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Identification of amphiphysin 1 as an endogenous substrate for CDKL5, a protein kinase associated with X-linked neurodevelopmental disorder

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ABSTRACT

Cyclin-dependent kinase-like 5 (CDKL5) is a Ser/Thr protein kinase predominantly expressed in brain and mutations of its gene are known to be associated with neurodevelopmental disorders such as X-linked West syndrome and Rett syndrome. However, the physiological substrates of CDKL5 that are directly linked to these neurodevelopmental disorders are currently unknown. In this study, we explored endogenous substrates for CDKL5 in mouse brain extracts fractionated by a liquid-phase isoelectric focusing. In conjunction with CDKL5 phosphorylation assay, this approach detected a protein band with an apparent molecular mass of 120 kDa that is remarkably phosphorylated by CDKL5. This 120-kDa protein was identified as amphiphysin 1 (Amph1) by LC-MS/MS analysis, and the site of phosphorylation by CDKL5 was determined to be Ser-293. The phosphorylation mimic mutants, Amph1(S293E) and Amph1(S293D), showed significantly reduced affinity for endophilin, a protein involved in synaptic vesicle endocytosis. Introduction of point mutations in the catalytic domain of CDKL5, which are disease-causing missense mutations found in Rett patients, resulted in the impairment of kinase activity toward Amph1. These results suggest that Amph1 is the cytoplasmic substrate for CDKL5 and that its phosphorylation may play crucial roles in the neuronal development.

Introduction

Protein kinases regulate a wide variety of cellular processes from cell proliferation and differentiation to apoptosis [1]. Cyclin-dependent kinase-like 5 (CDKL5[2], also known as STK9) is a Ser/Thr protein kinase originally identified in a transcriptional mapping project on the human chromosome Xp22 region [2]. Expression studies demonstrated that CDKL5 was expressed in several tissues, especially abundant in brain [2,3]. Mutations in the *CDKL5* gene have been connected to X-linked neurodevelopmental disorders involving severe mental retardation and seizures, which appears in the first postnatal months [3–9]. Furthermore, missense mutations in the catalytic domain of CDKL5 have been reported in patients with atypical variants of Rett syndrome (RTT[3]), infantile spasms and severe neurodevelopmental retardation [4,5,7,8]. RTT is a neurodevelopmental disorder predominantly affected in young females, presenting mental retardation, loss of acquired motor and vocal skills, and many other neurological problems. A majority of RTT is attributed to a mutation in the gene encoding for methyl-CpG-binding protein 2 (MeCP2[4]) [10–12].

In previous papers, MeCP2 was reported to be phosphorylated by CDKL5 *in vitro* [6,8]. On the contrary, Lin et al. reported that MeCP2 is not a direct substrate for CDKL5 [7]. Previously, we also examined phosphorylation of MeCP2 by CDKL5 and found that MeCP2 was weakly phosphorylated by CDKL5 [13]. Instead, DNA methyltransferase 1 (Dnmt1[5]) was more efficiently phosphorylated by CDKL5 than MeCP2, especially in the presence of DNA [13]. Although MeCP2 and Dnmt1 appeared to serve as substrates for CDKL5 *in vitro*, phosphorylation rates of these proteins were much lower than those expected for physiological substrates. MeCP2 is an exclusively nuclear protein, while CDKL5 shuttles between two main cellular compartments, the nucleus and the cytoplasm [3]. Intracellular localization of CDKL5 appears to vary in different brain areas and during development. In the early stage of development, relative concentration of CDKL5 is higher in the cytosolic fraction and then gradually increases in the nuclear fraction [3], suggesting

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[1] These authors contributed equally to this work.
[2] cyclin-dependent kinase-like 5.
[3] Rett syndrome.
[4] methyl-CpG-binding protein 2.
[5] DNA methyltransferase 1.

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that CDKL5 functions in both the nucleus and cytoplasm. Therefore, it is of great interest to identify the endogenous targets of CDKL5 to understand the role of CDKL5 in neurodevelopment.

To explore the endogenous substrate(s) for CDKL5, we introduced a new method that we have developed recently [14]. Mouse brain extracts were first fractionated by a liquid-phase isoelectric focusing, MicroRotofor, and the separated proteins were then phosphorylated by CDKL5 *in vitro*. With this approach, we identified a major substrate, with an apparent molecular mass of 120 kDa, as amphiphysin 1 (Amph1[6]), a brain specific protein involved in neuronal transmission and neuronal development. The present results demonstrate that Amph1 is the endogenous substrate for CDKL5 and suggest the potential role of CDKL5 in clathrin-mediated endocytosis in the central nervous system.

