Phosphorylation of Adult Type Sept5 (CDCrel-1) by Cyclin-dependent Kinase 5 Inhibits Interaction with Syntaxin-1*

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Increasing evidence implicates cyclin-dependent kinase 5 (Cdk5) in neuronal synaptic function. We searched for Cdk5 substrates in synaptosomal fractions prepared from mouse brains. Mass spectrometric analysis after two-dimensional SDS-PAGE identified several synaptic proteins phosphorylated by Cdk5-p35; one protein identified was Sept5 (CDCrel-1). Although septins were isolated originally as cell division-related proteins in yeast, Sept5 is expressed predominantly in neurons and is implicated in exocytosis. We confirmed that Sept5 is phosphorylated by Cdk5-p35 in vitro and identified Ser17 of adult type Sept5 (Sept5_v1) as a major phosphorylation site. We found that Ser17 of Sept5_v1 is phosphorylated in mouse brains. Coimmunoprecipitation from synaptosomal fractions and glutathione S-transferase-syntaxin-1 pulldown assays of Sept5_v1 expressed in COS-7 cells showed that phosphorylation of Sept5_v1 by Cdk5-p35 decreases the binding to syntaxin-1. These results indicate that the interaction of Sept5 with syntaxin-1 is regulated by the phosphorylation of Sept5_v1 at Ser17 by Cdk5-p35.

Cyclin-dependent kinase 5 (Cdk5)1 is a proline-directed Ser/Thr kinase that is activated by binding to a neuron-specific activator, p35 or p39 (1–3). In contrast to other members of the Cdk family, which are known as cell cycle promoting factors, Cdk5 plays a role in neuronal activities unrelated to cell cycle progression. Cdk5 is involved in neuronal migration during brain development and neurodegeneration in aged brains (4–8). Recent evidence indicates that Cdk5 also participates in synaptic transmission (9). Cdk5-p35 inhibits neurotransmitter release by phosphorylating the P/Q type calcium channels (10) and inhibits their endocytotic recycling by phosphorylation of dynamin 1 and amphiphysin 1 in the presynaptic region (11–13). Cdk5 is also thought to regulate exocytosis by inhibiting the interaction of Munc18 with syntaxin-1A by phosphorylation (14–16). Cdk5 also phosphorylates several postsynaptic proteins including NR2A subunit of N-methyl-d-aspartate (NMDA) receptor (17, 18), postsynaptic density-95 (19), protein phosphatase 1 inhibitor-1 (20), and DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) (21). However, the precise function of Cdk5-p35 in synaptic activity has not been elucidated fully.

Sept5 (also known as CDCrel-1) is a member of the septin family of cytoplasmic 40–60-kDa proteins comprising a highly conserved GTPase motif at the N terminus and a coiled-coil domain at the C terminus. Septins were first discovered in mutants of budding yeast (Saccharomyces cerevisiae) that cannot complete cell division (22). Yeast septins form hetero-oligomeric filaments that contribute to bud site selection and neck stability (23–25). Septins play an essential role in cytokinesis in Drosophila (26) and mammals (27). Mammalian septins are involved in many other cellular activities including membrane dynamics, apoptosis, and cytoskeletal remodeling (28–30). Several septins are also expressed in postmitotic neurons. Sept5 is expressed predominantly in neurons; localizes in presynaptic regions; interacts with syntaxin-1A, a component of the SNARE complex; and regulates neurotransmitter release (31, 32). Sept5 is thought to inhibit exocytosis by acting as a physical barrier, as shown by the observation that overexpression of Sept5 in HIT-T15 cells inhibits evoked human growth hormone secretion (32). Prolonged overexpression induces neurodegeneration of dopaminergic neurons in rat brain (33). Sept5 is a substrate for parkin, an E3 ubiquitin-protein ligase, whose mutation is a cause of autosomal recessive familial Parkinson disease (34). Thus, proper regulation of Sept5 is important for neuronal function and survival. However, the regulation of the functions of Sept5 is not understood at all.

We identified adult type Sept5 (Sept5_v1) as a Cdk5 substrate in the synaptosomal fractions using the combined meth-
Ods of two-dimensional SDS-PAGE and mass spectrometric analysis. We found Ser17 of Sept5_v1 at a phosphorylation site in vitro and in vivo and found that phosphorylation of Sept5_v1 at Ser17 decreases the binding to syntaxin-1. We hypothesize that Cdk5 modulates synaptic vesicle release by regulating the interaction between Sept5 and syntaxin-1.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies against CDCrel-1 (C-20), Cdk5 (DC17), p35 (C-19), and Myc tag (9E10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human CDCrel-1 (C354) antibody was purchased from MBL (Nagoya, Japan). Anti-dynamin (Clone 41) antibody was obtained from BD Transduction Laboratories (San Diego, CA). Anti-N-methyl-D-aspartate receptor 2A (NMDAR2A) antibody was purchased from Chemicon (Temecula, CA). Antibodies to syntaxin-1 (6D2, 10H5), SNAP-25 (BRO8), and VAMP-2 (3D10) were described previously (35, 36). Anti-Ser(P)17 antibody was generated by immunizing rabbits with the synthetic phosphopeptide of EWLLpSPRTQAC (AnyGen, Kwang-ju, Korea) and was affinity-purified using the peptide conjugated to Sepharose beads using SulfoLink (Pierce) to produce the negative nonphosphorylated peptide and positive phosphorylated peptide.

