
**Mechanisms of Signal Transduction:
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substrate for Cdk5/p35**

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Pctaire1 interacts with p35 and is a novel substrate for Cdk5/p35

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Summary

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase that plays important roles during central nervous system (CNS) development. Cdk5 kinase activity depends on its regulatory partners, p35 or p39, which are prominently expressed in the CNS. We have previously demonstrated the involvement of Cdk5 in the regulation of acetylcholine receptor expression at the neuromuscular junction, suggesting a novel functional role of Cdk5 at the synapse. Here we report the identification of Pctaire1, a member of Cdk-related kinase family, as a p35-interacting protein in muscle. Binding of Pctaire1 to p35 can be demonstrated by *in vitro* binding assay and co-immunoprecipitation experiments. Pctaire1 is associated with p35 in cultured myotubes and skeletal muscle, and is concentrated at the neuromuscular junction. Furthermore, Pctaire1 can be phosphorylated by Cdk5/p25 complex, and serine-95 is the major phosphorylation site. In brain and muscle of Cdk5 null mice, Pctaire1 activity is significantly reduced. Moreover, Pctaire1 activity is increased following pre-incubation with brain extracts and phosphorylation by Cdk5/p25 complex. Taken together, our findings demonstrate that Pctaire1 interacts with p35, both *in vitro* and *in vivo*, and that phosphorylation of Pctaire1 by Cdk5 enhances its kinase activity.

Introduction

Cyclin-dependent kinase 5 (Cdk5), a member of the Cdk family, is involved in cellular functions that are not related to the regulation of cell cycle progression (1). While other members of the family associate with cyclins, the serine/threonine kinase activity of Cdk5 requires the association with one of its regulatory units, p35 or p39. A C-terminal proteolytic product of p35, p25, has also been shown to activate Cdk5 (2). Although Cdk5 is ubiquitous in mammalian tissues, the expression of its activators is largely restricted to neurons. The temporal and spatial specificity of Cdk5 kinase activity is therefore due to the neuronal expression of p35 and p39 (3-6). Recent studies demonstrate that p35 transcript and p35-associated Cdk5 kinase activity can also be detected in muscle (7, 8). While the best-characterized role for Cdk5 is in regulating neuronal migration and axonal guidance as well as cytoskeletal dynamics, there is also evidence that links Cdk5 activity to the regulation of membrane transport and dopamine signaling (2).

In addition to its known functions in neuronal development, recent evidence suggests a novel role for Cdk5 in the modulation of post-synaptic functions. For example, Cdk5 regulates the expression of acetylcholine receptors in muscle during the formation of the neuromuscular junction (NMJ; 7). Cdk5 and its activators are localized to the NMJ in adult muscle and mediate the actions of neuregulin by associating with the ErbB receptors (7). In addition to playing a key role at the NMJ, Cdk5 has recently been demonstrated to regulate NMDA receptors in cultured hippocampal neurons, suggesting that Cdk5 is also

involved in modulating post-synaptic functions in the CNS (9).

To further elucidate the functional roles of Cdk5 at the NMJ, it is important to examine the proteins interacting with active Cdk5/p35 complex in post-synaptic sites. In this study, we have used p35 as a bait to perform a yeast two-hybrid screen using a muscle cDNA library. Pctaire1, a member of Cdk-related protein kinase family, is identified to be a p35-interacting protein. Pctaire1 and its related proteins, Pctaire2 and 3, are 50-60 kDa proteins consisting of a core kinase domain flanked by unique N-terminal and C-terminal domains. The catalytic domain of Pctaire proteins contains all the motifs and amino-acid residues conserved in the Cdk family (10). It has been suggested that the N-terminal domains of Pctaire define the specific function of the particular Pctaire family member (11). In this study, we demonstrate that Pctaire1 can be phosphorylated by the Cdk5/p35 complex. Two of the potential Cdk5 phosphorylation sites for Pctaire1 reside on the N-terminal region that shows no amino acid homology with other Cdks. Our results show that one of them, serine-95, is the major phosphorylation site. Taken together, our findings demonstrate that p35 and Pctaire1 interact with each other, both *in vitro* and *in vivo*, and that Pctaire1 is a novel substrate of Cdk5. While Cdk5/p25 alone cannot directly activate Pctaire1, we provide evidence that phosphorylation of Pctaire1 by Cdk5 complex enhances its kinase activity.

Experimental Procedures

Plasmids and antibodies

cDNA encoding full-length p35 was subcloned into the yeast GAL4 DNA-binding vector pAS2-1 (Clontech, Palo Alto, CA) to construct GAL4bd-p35 which was used as a bait in the yeast two-hybrid screen. Similarly, cDNAs encoding p25 and p10 were also subcloned into pAS2-1. Partial cDNA fragments of mouse Pctaire1 (encoding amino acids 1-161, 162-456, 457-496, 1-456 or 162-496 of Pctaire1) were amplified by PCR, subcloned into the GAL4 transcriptional activation vector pACT2 (Clontech), and used in the experiment described in Figure 1. Full-length mouse Pctaire1 was subcloned into expression vector pMT21 for overexpression experiments. The GST-Pctaire1 fusion construct was made by subcloning full-length mouse Pctaire1 into pGEX-6P-1 (Amersham, Uppsala, Sweden). Point mutations (S/T to A) as well as K194A mutation were generated by PCR using complementary primers containing the mutations and subcloned into pGEX-6P-1 and pMT21. All constructs were confirmed by sequencing. Antibodies specific for Cdk5, p35 and Pctaire1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). It is noteworthy that similar results for Pctaire1 could be obtained with a second Pctaire1 antibody raised against the peptide GEAPTRVAPGELSIR.

