or brain were not successful (data not shown). This could be due to a weak or transient interaction between the two proteins that could not be detected with the methodology used. Experiments to identify the protein(s) interacting with Pctaire1 in germ cells and neuronal cells, currently underway in our laboratory, will provide helpful insights in elucidating the function(s) of Pctaire1 in these terminally differentiated cells.

Materials and Methods

Sources of Tissues, Probes, and Abs. Normal tissues were obtained from Swiss Webster mice (Charles River, Wilmington, DE). Pctaire1 polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology, Inc. (Lake Placid, NY). The cDNA probe for *Pctaire1* has been described previously (16). [α-^{35}S]UTP and [γ-^{32}P]ATP were obtained from NEN Life Science Products (Wilmington, DE).

Immunoblot Analysis. Tissues or cells were homogenized in lysis buffer [50 mM Tris-HCl (pH8), 150 mM NaCl, 2 mM EGTA, 1 mM DTT, 10% glycerol, 1% NP40, 0.25% sodium deoxycholate, 1 mM Na_3VO_4, 50 mM NaF, 1 mg/ml apoprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride] with a Dounce homogenizer. Intact cells and debris were pelted by centrifugation at 10,000 $\times$ *g* for 15 min, and protein concentration was determined in the supernatants with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

For immunoblot analysis, protein lysates were mixed with Laemmli buffer (36) and boiled for 5 min. Samples were resolved by SDS-PAGE and electroblotted onto nitrocellulose membrane. The filter was blocked by soaking for 1 h in Blotto (1$\times$ TBS, 0.5% Tween 20, and 5% dried fat-free milk) or TBSB (1$\times$ TBS, 0.5% Tween 20, and 2.5% BSA), depending on the Ab used; incubated with the primary Ab diluted with Blotto (1:250 for anti-Pctaire1 Ab) or TBSB (1:100 for anti-HA Ab) overnight at 4°C; washed three times; incubated with secondary Ab (1:5000, horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG; Boehringer-Mannheim, Indianapolis, IN) for 1 h; and washed three times with TBST (1$\times$ TBS and 0.5% Tween 20). For detection, ECL Western blotting reagents (Amersham, Arlington Heights, IL) were used according to the manufacturer's recommendation.

PCR Analysis. Poly(A)+ RNAs were extracted from tissues using the Micro-FastTrack mRNA Isolation Kit from Invitrogen (Carlsbad, CA). cDNAs were synthesized by reverse transcription of 1 μg of mRNA using random primers provided with the cDNA Cycle Kit First-Strand cDNA Synthesis from Invitrogen (Carlsbad, CA).

PCR amplification of cDNA reverse-transcribed from 50 ng of mRNA was carried out for 30 cycles in a Perkin Elmer/Cetus thermal cycler programmed for 1 min at 95°C and 4 min at 70°C. The primers used were: upstream, ATCCCCCTGGCGCCGTTCCA (corresponding to nucleotides 101–120); and downstream, TGAAGCCTGGTCACTCTCCCCAT (nucleotides 600–578). PCR products were separated on a 2.5% agarose gel and visualized by ethidium bromide staining.

***In Situ* and Immunochemistry Analysis.** Testes were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated, and embedded in paraffin before sectioning (37). For analysis of brain tissues, mice were sacrificed by cervical dislocation just before perfusion first with saline and subsequently with 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight at 4°C. Tissues were cryoprotected in 30% sucrose, embedded in OCT (Tissue-Tek, Elkart, IN), and cut, using a cryostat, into 10-μm-thick sections for *in situ* hybridization analysis or 30-μm-thick sections for immunochemistry studies.

Pctaire1 cDNA was used as a template to generate ^{35}S-sense or antisense-labeled riboprobe using T3 or T7 polymerase (Promega, Madison, WI). Sections were processed for *in situ* hybridization analysis as described previously (16). Autoradiography was performed with Kodak NT/B2 emulsion. After 7–10 days exposure at 4°C, slides were developed, stained with H&E, mounted, and viewed on a Leitz photomicroscope using dark-field illumination.