Materials and methods

Materials

[γ-^{32}P]ATP (111 TBq/mmol) was purchased from PerkinElmer. HiTrap Chelating HP, glutathione Sepharose 4B and pGEX-6P-1 were obtained from GE Healthcare Bio-Sciences. ATP, myelin basic protein (MBP[7]), bovine serum albumin and an anti-phosphotyrosine antibody (PY20) were purchased from Sigma–Aldrich. Anti-amphiphysin antibody and anti-His_6 antibody were from Stressgen and Wako Pure Chemical Industries, respectively. An anti-phosphothreonine antibody (42H4) was obtained from Cell Signaling Technology. Microtubule-associated protein 2 (MAP2[8]) was prepared from rat brain as described previously [15]. Recombinant proteins encoding for mouse CDKL5(1-352) and GST-Dnmt1(1-290) were expressed in *Escherichia coli* and purified as described previously [13].

Plasmid constructions

Expression vectors for mouse Amph1 (pET-mAmph1), Amph2 (pET-mAmph2) endophilin A1 (pET-EndpA1) and drebrin A (pET-mDbnA) were constructed by PCR, with specific primers (for mAmph1: 5′-GGA TCC ATG GCC GAC ATC AAG ACG GG-3′ and 5′-CTC GAG CTC CAG GCG CCG CGT GAA GT-3′; for mAmph2: 5′-GGA TCC ATG GCA GAG ATG GGG AGC AAG-3′ and 5′-CTC GAG CTG CAC CCG CTC TGT AAA ATT CT-3′; for mEndpA1: 5′-GG GGA TCC ATG TCG GTG GCA GGG CTG AA-3′ and 5′-GG CTC GAG ATG GGG CAG AGC AAC CAG AAT-3′; for mDbnA: 5′-GGA TCC ATG GCC GGC GTC AGC TTC AG-3′ and 5′-CTC GAG ATC ACC ACC CTC GAA GCC CTC-3′), and mouse brain 5′-RACE cDNA library as a template. The BamHI(underlined)-XhoI(double underlined) fragments were subcloned into the BamHI/XhoI site of pET-23a(+) (Novagen) to generate His_6-fused proteins at their C-terminal ends.

For lambda protein phosphatase (λPPase[9]), the expression vector (pET-λPPase) was constructed by PCR using λDNA (TAKARA BIO) as a template and a set of primers (5′-AAA GAA TTC ATG CGC TAT TAC GAA AAA ATT GAT G-3′ and 5′-AAA CTC GAG TGC GCC TTC TCC CTG TAC CTG-3′). The EcoRI(underlined)-XhoI(double underlined) fragments were subcloned into the EcoRI/XhoI sites of pET-23a(+) to generate His_6-fused protein at its C-terminal end.

To generate pGEX-Amph1, the BamHI-XhoI fragment from pET-Amph1 was ligated into pGEX-6P-1. Point mutants of mouse Amph1 were prepared by an inverse PCR method [16] with the following primers (for S262A: 5′-GCA CCT CCA GAG GAG CCT TCT CC-3′ and 5′-TGG TGT CTT TGC AAT GCG AAG A-3′; for S268A: 5′-GCT CCC CTG CCC AGC CCC AC-3′ and 5′-AGG CTC CTC TGG AGG TGA TGG-3′; for S272A: 5′-GCA CCC ACG GCT AGT CCC AAC CA-3′ and 5′-GGG CAG GGG AGA AGG CTC CT-3′; for S276A: 5′-GCA CCC AAC CAC ACA TTA GCA CCT G-3′ and 5′-AGC CGT GGG GCT GGG CAG G-3′; for S285A: 5′-GCA CCT GCC CCA GTG CGA CCC A-3′ and 5′-TGC AGG TGC TAA TGT GTG GTT GG-3′; for S293A: 5′-GCA CCT TCA CAG ACA AGG AAA GGG-3′ and 5′-TCT GGG TCG CAC TGG GGC A-3′; for S293D: 5′-GAT CCT TCA CAG ACA AGG AAA GGG C-3′ and 5′-TCT GGG TCG CAC TGG GGC A-3′; for S293E: 5′-GAG CCT TCA CAG ACA AGG AAA GGG C-3′ and 5′-TCT GGG TCG CAC TGG GGC A-3′) (underlines indicate the sites of mutations), and pET-Amph1 as a template.