Yeast two-hybrid screen

Yeast two-hybrid screen was performed following the Matchmaker Two-hybrid Screen protocol (Clontech). p35 was used as a bait to screen a mouse muscle cDNA library that has been constructed in the GAL4 transcriptional activation vector (pACT2). Yeast strain Y190 was transformed with the bait and

the library plasmids and transformants were selected on SD-Trp-Leu-His plates. β -galactosidase activity of His⁺ colonies was assayed by filter assay. The Pctaire1 clone isolated from the two-hybrid screen encodes the full-length protein with 369 nucleotides at the 5' end of the initiation codon. A construct without this extra sequence still interacts with p35 (data not shown); therefore these extra nucleotides are not necessary for the interaction between p35 and Pctaire1. Subsequent two-hybrid interaction analyses were carried out by cotransformation of plasmids containing the GAL4 DNA-binding (pAS2-1) and activation (pACT2) domains into yeast. β -galactosidase activity was assayed by a filter assay.

Cell culture and transfection

COS-7 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum plus antibiotics. Mouse C2C12 cells were maintained and differentiated as previously described (12). COS-7 cells were transfected with Pctaire1 or p35 plasmids using calcium phosphate method.

Protein extraction, immunoprecipitation and Western blot analysis

COS-7 cells were lysed in lysis buffer A (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 0.5% NP-40). Brain and muscle tissues were homogenized in lysis buffer B (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EGTA, 1 mM DTT, 1% NP-40, 0.25% sodium deoxycholate, 50 mM NaF) with various protease inhibitors. Western blot analysis was performed as

previously described (7). For kinase assay, brain and muscle extracts from wild type and Cdk5 knockout mice were prepared in lysis buffer B as previously described (7). Immunoprecipitations of COS-7 cell lysates were performed in lysis buffer A. Two hundred μg of COS-7 cell lysates were incubated with corresponding antibody (2 μg) at 4°C overnight and then incubated with 40 μl protein-G sepharose at 4°C for 1 h. The samples were washed with buffer A and resuspended in SDS sample buffer. Immunoprecipitated proteins were detected by Western blot analysis. Membrane and cytosolic fractions of muscle extracts were prepared as described previously (7).

Fusion protein and pull-down assay

GST-Pctaire1 was expressed in BL21 strain and purified using a glutathione-sepharose 4B column following the instruction of the manufacturer (Amersham). The untagged form of Pctaire1 was produced by digesting GST-Pctaire1 with PreScission Protease (Amersham). For the binding assay, GST or GST-p25 was incubated with Pctaire1 and Glutathione-sepharose beads in 500 μl PBS. After incubation at 4°C overnight, the beads were washed five times with PBS and resuspended in SDS sample buffer. Samples were resolved by SDS-PAGE and immunoblotted using anti-Pctaire1 antibody. Binding between GST-Pctaire1 and Hisp25¹⁴⁵⁻¹⁷⁰ was carried out as described previously (13). For pull-down studies, 5 μg of GST or GST-Pctaire1 were first incubated with glutathione sepharose beads in PBS buffer supplemented with 5 $\mu\text{g}/\mu\text{l}$ of BSA.

After incubation at 4°C with end-to-end rotation for 1 h, the beads were washed three times with PBS. GST or GST-Pctaire1 bound to the beads was then incubated with 100 µg of COS-7 extracts overexpressing p35 in buffer A at 4°C for 1 h. The beads were washed 5 times with buffer A. The bound protein were resuspended in SDS loading buffer, resolved by SDS-PAGE and analyzed by Western blot analysis.

RNA extraction and Northern blot analysis

Total RNA was prepared from rat tissues by lithium chloride/urea extraction (12). Northern blot analysis was performed as previously described (14). Full-length cDNA fragment of Pctaire1 was used as the probe.

***In vitro* Phosphorylation Assay**

Pctaire1 overexpressed in COS-7 cells and immunoprecipitated with anti-Pctaire1 antibody was used as a substrate for recombinant Cdk5/p25 in the *in vitro* kinase assay. The kinase assay was done at 30°C for 30 min in kinase buffer (20 mM MOPS, pH 7.4, 15 mM MgCl₂, 100 µM ATP) containing 1 µCi γ -³²P ATP. Two hundred ng of recombinant proteins (including GST-Pctaire1, Pctaire1 cleaved from GST fusion, kinase-dead mutant, phosphorylation site mutants) were also utilized as the substrates for Cdk5/p25 in the same assay. The phosphorylated proteins were separated on a SDS-PAGE and visualized by autoradiography. Similarly, the kinase activity of Pctaire1 immunoprecipitated

from brain and muscle of rat as well as wild type and Cdk5 knockout mice was measured using histone H1 as the substrate. Recombinant Cdk5/p25 protein (0.5 μ g) was incubated with 0.5 μ g of purified GST-Pctaire1 proteins (WT and K194A mutant), and the activity of the mixture was measured in the same kinase assay. Purified GST-Pctaire1 proteins (WT and K194A) were pre-incubated with adult rat brain lysate for 2 hours at 4°C followed by pull-down with glutathione sepharose beads and the kinase activity was measured in the same assay using MBP or histone H1 as the substrate. In some experiments, GST-Pctaire1 was incubated with or without recombinant Cdk5/p25 complex for 5 or 10 minutes and Cdk5/p25 was removed by washing with buffer B prior to the kinase assay.

Immunohistochemistry

Adult rat muscle sections (10 μ m) were collected and fixed with 2% paraformaldehyde/5% sucrose in PBS for 15 min at room temperature. Double staining was performed by incubating the sections with rhodamine-conjugated α -bungarotoxin (10^{-8} M; Molecular Probe) and primary antibodies specific for Pctaire1 or p35 at 4°C overnight, followed by FITC-conjugated goat anti-rabbit antibody at room temperature for 1 h as described. The sections were then washed and mounted for confocal microscopy.