**Plasmids**—Myc adult type mouse Sept5 (Sept5_v1) was provided by Dr. Ted M. Dawson (The Johns Hopkins University of Medicine, Baltimore, MD) (34). cDNA of Sept5_v2 or Sept5_v1 excised with KpnI and NotI was cloned into a pcDNA3 vector (Invitrogen). Ala (S17A), Asp (S17D), and Glu (S17E) mutants of Ser17 in Sept5_v1 were generated by a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using pcDNA3-Sept5_v1 as a template according to the manufacturer’s protocol.

**Preparation of Synaptosomal Proteins from Mouse Brains**—Subcellular fractionation and preparation of synaptosomes were performed as described previously with some modifications (37). In brief, 12–15-week-old C57BL/6 mice were sacrificed by cervical dislocation followed by decapitation. The brains were homogenized with a Teflon pestle glass homogenizer in 10 volumes of HEPES buffer (5 mM HEPES, pH 7.5, 1 mM EGTA, 0.4 mM Pefabloc SC (Merck, Darmstadt, Germany), 1 μg/ml leupeptin, and 1 μM E64) containing 0.32 M sucrose. The homogenate (H) was centrifuged at 800 g for 10 min. The supernatant (S1) was centrifuged again at 8,500 x g for 15 min. The pellet (P2) was suspended in HEPES buffer containing 0.32 M sucrose and centrifuged at 9,000 x g for 15 min to yield the supernatant (S2) and pellet (P2’). The P2’ was homogenized in HEPES buffer containing 0.32 M sucrose with a Teflon pestle glass homogenizer. The homogenate was layered on top of a 1.2 and 0.8 M stepwise sucrose gradient and centrifuged at 100,000 x g for 1.5 h using an SPR28-5A rotor (Hitachi, Tokyo, Japan). Synaptosomes sedimented to the 0.8–1.2 M sucrose interface as a broad band with high turbidity.

To separate the detergent-soluble and -insoluble proteins, the synaptosomal proteins were suspended in MOPS buffer (10 mM MOPS, pH 6.8, 1 mM MgCl2, 0.1 mM EGTA, 0.1 mM EDTA, 0.4 mM Pefabloc SC, 1 μg/ml leupeptin, and 1 μM E64) containing 0.5% Nonidet P-40 and centrifuged at 100,000 x g for 1 h. All of the procedures were performed at 4°C throughout the preparation.

**In Vitro Phosphorylation of Synaptosomal Proteins by Cdk5-p35—**Cdk5-p35-His was purified from the extract of Sf9 cells infected with baculovirus encoding Cdk5 and p35-His as described previously (38). Synaptosomal proteins were phosphorylated by Cdk5-p35 in MOPS buffer containing 0.5% Nonidet P-40 and 0.1 mM [γ-32P]ATP for 15 min at 35°C. The reaction was stopped by cooling on ice, and the detergent-soluble supernatant and detergent-insoluble pellet fractions were separated by centrifugation at 100,000 x g for 1 h at 4°C. The proteins were analyzed on SDS-PAGE or two-dimensional PAGE, and the radioactivity was detected by a BAS2000 Image Analyzer (Fuji Film, Tokyo, Japan).

**Mass Spectrometric Analysis**—Protein spots were stained by ProteoSilver Plus silver stain kit (Sigma) according to the manufacturer’s protocol and excised from the two-dimensional PAGE gel. After washing, the gels were digested by incubation in 100 mM Tris-HCl, pH 8.8, with trypsin (39). The tryptic digests were analyzed by an LC-MS/MS system as described previously (40).

**Cell Culture, Preparation of Cell Extracts, and Immunoprecipitation for Phosphorylation**—COS-7 cells cultured in Dulbecco’s modified Eagle’s medium (KOHJIN BIO, Saitama, Japan) containing 10% fetal bovine serum (JRH, Lenexa, KS) were transfected with pcDNA3-Myc-Sept5 using a PolyFect transfection reagent (Qiagen). The cells were harvested 24 h after transfection. Myc-Sept5 was prepared from COS-7 cell extracts in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.4 mM Pefabloc SC, 1 μg/ml leupeptin, and 1 μM E64) by immunoprecipitation with anti-Myc antibody using protein G-Sepharose beads (GE Healthcare Bio-Science, Piscataway, NJ). Sept5 was also prepared from synaptosomes in RIPA buffer by immunoprecipitation with anti-Sept5 antibody using protein A-Sepharose beads (GE Healthcare Bio-Science). The beads were washed with RIPA buffer four times and with MOPS buffer containing 0.5% Nonidet P-40 four times, and the immunoprecipitated Sept5 was phosphorylated in the presence of 0.1 mM [γ-32P]ATP by Cdk5-p35.

**Coimmunoprecipitation of Syntaxin-1 with Sept5 from Synaptosomes**—The synaptosomes in MOPS buffer containing 0.5% Nonidet P-40 and 100 mM NaCl were incubated with anti-Sept5 for 1 h at 4°C, protein A-Sepharose was added, and the mixture was incubated for 1 h at 4°C. The beads were washed with MOPS buffer containing 0.5% Nonidet P-40 and 100 mM NaCl, and the binding of syntaxin-1 with Sept5 was examined by immunoblotting.

