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CYCLIN-DEPENDENT KINASE 5-DEPENDENT PHOSPHORYLATION OF PCTAIRE1 REGULATES DENDRITE DEVELOPMENT

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Abstract—Pctaire1, a Cdk-related protein kinase, is prominently expressed in terminally differentiated tissues, including the brain and the testis. We have previously shown that Pctaire1 regulates neurotransmitter release through phosphorylation of NSF, and its kinase activity is regulated by the Cdk5-dependent phosphorylation at Serine-95 (Ser95). Nonetheless, the functional roles of Pctaire1 in neurons during development remained poorly understood. In this study, we found that Pctaire1 is expressed along neurites and is concentrated at the growth cones of early differentiating hippocampal neurons. Upon maturation of these neurons, Pctiare1 is expressed as puncta and co-localized with synaptic marker in dendrites. Phosphorylation of Pctaire1 at Ser95 increases upon neuronal differentiation, concurrent with the elevation in Cdk5 activity. Knockdown of Pctaire1 abolishes dendrite development, and more importantly, expression of Ser95 phosphorylation-deficient mutant of Pctaire1 also reduces dendrite complexity, suggesting that Cdk5 regulates Pctaire1 functions in differentiating neurons. Together, our findings demonstrate that Cdk5-dependent phosphorylation of Pctaire1 at Ser95 plays an important role in dendrite development.

Key words: cyclin-dependent kinase 5, p35, neurite, dendrite, neuronal development.

Neuronal morphological changes that are important for neural developmental processes such as neuron migration and synapse formation are tightly controlled by concerted phosphorylation of signaling proteins or cytoskeletal proteins. Thus, the precise interplay among protein kinases is essential for integrating multiple signaling pathways in neuronal morphogenesis. Indeed, phosphorylation of kinases themselves contributes as one of the major mechanisms for regulating their activities. Pctaire1, a cyclin-dependent kinase (Cdk)-related protein, was originally identified as a Cdc2-like kinase (Meyerson et al., 1992; Okuda et al., 1992). Pctaire1 is ubiquitously expressed in mammalian tissues, and is prominently found in highly differentiated tissues such as the brain and the testis (Hirose et al., 1997; Besset et al., 1999). Although Pctaire1 has been identified more than a decade ago, its precise functions in the nervous system remained largely unknown. The lack of knockout or transgenic animal models of Pctaire1 hinders the understanding of its *in vivo* functions. Nonetheless, through its interaction with various synaptic vesicle-associated proteins including N-ethylmaleimide-sensitive fusion protein (NSF), syntaxin1, synaptotagmin and synapsinI, Pctaire1 is suggested to regulate vesicle transport at presynaptic terminals in the adult rat brain (Liu et al., 2006). Moreover, Pctaire family members interact with Cables and 14-3-3 proteins, implicating its roles in neurite outgrowth and neuronal migration (Sladeczek et al., 1997; Zukerberg et al., 2000; Yamochi et al., 2001; Kajiwara et al., 2009). Consistent with this notion, Pctaire1 regulates neurite outgrowth in a neuroblastoma cell line, N2A (Graeser et al., 2002). However, the precise functions of Pctaire1 in neuronal development remained obscure.

The mechanisms by which Pctaire1 activity is regulated were also enigmatic (Graeser et al., 2002). Similar to other members of the Cdk family, Pctaire1 requires specific activator(s) to trigger its activity (Cheng et al., 2002; Graeser et al., 2002). Cyclin (CYY-1) has recently been shown to be an essential activator for Pctaire (PCT-1) in *Caenorhabditis elegans* (Ou et al., 2010). Importantly, Pctaire1 activity is tightly regulated by its phosphorylation at distinct sites *in vitro* (Cheng et al., 2002; Graeser et al., 2002). Phosphorylation of Pctaire1 at Ser153 by PKA reduces its kinase activity (Graeser et al., 2002), whereas Cdk5-mediated phosphorylation of Pctaire1 at Ser95 enhances its activity (Cheng et al., 2002). However, the temporal regulation of Pctaire1 phosphorylation and their roles in neurons remained unclear.