Results

Identification of Pctaire1 as a p35-interacting protein

Pctaire1 was isolated as a positive clone from a mouse muscle cDNA library by yeast two-hybrid screen using the full-length p35 as the bait. Pctaire1 encodes a polypeptide of 496 amino acids, containing a 295-amino acid core kinase region that is similar to Cdc2, a 161-amino acid N-terminal extension and a 40-amino acid C-terminal extension (15). As a first step to dissect the regions of Pctaire1 that interact with p35, different constructs containing combinations of the N-terminal, kinase, and C-terminal domains of Pctaire1 were made. The ability of these constructs to interact with p35 in yeast was examined (Fig. 1). Our findings demonstrated that the full length Pctaire1 interacted strongly with p35, while the individual domains did not bind to p35. In addition to the full length Pctaire1, the construct comprising the kinase region and the C-terminal region also bound to p35, albeit to a lesser extent. It is noteworthy that in our yeast two-hybrid assay, binding was neither observed between Pctaire2 and p35, nor between Pctaire1 and Cdk5 (data not shown). To further characterize the interaction between p35 and Pctaire1, the N-terminal (p10) and C-terminal (p25) regions of p35 were tested for their ability to bind to Pctaire1. The p25 protein is a cleavage product of p35 that activates Cdk5 kinase robustly but lacks the amino-terminal 100 residues of p35 necessary for membrane targeting (16). We found that Pctaire1 interacted with the fragment encoding the C-terminal p25 protein and not the N-terminal region of p35 (Fig. 1).

Pctaire1 interacts with p35 both *in vitro* and *in vivo*

To test whether Pctaire1 binds to p35 directly, *in vitro* binding assay was performed. Pctaire1 was cleaved from GST-Pctaire1 fusion protein and purified Pctaire1 was incubated with GST or GST-p25. Prominent binding between p25 and Pctaire1 was detected (Fig. 2A, upper panel). A region of 26 amino acid residues (residues 145 to 170) of p35 has been identified to contain the binding sites of a number of p35-binding proteins (13). We also examined whether Pctaire1 binds to this region by using GST-Pctaire1 and His-p25¹⁴⁵⁻¹⁷⁰ in the binding assay. Pctaire1 also binds to this region (Fig. 2A, middle panel).

The association between Pctaire1 and p35 was also observed using an *in vitro* pull-down assay. A cell lysate of COS-7 cells overexpressing p35 was incubated with GST or GST-Pctaire1 protein. The recombinant Pctaire1, but not GST, was able to pull-down p35 protein from the transfected cell lysate (Fig. 2A, lower panel). To examine whether the interaction between Pctaire1 and p35 could be detected in mammalian cells, COS-7 cells were transiently transfected with both Pctaire1 and p35. Pctaire1 was immunoprecipitated from the cell lysate with Pctaire1 antibody, and the presence of p35 in the complex was analyzed by immunoblotting with p35 antibody. We found that Pctaire1 antibody, but not normal IgG, co-immunoprecipitated Pctaire1 with p35 from the COS-7 extract (Fig. 2B). Similarly, positive interaction was observed between Pctaire1 and p35 when p35 antibody was used for co-immunoprecipitation (Fig. 2B).

Pctaire1 associates with p35 in cultured myotubes and skeletal muscle

We have examined the expression of Pctaire1 in C2C12 myoblasts and differentiated myotubes. Western blot analysis revealed that the expression of Pctaire1 was increased in C2C12 myotubes (Fig. 3A). Association between Pctaire1 and p35 was demonstrated by co-immunoprecipitation experiment (Fig. 3A). To test whether Pctaire1 interacts with p35 in muscle, co-immunoprecipitation was performed using muscle extracts. While Pctaire1 expression could be observed in both membrane and cytosolic fractions, p35 could only be detected in the membrane-enriched fractions (Fig. 3B; 7). Pctaire1 can be co-immunoprecipitated by p35 antibody in membrane fraction of E21 muscle (Fig. 3B). These results suggested that Pctaire1 could interact with p35 *in vitro* as well as *in vivo*.

Pctaire1 is expressed in muscle during development

To examine the spatial and temporal profiles of Pctaire1 in rat tissues, Northern blot analysis was performed. It was previously reported that expression of Pctaire1 was prominently detected in adult rat brain, eye, skeletal muscle, heart, and reproductive organs (10, 15). The level of Pctaire1 transcripts in skeletal muscle was higher during embryonic and early postnatal stages and subsequently decreased in later stages of development (Fig. 4A). On the other hand, the mRNA expression of Pctaire1 in brain was more prominent during postnatal stages. An antibody raised against a peptide of Pctaire1 recognized a doublet in muscle and brain lysates (Fig. 4B). Both bands can be totally blocked by preincubation with the Pctaire1 peptide (data not shown). Pctaire1 detected in adult muscle apparently migrated slower than that in E18 and P5 muscle. The

band was downshifted after phosphatase treatment (data not shown), suggesting that Pctaire1 is phosphorylated in adult muscle. While Cdk5 protein was expressed at high level in embryonic muscle, the expression of p35 protein was much lower (Fig. 4B) and could only be detected in the membrane-enriched fractions of E18 muscle (7).

The kinase activity of Pctaire1 during development was also examined. When histone H1 was used as the substrate, higher level of Pctaire1 kinase activity was seen in embryonic muscle (E18) than in P5 and adult muscle, whereas the kinase activity remained very high throughout brain development (Fig. 4C).

Pctaire1 protein is concentrated at the neuromuscular synapse in adult muscle

To compare the subcellular localization of Pctaire1 with p35 in adult muscle, immunohistochemical analysis was performed. Pctaire1 staining was localized at the sarcolemma of muscle and concentrated at the NMJ co-stained with α -bungarotoxin. The staining for Pctaire1 was localized subjacent to AChRs and is broader than that observed for AChR. Compared to Pctaire1, p35 was found to be more restricted to the NMJ regions in muscle (Fig. 5).

