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Critical Determinants of Substrate Recognition by Cyclin-Dependent Kinase-like 5 (CDKL5)

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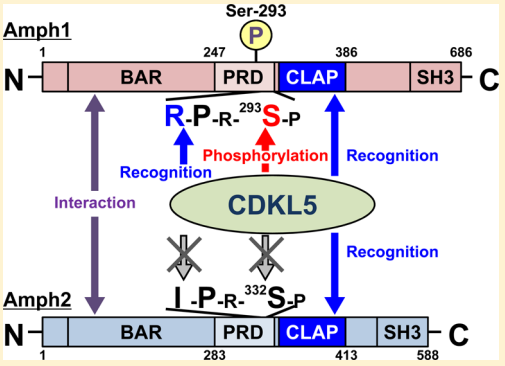
ABSTRACT: Cyclin-dependent kinase-like 5 (CDKL5) is a Ser/Thr protein kinase known to be associated with X-linked neurodevelopmental disorders. In a previous study, we identified amphiphysin 1 (Amph1) as a potential substrate for CDKL5 and identified a single phosphorylation site at Ser-293. In this study, we investigated the molecular mechanisms of substrate recognition by CDKL5 using Amph1 as a model substrate. Amph1 served as an efficient CDKL5 substrate, whereas Amph2, a structurally related homologue of Amph1, was not phosphorylated by CDKL5. The sequence around the Amph1 phosphorylation site is RPR^{293}SPSQ, while the corresponding sequence in Amph2 is IPK^{332}SPSQ. To define the amino acid sequence specificity of the substrate, various point mutants of Amph1 and Amph2 were prepared and phosphorylated by CDKL5. Both Amph2(I329R) and Amph1 served as efficient CDKL5 substrates, but Amph1(R290I) did not, indicating that the arginyl residue at the P −3 position is critical for substrate recognition. With regard to prolyl residues around the phosphorylation site of Amph1, Pro-291 at the P −2 position, but not Pro-294 at the P +1 position, is indispensable for phosphorylation by CDKL5. Phosphorylation experiments using various deletion mutants of Amph1 revealed that the proline-rich domain (PRD) (amino acids 247−315) alone was not phosphorylated by CDKL5. In contrast, Amph1(247−385), which comprised the PRD and CLAP domains, served as an efficient CDKL5 substrate. These results, taken together, suggest that both the phosphorylation site sequence (RPXSX) and the CLAP domain structure in Amph1 play crucial roles in recognition and phosphorylation by CDKL5.



Cyclin-dependent kinase-like 5 (CDKL5, also known as STK9) is a Ser/Thr protein kinase predominantly expressed in the brain.[1,2] Human CDKL5 was originally identified in a transcriptional mapping project on the human chromosome Xp22 region.[1] Mutations in the *CDKL5* gene have been associated with X-linked neurodevelopmental disorders involving severe mental retardation and seizures, which appear in the first postnatal months.[2−8] CDKL5 is a large protein (110 kDa) containing a Ser/Thr kinase domain within its N-terminus and a large C-terminal domain that may play important roles in cellular localization and protein−protein interaction.[6,7] Although the mutations are found throughout the coding region of CDKL5, missense mutations are found especially within the kinase domain of CDKL5 in patients with atypical variants of Rett syndrome (RTT).[3,4,6,7] Therefore, it is of great interest to investigate the endogenous targets and the kinase activity of CDKL5 to understand the physiological significance of the kinase.

In previous studies, methyl-CpG-binding protein 2 (MeCP2),[5,7] DNA methyltransferase 1 (Dnmt1),[9] and netrin-G1 ligand (NGL-1)[10] have been reported to be phosphorylated by CDKL5. Furthermore, the cause of RTT has been reported to be attributed to a mutation in the gene encoding MeCP2.[11−13] Therefore, it is tempting to speculate about the relationship between the onset of RTT and mutation of CDKL5 on the basis of phosphorylation of MeCP2 by CDKL5. However, because phosphorylation of MeCP2 by CDKL5 was relatively weak,[9] it is not reasonable to assume that MeCP2 is a direct substrate of CDKL5. Therefore, we attempted to explore an endogenous substrate for CDKL5 using a new method that we have previously developed.[14] With this approach, we identified amphiphysin 1 (Amph1) as an efficient CDKL5 substrate in the mouse brain.[15] Amph1 is a cytoplasmic protein that plays important roles in neuronal transmission and neuronal development through clathrin-mediated endocytosis.[16,17] CDKL5 is known to be localized not only in the nucleus but also in the cytoplasm, and cellular localization of CDKL5 has been reported to change during the process of development.[2] Furthermore, a lack of Amph1 is known to cause seizures and striking learning deficits.[18] Taken together, Amph1 could be one of the cytoplasmic targets of CDKL5, and possibly a critical molecular component of the pathogenesis underlying CDKL5-related neurodevelopmental disorders.

Wang et al. reported that mice lacking CDKL5 show autistic-like phenotypes and impairment of neuronal development.[19] Although many signal transduction pathways, including the AKT pathway, were disrupted in *CDKL5* knockout mice, the mechanism of disruption of kinome profile and the direct downstream target of CDKL5 are still unclear. To clarify the physiological substrates of CDKL5, it is important to elucidate

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the detailed molecular mechanisms of substrate recognition by CDKL5. Therefore, in this study, we analyzed the molecular basis of substrate recognition by CDKL5 using catalytically active CDKL5(1−352) and Amph1/Amph2 as model substrates. Here, we report that CDKL5 recognizes not only the sequence (RPXSX) around the phosphorylation site but also the different region (CLAP domain) of Amph1 as a docking site.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (111 TBq/mmol) was purchased from PerkinElmer. HiTrap Chelating HP, protein G-Sepharose 4B Fast Flow, glutathione Sepharose 4B, and pGEX-6P-1 were obtained from GE Healthcare Bio-Sciences. ATP, myelin basic protein (MBP), and bovine serum albumin were purchased from Sigma-Aldrich. Anti-CDKL5 antibody and anti-His$_6$ antibody were obtained from abcam and Wako Pure Chemical Industries, respectively. Recombinant proteins encoding Amph1,[15] Amph1(S293A),[15] Amph2,[15] CDKL5(WT),[9] CDKL5(1−352),[9] glutathione *S*-transferase (GST)-Amph1,[15] Dyrk1A,[20] and ERK2[21] were expressed in *Escherichia coli* and purified as described previously. Antibody against phospho-Amph1 at Ser-293 was produced by immunizing BALB/c mice using antigenic phosphopeptide as described previously.[15]

Construction of Plasmids. To generate pET-MEK1, the following primers were used for polymerase chain reaction (PCR) with the rat brain 5′-RACE cDNA library as a template: 5′-GCT AGC ATG CCC AAG AAG AAG CCG ACG C-3′ and 5′-CTC GAG GAT GCT GGC AGC GTG GGT TGG-3′. The PCR fragment was ligated into NheI and XhoI sites of pET-23a. Plasmid pGEX-MEK1-His$_6$ was generated using primers (5′-GGA TCC ATG CCC AAG AAG AAG CCG ACG-3′ and 5′-TTT GTC GAC TCA GTG GTG GTG GTG GTG GTG-3′) and pET-MEK1 as a template. The PCR fragment was digested with BamHI and SalI and ligated into pGEX-6P-1. A constitutively active mutant of MEK, pGEX-MEK1(S218D/S222E)-His$_6$, was constructed by an inverse PCR method[22] with sense (5′-GCC AAC TCC TTC GTG GGA ACA AGG TCC T-3′) and antisense (5′-CAT GGA GTC AAT TAG CTG CCC GCT GAC-3′) primers and pGEX-MEK1-His$_6$ as a template.