**GST-Syntaxin-1A Pulldown Assay**—GST-syntaxin-1A was expressed in Escherichia coli strain BL21 and purified from the cell extract by glutathione-Sepharose beads (GE Healthcare Bio-Science). The extracts of COS-7 cells expressing Sept5_v1, either WT, S17A, S17D, or S17E, with or without Cdk5-p35, were incubated with GST-syntaxin-1A immobilized on glutathione-Sepharose beads for 1 h at 4°C. The beads were washed with MOPS buffer containing 0.5% Nonidet P-40 and 100 mM NaCl, and the bound proteins were analyzed by immunoblotting.
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FIGURE 1. Immunoblotting of isolated synaptosomes with antibodies to synaptic proteins and Cdk5-p35. A, enrichment of Cdk5 and p35 in the synaptosomal fractions. Synaptosomes were prepared as described under “Experimental Procedures.” Immunoblots of VAMP-2 and dynamin 1 as presynapse markers and NR2A as a postsynapse marker are shown together with those of anti-Cdk5 and anti-p35 antibodies. H, brain homogenate; S1, postnuclear fraction; P1, nuclear (and debris) fraction; S2, cytosolic fraction; P2, crude synaptosomal fractions; S2’, cytosolic fraction after recentrifugation; P2’, crude synaptosomal fractions after recentrifugation; Top, light membrane fraction containing myelin, endoplasmatic reticulum, and Golgi apparatus; Synaptosome, synaptosomal fraction; Bottom, heavy membrane fraction containing mitochondria. B, immunoblots of detergent-soluble and -insoluble synaptosomal proteins. Synaptosomes were suspended with MOPS buffer containing 0.5% Nonidet P-40 and centrifuged at 100,000 × g for 1 h. The supernatant (sup) and pellet (ppt) were immunoblotted with antibodies against VAMP-2, dynamin 1, NR2A, Cdk5, and p35.

RESULTS

Subcellular Fractionation of Cdk5-p35 in Synaptosomes of Mouse Brains—Synaptosomes were prepared from mouse brains by the method described under “Experimental Procedures.” Successful fractionation of synaptosomes was confirmed by the enrichment of synaptic proteins such as VAMP-2, dynamin 1, and NR2A (Fig. 1A). Both Cdk5 and p35 were detected in the isolated synaptosomal fractions, as reported previously (Fig. 1A) (13, 41). p35 was more concentrated in synaptosomes than was Cdk5, which was distributed more evenly in other fractions. Synaptosomal proteins were separated further into detergent-soluble and -insoluble fractions by centrifugation in the presence of Nonidet P-40. Consistent with previous observations, presynaptic proteins (e.g. VAMP-2) were enriched in the Nonidet P-40-soluble fraction, and the Nonidet P-40-insoluble fraction contained the postsynaptic density proteins (e.g. NR2A) and detergent-resistant membrane components and cytoskeletal proteins. Dynamin 1 was enriched into the Nonidet P-40-insoluble fraction (Fig. 1B). More Cdk5 and p35 was found in the Nonidet P-40-insoluble fraction than in the Nonidet P-40-soluble fraction, implicating the presence of more substrate proteins in the detergent-resistant membranes, cytoskeletons, or postsynaptic density (42).

Phosphorylation of Synaptosomal Proteins by Cdk5-p35 and Identification of Sept5 as a Phosphorylated Protein—To identify the substrates of Cdk5, we incubated synaptosomal proteins with exogenously added Cdk5-p35 in the presence of [γ-32P]ATP in MOPS buffer containing 0.5% Nonidet P-40. Synaptosomal proteins were separated into Nonidet P-40-soluble and Nonidet P-40-insoluble fractions by centrifugation at 100,000 × g for 1 h, and the resulting proteins were resolved by SDS-PAGE, and phosphorylation was detected by autoradiography (ARG) after SDS-PAGE. B, silver staining (Silver, upper panels) and autoradiographs (ARG, lower panels) of synaptosomal proteins phosphorylated in the absence (left panels) or presence (right panels) of exogenous Cdk5-p35. Spots 1–4 show that phosphorylation was enhanced in the presence of Cdk5-p35 and were chosen for mass spectrometric analysis.

with exogenously added Cdk5-p35 in the presence of [γ-32P]ATP for 15 min at 35 °C and separated into Nonidet P-40-soluble (sup) and -insoluble fractions (ppt). Proteins were detected by Coomassie Brilliant Blue (CBB) staining, and phosphorylation was detected by autoradiography (ARG) after SDS-PAGE. B, silver staining (Silver, upper panels) and autoradiographs (ARG, lower panels) of synaptosomal proteins phosphorylated in the absence (left panels) or presence (right panels) of exogenous Cdk5-p35. Spots 1–4 show that phosphorylation was enhanced in the presence of Cdk5-p35 and were chosen for mass spectrometric analysis.

To separate individual phosphorylated proteins, the phosphorylated Nonidet P-40-insoluble fraction was resolved by two-dimensional PAGE, visualized by silver stain, and autoradiographed (Fig. 2B). Four protein spots showing significant

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FIGURE 2. Phosphorylation of synaptosomal proteins by Cdk5-p35. A, SDS-PAGE of synaptosomal proteins phosphorylated by Cdk5-p35. Synaptosomal proteins were incubated with Cdk5-p35 in the presence of [γ-32P]ATP in MOPS buffer containing 0.5% Nonidet P-40. Synaptosomal proteins were separated into Nonidet P-40-soluble and Nonidet P-40-insoluble fractions by centrifugation at 100,000 × g for 1 h, and the resulting proteins were resolved by SDS-PAGE, and phosphorylation was detected by autoradiography. The addition of Cdk5-p35 increased phosphorylation significantly (Fig. 2A, right panel). Stronger phosphorylation signals were detected in the Nonidet P-40-insoluble fraction than in the Nonidet P-40-soluble fraction (Fig. 2A, right panel), as was expected.