Pctaire1 is a substrate of Cdk5

Cdk5 is a proline-directed kinase that phosphorylates serine and threonine residues immediately upstream of the proline residue. Since we were able to identify three potential Cdk5 phosphorylation sites (S/T)PX(K/H/R) in Pctaire1, the ability of active Cdk5 to phosphorylate Pctaire1 was examined. The purified Cdk5/p25 recombinant protein was able to phosphorylate Pctaire1,

either in the form of GST-Pctaire1 fusion protein or after cleavage (Fig. 6A). GST alone was not phosphorylated by Cdk5/p25 complex (data not shown). Moreover, active Cdk5 was also able to phosphorylate Pctaire1 immunoprecipitated from COS-7 cells overexpressing Pctaire1 (Fig. 6B). These results suggest that Pctaire1 is a novel substrate identified for Cdk5.

To determine which of the three potential sites can be phosphorylated by Cdk5, we generated point mutations (S/T to A) for each of the sites (S95, S138 and T380) as well as double mutations or triple mutations. GST-Pctaire1 fusion proteins containing these mutations were purified and used as substrates for the *in vitro* kinase assay. Mutation of serine-95 to alanine completely abolished the phosphorylation of Pctaire1 by Cdk5, as seen in single mutant as well as in double or triple mutant containing this mutation (Fig. 6C). Phosphorylation was slightly decreased in S138A mutant, whereas no change was seen in T380A mutant. Similar result was obtained when Pctaire1 containing these mutations were overexpressed in COS-7 cells and used as the substrate for the kinase assay (data not shown). This result demonstrates that Pctaire1 is a novel substrate of Cdk5 and that serine-95 is the major Cdk5 phosphorylation site.

To rule out the possibility that Pctaire1 phosphorylation is a result of autophosphorylation, we mutated the catalytic lysine-194 to alanine in the ATP binding pocket. The inactive Pctaire1 protein with this mutation can still be phosphorylated in the presence of Cdk5, in the same manner as the wild type protein (Fig. 6D). Therefore, the phosphorylation of Pctaire1 in the presence of Cdk5 is not mediated by its own kinase activity.

Pctaire1 kinase activity is decreased in Cdk5 knockout mice

To further establish the kinase-substrate relationship between Cdk5 and Pctaire1, we compared the kinase activity of Pctaire1 in wild type and Cdk5 knockout mice. Pctaire1 immunoprecipitated from brain or muscle extracts of both wild type and Cdk5 knockout mice was used in the kinase assay with histone H1 as the substrate. Phosphorylation of histone H1 by Pctaire1 was significantly reduced in Cdk5 knockout mice than in wild type mice for both muscle and brain extracts (Fig. 7). Cdk5 could not be detected in the immunoprecipitates by Western blotting (data not shown). This result suggests that Cdk5 might be involved in regulating the kinase activity of Pctaire1 *in vivo*.

Cdk5 enhances Pctaire1 activity

Purified bacterial expressed GST-Pctaire1 does not have any kinase activity, and pre-incubation of Pctaire1 with recombinant Cdk5/p25 complex could not increase the kinase activity (data not shown; 17). To test whether Pctaire1, like Cdk5, depends on an activator protein for kinase activity, we pre-incubated purified wild type and kinase dead mutant of GST-Pctaire1 with adult brain lysate. After pre-incubation, the Pctaire1 protein was affinity precipitated and tested for kinase activity. We found that high level of kinase activity was displayed by the wild type Pctaire1, and not the mutant protein (Fig. 8A). Moreover, no kinase activity was detected with Pctaire1 samples pre-incubated with COS-7 cells or adult rat muscle lysates (data not shown; 18). These results are compatible with the suggestion that Pctaire1 depends on brain-specific cellular activator for kinase activity.

Since we have shown that Cdk5 phosphorylates Pctaire1, and that Pctaire1 activity decreases in Cdk5 knockout mice, it is interesting to know whether Cdk5 can regulate Pctaire1 activity. To test this possibility, wild type as well as the kinase dead mutant of Pctaire1 that had been pre-incubated with brain extract were further incubated with recombinant Cdk5/p25 complex under protein phosphorylating conditions. The recombinant Cdk5/p25 complex was then removed by stringent wash and Pctaire1 activity was examined. MBP instead of histone H1 was used as the substrate protein since it is a poor substrate for Cdk5.

We found that Pctaire1 activity was increased after 10 or even 5 minutes of incubation with Cdk5/p35 complex, whereas the activity of its kinase dead mutant was not affected, suggesting that Cdk5 phosphorylation on Pctaire1 could enhance its activity (Fig. 8B). The use of MBP as the substrate, together with the stringent wash which resulted in undetectable Cdk5 in the precipitates, ruled out the possibility of Cdk5 contamination in the MBP phosphorylation assay. The mutation which changes serine-95 of Pctaire1 to alanine completely abolished the phosphorylation of Pctaire1 by Cdk5 (Fig. 6C). When GST-Pctaire1 harboring this mutation was used in the same assay, no significant increase in the phosphorylation of MBP was seen (Fig. 8C). These results suggested that Cdk5 phosphorylation on Pctaire1 could enhance Pctaire1 activity, which is consistent with the *in vivo* data from Cdk5 null mice.

Discussion

In the present study, Pctaire1 was identified as an interacting protein with p35 in a yeast two-hybrid screen. Our findings demonstrate that p35 and Pctaire1 interact with each other, both *in vitro* and *in vivo*. The full interaction between p35 and Pctaire1 requires the presence of the N-terminal, core kinase and C-terminal regions, suggesting that the three-dimensional structure of Pctaire1 is important for its association with p35. This finding is consistent with previous reports on the interaction between Pctaire1 and p11 or 14-3-3 proteins (19, 20), i.e. both the N- and C-terminal domains are required. We also found that Pctaire1 can be phosphorylated by Cdk5/p25 on serine-95, and is therefore a novel substrate of active Cdk5. Although the precise significance of this interaction remains to be elucidated, our findings suggest that Cdk5 regulates the kinase activity of Pctaire1.