To generate pGEX-Amph2, the BamHI−XhoI fragment from pET-Amph2[15] was ligated into pGEX-6P-1. Six point mutants of Amph1 [Amph1(R290I), Amph1(R290K), Amph1-(R292K), Amph1(R290/292K), Amph1(P291A), and Amph1-(P294A)] were prepared by an inverse PCR with sense primers [5′-ATA CCC AGA TCA CCT TCA CAG ACA AGG-3′ for Amph1(R290I), 5′-AAA CCC AGA TCA CCT TCA CAG ACA AGG-3′ for Amph1(R290K), 5′-CGA CCC AAA TCA CCT TCA CAG ACA AGG-3′ for Amph1(R292K), 5′-AAA CCC AAA TCA CCT TCA CAG ACA AGG-3′ for Amph1(R290/292K), 5′-CGA GCT AGA TCA CCT TCA CAG ACA AGG-3′ for Amph1(P291A), and 5′-CGA CCC AGA TCA GCT TCA CAG ACA AGG-3′ for Amph1(P294A), where underlines indicate the sites of mutations] and an antisense primer (5′-CAC TGG GGC AGG AGA TGC AG-3′), and pET-Amph1 as a template. Two point mutants of Amph1 [Amph1(S293T) and Amph1(S293Y)] were prepared by an inverse PCR with sense primers [5′-ACA CCT TCA CAG ACA AGG AAA GGG-3′ for Amph1(S293T) or 5′-TAT CCT TCA CAG ACA AGG AAA GGG-3′ for Amph1(S293Y), where underlines indicate the sites of mutations] and an antisense primer (5′-TCT GGG TCG CAC TGG GGC A-3′), and pET-Amph1 as a template. pET-Amph2(I329R) was prepared by an inverse PCR with the primers (5′-AGG CCC AAG TCC CCA TCT CAG CTC-3′ and 5′-GGT AGC CCC GGG TGA GGC-3′, where underlines indicate the site of mutation) and pET-Amph2 as a template.

Plasmids for the kinase-dead (KD) CDKL5 and CDKL5(1−352), in which Lys-42 and Lys-43 were replaced with arginyl residues [CDKL5(KD) and CDKL5(1−352/KD), respectively], were constructed by an inverse PCR using a sense primer (5′-TTC AAG GAC AGC GAA GAA AAT GA-3′) and an antisense primer (5′-TCT TCT GAT TGC CAC AAT TTC ATG TG-3′) (underline indicates the sites of mutations) and pET-CDKL5 and pET-CDKL5(1−352) as templates, respectively. Expression plasmid pET-CDKL5(1−800) was prepared by an inverse PCR with the primers (5′-ATC AGG ACC GTC AGT GTT GGG CA-3′ and 5′-GTC GAC AAG CTT GCG GCC-3′) and pET-CDKL5 as a template. An N-terminal deletion mutant of GST-Amph1, GST-Amph1(247−686), was prepared by an inverse PCR with a sense primer (5′-GGA GCT CCC AGT GAC TCG GG-3′) and an antisense primer (5′-GGA TCC CAG GGG CCC CTG-3′) and pGEX-Amph1 as a template. Two C-terminal deletion mutants of GST-Amph1, GST-Amph1(1−314) and GST-Amph1(1−385), were prepared by an inverse PCR with a sense primer (5′-TAG CTC GAG CGG CCG CAT-3′) and antisense primers [5′-TTC CTT TGT TGG GGT GAC TTT AGG-3′ for GST-Amph1(1−314) and 5′-CCA TAA GTC CCA GGG CAA TGT C-3′ for GST-Amph1(1−385)] and pGEX-Amph1 as a template. Plasmids pGEX-Amph1(247−314) and pGEX-Amph1(247−385) were prepared by an inverse PCR with a sense primer (5′-TAG CTC GAG CGG CCG CAT-3′) and antisense primers [5′-TTC CTT TGT TGG GGT GAC TTT AGG-3′ for GST-Amph1(247−314) and 5′-CCA TAA GTC CCA GGG CAA TGT C-3′ for GST-Amph1(247−385)] and pGEX-Amph1(247−686) as a template.

To generate pET-Septin 4, the following primers were used for PCR with mouse brain 5′-RACE cDNA library as a template: 5′-GCT AGC ATG GAC CAT TCA CTG GGA TGG C-3′ and 5′-CTC GAG GTT TGC TGA AGG GAG GGG GAA-3′. The PCR fragment was ligated into NheI and XhoI sites of pET-23a. To produce the cytoplasmic domain (CD) of mGluR5 (amino acids 2530−3609), pET-mGluR5-(CD) was generated. To generate pET-mGluR5(CD), the following primers were used for PCR with the mouse brain 5′-RACE cDNA library as a template: 5′-GGA TCC ATG CAC GTA GGA GAT GGC AAG TCA-3′ and 5′-AAG CTT CAA CGA TGA AGA ACT CTG CGT GTA ATC-3′. The PCR fragment was ligated into BamHI and HindIII sites of pET-23a.

Expression and Purification of Recombinant Proteins. Various mutants of CDKL5 and point mutants of Amph1 were expressed and purified essentially as described previously.[9,15] To produce constitutively active MEK1, pGEX-MEK1(S218D/S222E)-His$_6$ was introduced into *E. coli* strain BL21(DE3) cells (Stratagene). The transformed bacteria were grown at 37 °C to an A_{600} of 0.5, and the expression of the protein was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After a 20 h culture at 25 °C, the bacteria were harvested by centrifugation and suspended in buffer A [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 40]. After the harvest, the bacterial cells were sonicated and cell debris was removed by centrifugation (20000*g*) at 4 °C for 10 min. Subsequently, the supernatant was loaded on a HiTrap Chelating HP column (1 mL) pre-equilibrated with

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buffer A. The column was washed in a stepwise manner with buffer A, buffer A containing 20 mM imidazole, and buffer A containing 50 mM imidazole and then eluted with buffer A containing 200 mM imidazole. In the case of Septin 4 and mGluR5(CD), BL21(DE3) cells carrying pET-Septin 4 or pET-mGluR5(CD) were grown at 18 °C for 24 h or at 37 °C for 6 h without being induced. The cells were harvested and lysed, and then the recombinant proteins were purified with a HiTrap Chelating HP column as described above.

For the recombinant GST-Amph2, GST-Amph1(247−686), GST-Amph1(247−385), and GST-Amph1(247−314) fusion proteins, BL21(DE3) cells transformed with pGEX-Amph2, pGEX-Amph1(247−686), pGEX-Amph1(247−385), and pGEX-Amph1(247−315) were grown at 37 °C to an A_{600} of 0.5, and then IPTG was added to a final concentration of 0.1 mM to induce expression. After an 8 h culture at 37 °C, the bacteria were harvested and homogenized in buffer A as described above. For the recombinant GST-Amph1(1−314) and GST-Amph1(1−385) fusion proteins, BL21(DE3) cells transformed with pGEX-Amph1(1−314) and pGEX-Amph1(1−385) were grown at 37 °C to an A_{600} of 0.5, and then IPTG was added to a final concentration of 0.1 mM to induce expression. After a 24 h culture at 18 °C, the bacteria were harvested and homogenized in buffer A as described above. The cell lysate was loaded on a glutathione Sepharose 4B column (1 mL) pre-equilibrated with buffer A. The column was washed with 10 mL of buffer A, and the GST fusion protein was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione. The purified fractions were pooled, dialyzed against buffer A, and stored in aliquots at −30 °C until they were used.

Preparation of the Crude Extract from Mouse Brain. Brain extracts were prepared from male ddY mice (Japan SLC). Whole brain was suspended in 3 volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 0.5 mM EGTA, 0.05% Tween 40, and 1 mM phenylmethanesulfonyl fluoride and homogenized by a Teflon/glass homogenizer. The homogenate was centrifuged at 20000*g* and 2 °C for 30 min, and the resultant supernatant was used as a crude extract from mouse brain. Prior to *in vitro* kinase assays, the extract was heat-treated at 60 °C for 30 min to inactivate endogenous protein kinases and phosphatases.

Cell Culture, Transfection, and Immunoprecipitation. Expression vectors for transfection [pcFLAG-CDKL5(WT) and pcFLAG-CDKL5(KD)] were constructed essentially as described previously.[9] These expression vectors were designed to produce recombinant proteins with myc and His_6 tags on their C-terminal ends.[23] Transfection of expression plasmids into 293T cells was performed using Polyethylenimine Max (Polysciences) according to the manufacturer's instructions. Briefly, 293T cells (5×10^6) were plated in 35 mm dishes in 2 mL of Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% fetal calf serum 24 h before transfection. Transfection was performed by incubation of the 293T cells in 1 mL of DMEM containing 5% fetal calf serum, 6 μL of Polyethylenimine Max, and 2.5 μg of plasmid DNA for 24 h. Cells were collected 24 h after transfection and homogenized with IP buffer [20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 M NaCl, and 1 mM phenylmethanesulfonyl fluoride]. The homogenates were centrifuged at 20000*g* for 30 min, and the supernatants were harvested. Supernatants, thus obtained, were mixed and incubated at 4 °C for 2 h with 20 μL of protein G-Sepharose 4B (GE Healthcare Bio-Sciences), which had previously been treated with 0.5 μg of anti-myc antibody (Invitrogen) for 2 h. The samples were washed three times with IP buffer and three times with kinase assay buffer [20 mM Hepes-NaOH (pH 7.4), 10 mM $MgCl_2$, and 1 mM dithiothreitol], and then protein kinase activity was determined using 100 μM [γ-^{32}P]ATP.