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increased phosphorylation in the presence of Cdk5-p35 were analyzed by LC mass spectrometry after trypsin digestion. The major protein contained in each spot was identified as follows: dynamin 1 in spot 1, CRMP-2 in spot 2, synapsin 2 in spot 3, and Sept5 in spot 4. Peptide sequences obtained from each spot by mass spectrometry and sequence coverage are listed in Table 1.

Phosphorylation of Ser17 in the Adult Type Isoform of Sept5 by Cdk5-p35

—Sept5 binds synaptic vesicles prepared by a slightly different procedure than ours (32). We confirmed the fractionation of Sept5 in our synaptosomal fraction by immunoblotting with anti-Sept5 antibody. Sept5 was distributed widely from the S1 to P2 fractions in the subcellular preparation and was recovered in the synaptosomal fraction when the crude synaptosomes (P2) were fractionated by sucrose density gradient centrifugation (Fig. 4). In synaptosomes treated with Nonidet P-40, Sept5 was found in both Nonidet P-40-insoluble and Nonidet P-40-soluble fractions (Fig. 4B). Further, when the region around spot 4 in two-dimensional PAGE was immunoblotted with anti-Sept5, the reaction was detected at spot 4 with additional spots of several different isoelectric points and two distinct electrophoretic mobilities (data not shown).

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The Sept5 gene generates two isoforms of CDCrel-1F (fetal type) and CDCrel-1A (adult type) with distinct N-terminal amino acid sequences (Fig. 4C) (45). Following the standard nomenclature (46), we hereafter refer to shorter CDCrel-1F as Sept5_v2 and to longer CDCrel-1A as Sept5_v1. To confirm whether Sept5 is phosphorylated by Cdk5, both types of Sept5 were expressed in COS-7 cells and incubated with (+) and without (−) Cdk5-p35. Immunoblotting and phosphorylation of immunoprecipitated Sept5_v1 are shown in the left and right panels, respectively. The control experiment of mock transfection is indicated by MOCK. The phosphorylation of Sept5_v1 at Ser17 by Cdk5-p35 in vitro. A and B, immunoblots showing the fractionation of Sept5 in the synaptosomal fractions, and the detergent-soluble and -insoluble fractions. Preparation of the synaptosomal fractions (A), and the Nonidet P-40-soluble and -insoluble fractions (B) are described under “Experimental Procedures.” C, the molecular structure and the N-terminal amino acids sequences of Sept5_v2 and Sept5_v1. Sept5 is composed of the N-terminal variable region, the central conserved GTPase motif, and the C-terminal coiled-coil domain. Sept5_v2 and Sept5_v1 are alternatively spliced isoforms: Sept5_v2 comprises 369 amino acids, 18 of which are distinct at the N terminus, and Sept5_v1 comprises 378 amino acids, 27 of which are distinct at the N terminus. Thr13 in Sept5_v2; Ser17 in Sept5_v1; Thr119, Ser170, Ser336, and Thr345 in both isoforms are (S/T)P Cdk5 phosphorylation consensus sites. The arrowhead indicates Ser17 of Sept5_v1, and the Cdk5 phosphorylation site identified. D, phosphorylation of Sept5_v1 by Cdk5-p35. Sept5_v2 and Sept5_v1 were expressed in COS-7 cells, and their immunoprecipitates were incubated with (+) and without (−) Cdk5-p35 in vitro. Immunoblotting and phosphorylation of immunoprecipitated Sept5_v1 are shown in the left and right panels, respectively. The control experiment of mock transfection is indicated by MOCK.

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### Table 1: Peptide sequences identified by mass spectrometry

| Spot | Protein | Peptide sequence | Sequence coverage (%) |
|------|---------|------------------|-----------------------|
| 1    | Dynamin 1 | 5-OMEDO[L]FNYE-R15 | 13 |
| 2    | CRMP-2 | 24-VNDQ5KQYMDLGK-43 | 38 |
| 3    | Synapsin 2 | 61-GEKNQQGKST-80 | 90 |
| 4    | Sept5 | 247-312 | 18 |

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**FIGURE 3.** LC-MS/MS spectrum of a Sept5 peptide. The doubly charged ions of the tryptic peptide (m/z = 859.95) from spot 4 was analyzed by nanoelectrospray ionization-MS/MS (40). The y-type product ion series with the amino acid sequence was indicated. The data base analysis of the MS/MS spectrum showed that it corresponded to the sequence ESAPAVIGSNTVVEAK of Sept5 at residues 249–265.