To obtain the point mutants of CDKL5(1-352), the following primer sets were used for inverse PCR with pET-CDKL5(1-352) as a template; for CDKL5(C152F): 5′-TTT GAC TTT GGT TTT GCT CGC A-3′ and 5′-TAG TTT CAG GAC ATC ATT GTG GCT G-3′; for CDKL5(R175S): 5′-AGC TGG TAC CGA TCC CCG GA-3′ and 5′-GGT AGC CAC ATA CTC TGT ATA ATT AGC AT-3′; for CDKL5(P180L): 5′-AGG TGG TAC CGA TCC CTG GA-3′ and 5′-GGT AGC CAC ATA CTC TGT ATA ATT AGC AT-3′ (underlines indicate the sites of mutations). The 5′-ends of each PCR fragment were phosphorylated by T4 polynucleotide kinase (Nippon Gene) and self-ligated using T4 DNA ligase (Nippon Gene).

Expression and purification of recombinant proteins

To produce His-tagged proteins, pET-mAmph1, pET-mDbnA and expression vectors for point mutants of Amph1 were introduced into *E. coli* strain BL21(DE3)-RIL cells (Stratagene). The transformed bacteria were grown at 37 °C to an A_{600} of 0.5, and the expression of the proteins were induced by adding isopropyl β-D-galactoside (IPTG) to a final concentration of 0.1 mM. After 6-h culture at 37 °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]. For recombinant endophilin, BL21(DE3) cells transformed with the pET-EndpA1 were cultured at 37 °C for 9 h without IPTG induction and collected as described above. After the harvest, the bacterial cells were sonicated and cell debris was removed by centrifugation (20,000*g*) at 4 °C for 10 min. Subsequently, the supernatant was loaded on a HiTrap Chelating HP column pre-equilibrated with buffer A. The column was washed in stepwise with buffer A, buffer A containing 20 mM imidazole, buffer A containing 50 mM imidazole, and then eluted with buffer A containing 200 mM imidazole.

For the recombinant GST-Amph1 fusion protein, BL21(DE3) cells transformed with pGEX-Amph1 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 the expression. After 6-h culture at 37 °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 by 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 use.

For the production of λPPase in *E. coli*, pET-λPPase was introduced into BL21(DE3) cells. The transformed bacteria were grown at 25 °C to an A_{600} of 0.5, and then IPTG was added to a final concentration of 0.4 mM. After 16-h culture at 25 °C, the bacteria were harvested by centrifugation and suspended in buffer A and purified using a HiTrap Chelating HP column as described above. The purified λPPase fractions were pooled, dialyzed against 40 mM Tris–HCl buffer (pH 7.5) containing 200 mM NaCl, 0.2 mM $MnCl_2$, 4 mM EGTA and 0.1% Tween 40. The dialyzed λPPase were diluted by an equal volume of glycerol and stored in aliquots at −80 °C until use.

[6] amphiphysin 1.
[7] myelin basic protein.
[8] microtubule-associated protein 2.
[9] lambda protein phosphatase.

Preparation of 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 phenylmethylsulfonyl fluoride, and homogenized by a Teflon/glass homogenizer. The homogenate was centrifuged at 20,000*g* at 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.

Fractionation of crude extracts by MicroRotofor

A liquid-phase isoelectric focusing using MicroRotofor (Bio-Rad) was carried out essentially as described previously [17]. Mouse brain was homogenized in 3 volumes of 50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl, 0.5% Triton X-100, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 20,000*g* at 2 °C for 30 min. Proteins were precipitated from the resultant supernatant by trichloroacetic acid in a final concentration of 10%, and the precipitate was rinsed with acetone and solubilized with 3 ml of IEF buffer (7 M urea, 2 M thiourea, 5 mM dithiothreitol, 4% CHAPS and 2% Pharmalyte pH 3–10). The protein (3 mg) solution was subjected to the MicroRotofor and electrophoresed for 2.5 h at a constant power of 1 W at room temperature. After the electrophoresis, protein fractions from each compartment (200 μl) were harvested and an equal volume of 20% trichloroacetic acid was added. The proteins were precipitated by centrifugation at 20,000*g* for 10 min and then washed twice with 400 μl of acetone to remove remaining trichloroacetic acid in the precipitates. The precipitates were air-dried and dissolved in 50 μl of 40 mM Hepes–NaOH buffer (pH 7.4) containing 0.05% Tween 40 with sonication and used for protein kinase assay.

Identification of an endogenous substrate for CDKL5 in mouse brain

A 120-kDa protein band corresponding to the radioactive band detected in Fraction 2 of MicroRotofor was excised, followed by in-gel digestion with 10 μg/ml trypsin (Promega) [18]. The digested peptides were eluted with 0.1% formic acid and subjected to LC-MS/MS analysis. LC-MS/MS analysis was performed on an LCMS-IT-TOF instruments (Shimadzu) interfaced with a nano reverse-phase liquid chromatography system (Shimadzu) and the obtained MS/MS data were analyzed with Mascot software (Matrix Science) according to the method described previously [19].