In this study, we found that Pctaire1 is expressed at the growth cones of early differentiating neurons, and is enriched at synapses of differentiated neurons. Activity of Pctaire1 increased in early differentiating neurons and in the postnatal rat brain, with its temporal profile similar to that of the Cdk5-dependent phosphorylation of Pctaire1 at Ser95, suggesting that the specific phosphorylation of Pctaire1 by Cdk5 regulates its activity. Suppressing the expression of Pctaire1 in neurons perturbed initial neurite extension, and significantly inhibited dendrite outgrowth and maintenance at a later stage. Interestingly, the Ser95 phosphorylation of Pctaire1 and its activity is important for the proper function of Pctaire1 in dendrite development. To-

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Abbreviations: Cdk, cyclin-dependent kinase; DIV, days *in vitro*; GFP, green fluorescent protein.

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gether, we demonstrated that Pctaire1 is critically required for dendrite development in a Cdk5-dependent manner.

EXPERIMENTAL PROCEDURES

Constructs and antibodies

Expression constructs encoding different mutants of Pctaire1 were generated as described (Cheng et al., 2002). The shRNA target sequence for mouse Pctaire1 is 5′ acctcaatactatcccaac 3′. The complementary oligonucleotides for corresponding sequences were annealed and subcloned into the pSUPER vector. The knockdown efficiency of pSUPER Pctaire1 shRNA construct was confirmed by overexpressing pSUPER Pctaire1 shRNA construct in HEK 293T cells or in hippocampal neurons, and then examined by Western blot analysis or immunocytochemical analysis, respectively.

Antibodies specific for Cdk5 (C-8) and p35 (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for MAP2 and α-tubulin were from Sigma-Aldrich (St. Louis, MO, USA) and Tau-1 antibody from Millipore (Billerica, MA, USA). Rhodamine-conjugated phalloidin was purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against p39 (KGRRPGGLPEE, a.a.14–24) and Pctaire1 (VQSPVRVRMRNHPPRK, a.a.93–108), as well as phospho-specific antibody against Pctaire1 at Ser95 [pPctaire; VQ[pS] PVRVRMRNHPPRK, a.a.93–108] were custom generated. Recombinant proteins encoding histone H1 and myelin basic protein (MBP) used in *in vitro* kinase assay were purchased from Millipore (Billerica, MA, USA).

Cell cultures and transfection

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics. Primary cortical and hippocampal neurons were prepared from embryonic (E) day 18–19 mouse embryos and plated on culture dishes or coverslips coated with poly-D-lysine (12.5 μg/ml; Sigma-Aldrich, St. Louis, MO, USA). Cortical neurons were fed with Neurobasal medium supplemented with 2% B27 (Invitrogen, Carlsbad, CA, USA). Hippocampal neurons were seeded on 18 mm coverslips coated with poly-D-lysine (50 μg/ml) at two densities: high and low density (2.5×10^5 and 0.5×10^5/18 mm coverslip) for calcium phosphate transfection and immunocytochemical analysis, respectively.

HEK 293T cells were transiently transfected with different combination of plasmids as indicated using LipofectAMINE PLUS reagents according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Hippocampal neurons and cortical neurons were transfected with various cDNA constructs or pSUPER shRNA constructs plus green fluorescent protein (GFP) using calcium phosphate transfection (Sala et al., 2001). The morphology of GFP-expressing neurons was visualized under confocal microscopy (Fluoview 300, Olympus Imaging Corp, Tokyo, Japan). The total neurite length and dendritic complexity was quantified by Metamorph (Molecular Devices, Inc., Sunnyvale, CA, USA) and Sholl analysis using Image-J software (National Institute of Health), respectively.