Based on the yeast two-hybrid screen, previous studies have identified several proteins that interact with Cdk5/p35, including neurofilament, tau, cables and nudel (21-23). Our identification of Pctaire1 as a novel substrate for Cdk5 provides the first demonstration of a Cdk-related kinase, other than Cdk5, that interacts with p35. Analysis of the deduced amino acid sequence of Pctaire1, a Cdc2-like kinase, identifies the presence of domains conserved among Cdks, including kinase subdomains with ~52% similarity to Cdc2 kinases (20). On the other hand, it exhibits several features that are distinct from other members of the Cdk family. For example, the size of Pctaire1 is twice that of other Cdks (i.e. ~ 60 kDa compared to ~ 30 kDa) due to the presence of N-terminal and C-

terminal extensions. Unlike other Cdks, and similar to Cdk5, Pctaire1 is not involved in the regulation of cell cycle progression. The expression of Pctaire1 is ubiquitous, and is prominently expressed in highly differentiated tissues, including brain, eye, skeletal muscle and heart (18). In particular, Pctaire1 is abundant in the cytoplasm of terminally differentiated cells, such as elongated spermatids in testis, and postmitotic neurons in brain.

Although Pctaire1 is expressed in both neuronal and non-neuronal cells, their precise functions are still unknown. Kinase activity has been detected in both brain and testis (18, 20), suggesting a role for Pctaire1 in the process of differentiation in these tissues. However, purified Pctaire1 does not exhibit kinase activity (unpublished observation; 17), suggesting that Pctaire1 activation requires binding to a regulatory partner. We report here that the kinase activity of Pctaire1 depends on a brain-specific activator, consistent with the observation that Pctaire1 activity is much higher in adult brain than in adult muscle. Thus, like the other members of the Cdk family, such as p35 for Cdk5 or cyclins for other Cdks, Pctaire1 needs to associate with specific activator(s) in order to trigger its activity. While p35 is not the activator of Pctaire1 (unpublished observation; 5), we have provided several lines of evidence to demonstrate that Cdk5/p35 is involved in the regulation of Pctaire1 activity. We found that Pctaire1 is phosphorylated by Cdk5/p25 and that its kinase activity is significantly reduced in brain and muscle of Cdk5 null mice. In addition, Pctaire1 activity can be further enhanced by incubation with Cdk5/p25. Taken together, our findings suggest that Pctaire1 activity is regulated by Cdk5 *in vivo*.

It is possible that p35 may act as a substrate-targeting protein for Cdk5 to phosphorylate Pctaire1, which in turn modulate the activity of Pctaire1. The identification of the brain-specific activator of Pctaire1 will be critical in order to delineate the biological functions of Pctaire1 in Cdk5 signaling and to facilitate our understanding of the regulation of this protein.

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Footnotes

Abbreviations: AChR, acetylcholine receptor; Cdk, cyclin-dependent kinase; CNS, central nervous system; DTT, dithiothreitol; GST, glutathione-s-transferase; NMJ, neuromuscular junction; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Figure Legends

Fig. 1. Mapping of the interaction domain between Pctaire1 and p35. The indicated domains of Pctaire1 were cloned into vector pACT2 and cotransformed into yeast with p35 construct in pAS2-1. The interaction was examined by filter assay. p10 comprises the N-terminal (N-ter) 98 amino acids of p35, whereas p25 contains the C-terminal (C-ter) region of p35. The Pctaire1 (Pct1) construct containing both the kinase and the C-terminal domains binds to p35 and p25 (+), but weaker than the full-length Pctaire1 (+++). The remaining constructs did not bind to p35 (-).

Fig. 2. Association of p35 with Pctaire1 *in vitro* and *in vivo*. (A) Pctaire1 binds to p25 *in vitro*. Top panel: Purified GST-p25 protein, but not GST, binds to Pctaire1 protein. The loading control of Pctaire1 was indicated on the left lane. Middle panel: Pctaire1 binds to residues 145 to 170 of p35. GST-Pctaire1 and His-p25¹⁴⁵⁻¹⁷⁰ were used in the binding assay. His-p25¹⁴⁵⁻¹⁷⁰ was detected using anti-His antibody. Bottom panel: p35 overexpressed in COS-7 cells were pulled down by purified GST-Pctaire1. The total cell lysate was included on the left lane. (B) Association of p35 with Pctaire1 in COS-7 cells. p35 and Pctaire1 were cotransfected into COS-7 cells. The cell lysates from transfected cells were immunoprecipitated with p35 antibody and immunoblotted with Pctaire1

antibody (top panel). The rabbit normal IgG was used as a negative control. The cell lysates were also immunoprecipitated with Pctaire1 antibody and immunoblotted with p35 antibody (bottom panel).

Fig. 3. Association of p35 with Pctaire1 in muscle. (A) Association of p35 with Pctaire1 in C2C12 cells. Western blot of C2C12 myoblasts (B) and myotubes (T) using Pctaire1 or p35 antibodies was shown on the left panel. C2C12 myotube lysates were immunoprecipitated with p35 antibody and immunoblotted with Pctaire1 antibody (right panel). (B) Association of p35 and Pctaire1 in embryonic muscle. Western blot of membrane (M) and cytosolic (C) fractions of E21 muscle using p35 or Pctaire1 antibodies was shown on the left panel. Membrane fractions of muscle extracts were immunoprecipitated using p35 antibody and immunoblotted with Pctaire1 antibody (right panel).

Fig. 4. Temporal and spatial expression profiles of Pctaire1. (A) Northern blot analysis of Pctaire1 in developing rat muscle and brain. Ad, adult. Two transcripts were identified as indicated by the arrowheads. (B) Western blot analysis of Pctaire1, p35 and Cdk5 in developing rat muscle and brain. (C) Kinase activity of Pctaire1 in developing muscle and brain. Pctaire1 was immunoprecipitated from muscle or brain extracts and subjected to *in vitro* kinase assay using histone H1 as the substrate.