Ser-293 Peptide. Amph1(287−300) peptide (Ser-293 peptide, APVRPRSPSQTRKGC) corresponding to amino acid residues 287−300 of Amph1 was synthesized using a Shimadzu PSSM-8 automated peptide synthesizer.[24] The peptide was purified by reverse-phase high-performance liquid chromatography on a C18 column, and its purity was confirmed by time-of-flight mass spectrometry.

Protein Phosphorylation. Phosphorylation of proteins by CDKL5 was conducted using the full-length CDKL5 or truncated kinase, CDKL5(1−352). The standard phosphorylation mixture (10 μL) contained 20 mM Hepes-NaOH (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP, substrate proteins, and an appropriate amount of CDKL5(1−352). The reaction was started by the addition of kinases and the mixture incubated at 30 °C for the indicated times. After incubation, the reaction was stopped by adding an equal volume of 2× SDS sample buffer. The mixtures were electrophoresed via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the phosphorylated proteins were detected by autoradiography. For detection of the phosphorylated proteins by Western blotting, cold ATP was used instead of [γ-^{32}P]ATP to phosphorylate proteins.

To determine the stoichiometry of phosphate incorporation, Amph1 was phosphorylated by CDKL5(1−352) in the reaction mixture mentioned above in a final volume of 70 μL. After incubation at 30 °C for 10, 30, 60, 90, and 120 min, a 10 μL aliquot of the mixture was withdrawn, spotted onto a 2 cm square of Whatman 3MM chromatography paper, and immediately placed in 5% trichloroacetic acid containing 1% sodium diphosphate. The level of incorporation of [^{32}P]-phosphate into Amph1 was measured by a liquid scintillation counter. The phosphate incorporated into Amph1 was calculated as moles of phosphate per mole of Amph1 by subtracting the radioactivity in CDKL5(1−352) from that in CDKL5(1−352) and Amph1.

To determine the stoichiometry of incorporation of phosphate into the Ser-293 peptide, it was phosphorylated by CDKL5(1−352) in an aforementioned reaction mixture in a final volume of 70 μL. After incubation at 30 °C for 10, 30, 60, 90, and 120 min, a 10 μL aliquot of the mixture was withdrawn, spotted onto a 2 cm square of the phosphocellulose paper (Whatman P-81), and immediately placed in 75 mM phosphoric acid. The level of incorporation of [^{32}P]phosphate into the peptide was measured by a liquid scintillation counter. The phosphate incorporated into the Ser-293 peptide was calculated as moles of phosphate per mole of peptide by subtracting the radioactivity in CDKL5(1−352) from that in CDKL5(1−352) and the Ser-293 peptide.

SDS−PAGE and Western Blotting. SDS−PAGE was performed essentially according to the method of Laemmli[25] on a slab gel consisting of a 7, 10, or 12% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Hibond-ECL, GE Healthcare Bio-Sciences), and immunoreactive protein bands were detected according to the previously described method.[26]

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GST Pull-Down Assay. For pull-down experiments, GST or GST fusion proteins (10 μg) were absorbed on glutathione Sepharose 4B (20 μL) at 4 °C for 2 h. The glutathione Sepharose was washed five times with ice-cold buffer A and added purified recombinant CDKL5(1−352/KD) (10 μg) in 200 μL of buffer A. After incubation at 4 °C for 2 h, the samples were washed five times with ice-cold buffer A and eluted with 20 μL of 2× SDS−PAGE sample buffer. The pulled-down samples thus obtained were electrophoresed via SDS−PAGE and analyzed by Western blotting.

Other Methods. Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard.[27] Nucleotide sequences were determined by the dideoxynucleotide chain termination method with BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems) and a DNA sequencer (model 3100, Applied Biosystems).

RESULTS

Selective Phosphorylation of Ser-293 in Amph1 by CDKL5. CDKL5 is a large protein with an estimated molecular mass of 110 kDa; one-third of its N-terminal region consists of a Ser/Thr kinase domain, and two-thirds of its C-terminal region is believed to be a regulatory domain that may regulate cellular localization and kinase activity. Because it is difficult to obtain a constitutively active full-length CDKL5 by *E. coli* expression systems, we mainly used an N-terminal truncation mutant, CDKL5(1−352), that contains solely the kinase domain but lacks a large C-terminal domain. In our previous study, we found that CDKL5(1−352) phosphorylated a single site at Ser-293 in the PRD of Amph1.[15] Although this site is known to be phosphorylated not only by CDKL5 but also by ERK1/2 and Dyrk1A, the latter kinases have been reported to phosphorylate multiple sites in addition to Ser-293 (Figure 1A).[28,29] Therefore, we compared the phosphorylation of Amph1 by these three protein kinases: CDKL5(1−352), Dyrk1A, and prephosphorylated ERK2 by active MEK. When Amph1 was incubated with these kinases under standard phosphorylation conditions, it was significantly phosphorylated (Figure 1B). When Amph1(S293A), a mutant in which Ser-293 was replaced with Ala, was used as a substrate, it was not phosphorylated by CDKL5(1−352) at all, whereas it was significantly and weakly phosphorylated by ERK2 and Dyrk1A, respectively (Figure 1B, top panel). Under these conditions, constitutively active MEK did not phosphorylate either Amph1(WT) or Amph(S293A). Phosphorylation at Ser-293 by these kinases was further confirmed by Western blotting with an antibody that detects phospho(Ser-293) (Figure 1B, middle panel). These results indicate that CDKL5(1−352) phosphorylated Amph1 exclusively at Ser-293 and that Dyrk1A preferentially phosphorylated Ser-293, while ERK2 had multiple phosphorylation sites in addition to Ser-293.

Substrate Specificity of ERK2, Dyrk1A, and Truncated Forms of CDKL5. The experiments mentioned above suggest that CDKL5 may exhibit a substrate specificity different from those of ERK2 and Dyrk1A. In the next experiment, therefore, we examined in more detail the substrate specificity of CDKL5 using Amph1, MBP, and a mouse brain extract. Because the brain extract contains various endogenous protein kinases and phosphatases, it was pretreated at 60 °C for 30 min to inactivate the kinases and phosphatases. The detailed functional role of the C-terminal region of CDKL5 remains unknown. Therefore, to examine whether the C-terminal truncation of

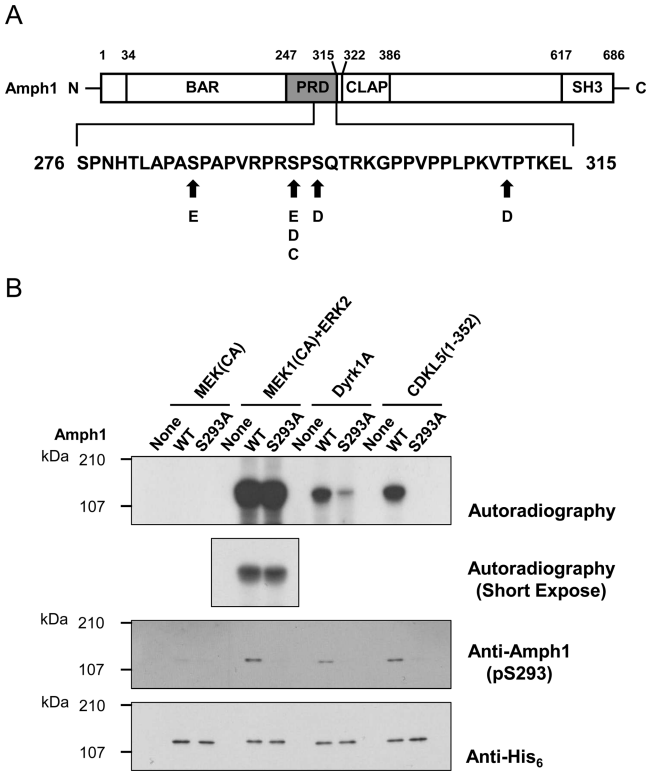

Figure 1. (A) Primary structure of Amph1. Arrows in PRD(276−315) show the sites of phosphorylation by (C) CDKL5, (D) Dyrk1A, and (E) ERK1/2. (B) Phosphorylation of Amph1 and Amph1(S293A) by various protein kinases. Approximately 400 ng of Amph1(WT) or Amph1(S293A) was phosphorylated in a reaction mixture (10 μL) containing 100 μM ATP and constitutively active MEK (0.1 ng), preactivated ERK2 (1 ng) with MEK (0.1 ng), Dyrk1A (1 ng), or CDKL5(1−352) (100 ng). After a 60 min incubation at 30 °C, the reaction was stopped by adding an equal volume of 2× SDS sample buffer. The samples were electrophoresed via SDS−PAGE and analyzed by Western blotting with an anti-Amph1(pS293) antibody (middle) or an anti-His_6 antibody (bottom). Also, Amph1 and its mutant were phosphorylated by protein kinases in the presence of [γ-^{32}P]ATP, and phosphorylated proteins were detected by autoradiography (top).