Protein phosphorylation

Phosphorylation of proteins by CDKL5 was carried out using the truncated kinase, CDKL5(1-352), since it showed much higher kinase activity than the full length CDKL5 [13]. 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). Phosphorylation was carried out either in the presence or absence of mouse genomic DNA (37 μg/ml). The reaction was started by the addition of CDKL5(1-352) and 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 on 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 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 Whatman 3MM chromatography paper (Whatman), and immediately placed in 5% trichloroacetic acid containing 1% sodium diphosphate. [^{32}P]phosphate incorporation into Amph1 was measured by a liquid scintillation counter. The phosphate incorporated into Amph1 was calculated as mol phosphate/mol Amph1 by subtracting the radioactivity into CDKL5(1-352) from that into CDKL5(1-352) plus Amph1.

Dephosphorylation of Amph1 by λPPase

Dephosphorylation of Amph1 was carried out at 30 °C for 60 min in a standard reaction mixture (50 μl) consisting of 40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2 mM $MnCl_2$, 2 mM dithiothreitol, 0.01% Tween 40, 500 ng of phospho-Amph1 and 5 ng of λPPase, and the reaction was stopped by adding an equal volume of 2× SDS sample buffer. The mixtures were subjected to SDS–PAGE and analyzed by Western blotting.

Production of anti-Amph1(pS293) antibody

An antigenic phosphopeptide (APVRPRpSPSQTRKGC) corresponding to amino acid residues 287–300 of Amph1 was synthesized using a Shimadzu PSSM-8 automated peptide synthesizer [20]. The peptide was purified by reverse-phase HPLC on a C18 column and its purity was confirmed by time-of-flight mass spectrometry (Microflex AI, Bruker Daltonics). Purified peptide was coupled to keyhole limpet hemocyanin by a heterobifunctional reagent, N-(6-maleimidocaproyloxy)succinimide (Dojindo Laboratories), through its C-terminal cysteinyl residues as described previously [21]. An antibody that detects phospho-(Ser-293) of Amph1, designated as anti-Amph1(pS293), was produced by immunizing BALB/c mice with a phosphopeptide conjugate essentially according to the procedure described previously [22].

SDS–PAGE and Western blotting

SDS–PAGE was performed essentially according to the method of Laemmli [23] on a slab gel consisting of an 8% or 10% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Hi-bond-ECL, GE Healthcare) and immunoreactive protein bands were detected according to the previously described method [22].

GST pull-down assay

For pull-down experiments, GST or GST-Amph1s (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 endophilin or Amph2 (10 μg) in 200 μl of buffer A. After incubation at 4 °C for 2 h, samples were washed 5 times with the ice cold buffer A, eluted with 20 μl of 2× SDS sample buffer and analyzed by SDS–PAGE, followed by Western blotting.

Other methods

Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard [24]. Nucleotide sequences were determined by the dideoxynucleotide chain termination method with a BigDye Terminator Cycle Sequencing Ready Reaction Kit Ver. 3.1 (Applied Biosystems) and a DNA Sequencer (model 3100, Applied Biosystems).

Results

Identification of a major substrate for CDKL5 in mouse brain

Although CDKL5 is known to be closely correlated with X-linked neurodevelopmental disorders, a direct cellular target of CDKL5 is still unknown. To find out endogenous substrates for CDKL5, we searched for new candidates in mouse brain extracts, because CDKL5 is predominantly expressed in neurons and its mutation causes severe neuronal dysfunctions. Since the brain extract contains many protein kinases and their substrates, the crude extract itself will confound to screen the protein substrates for CDKL5. When the heat-treated brain extract (60 °C for 30 min) was incubated with CDKL5(1-352), a constitutively active form of CDKL5 [13], in the presence of [γ-^{32}P]ATP, no radioactive band was observed by autoradiography (data not shown). The lack of detection of CDKL5 substrates could be due to the substrate too diluted in this preparation.

To gain more sensitivity to detect the substrates, we fractionated the mouse brain extract into 10 fractions by employing MicroRotofor (Fig. 1(A)), a liquid-phase isoelectric focusing, and carried out the CDKL5 *in vitro* kinase assays. When the fractionated samples were incubated with CDKL5(1-352) in the presence of [γ-^{32}P]ATP, followed by autoradiography, one major phospho-protein band with an apparent molecular mass of 120 kDa on SDS–PAGE was detected (Fig. 1(B)). The most significantly phosphorylated protein by CDKL5(1-352) was detected in Fraction 2, in which acidic proteins are supposed to migrate. A faint protein band corresponding to the position of the phosphorylated protein band was observed in a silver-stained gel (Fig. 1(A), shown by rectangle box). Since there were only few protein bands around this position, the corresponding protein band was excised, digested in gel by trypsin and subjected to LC-MS/MS analysis. Three possible candidates, MAP2, drebrin A and Amph1, were predicted by the analysis of MS/MS data with Mascot software (Table 1). All these three proteins are acidic proteins with pI values between 4.2 and 4.7, consistent with pH of Fraction 2. Among the candidates, intact molecular mass of MAP2 is almost 200 kDa, suggesting that proteolytic fragment(s) of MAP2 was included in this preparation.