Fig. 5. Immunohistochemical localization of Pctaire1 and p35 in rat skeletal muscle. Pctaire1 protein is localized at the sarcolemma of muscle and predominantly concentrated at the neuromuscular synapse of adult rat. Immunohistochemical analysis for Pct1 and p35 (green, left panels) and AChR (red, middle panels) and merged images (right panels) were shown. Scale bar: 50 μm .

Fig. 6. *In vitro* phosphorylation of Pctaire1 by Cdk5/p25 complex. (A) Purified GST-Pctaire1 and Pctaire1 cleaved from GST fusion were utilized as the substrates for the recombinant Cdk5/p25 complex (+) in the kinase assay; - indicates control without addition of Cdk5/p25. (B) Pctaire1 overexpressed in COS-7 cells and immunoprecipitated by Pctaire1 antibody was used as the substrate in the kinase assay. The addition of Cdk5/p25 complex (+) increased the phosphorylation of Pctaire1. (C) Wild type (WT) Pctaire1 or Pctaire1 harboring the S95A (1), S138A (2), or T380A (3) single, double (12, 23 or 13) or triple (123) mutations were expressed as GST-fusion protein and used as substrates in the kinase assay. Purified proteins were shown in Coomassie blue stained gel (bottom). (D) Wild type (WT) Pctaire1 or kinase-dead (K194A) Pctaire1 were expressed as GST-fusion protein and used as substrates in the kinase assay with increasing amounts of Cdk5/p25 (0.25 to 1 μg).

Fig. 7. Pctaire1 kinase activity in E17.5 wild type and Cdk5 knockout mice.

Pctaire1 was immunoprecipitated from both brain and muscle extracts of wild

type (+/+) and Cdk5 knockout (-/-) mice and its kinase activity was examined using histone H1 as substrate (top panel). Western blot analysis of the same extracts using Pctaire1 antibody and Cdk5 antibody was also shown (middle and bottom panels).

Fig. 8. Effect of Cdk5/p25 on Pctaire1 activity. (A) Adult brain has potential activator(s) for Pctaire1. Equal amount of purified GST-Pctaire1 proteins, WT and K194A, were pre-incubated with adult rat brain lysate. The proteins were then pulled down by glutathione sepharose beads and the kinase activity was measured by *in vitro* kinase assay using histone H1. (B) Cdk5/p25 complex increases Pctaire1 activity. Equal amount of purified GST-Pctaire1 proteins, WT and K194A, were pre-incubated with adult rat brain lysate. Both proteins were then pulled down and incubated with (+) or without (-) recombinant Cdk5/p25 complex in the kinase buffer. After 5 minute (upper panel) or 10 minute (lower panel) incubation, Cdk5/p25 was removed by stringent washing and the activity of GST-Pctaire1 was measured by *in vitro* kinase assay using MBP as substrate. (C) Cdk5 phosphorylation on Pctaire1 increases its activity. Equal amount of GST-Pctaire1 proteins, WT and S95A, were used in the assay as described in (B).

Fig. 1

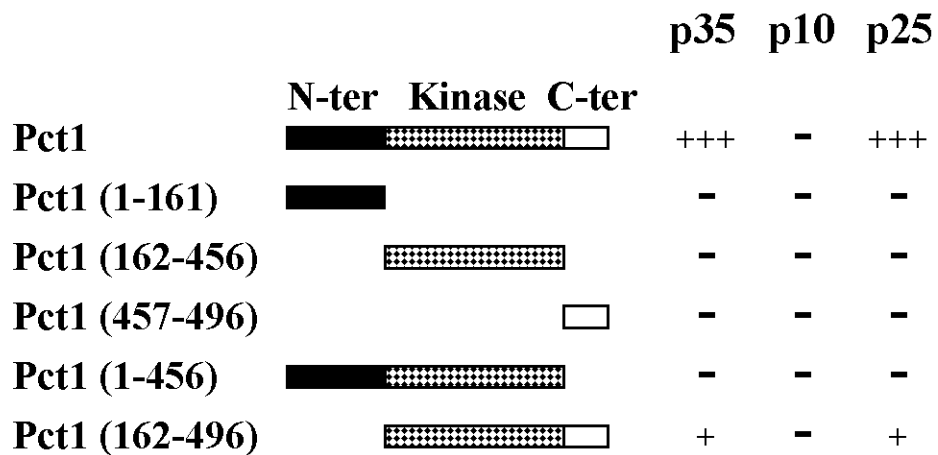
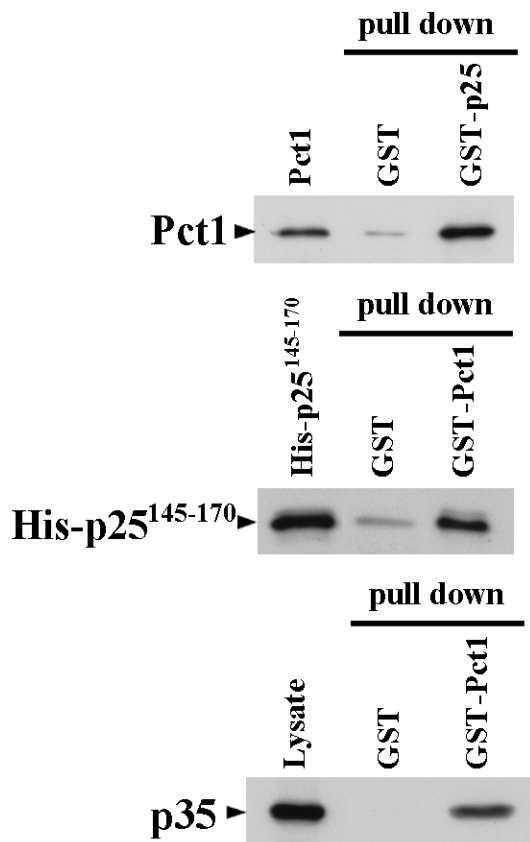