CDKL5 affects substrate specificity, protein substrates were phosphorylated using different C-terminal truncation mutants of CDKL5. Both CDKL5(1−352) and CDKL5(1−800) efficiently phosphorylated Amph1, but they did not phosphorylate MBP or endogenous proteins in brain extract (Figure 2A). CDKL5(FL), which was pulled down from 293T cells, phosphorylated Amph1, but not MBP or proteins in brain extract as in cases of truncated forms of CDKL5 (Figure 2A). These results suggest that truncation of the C-terminal region did not affect the substrate specificity of CDKL5.

Phosphorylation of the heat-treated brain extract was compared using the three protein kinases, CDKL5, ERK2, and Dyrk1A. When the heat-treated brain extract was incubated with activated ERK2 or Dyrk1A in the presence of [γ-^{32}P]ATP, various phosphorylated protein bands were observed via autoradiography (Figure 2B), while essentially no phosphorylation of endogenous proteins was detected when it was incubated with CDKL5 (Figure 2A). MBP, which is often used as a substrate for *in vitro* kinase assays, was significantly phosphorylated by ERK2 and Dyrk1A, but not by CDKL5 (Figure 2A,B). These results suggest that CDKL5 has a

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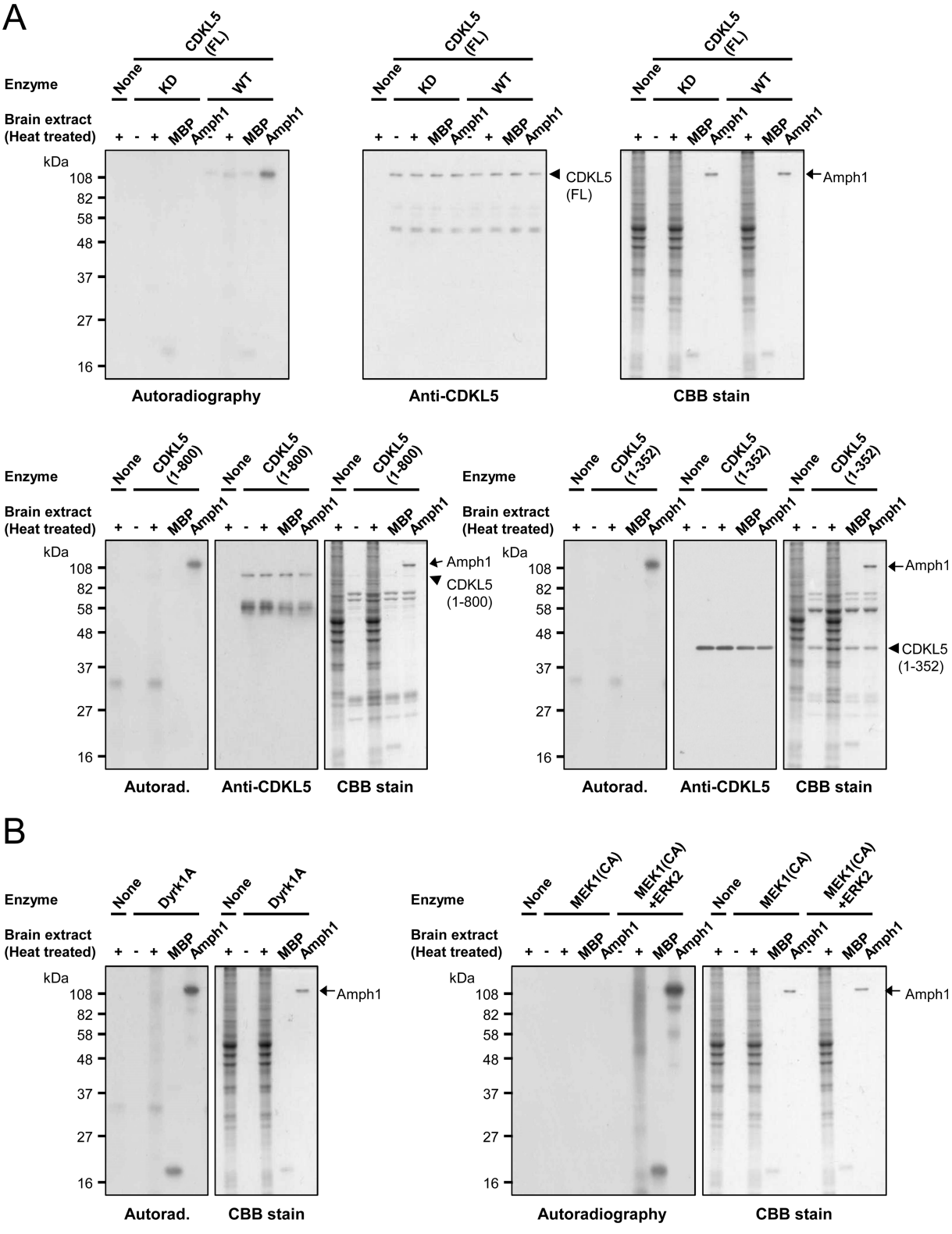

Figure 2. Substrate specificity of CDKL5 (A), ERK2, and Dyrk1A (B). Crude extract from mouse brain was incubated at 60 °C for 30 min to inactivate endogenous protein kinases. Heat-treated brain extract (10 μg), MBP (100 ng), or Amph1 (400 ng) was incubated with the indicated protein kinases in a standard reaction mixture (10 μL) containing 100 μM [γ-^{32}P]ATP. Immunoprecipitated CDKL5(FL/WT) or CDKL5(FL/KD) and purified CDKL5(1−800) (100 ng), CDKL5(1−352) (100 ng), Dyrk1A (1 ng), MEK (0.1 ng), or ERK2 (1 ng) preactivated by MEK (0.1 ng) were used for phosphorylation. After incubation at 30 °C for 60 min, the reaction was stopped by adding an equal volume (10 μL) of 2× SDS sample buffer. The reaction mixtures were subjected to SDS−PAGE, and the radioactive protein bands were detected by autoradiography and analyzed by Western blotting with an anti-CDKL5 antibody and protein staining with Coomassie brilliant blue.