**FIGURE 4.** Phosphorylation of Sept5_v1 at Ser17 by Cdk5-p35 in vitro. A and B, immunoblots showing the fractionation of Sept5 in the synaptosomal fractions, and the detergent-soluble and -insoluble fractions. Preparation of the synaptosomal fractions (A), and the Nonidet P-40-soluble and -insoluble fractions (B) are described under “Experimental Procedures.” C, the molecular structure and the N-terminal amino acids sequences of Sept5_v2 and Sept5_v1. Sept5 is composed of the N-terminal variable region, the central conserved GTPase motif, and the C-terminal coiled-coil domain. Sept5_v2 and Sept5_v1 are alternatively spliced isoforms: Sept5_v2 comprises 369 amino acids, 18 of which are distinct at the N terminus, and Sept5_v1 comprises 378 amino acids, 27 of which are distinct at the N terminus. Thr13 in Sept5_v2; Ser17 in Sept5_v1; Thr119, Ser170, Ser336, and Thr345 in both isoforms are (S/T)P Cdk5 phosphorylation consensus sites. The arrowhead indicates Ser17 of Sept5_v1, and the Cdk5 phosphorylation site identified. D, phosphorylation of Sept5_v1 by Cdk5-p35. Sept5_v2 and Sept5_v1 were expressed in COS-7 cells, and their immunoprecipitates were incubated with (+) and without (−) Cdk5-p35 in vitro. Immunoblotting and phosphorylation of immunoprecipitated Sept5_v1 are shown in the left and right panels, respectively. The control experiment of mock transfection is indicated by MOCK.

**E.** Phosphorylation of Sept5_v1 at Ser17 by Cdk5-p35. WT and S17A mutant of Sept5_v1 were expressed in COS-7 cells, and their immunoprecipitates were incubated with (+) and without (−) Cdk5-p35 in vitro. Immunoblotting and phosphorylation of immunoprecipitated Sept5_v1 are shown in the left and right panels, respectively. The control experiment of mock transfection is indicated by MOCK.

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Phosphorylation of Sept5 by Cdk5

Phosphorylation of Sept5_v1 at Ser^{17} in COS-7 Cells by Cdk5-p35 and in the Mouse Brain. A. A phosphorylation of Ser^{17} in Sept5_v1 by Cdk5-p35 in COS-7 cells. WT and S17A mutant (SA) of Sept5_v1 were transfected in COS-7 cells with (+) and without (−) Cdk5-p35. The extracts were probed with anti-Sept5, anti-Ser(P)^{17}, anti-Cdk5, and anti-phosphatase antibodies. B. Phosphorylation of Sept5_v1 by Cdk5-p35. Sept5 was prepared from the synaptosomal fractions (Synaptosome) by immunoprecipitation (IP) with normal rabbit IgG (Cont) and with anti-Sept5 antibody (Sept5). Sept5 was incubated with (+) and without (−) Cdk5-p35 in the presence (+) and absence (−) of roscovitine (Ros) in vitro. The lower panel is an autoradiograph of Sept5 phosphorylated by Cdk5-p35. C. Phosphorylation of Ser^{17} on Sept5_v1 in the synaptosomes by Cdk5-p35. Synaptosomal proteins were incubated in the presence (+) and absence (−) of exogenously added Cdk5-p35. Phosphorylation of Sept5_v1 was examined by immunoblotting with anti-Ser(P)^{17} antibody (pS17). Immunoblotting with anti-Sept5 antibody is shown in the upper row. D. Phosphorylation of Sept5_v1 at Ser^{17} in the mouse brain. The brain extract prepared from mouse brains at postnatal day 10 was incubated in the presence (+) and absence (−) of phosphatase inhibitors (PPase inh; 20 mM NaF, 10 mM β-glycerophosphate, and 100 nM microcystin-LR). Immunoblots with anti-Sept5 antibody and anti-Ser(P)^{17} antibody are shown in the upper and lower panels, respectively. Absorption with phosphopeptide used for immunization (pS17 pep) is shown in the right panel.

showed phosphorylation signals, but no signal was observed in a control sample (mock transfection) or samples incubated without Cdk5-p35 (Fig. 4D, right panel). We could not see phosphorylation of Sept5_v2 more than background levels. These results indicate that Sept5_v1 is phosphorylated preferentially by Cdk5.

Cdk5 is a proline-directed protein kinase that phosphorylates Ser/Thr residues at the N terminus next to Pro. There is a consensus motif in the unique N-terminal sequence of Sept5_v1. To determine whether Ser^{17} of Sept5_v1 is the phosphorylation site, the Ala mutant of Ser^{17} (S17A) of Sept5_v1 was phosphorylated by Cdk5-p35 in vitro. Mutation of Ser^{17} to Ala reduced phosphorylation of Sept5_v1 dramatically (Fig. 4E, right panel). The amount of Sept5_v1 in immunoprecipitates is shown in the left panel of Fig. 4E. A faint signal was observed in S17A mutant of Sept5_v1, suggesting that some of (S/T)P sequences in the C-terminal common domain could also be phosphorylated slightly. These results indicate that Ser^{17} of Sept5_v1 is the primary phosphorylation site for Cdk5-p35.

Phosphorylation of Sept5_v1 at Ser^{17} in COS-7 Cells by Cdk5-p35 and in Mouse Brains—To address the physiological relevance of Cdk5 phosphorylation of Sept5_v1 at Ser^{17}, we raised an antibody to phosphorylated Ser^{17} (anti-Ser(P)^{17}; Fig. 5A). Fig. 5A shows phosphorylation of Sept5_v1 at Ser^{17} in COS-7 cells and the specificity of the anti-Ser(P)^{17} antibody. Sept5_v1 WT might be phosphorylated at Ser^{17} in COS-7 cells to some extent by endogenous Cdks. The anti-Ser(P)^{17} reaction of Sept5_v1 increased markedly when coexpressed with Cdk5-p35, although a weak signal was detected in Sept5_v1 transfected alone (Fig. 5A, second row). Anti-Ser(P)^{17} antibody recognized the shifted upper band of Sept5 (Fig. 5A, second row), implying that phosphorylation induces some conformational changes. In contrast, Sept5_v1 S17A did not react with anti-Ser(P)^{17} antibody even when coexpressed with Cdk5-p35 (Fig. 5A, fourth lane of the second row). These results indicate that anti-Ser(P)^{17} antibody specifically recognizes Sept5_v1 phosphorylated at Ser^{17}.