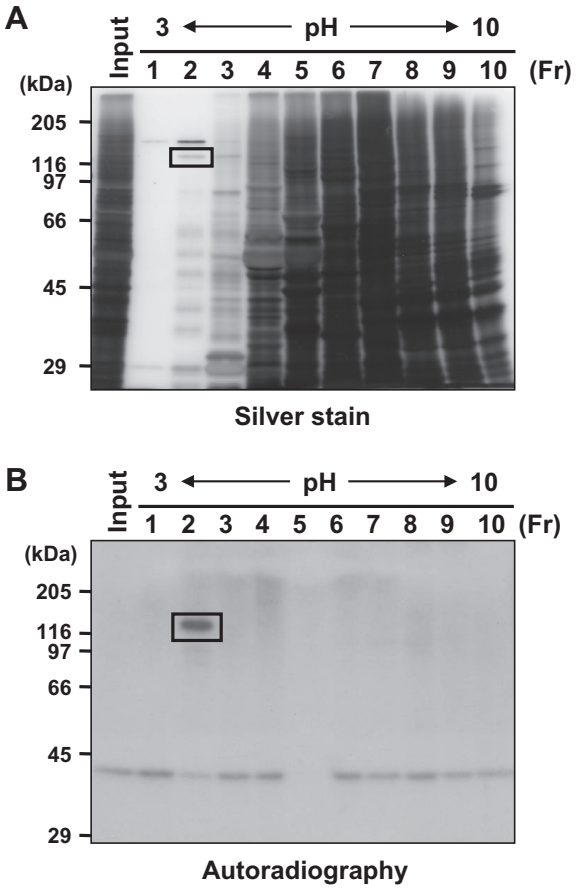


Fig. 1. Detection of endogenous substrates for CDKL5 from the fractionated mouse brain extract. (A) Crude extract (2.5 mg) from mouse brain was applied to MicroRotofor and separated into 10 fractions by a pH gradient (pH 3–10). Each fraction (5 μl) was applied to SDS–PAGE and protein bands were visualized by silver staining. (B) An aliquot of each fraction (5 μl) was subjected to the kinase assay with CDKL5(1-352) (100 ng) and 100 μM [γ-^{32}P]ATP. After incubation at 30 °C for 60 min, the reaction was stopped by adding an equal volume of 2× SDS sample buffer, applied to SDS–PAGE and radioactive protein bands were detected by autoradiography. The protein band indicated by the rectangle box in Fraction 2 was excised from the silver-stained gel and subjected to LC-MS/MS analysis.

Table 1
List of possible substrates for CDKL5 obtained from MicroRotofor fraction 2.

Protein name	NCBI ID	Mass (Da)	pI	Mascot score
MAP2	gi|187466393	198904	4.66	1159
Drebrin A	gi|6694227	74967	4.24	641
Amphiphysin1	gi|41281852	72243	4.40	598