Sections of adult testis were deparaffinized in xylene and progressively rehydrated in a graded series of ethanol/water mixtures. After boiling in 0.01 M sodium citrate (pH 6.0) for 10 min in a microwave, sections were treated with 0.3% H_2O_2 in methanol to destroy any endogenous peroxydase activity from the tissue, washed twice in PBST (1$\times$ PBS and 0.1% Triton X-100), and blocked for 1 h in PBST and 2.5% goat serum. The slides were incubated with anti-Pctaire1 Ab (Upstate Biotechnology, Inc., Lake Placid, NY; 1:500) overnight at 4°C, washed three times with PBST, and stained with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Testis sections were counterstained with hematoxylin.

Floating sections of brain were treated as described above with the omission of the deparaffinization/rehydration and the boiling steps. No counterstaining was performed with brain sections.

***In Vitro* Pctaire1 Kinase Assay.** For immunoprecipitation of Pctaire1, 500 μg of protein were incubated with anti-Pctaire1 Ab (1:50) in the lysis buffer for 2 h at 4°C, followed by incubation with 3 μg of protein A-Sepharose for 2 h. Immune complexes were collected by centrifugation and washed three times with the lysis buffer and once with the kinase assay buffer [50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 0.2 mM Na_3VO_4, and 1 mM DTT].

The assay for *in vitro* Pctaire1 kinase activity was carried out for 20 min at 30°C in the kinase assay buffer supplemented with 10 mM ATP, 5 μM protein kinase A inhibitor, 10 μM calmidozium, and 70 μg/ml MBP (Life Technologies, Inc., Gaithersburg, MD), and 3 μCi of [γ-^{32}P]ATP in a volume of 30 μl. The reaction was stopped by the addition of 30 μl of 2$\times$ Laemmli sample buffer and boiled for 3 min. Reaction products were visualized by SDS-PAGE followed by autoradiography.

Pctaire1 Constructs. A cDNA construct was generated that contained the open reading frame of murine Pctaire1 and two HA epitope tags (YDVPDYA) at the COOH-terminal end. A *Nco*I site was created by PCR just before the stop codon of the Pctaire1 sequence. The primers used were: upstream, GAGATTGATATCGCCGCCATGAAGATGGGATCT; and downstream, AAACTTGGCCATGGACTCGGTATCCACCACACG. Amplification was carried out for 25 cycles in a Perkin Elmer/Cetus thermal cycler programmed for 1 min at 95°C, 30 s at 60°C, and 1 min at 72°C. The PCR products were cut with *Eco*RV and *Nco*I and subcloned into pBluescriptKS+ carrying two HA epitopes downstream of the *Nco*I site vector (generously provided by Dr. J. Kitajewski, Columbia University).

To generate a construct that encoded the same NH_2-terminus as found *in vivo*, we replaced the *Pst*I/*Bgl*II fragment of the Pc-1b clone (9) with the *Pst*I/*Bgl*II fragment of a cDNA obtained from adult testis mRNAs by RT-PCR. Briefly, the cDNA obtained by reverse transcription from adult testis mRNA were amplified by PCR for 30 cycles in a Perkin Elmer/Cetus thermal cycler programmed for 1 min at 95°C and 4 min at 70°C. The primers used were: upstream, ATCCCCCTGGCGCCGTTCCA (nucleotides 101–120); and downstream, GGTCAGCTTCTCAAGGTAGC (nucleotides 747–728). PCR products were then double digested with *Pst*I and *Bgl*II enzymes for subcloning into the Pc-1bHA/pBKS+ plasmid.

The resulting construct, Pctaire1HA, containing two HA epitopes at the COOH terminus and an NH_2 terminus identical to that found *in vivo*, was then subcloned in pcDNA3 vector (Invitrogen, Carlsbad, CA) for use in transfection experiments.