Western blot analysis, kinase assay and immunocytochemical analysis

Proteins were extracted using different lysis buffers supplemented with various protease inhibitors. HEK 293T cells and cultured neurons were lyzed in lysis buffer A (in mM) (Tris, pH 8.5, 100; NaCl, 100; EDTA, 1) with 0.5% Nonidet P-40 and various protease inhibitors. Rat brain tissues were homogenized in lysis buffer B (in mM) (Tris, pH 8, 50; NaCl, 150; EGTA, 2; DTT, 1; NaF, 5) with 1% Nonidet P-40 and 0.25% sodium deoxycholate. Kinase assay was performed as previously described (Fu et al., 2007). Densitometric quantification of protein band intensity was performed using the NIH Image J program. Statistical analysis was performed using Student's *t*-test. All experiments were performed for three times.

To examine the endogenous expression of Pctaire1, neurons were fixed with 4% paraformaldehyde/5% sucrose at 37 °C for 20 min. Immunostaining was performed as previously described (Fu et al., 2007).

RESULTS

Pctaire1 is prominently expressed in the nervous system (Hirose et al., 1997; Besset et al., 1999) but its functional roles in neurons remained unclear. Thus, we first examined the subcellular distribution of Pctaire1 in cultured neurons at different developmental stages. While basal Pctaire1 was expressed in the soma and minor neurites of early differentiating hippocampal neurons (3 days *in vitro* (DIV)), its expression was prominently detected in axons, being co-localized with the axonal marker Tau-1 (Fig. 1a). Interestingly, Pctaire1 was enriched at the filopodia of growth cones (Fig. 1b), as revealed by its co-localization with F-actin filament, suggesting that Pctaire1 is involved in axon extension or growth cone turning. Upon differentiation, Pctaire1 expression was ubiquitously detected in the mature hippocampal neurons, and as clusters in the dendrites (Fig. 1c, d). These Pctaire1 clusters were partially co-localized with the presynaptic protein synaptophysin (Fig. 1d), suggesting that Pctaire1 is concentrated at the synapses. The regulation of subcellular distribution of Pctaire1 in cultured neurons upon differentiation suggests that Pctaire1 may exert distinct functional roles during neuronal development.

We have previously demonstrated that Pctaire1 exists as a signaling complex with Cdk5/p35 and is phosphorylated by Cdk5 at Ser95 residue *in vitro* (Cheng et al., 2002). The Cdk5-dependent phosphorylation of Pctaire1 is suggested to enhance its kinase activity (Cheng et al., 2002). To study the regulation of phosphorylated Pctaire1 at Ser95 (pSer95) in neurons, we generated a phospho-specific antibody and confirmed the specificity of this antibody (pPctaire1) using *in vitro* phosphorylation assay. Recombinant proteins encoding for GST-Pctaire1 (WT) or its mutants (S95A or T380A) were subjected to *in vitro* phosphorylation by the Cdk5/p25 complex. Robust phosphorylation of WT Pctaire1 at Ser95 by active Cdk5/p25 was observed using the pPctaire1 antibody, whereas basal phosphorylation of WT Pctaire1 was not detected (Fig. 2a). Mutation of Pctaire1 on Ser95, but not Thr380, to Ala abolished this specific phosphorylation (Fig. 2a), further confirming the specificity of this antibody. Whereas basal phosphorylation of Pctaire1 was detected when overexpressed in COS-7 cells, phosphorylation of Pctaire1 at Ser95 was significantly induced upon co-transfection of p35 or Cdk5/p35 (Fig. 2b). On the contrary, co-expression of the dominant negative form of Cdk5 (DN Cdk5)/p35 failed to induce Pctaire1 phosphorylation at Ser95, and mutating Ser95 on Pctaire1 abolished this specific phosphorylation, thus confirming the Ser95 phosphorylation of Pctaire1 by Cdk5 in mammalian cells (Fig. 2b).

We then examined the temporal regulation of phosphorylated Pctaire1 at Ser95 in neurons, and correlated this profile with the change of Pctaire1 activity. Total and