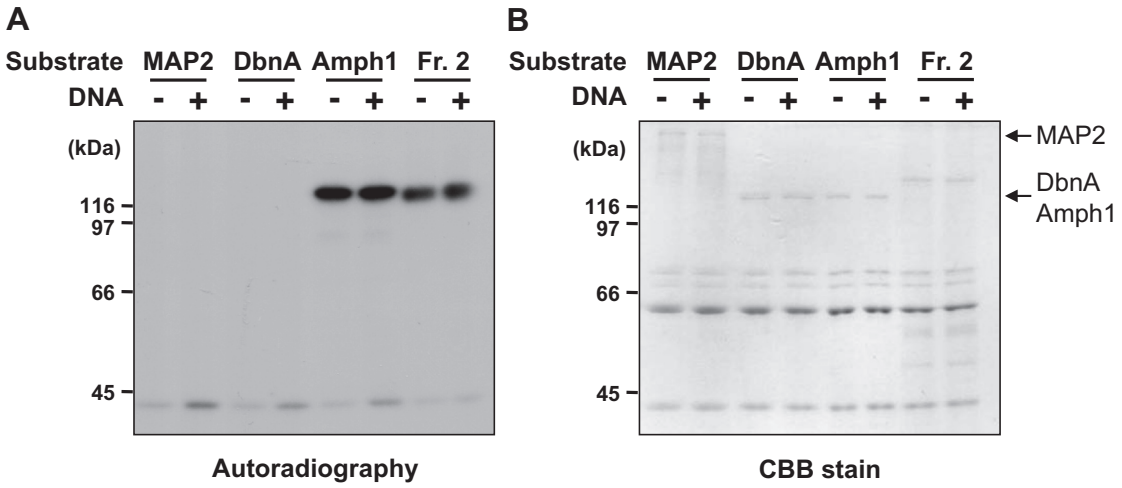
To examine which protein serves as an efficient substrate for CDKL5, we tried to produce all three proteins by *E. coli* expression systems. While Amph1 and drebrin A were successfully expressed in *E. coli*, expression of MAP2 in *E. coli* was totally unsuccessful. Therefore, MAP2 was purified from rat brain. Purified recombinant proteins were subjected to the kinase assays with CDKL5(1-352) in comparison with the phosphorylated protein in Fraction 2. As shown in Fig. 2(A), Amph1 was found to be efficiently phosphorylated by CDKL5(1-352) either in the presence or absence of DNA. In contrast, MAP2 and drebrin A were not phosphorylated by CDKL5(1-352) at all. The entire amino acid sequence of Amph1 and identified peptides by LC-MS/MS analysis were shown in Fig. 2(C). Although the molecular size of phosphorylated band (120 kDa) on SDS–PAGE is considerably higher than the predicted size of Amph1 (72243 Da), phosphorylated band exactly coincided with the band observed by phosphorylation of Fraction 2 with CDKL5(1-352). These results implicate that Amph1 migrated as 120-kDa protein exhibiting higher molecular size than predicted from its molecular mass. These results, taken together, indicate that an efficient substrate for CDKL5 found in the MicroRotofor fraction (Fraction 2) was identified as Amph1.

Next, we compared the efficiency of phosphorylation of recombinant Amph1 to that of other *in vitro* substrates reported previously. In our previous study, we showed that MeCP2 and Dnmt1 were phosphorylated by CDKL5 especially in the presence of DNA [13]. As shown in Fig. 3(A), MeCP2 and Dnmt1 were weakly phosphorylated by CDKL5(1-352) and phosphorylation was enhanced by the addition of DNA, while Amph1 was significantly phosphorylated irrespective of the presence of DNA. Furthermore, the phosphorylation rate of Amph1 was much more significant than MeCP2 and Dnmt1 (Fig. 3(A)). Phosphate incorporation into Amph1 was estimated to be approximately 0.4 mol phosphate/mol protein (Fig. 3(C)).

Determination of phosphorylation site(s) of Amph1 by CDKL5

It has already been reported that Amph1 was phosphorylated by some other protein kinases *in vitro* [25–30]. Multiple phosphorylation sites in Amph1 are known to be mainly localized in a region called the proline-rich domain (PRD[10]) as illustrated in Fig 4(A). Therefore, in the next experiment, we examined the phosphorylation

[10] proline-rich domain.