Cell Culture and Transfection. Cos7 cells were cultured in DMEM (Cellgro, Herndon, VA) supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2.

pcDNA3 vector and pcDNA/Pctaire1HA constructs were transfected in Cos7 cells using the calcium phosphate method as described by Pear (38) and cultured for 36 h. Cell protein extracts were used as a check for expression of the construct by immunoblotting with anti-HA antibody, and they were tested in the *in vitro* kinase assay.

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References

1. Solomon, M. J. Activation of the various cyclin/cdc2 protein kinases. Curr. Opin. Cell Biol., *5:* 180–186, 1993.

2. Hartwell, L. H. *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev., *38:* 164–198, 1974.

3. Hunt, T. Maturation promoting factor, cyclin and the control of M-phase. Curr. Opin. Cell Biol., *1:* 268–274, 1989.

4. Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L. H. A family of human cdc2-related protein kinases. EMBO J., *11:* 2909–2917, 1992.

5. Brambilla, R., and Draetta, G. Molecular cloning of PISSLRE, a novel putative member of the cdk family of protein serine/threonine kinases. Oncogene, *9:* 3037–3041, 1994.

6. Grana, X., Claudio, P. P., De Luca, A., Sang, N., and Giordano, A. PISSLRE, a human novel CDC2-related protein kinase. Oncogene, *9:* 2097–2103, 1994.

7. Grana, X., De Luca, A., Sang, N., Fu, Y., Claudio, P. P., Rosenblatt, J., Morgan, D. O., and Giordano, A. PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein *in vitro*. Proc. Natl. Acad. Sci. USA, *91:* 3834–3838, 1994.

8. Bunnell, B. A., Heath, L. S., Adams, D. E., Lahti, J. M., and Kidd, V. J. Increased expression of a 58-kDa protein kinase leads to changes in the CHO cell cycle (Published erratum in Proc. Natl. Acad. Sci. USA, *88:* 2612, 1991). Proc. Natl. Acad. Sci. USA, *87:* 7467–7471, 1990.

9. Okuda, T., Cleveland, J. L., and Downing, J. R. PCTAIRE-1 and PCTAIRE-3, two members of a novel cdc2/CDC28-related protein kinase gene family. Oncogene, *7:* 2249–2258, 1992.

10. Lahti, J. M., Xiang, J., Heath, L. S., Campana, D., and Kidd, V. J. PITSLRE protein kinase activity is associated with apoptosis. Mol. Cell. Biol., *15:* 1–11, 1995.

11. Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F., and Tsai, L. H. The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. Genes Dev., *10:* 816–825, 1996.

12. Tsai, L. H., Takahashi, T., Caviness, V. S., Jr., and Harlow, E. Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development (Camb.), *119:* 1029–1040, 1993.

13. Zhang, Q., Ahuja, H. S., Zakeri, Z. F., and Wolgemuth, D. J. Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. Dev. Biol., *183:* 222–233, 1997.

14. Mottram, J. C., and Smith, G. A family of trypanosome cdc2-related protein kinases. Gene, *162:* 147–152, 1995.

15. Michaelis, C., and Weeks, G. The isolation from a unicellular organism, *Dictyostelium discoideum*, of a highly-related *cdc2* gene with characteristics of the PCTAIRE subfamily. Biochim. Biophys. Acta, *1179:* 117–124, 1993.

16. Rhee, K., and Wolgemuth, D. J. Cdk family genes are expressed not only in dividing but also in terminally differentiated mouse germ cells, suggesting their possible function during both cell division and differentiation. Dev. Dyn., *204:* 406–420, 1995.

17. Gao, C. Y., Chauthaiwale, V. M., Rampalli, A. M., and Zelenka, P. S. Expression of alternatively spliced PCTAIRE-1 mRNA in PC12 cells and neonatal rat brain. Gene, *176:* 243–247, 1996.