Fig. 2

A



B

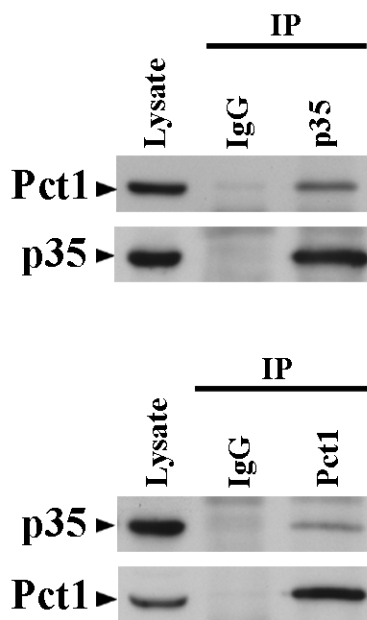


Fig. 3

A

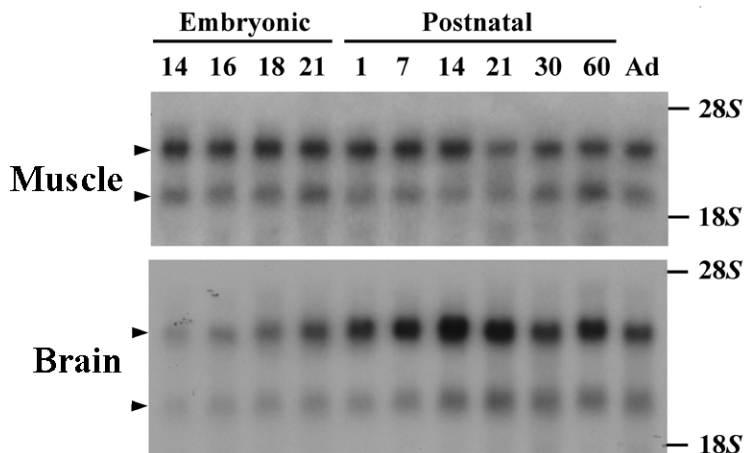


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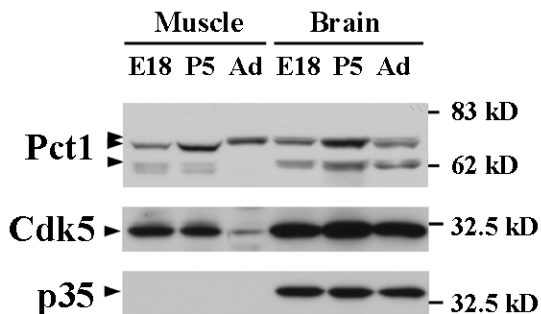