C

Amphiphysin1

1 MADIKTGIFAKNVQKRLNRAQEKVLQKLGKADETKDEQFEEYVQNFKRQEAEGTRLQREL

61 RGYLAAIKGMQEASMKLTESLHEVYEPDWYGREDVKMVGEKCDVLWEDFHQKLVDGSLLT

121 LDTYLGQFPDIKNRIAKRSRKLVDYDSARHHLEALQSSKRKDESRISKAEEEFQKAQKVF

181 EEFNVDLQEELPSLWSSRVGFYVNTFKNVSSLEAKFHKEIAVLCHKLYEVMTKLGDQHAD

241 KAFSIQGAPSDSGPLRIAKTPSPPEEPSPLPSPTASPNHTLAPASPAPVRPRSPSQTRKG

301 PPVPPLPKVTPTKELKQENIINFFEDNFVPEINVTTPSQNEVLEVKKEETLLDLDFDPFK

361 PDVAPAGSAAATHSPMSQTLPWDLWTTSTDLVQPASGGSFNDFTQAQDTSLFTMQTDQNM

421 AETEQALPTEPQAEEPPATAAAPTAGLDLGLEMEEPKEEAVIPPATDTGETVETAVPTEG

481 APVEEAEAEKAALPAGEGGSPEGAKIDGESTELAISESPQPVEPEAGAPQVIPSVVIEPA

541 SNHEGEGEHQETATGTEPREAAEDVAAQGSAGEKQEVATEPTPLDSQATLPASAGAVDAS

601 LSAGDATQELPPGFLYKVETLHDFEAANSDELNLQRGDVVLVVPSDSEADQDAGWLVGVK

661 ESDWLQYRDLATYKGLFPENFTRRLE

Fig. 2. Identification and phosphorylation of an endogenous substrate for CDKL5. (A) The MicroRotofor fraction (Fraction 2; 5 μl), MAP2 (100 ng), drebrin A (100 ng) or Amph1 (100 ng) was incubated in the standard reaction mixture containing CDKL5(1-352) (100 ng) and 100 μM [γ-^{32}P]ATP. The assays were carried out either in the presence (+) or absence (−) of mouse genomic DNA (37 μg/ml). After incubation at 30 °C for 60 min, the reaction was stopped by adding an equal volume of 2× SDS sample buffer, applied to SDS–PAGE and the radioactive protein bands were detected by autoradiography. (B) Protein staining pattern with Coomassie brilliant blue was shown. (C) Amino acid sequence of Amph1 and the identified peptide sequences by LC-MS/MS analysis were indicated by underlines.

site(s) of Amph1 by CDKL5. When phosphorylated Amph1 by CDKL5(1-352) was analyzed by anti-phosphotyrosine anitibody (PY20) and anti-phosphothreonine antibody (42H4), no immunoreactive band was detected (Fig. 4(B)). These results suggest that the phosphorylation by CDKL5 may be attributed to Ser residue(s). To identify the site(s) for phosphorylation, we prepared point mutants of Amph1, whose Ser residues in the PRD (Ser-262, Ser-268, Ser-272, Ser-276, Ser-285 and Ser-293) were replaced by non-phosphorylatable Ala residues (Fig. 4(A)). Wild type and point mutants of Amph1 were expressed in *E. coli* and purified proteins were assessed for the kinase assay with CDKL5(1-352). When Amph1(WT), Amph1(S262A), Amph1(S268A), Amph1(S272A), Amph1(S276A), Amph1(S285A) and Amph1(S293A) were phosphorylated by CDKL5(1-352), no phosphate incorporation into Amph1(S293A) was observed, while Amph1(WT) and other point mutants were equally phosphorylated (Fig. 4(C)). These results clearly demonstrate that phosphorylation of Amph1 by CDKL5 occurred exclusively at Ser-293.

To further confirm the phosphorylation of Amph1 at Ser-293, phosphorylation site-specific antibody was generated. A phosphopeptide corresponding to amino acid residues, Amph1(287–300) with phosphorylated Ser-293 (Fig. 4(A), indicated by underline), was synthesized and used for production of anti-Amph1(pS293) antibody. When Amph1 was phosphorylated with CDKL5(1-352), 120-kDa protein band was detected by anti-Amph1(pS293) antibody, and this band disappeared after phosphatase treatment (Fig. 5(A)). Furthermore, time-dependent phosphorylation at this site by CDKL5 was observed when detected by anti-Amph1(pS293) antibody (Fig. 5(B)). These results also indicate that the phosphorylation site of Amph1 by CDKL5 is Ser-293 in the PRD.

Effect of phosphorylation at Ser-293 of Amph1 on binding to other proteins

Amph1 is a protein involved in clathrin-mediated endocytosis together with endophilin. Since endophilin is known to bind to