18. Oakberg, E. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am. J. Anat., *99:* 391–413, 1956.

19. Russel, L. D., Ettlin, R. A., Sinha Hikim, A. P., and Clegg, E. D. Histological and histopathological evaluation of the testis. Clearwater, FL: Cache River Press, 1990.

20. Martin, J. H. The limbic system. *In:* S. Muir (ed.), Neuroanatomy Text and Atlas, pp. 376–396. Norwalk, CT: Appleton & Lange, 1989.

21. van den Heuvel, S., and Harlow, E. Distinct roles for cyclin-dependent kinases in cell cycle control. Science (Washington, DC), *262:* 2050–2054, 1993.

22. Hirose, T., Tamaru, T., Okumura, N., Nagai, K., and Okada, M. PCTAIRE 2, a Cdc2-related serine/threonine kinase, is predominantly expressed in terminally differentiated neurons. Eur. J. Biochem., *249:* 481–488, 1997.

23. Brito, M., Figueroa, J., Maldonado, E. U., Vera, J. C., and Burzio, L. O. The major component of the rat sperm fibrous sheath is a phosphoprotein. Gamete Res., *22:* 205–217, 1989.

24. Olson, G. E., Hamilton, D. W., and Fawcett, D. W. Isolation and characterization of the fibrous sheath of rat epididymal spermatozoa. Biol. Reprod., *14:* 517–530, 1976.

25. Olson, G. E., and Sammons, D. W. Structural chemistry of outer dense fibers of rat sperm. Biol. Reprod., *22:* 319–332, 1980.

26. Vera, J. C., Brito, M., Zuvic, T., and Burzio, L. O. Polypeptide composition of rat sperm outer dense fibers. A simple procedure to isolate the fibrillar complex. J. Biol. Chem., *259:* 5970–5977, 1984.

27. Sladeczek, F., Camonis, J. H., Burnol, A. F., and Le Bouffant, F. The Cdk-like protein PCTAIRE-1 from mouse brain associates with p11 and 14-3-3 proteins. Mol. Gen. Genet., *254:* 571–577, 1997.

28. Matsushita, M., Matsui, H., Itano, T., Tomizawa, K., Tokuda, M., Suwaki, H., Wang, J. H., and Hatase, O. Developmental changes of cyclin dependent kinase 5 subcellular localization in the rat cerebellum. Neuroreport, *6:* 1267–1270, 1995.

29. Pigino, G., Paglini, G., Ulloa, L., Avila, J., and Caceres, A. Analysis of the expression, distribution and function of cyclin dependent kinase 5 (cdk5) in developing cerebellar macroneurons. J. Cell Sci., *110:* 257–270, 1997.

30. Sheetz, M. P., Pfister, K. K., Bulinski, J. C., and Cotman, C. W. Mechanisms of trafficking in axons and dendrites: implications for development and neurodegeneration. Prog. Neurobiol., *55:* 577–594, 1998.

31. Nixon, R. A. The slow axonal transport of cytoskeletal proteins. Curr. Opin. Cell Biol., *10:* 87–92, 1998.

32. Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J. Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G_1 cyclins. Cell, *71:* 323–334, 1992.

33. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. The structural basis for 14-3-3: phosphopeptide binding specificity. Cell, *91:* 961–971, 1997.

34. Perego, L., and Berruti, G. Molecular cloning and tissue-specific expression of the mouse homologue of the rat brain 14-3-3 θ protein: characterization of its cellular and developmental pattern of expression in the male germ line. Mol. Reprod. Dev., *47:* 370–379, 1997.

35. Aitken, A. 14-3-3 and its possible role in co-ordinating multiple signaling pathways. Trends Cell Biol., *6:* 341–347, 1996.

36. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.

37. Chapman, D. L., and Wolgemuth, D. J. Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis. Development (Camb.), *118:* 229–240, 1993.

38. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA, *90:* 8392–8396, 1993.