To determine whether synaptosomal Sept5 can be phosphorylated by Cdk5, Sept5 was prepared from synaptosomes by immunoprecipitation (Fig. 5B, upper panel) and incubated with exogenous Cdk5-p35 in the presence of [γ-^{32}P]ATP. The phosphorylation was detected only when Sept5 was immunoprecipitated with anti-Sept5 antibody and incubated with Cdk5-p35 (Fig. 5B, lower panel). The phosphorylation was attenuated in the presence of roscovitine, a Cdk5 inhibitor. The synaptosomal fractions contained two bands of Sept5 (Fig. 5C, left lane of the upper panel). The upper band was shifted upward by incubation with Cdk5-p35. Anti-Ser(P)^{17} antibody recognized the shifted upper band of Sept5 (Fig. 5C, lower panel) as well as Sept5_v1 coexpressed with Cdk5-p35 in COS-7 cells (Fig. 5A). The upper band might be Sept5_v1, and the lower band might be Sept5_v2, which is also expressed in adult mouse brains (data not shown). No reaction was observed after incubation without Cdk5-p35 (Fig. 5, B and C).

To determine whether Ser^{17} is phosphorylated in the brain, we prepared brain extracts from mice at postnatal day 10 and measured Ser^{17} phosphorylation using immunoblotting with anti-Ser(P)^{17} antibody. Anti-Ser(P)^{17} antibody reacted with Sept5 (Fig. 5D), and the reaction was lost by incubating the brain extract at 37 °C for 1 h, but not in the presence of phosphatase inhibitors (Fig. 5D, PPase inh). These results indicate that the reaction is phosphorylation-dependent. Absorption with antigen peptide showed that the reaction was specific (Fig. 5D, right panel).

The Effect of Phosphorylation of Sept5_v1 by Cdk5 on Its Interaction with Syntaxin-1—Previous studies have shown that Sept5 binds to syntaxin-1A (32). We tested whether Ser^{17} phosphorylation of Sept5 by Cdk5-p35 regulates its association with syntaxin-1A. Immunoprecipitation of syntaxin-1 with Sept5 from the isolated synaptosomes was examined after incubation with and without Cdk5-p35. Phosphorylation of Sept5_v1 at Ser^{17} by Cdk5-p35 was confirmed by immunoblotting with anti-Ser(P)^{17} antibody (Fig. 6A, second row of the left panel). Phosphorylation did not affect the amount of Sept5 immunoprecipitated with anti-Sept5 antibody (Fig. 6A, first row of the right panels). Syntaxin-1 immunoprecipitated specifically with anti-Sept5 antibody (Fig. 6A, Sp). Among other t-SNARE components, SNAP-25 also immunoprecipitated with Sept5, but VAMP-2 did not. Incubation with Cdk5-p35 significantly reduced the amount of syntaxin-1 and SNAP-25 in the Sept5 immunoprecipitate. As expected, roscovitine suppressed the Cdk5-p35-induced dissociation of syntaxin-1 and SNAP-25.
Phosphorylation of Sept5 by Cdk5

(A) Input ppt

- + + + +

Cdk5-p35 Ros

- + + + +

Sept5 v1

- + + + +

pS17

- + + + +

syntaxin-1

- + + + +

SNAP-25

- + + + +

VAMP-2

(B) Syndtin-1 in the GST-pulldown assay. Sept5 was expressed in COS-7 cells and the GST-syntaxin-1A pull-down assay was performed as described above. Sept5_v1 bound to GST-syntaxin-1A was analyzed by immunoblotting with anti-Sept5 antibody (lower row). Inputs are shown in the upper panel. F, the amount of Sept5_v1 mutants pulled down by GST-syntaxin-1A. The binding ratios of Sept5_v1 coexpressed with Cdk5-p35 is shown relative to that without Cdk5-p35. The binding ratio of Sept5_v1 coexpressed with Cdk5-p35 was 56.2 ± 4.0% of the value expressed without Cdk5-p35. Phosphorylation of Sept5_v1 preferentially between two isoforms of CRMP-2 as known substrates (12, 13, 43), Cdk5-p35 phosphorylated Sept5_v1 WT, which is consistent with Sept5_v1 WT (Fig. 6A). After phosphorylation, the amount of syntaxin-1 coimmunoprecipitated with Sept5 declined to ~52% of the control value (Fig. 6B).