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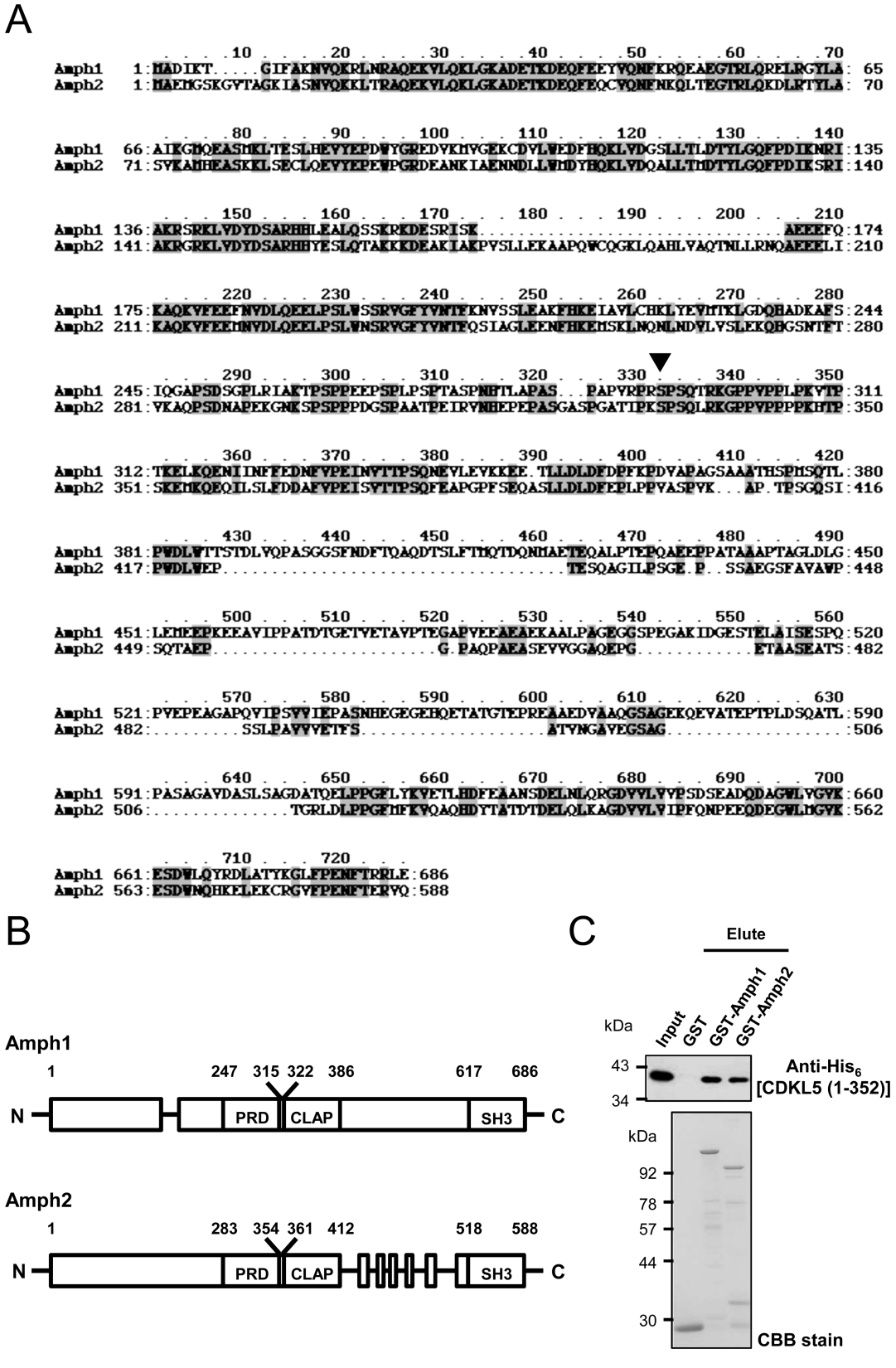

Figure 3. (A) Alignment of amino acid sequences of Amph isoforms. The arrowhead indicates the positon of Ser-293 of Amph1. (B) Primary structures of Amph1 and Amph2. (C) GST, GST-Amph1, or GST-Amph2 (10 μg each) was incubated with glutathione Sepharose 4B (20 μL) at 4 °C for 2 h. The glutathione Sepharose was washed extensively with washing buffer and then incubated with CDKL5(1−352/KD) at 4 °C for 2 h. After the glutathione Sepharose was washed five times with washing buffer, 2× SDS sample buffer (20 μL) was added and bound proteins were eluted by vortexing and boiling for 90 s. Eluted samples were electrophoresed via SDS−PAGE and analyzed by Western blotting with an anti-His_6 antibody (top) and protein staining with Coomassie brilliant blue (bottom).

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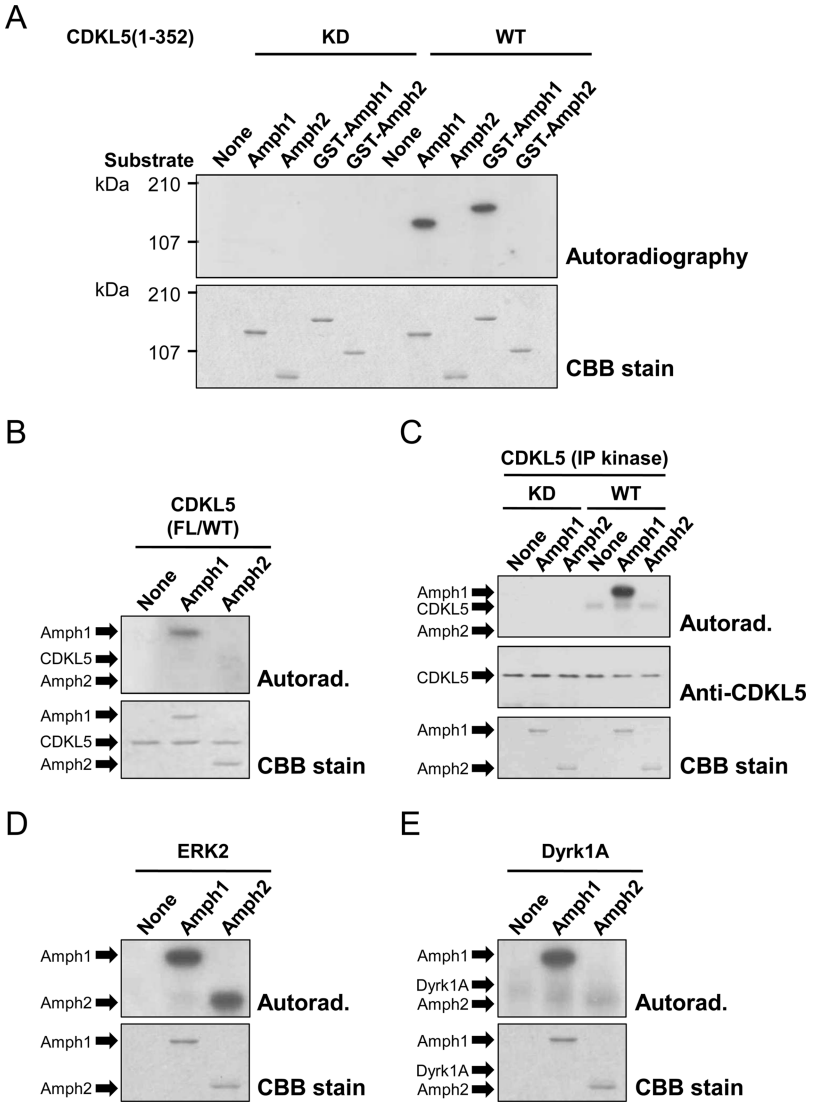

Figure 4. Amph1 or Amph2 (400 ng) was incubated in a standard reaction mixture containing CDKL5(1−352) (100 ng) (A), full-length CDKL5 (400 ng) purified from *E. coli* (B), full-length CDKL5(WT) or CDKL5(KD) immunoprecipitated from 293T cells (5 μL of Sepharose) (C), activated ERK2 (5 ng) (D), or Dyrk1A (25 ng) (E) and 100 μM [γ-^{32}P]ATP. After a 60 min incubation at 30 °C, the reaction was stopped by adding an equal volume of 2× SDS sample buffer and the mixture electrophoresed via SDS−PAGE. Phosphorylated proteins were detected by autoradiography (top). The protein staining patterns of Coomassie brilliant blue are shown in the bottom panels.

substrate specificity much more restricted than those of ERK2 and Dyrk1A.

Phosphorylation of Amph1 and Amph2 by CDKL5. Amph1 is known to play important roles in clathrin-mediated endocytosis. Amph2 is an isoform of Amph1 and has domain structures similar to those of Amph1 (Figure 3A,B). GST pull-down experiments demonstrated that both Amph1 and Amph2 could be pulled down with CDKL5(1−352), indicating that both Amph1 and Amph2 have binding affinity for the kinase domain of CDKL5 (Figure 3C). Comparison of the amino acid sequences of these proteins revealed that Ser-293, the site of phosphorylation in Amph1 by CDKL5(1−352), is conserved in Amph2 as Ser-332 (Figure 3A, arrowhead), suggesting that Amph2 may also be phosphorylated by CDKL5. Unexpectedly, however, Amph2 was not phosphorylated by CDKL5(1−352) at all, while Amph1 was significantly phosphorylated (Figure 4A). Both GST fusion Amph2 and His_6-tagged Amph2 showed the same result. When the full-length CDKL5 prepared from the *E. coli* expression system was used, only Amph1 was selectively phosphorylated as in the case of CDKL5(1−352) (Figure 4B). Immunoprecipitated full-length CDKL5(WT) from 293T cells also clearly phosphorylated Amph1, but not Amph2 (Figure 4C). Activated ERK2 significantly phosphorylated not only Amph1 but also Amph2 (Figure 4D), while Dyrk1A phosphorylated Amph1 more efficiently than it phosphorylated Amph2, similar to the case of CDKL5 (Figure 4E).