Fig. 4

A



B



C

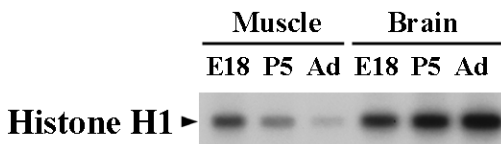


Fig. 5

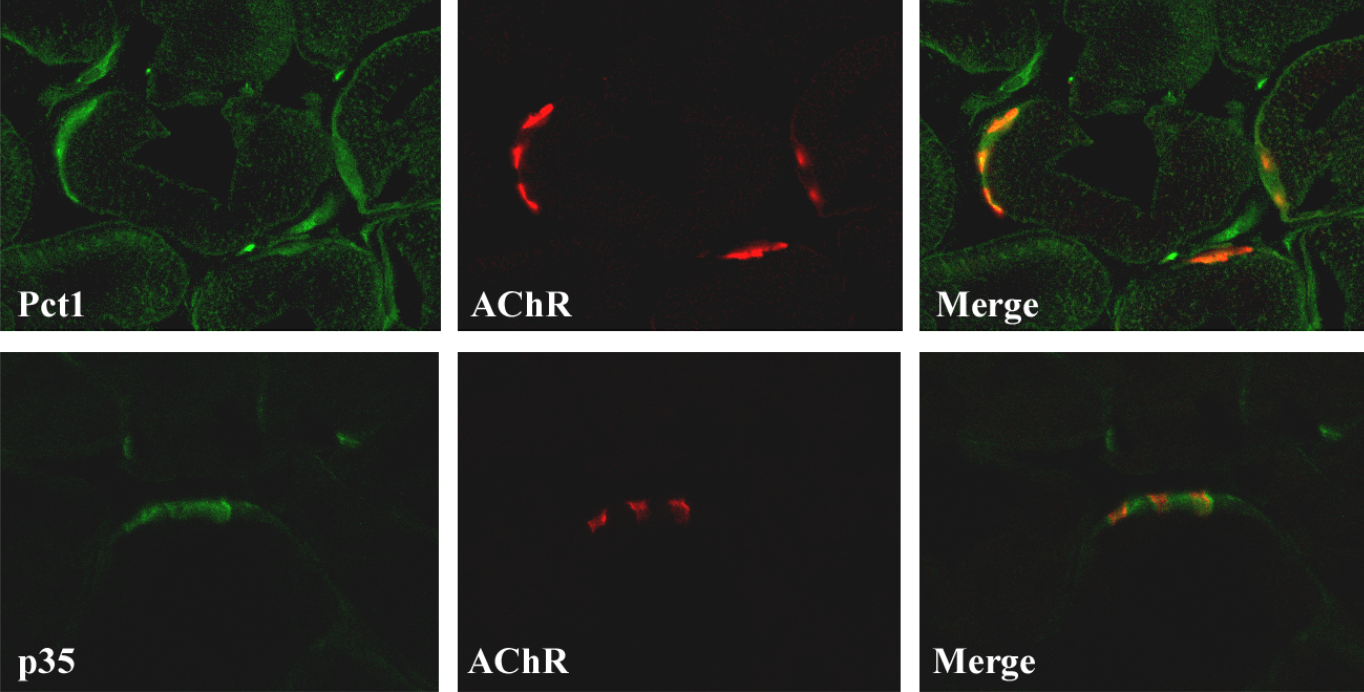


Fig. 6

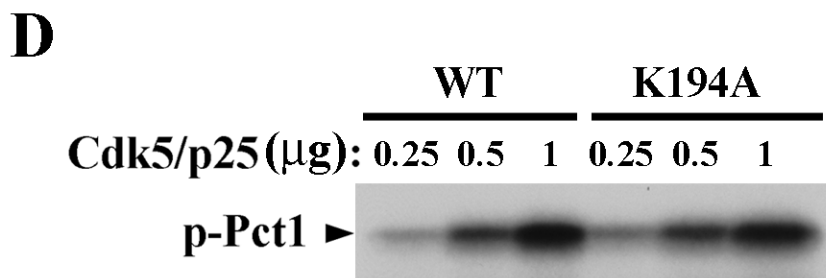
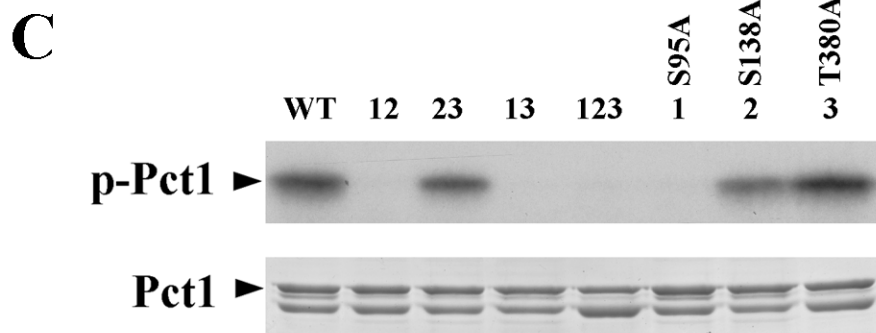
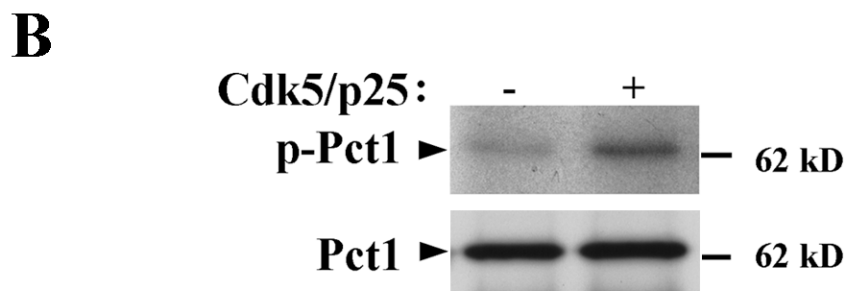
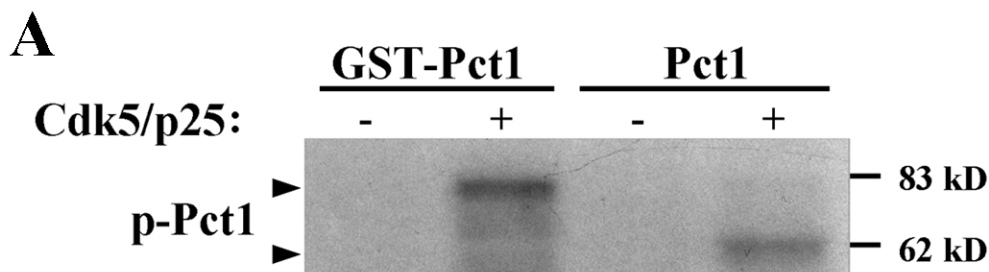


Fig. 7

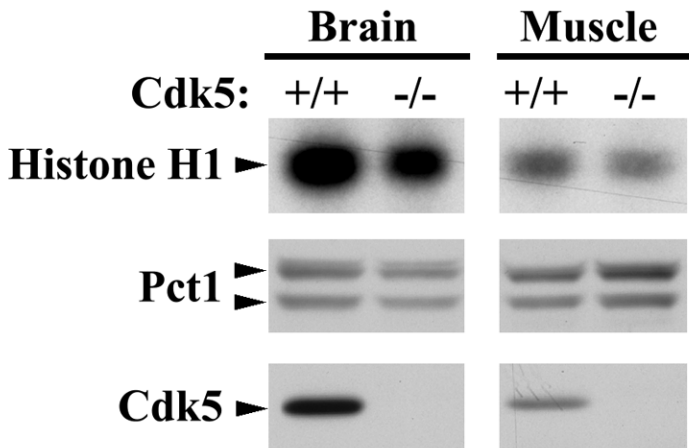
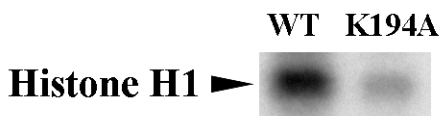
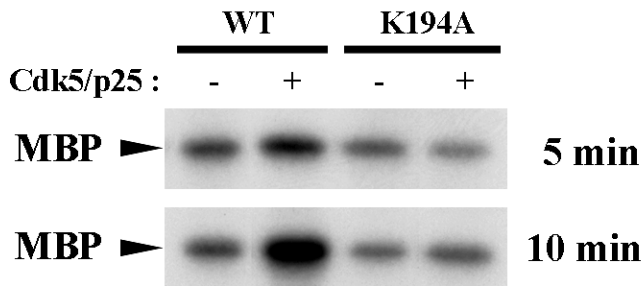


Fig. 8

A



B



C