To test the direct effect of the Ser^{17} phosphorylation on the interaction with syntaxin-1, we carried out the GST-syntaxin-1A pulldown assay. GST-syntaxin-1A immobilized on glutathione-Sepharose was incubated with Sept5_v1 WT coexpressed with and without Cdk5-p35 in COS-7 cells, and the binding of Sept5_v1 was detected by immunoblotting with anti-Sept5 antibody after SDS-PAGE. Coexpression of Cdk5-p35 decreased the binding of Sept5_v1 to syntaxin-1A to ~56% of the control level, in which Sept5_v1 alone was expressed (Fig. 6D). Cdk5 interacts with syntaxin-1 (14). To exclude the possibility that Cdk5 physically mediates the binding of Sept5 to syntaxin, we carried out the GST-syntaxin-1A pulldown assay using Asp or Glu mutants of Ser^{17} (S17D or S17E) as the phosphorylation-mimicking Sept5_v1 and the Ala mutant of Ser^{17} (S17A) as the unphosphorylated Sept5_v1. S17D and S17E mutants showed reduced binding to syntaxin-1A (Fig. 6E). The binding was ~60–70% that of Sept5_v1 WT (Fig. 6F), similar to the binding ratios observed with Sept5_v1 coexpressed with Cdk5-p35. In contrast, the binding to GST-syntaxin-1A was greater for Sept5_v1 S17A than for Sept5_v1 WT, which is consistent with Sept5_v1 WT was phosphorylated slightly by endogenous kinases in COS-7 cells (Fig. 5A). The coimmunoprecipitation from isolated synaptosomes and in vitro binding assays using the GST-syntaxin-1A both indicate that phosphorylation of Sept5_v1 at Ser^{17} by Cdk5 reduces the binding ability of Sept5_v1 to syntaxin-1A.

DISCUSSION

Increasing evidence shows that Cdk5 plays a role in synaptic activity. Although several Cdk5 substrates have been identified in pre- and postsynaptic regions (10–21), the roles of Cdk5 in synaptic activity are not fully understood. We searched for the unidentified Cdk5 substrates in the synaptosomal fractions using a proteomic approach involving the in vitro phosphorylation by Cdk5, separation of phosphorylated proteins on two-dimensional PAGE, and mass spectrometric analysis. We identified Sept5 as a novel Cdk5 substrate, as well as dynamin 1 and CRMP-2 as known substrates (12, 13, 43). Cdk5-p35 phosphorylated Sept5_v1 preferentially between two isoforms of Sept5_v1 WT and Sept5_v2. Ser^{17} of Sept5_v1 was phosphorylated by Cdk5 in vitro, and the site was shown to be phosphorylated in mouse brains using the phosphospecific antibody. Phosphorylation of Sept5 significantly decreased the binding to syntaxin-1. We now discuss the role of Sept5_v1 phosphorylation by Cdk5 in relation to SNARE-mediated exocytosis.

We employed the proteomic approach to identify substrates for Cdk5 in the synaptosomal fractions. Our approach included two-dimensional PAGE and mass spectrometric analysis of isolated synaptosomal proteins phosphorylated in vitro by recombinant Cdk5-p35. A similar approach has been used recently for other protein kinases to understand the complicated phosphorylation cascades (for examples, see Refs. 47 and 48). We identified several known substrates such as dynamin 1 and CRMP-2, indicating that this method is useful for identifying substrates for Cdk5. The Sept5_v1 identified here is a substrate from Sept5 (Fig. 6A). After phosphorylation, the amount of syntaxin-1 coimmunoprecipitated with Sept5 declined to ~52% of the control value (Fig. 6B).
for Cdk5 \textit{in vitro} and probably \textit{in vivo}. Although we have analyzed only four phosphorylation spots on two-dimensional PAGE by mass spectrometric analysis, more proteins could be identified as substrates for Cdk5 by comprehensive analysis of the phosphorylation spots.

Cdk5 and p35 were enriched in the synaptosomal fractions, as reported previously (10, 19, 49). More Cdk5-p35 was recovered in the detergent-insoluble fraction composed mainly of postsynaptic density proteins and cytoskeletons. The functions of Cdk5-p35 have been demonstrated in the presynaptic terminal (10–16), and the postsynaptic functions have been reported recently (9, 17, 18). More phosphorylation was observed in the detergent-insoluble fraction where endogenous Cdk5-p35 was more abundant. Substrate proteins would also gather in the same compartment where Cdk5-p35 is localized. The synaptosomal fractions contained endogenous Cdk5-p35, but we added recombinant Cdk5-p35 in the phosphorylation reaction to enhance Cdk5-dependent phosphorylation signals. Although the \textit{in vitro} reaction with purified proteins often causes artificial phosphorylation not observed \textit{in vivo}, the addition of exogenous Cdk5-p35 here did not induce artificial phosphorylation, at least using our methods of analysis. Cdk5 might have strict substrate specificity \textit{in vitro} as well as \textit{in vivo}. Alternatively, proteins in the synaptosomal fractions that we used for \textit{in vitro} phosphorylation might maintain the steric conformation that is phosphorylated mainly \textit{in vivo}.