Determination of the Critical Amino Acid Sequence around the CDKL5 Phosphorylation Site in Amph1. The amino acid sequence around the phosphorylation site in Amph1 was compared with the corresponding sequence in Amph2 (Figure 5A). The amino acid sequence around Ser-293 in Amph1 is $RPR^{293}SPSQ$, while the corresponding sequence in Amph2 is $IPK^{332}SPSQ$. The most noticeable difference between these sequences is the amino acid residues at the P −3 position. The amino acid residue at the P −3 position in Amph1 is Arg, a positively charged amino acid, while the corresponding site in Amph2 is Ile, a typical hydrophobic residue. Considering the

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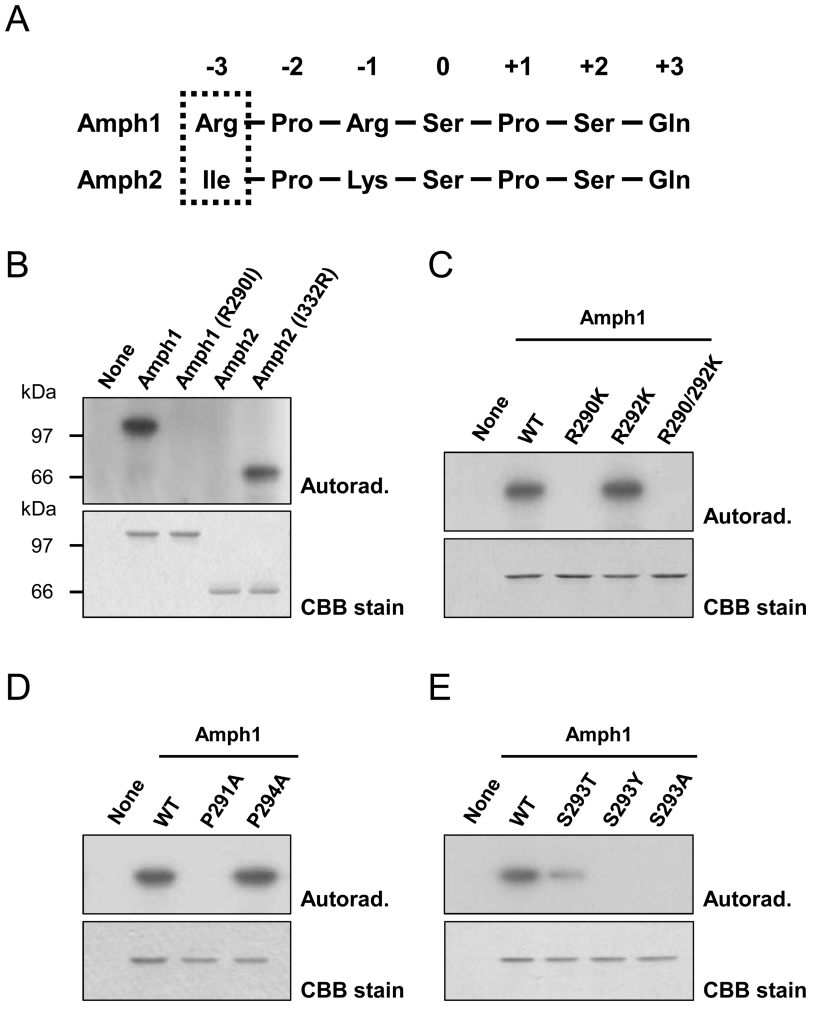

Figure 5. (A) Comparison of the amino acid sequence around the phosphorylation site of Amph1 with that of Amph2. (B) Amph1, Amph1(R290I), Amph2, or Amph2(I329R) (400 ng) was incubated in a standard reaction mixture containing CDKL5(1−352) (100 ng) and 100 μM [γ-^{32}P]ATP. After a 60 min incubation at 30 °C, the reaction was stopped by adding an equal volume of 2× SDS sample buffer and the mixture electrophoresed via SDS−PAGE. Phosphorylated proteins were detected by autoradiography (top). The protein staining pattern by Coomassie brilliant blue is shown in the bottom panel. (C−E) The wild type and various point mutants of Amph1 (400 ng) indicated in the figure were incubated in a standard reaction mixture containing CDKL5(1−352) (100 ng) and 100 μM [γ-^{32}P]ATP. After a 60 min incubation at 30 °C, the reaction was stopped and the phosphorylation of proteins was analyzed as described for panel B.

fact that the P −3 position in the consensus sequence around the phosphorylation site for Dyrk1A is Arg,[30] it is conceivable that the positively charged Arg residue at this position may also be important for phosphorylation by CDKL5. To examine this possibility, we prepared Amph mutants at the P −3 position, Amph1(R290I) and Amph2(I329R), and examined their phosphorylation by CDKL5(1−352). Amph1(R290I) was not phosphorylated by CDKL5(1−352) at all, but Amph2(I329R) was significantly phosphorylated by CDKL5(1−352) as in case of Amph1(WT) (Figure 5B). We also prepared Amph1(R290 K), whose Arg residue at the P −3 position was replaced with Lys, another positively charged residue, but this mutant did not serve as a substrate for CDKL5(1−352). Another Arg residue at the P −1 position is not critical for phosphorylation, because Amph1(R292 K) was efficiently phosphorylated by CDKL5(1−352) at a level similar to that of Amph1(WT) (Figure 5C). These results indicate that the Arg residue at the P −3 position is a critical amino acid residue for phosphorylation by CDKL5.

Many protein kinases that belong to CMGC family are known to be proline-directed protein kinases. To examine whether the Pro residue at the P −2 or P +1 position in Amph1 is essential for phosphorylation by CDKL5, two point mutants, Amph1(P291A) and Amph1(P294A), were prepared and phosphorylated by CDKL5(1−352). Among these mutants, Amph1(P294A) served as an efficient substrate for CDKL5(1−352), whereas Amph1(P291A) did not (Figure 5D). These results indicate that Pro residue at the P −2 position, but not the P +1 position, is indispensable for phosphorylation by CDKL5.

Although CDKL5 is a member of the Ser/Thr protein kinase family, it has also been reported to be activated by autophosphorylating the TEY motif in the activation loop of the kinase.[31,32] Therefore, in the next experiment, we examined the amino acid preference of CDKL5 in the Amph1 phosphorylation site. We prepared Amph1(S293T) and Amph1(S293Y) and compared the phosphorylation of these mutants with that of Amph1(WT). Amph1(S293T) was weakly phosphorylated by CDKL5(1−352), but Amph1(S293Y) was not phosphorylated at all (Figure 5E). Therefore, although CDKL5 is a Ser/Thr protein kinase, it prefers the Ser residue to the Thr residue as a phosphorylation site.

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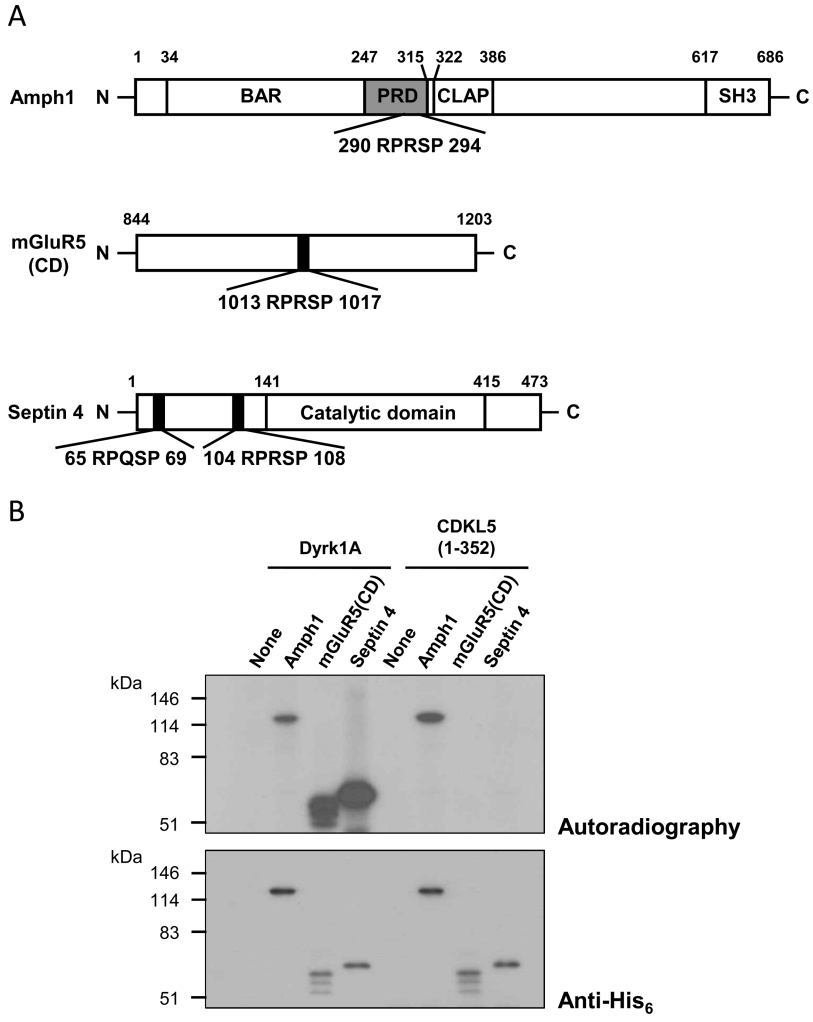

Figure 6. (A) Primary structure of Amph1, mGluR5(CD), and Septin 4. The positions of the RPXSP consensus sequences are also shown. (B) Amph1, mGluR5(CD), or Septin 4 (200 ng each) was incubated in a standard reaction mixture containing CDKL5(1−352) (100 ng) and 100 μM [γ-^{32}P]ATP. After a 60 min incubation at 30 °C, the reaction was stopped by adding an equal volume of 2× SDS sample buffer and the mixture electrophoresed via SDS−PAGE. Phosphorylated proteins were detected by autoradiography (top) and Western blotting with an anti-His$_6$ antibody (bottom).

Phosphorylation of Proteins with a Consensus Phosphorylation Sequence. From our experimental data, RPXSX was identified as a putative consensus sequence around the CDKL5 phosphorylation site. To examine other possible CDKL5 substrates, we prepared two recombinant proteins possessing the consensus phosphorylation sequence. These are the cytoplasmic domain of metabotropic glutamate receptor 5 [mGluR5(CD)], which is a subtype of group I mGluRs, and Septin 4, which has GTPase activity and correlates with membrane dynamics during cell division. When these proteins were incubated with Dyrk1A in the presence of [γ-^{32}P]ATP, they were both markedly phosphorylated. In contrast, these proteins were not phosphorylated by CDKL5(1−352) (Figure 6B). On the other hand, Amph1 was phosphorylated by both CDKL5(1−352) and Dyrk1A. These results suggest that CDKL5 recognizes not only the primary sequence around the phosphorylation site but also some other structural features in Amph1.

Determination of the Essential Domain in Amph1 for Recognition by CDKL5. When a peptide substrate containing Ser-293 (Ser-293 peptide, APVRPRSPSQTRKGC) was synthesized and phosphorylated by CDKL5(1−352), no incorporation of phosphate into the peptide was observed. Under the same conditions, Amph1 was significantly phosphorylated (Figure 7A), suggesting that another part of Amph1 is necessary for recognition by CDKL5. In contrast to CDKL5(1−352), Dyrk1A phosphorylated the Ser-293 peptide to some extent (data not shown). To investigate which region of Amph1 is necessary for recognition by CDKL5, we prepared various deletion mutants of Amph1 and used them in the phosphorylation assay using CDKL5(1−352) (Figure 7B). Amph1(1−385) was significantly phosphorylated by CDKL5(1−352) as in case of Amph1(FL). In contrast, Amph1(1−314), which also contains the phosphorylation site Ser-293, served as a poor CDKL5 substrate (Figure 7C). Although Amph1(247−686) and Amph1(247−385) served as efficient substrates for CDKL5(1−352), Amph1(247−314), which contains the PRD, was not phosphorylated by CDKL5(1−352) (Figure 7C). These results, taken together, suggest that the CLAP domain (amino acids 322−385) in Amph1 in addition to the PRD is essential for selective recognition and/or phosphorylation by CDKL5.

In the next experiment, we examined whether CDKL5 interacted with Amph1 through the CLAP domain. The pull-

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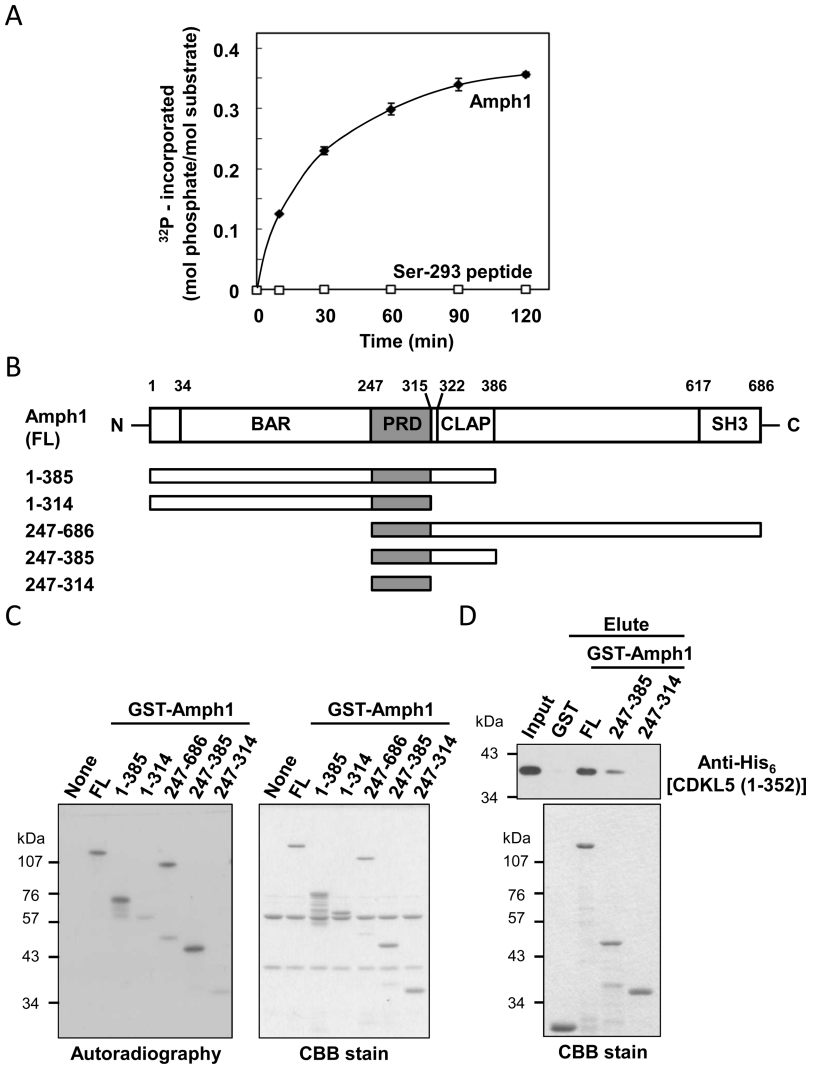