Septins are a family of GTP-binding proteins conserved from yeast to mammalian cells (30). Septins contain the P-loop nucleotide-binding consensus sequence for the GTP binding near the N terminus and have a coiled-coil domain at the C terminus. Septins function in variety of cellular activities including cell division and secretion (28, 30). Although the regulation of the functions of septins is not understood fully, phosphorylation could be one of the regulatory mechanisms. For example, phosphorylation of budding yeast septins, Cdc3, Cdc10, and Cdc11, by p21-activated protein kinase (Cla4) regulates the collar formation (50). Two Ser residues near the C terminus of Cdc3 are also phosphorylated by a Cdk, Cdc28, resulting in disassembly of the septin ring at the end of the G1 phase of the cell cycle (51). Phosphorylation of Sept3, a mammalian brain septin, by cGMP-dependent protein kinase regulates its subcellular localization in neurons (52). Sept5 is also phosphorylated in the N-terminal polybasic region by protein kinase C in response to agonist such as thrombin, phorbol 12-myristate 13-acetate, and collagen in platelets (53). Thus, septins appear to be phosphorylated at different sites by different protein kinases in response to different stimulation. Comprehensive mass spectrometric analysis of phosphoproteins suggests that phosphorylation of Sept5 occurs in the mouse brain (54), but the details are not known. Our results are the first clear evidence to demonstrate the site, protein kinase, and function of Sept5 phosphorylation in the brain.

Sept5 exists in two isoforms, Sept5_v1 and Sept5_v2 (45), which are alternatively spliced isoforms that differ in their N-terminal amino acid sequences. Sept5_v2 has a specific 18-amino acid stretch, Sept5_v1 has a specific 27-amino acid stretch, and both share the identical following 351 amino acids, including the GTP-binding and C-terminal coiled-coil domains (Fig. 4C). Sept5_v2 is expressed more than Sept5_v1 at postnatal day 8, and Sept5_v1 is expressed more than Sept5_v2 at postnatal day 50 (45). The functional difference, if any, between the two isoforms has not been addressed. We found specific phosphorylation of Sept5_v1 at Ser\textsuperscript{15} \textit{in vitro} and in mouse brain. Considering that phosphorylation of Sept5_v1 by Cdk5 decreased the binding to syntaxin-1, Sept5_v1 may participate specifically in the regulation of exocytosis.

Recently another group found phosphorylation of human SEPT5_v2 at Ser\textsuperscript{327}, the different site from ours, by Cdk5.\textsuperscript{4} The reason why we could not detect the phosphorylation of Ser\textsuperscript{336} in mouse Sept5_v1, corresponding to Ser\textsuperscript{327} in hSEPT5_v2, is not clear. It may be due to the lower phosphorylation at Ser\textsuperscript{336} relative to Ser\textsuperscript{17} in mouse Sept5_v1. Even though the phosphorylation sites were different, both phosphorylation reduced the binding to syntaxin-1. Considering the previous result that Sept5 binds syntaxin-1 at both sides of the N and C termini (55), it may be reasonable that phosphorylation at both sites inhibits the binding partially.

Sept5 appears to act as an inhibitor of neurotransmitter release. Sept5 inhibits exocytosis when overexpressed in HIT-T15 cells (32), and the platelet secretion response increases in Sept5\textsuperscript{A/L} mutant mice (53). This inhibitory action is thought to be mediated by interaction with syntaxin, a component of the SNARE complex, which also includes SNAP-25 and VAMP-2 and is involved in membrane docking (32, 55). Regulation of the assembly and disassembly of the SNARE complex is the central issue in neurotransmitter release. Beites \textit{et al.} (55) suggested that Sept5 regulates the availability of SNARE proteins after exocytosis by showing that Sept5 can bind to syntaxin-1 in the SNARE complex but not to syntaxin-1 in the α-SNAP-SNARE complex. We did not detect VAMP-2 in the Sept5 immunoprecipitates, which might be explained by the different materials used for immunoprecipitation. We used isolated synaptosomes for immunoprecipitation in the presence of detergent, whereas Beites \textit{et al.} (55) used the detergent-soluble P2 fraction. Syntaxin-1-SNAP-25 (t-SNARE) might not have been incorporated into the SNARE complex with VAMP-2 in our isolated synaptosomes.

We found that the phosphorylation of Sept5_v1 by Cdk5 reduced the binding to syntaxin. This resembles the role of the phosphorylation of Munc18 by Cdk5 (15). Phosphorylation of Munc18 by Cdk5 suppresses the binding to syntaxin-1A and promotes the docking process of the membrane vesicle to its target membranes (15, 16). However, Sept5 appears to regulate different steps of the exocytotic cycle from Munc18. Whereas Munc18 suppresses the formation of SNARE complex, Sept5 may be involved in the disassembly of SNARE complex (55). In addition to these syntaxin-1-binding proteins, several recent papers have described the positive and negative roles of Cdk5 in several different steps of exocytosis. For example, Cdk5 is thought to stimulate the approach of secretory granules to the plasma membranes by reorganizing cortical actin through phosphorylation of Trio RacGEF (56). Cdk5 is required to elicit the maximum GTP-induced secretory response of neutrophils (57). In contrast, Cdk5 inhibits insulin secretion.

\textsuperscript{4} N. D. Amin, S. Kesavapany, J. Kanungo, Y. L. Zheng, R. K. Sihag, W. Albers, P. Grant, and H. C. Grant, personal communication.
secretion by phosphorylation voltage-dependent Ca\textsuperscript{2+} channel in pancreatic \( \beta \)-cells (58). Cdk5 activates PCTAIRE-1, a Cdc2-related kinase, by phosphorylation, and then PCTAIRE-1 phosphorylates NSF to reduce its oligomerization, resulting in inhibition of Ca\textsuperscript{2+}-dependent human growth hormone release in PC12 cells (59). Taken together, these data indicate that Cdk5 is closely involved in the regulation of exocytosis. The regulation of the interaction between Sept5 and syntaxin by Cdk5 is important in understanding the exocytotic mechanism.

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