Figure 7. (A) Time course of incorporation of phosphate into the Amph1 or Ser-293 peptide. Three independent experiments were conducted, and the data are present as means ± the standard deviation. (B) Schematic representation of the Amph1 deletion mutants used in this study. (C) Phosphorylation of various deletion mutants of Amph1 by CDKL5. Amph1 mutants (400 ng) indicated in the figure were incubated in a standard reaction mixture containing CDKL5(1−352) (100 ng) and 100 μM [γ-^{32}P]ATP. After a 60 min incubation at 30 °C, the reaction was stopped by adding an equal volume of 2× SDS sample buffer and the mixture electrophoresed via SDS−PAGE. Phosphorylated proteins were detected by autoradiography (left). The Coomassie brilliant blue protein staining pattern is shown in the right panel. (D) Pull-down experiment using GST-Amph1. GST, GST-Amph1(FL), GST-Amph1(247−385), or GST-Amph1(247−314) (10 μg each) was incubated with glutathione Sepharose 4B (20 μL) at 4 °C for 2 h. The glutathione Sepharose was washed five times with washing buffer and then incubated with CDKL5(1−352/KD) at 4 °C for 2 h. After the glutathione Sepharose was washed five times with washing buffer, 2× SDS sample buffer (20 μL) was added and bound proteins were eluted by vortexing and boiling for 90 s. Eluted samples were electrophoresed via SDS−PAGE and analyzed by Western blotting with an anti-His_6 antibody (top) or protein staining with Coomassie brilliant blue (bottom).

down experiments were conducted using the GST fusion proteins of Amph1. GST-Amph1(FL) clearly coprecipitated with CDKL5, and GST-Amph1(247−385), which is composed of the PRD and CLAP domains, also bound to CDKL5(1−352) (Figure 7D). In contrast, GST alone and GST-Amph1(287−314), which contains the PRD but not the CLAP domain, could not bind to CDKL5(1−352) at all. These results suggest that the CLAP domain in Amph1 serves as a docking site for binding of Amph1 to CDKL5.

■ DISCUSSION

There are more than 500 protein kinases in mammals,[33] and these kinases are believed to phosphorylate their own target proteins as physiological substrates. Casein kinase 1/2, cAMP-dependent protein kinase, CaM kinases I, II, and IV, and MAP kinases are known as multifunctional protein kinases that phosphorylate a wide variety of protein substrates. In contrast, protein kinases such as myosin light chain kinase and CaM kinase III have quite restricted substrate specificities and selectively phosphorylate myosin light chain[34] and elongation factor-2,[35] respectively. MAP-kinase kinase and CaM-kinase kinases are also known as dedicated kinases and specifically phosphorylate their downstream kinases to transmit cellular signaling correctly. However, the physiological substrates of many protein kinases remain unknown. Identifying the correct substrates of protein kinases is one of the most important issues for understanding their physiological functions in the cellular responses to various stimuli. So far, MeCP2,[5,7] Dnmt1,[9] and NGL-1[10] have been reported as possible CDKL5 substrates, but the phosphorylation of these proteins by CDKL5 was rather

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weak (ref 15 and unpublished results). Therefore, we have searched for other possible substrates of CDKL5 with the aid of a unique technique that we developed[14] and identified Amph1 as an efficient substrate for CDKL5. Although no other efficient substrate has been found to date, it is still unclear whether Amph1 is the physiological substrate in brain. To understand the real target of CDKL5, we investigated the molecular mechanisms of substrate recognition and phosphorylation by constitutively active CDKL5(1−352) using Amph1 as a model substrate.

In a previous study, we identified the phosphorylation site of Amph1 by CDKL5(1−352) as a single site at Ser-293.[15] Because Ser-293 in Amph1 has also been reported to be phosphorylated by ERK2[28] and Dyrk1A,[29] the substrate specificity of these kinases was compared with that of CDKL5 to characterize their physiological significance. ERK2 and Dyrk1A appeared to phosphorylate many proteins in the heat-treated mouse brain extract, whereas almost no protein phosphorylation by CDKL5 was detected with this preparation. MBP, a widely used substrate for *in vitro* assays for various protein kinases, was significantly phosphorylated by ERK2 and Dyrk1A, but not by CDKL5 (Figure 2). ERK2 has been reported to phosphorylate Thr-97 (TPRTP) in MBP, and Dyrk1A appeared to phosphorylate multiple sites, including Ser-164 (RSGSP) in the C-terminal region of MBP.[30] The Arg residue at the P −3 position in the substrate is important for substrate recognition by Dyrk1A[30,36] and by CDKL5(1−352) (Figure 5). On the basis of X-ray crystallographic study, the Arg residue at the P −3 position in the substrate was found to interact with Glu-291 and Glu-353 in Dyrk1A.[37] These acidic residues in Dyrk1A correspond to Glu-139 and Glu-203, respectively, in CDKL5. In contrast, the equivalent residues in ERK2 are Ser-151 and Glu-218, making its substrate specificity different from that of Dyrk1A and CDKL5. The consensus phosphorylation sequence by CDKL5 was predicted to be RPXSX; hence, CDKL5 could not phosphorylate MBP, because this consensus sequence does not exist in MBP. These results suggest that CDKL5 shows substrate specificity more restricted than that of other CMGC family protein kinases such as ERK2 and Dyrk1A.

Dyrk1A and ERK2 are known to phosphorylate synthetic peptide substrates as well as protein substrates. In contrast, CDKL5 could not phosphorylate a peptide substrate corresponding to the phosphorylation site of Amph1, APVRPR^{293}SPSQTRKGC, which was readily phosphorylated by Dyrk1A (data not shown). Therefore, additional experiments using various deletion mutants of Amph1 were conducted to clarify the critical determinants for a CDKL5 substrate. Phosphorylation experiments using various Amph1 mutants revealed that the CLAP domain (amino acids 322−385) in addition to the PRD is important for recognition by CDKL5(1−352), making it a good substrate for this kinase. Furthermore, pull-down experiments revealed that Amph1-(PRD+CLAP domain) could bind to CDKL5(1−352), but Amph1(PRD) without the CLAP domain failed to interact with the kinase. These results, taken together, suggest that CDKL5 recognizes not only the amino acid sequence around the phosphorylation site (RPXSX) but also the structural features of a distinct site (CLAP domain) of Amph1, which serves as a docking site for CDKL5.

CDKL5 has been found to bind and phosphorylate the cell adhesion molecule NGL-1.[10] This phosphorylation promotes a stable association between NGL-1 and PSD95, thereby inducing correct dendritic spine formation and synaptic activity. The amino acid sequence (RMNSK) of the phosphorylation site of NGL-1 in its cytoplasmic domain does not coincide with our consensus phosphorylation sequence (RPXSX) of CDKL5. To compare phosphorylation of these proteins by an *in vitro* kinase assay, we prepared the C-terminal cytoplasmic domain of NGL-1 using the *E. coli* expression system. Under our experimental conditions, Amph1 served as a much more efficient substrate for CDKL5(1−352) than for NGL-1 (data not shown). The differences in the phosphorylation of these proteins *in vitro* may be attributed to the differences in the sequence around the phosphorylation site and the lack of a docking site such as the CLAP domain in NGL-1. However, further studies are necessary to assess phosphorylation of Amph1 by CDKL5 in comparison with that of NGL-1 *in vivo*.

Amph1 and Amph2 are homologous proteins with similar domain structures, including the BAR domain, PRD, the CLAP domain, and the SH3 domain.[16,17,38,39] These proteins are strongly expressed in the brain,[38,39] are known to form heterodimers, and positively regulate clathrin-mediated endocytosis.[17,38] Although Amph1 and Amph2 have similar structural features and similar characteristic properties, only Amph1 was phosphorylated by CDKL5(1−352). Upon phosphorylation of Amph1 by CDKL5(1−352), the binding affinitiy of Amph1 for endophilin drastically decreased, while the binding affinity of Amph1 for Amph2 was not altered.[15] The kinase activity of CDKL5 appears to play an important role in the process of neuronal development, though the detailed mechanisms are still unknown. Mutations in the *CDKL5* gene are known to cause neurodevelopmental disorders such as RTT.[31,32] However, the physiological significance of the selective phosphorylation of Amph1 without phosphorylating Amph2 by CDKL5 is not clear at this point. Restricted phosphorylation of Amph1 may correlate with the mechanisms of fine control of endocytotic processes in neuronal cells by which neuronal development may be stringently regulated. The molecular mechanisms underlying the neurodevelopmental control through phosphorylation of Amph1 by CDKL5 will be the next issue to be elucidated.

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Notes

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ABBREVIATIONS

Amph1, amphiphysin 1; CDKL5, cyclin-dependent kinase-like 5; CLAP, clathrin and adaptor AP2 binding; Dnmt1, DNA methyltransferase 1; Dyrk1A, dual-specificity tyrosine phosphorylation-regulated kinase 1A; ERK, extracellular signal-

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regulated kinase; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; MBP, myelin basic protein; MeCP2, methyl CpG-binding protein 2; PRD, proline-rich domain.

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