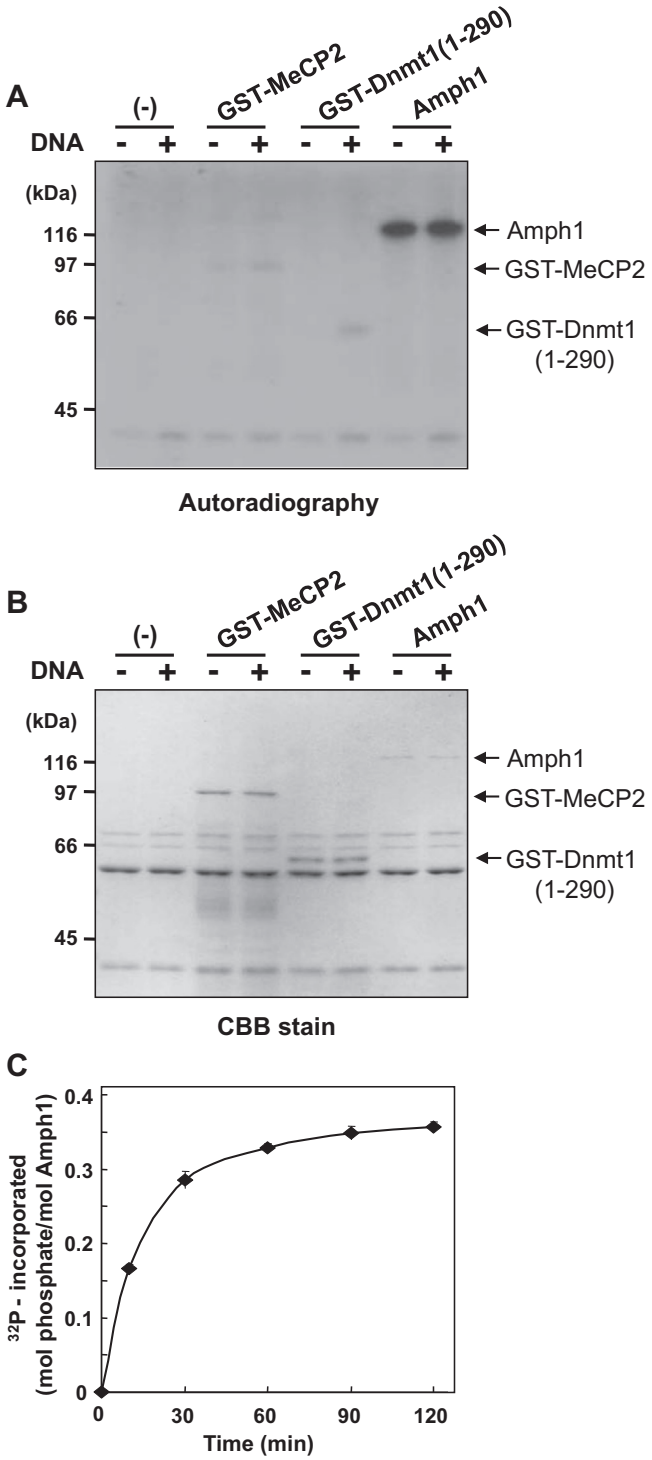


Fig. 3. Phosphorylation of recombinant Amph1, MeCP2 and Dnmt(1-290). (A) GST-MeCP2 (100 ng), GST-Dnmt1(1-290) (100 ng) and Amph1 (25 ng) were phosphorylated in the standard reaction mixture containing CDKL5(1-352) (100 ng) and 100 μM [γ-^{32}P]ATP. Phosphorylation was carried out either in the presence (+) or absence (−) of mouse genomic DNA (37 μg/ml). After incubation at 30 °C for 60 min, the reaction was stopped by adding an equal volume of 2× SDS sample buffer, electrophoresed on SDS–PAGE and analyzed by autoradiography. (B) Protein staining pattern with Coomassie brilliant blue was shown. (C) Time course of phosphate incorporation into Amph1. Three independent experiments were carried out and the data present means ± SD.

Amph1 through PRD region [29], we examined the effect of phosphorylation at Ser-293 on their protein–protein interaction. To clearly observe the effect of single site phosphorylation at Ser-293, phosphorylation mimic mutants whose Ser-293 was replaced by Glu or Asp were generated. When GST-Amph1(WT), Amph1(S293E) or Amph1(S293D) was incubated with endophilin and GST pull-down experiments were carried out, endophilin was coprecipitated with GST-Amph1(WT), but not with GST alone or phosphorylation mimic mutants (Fig. 6(A)). Endophilin was also coprecipitated with GST-Amph1(S293A), another point mutant at Ser-293, as in case of GST-Amph1(WT) (Fig. 6(A)). In contrast to endophilin, Amph2, which is known to form a heterodimer with Amph1 through their N-terminal domain, was pulled down either with Amph1(WT) or with Amph1 mutants, but not with GST alone (Fig. 6(B)). These results suggest that phosphorylation of single site at Ser-293 in the PRD has an inhibitory effect on endophilin binding, but not heterodimerization with Amph2.

Correlation between CDKL5 mutations associated with RTT and Amph1 phosphorylating activity

A number of genetic studies demonstrated that the X-linked *CDKL5* gene is mutated in the patients with RTT-like features [3–9]. Although the mutations are found throughout the coding region of CDKL5, missense mutations are found almost exclusively within the kinase domain [4,5,7,8]. To characterize the functional consequence of the naturally occurring mutations, we introduced point mutations in the catalytic domain of CDKL5 and examined their kinase activities. Among the missense mutations found in the *CDKL5* gene, we chose mutations at Cys-152, Arg-175 and Pro-180 (Fig. 7(A)), since these amino acids are highly conserved across the species and their mutations are known to cause severe neurodevelopmental disorders [4,5,8]. As shown in Fig. 7(B), all of these point mutants of CDKL5(1-352), CDKL5(C152F), CDKL5(R175S) and CDKL5(P180L), showed no phosphorylating activity toward Amph1, MeCP2 and Dnmt1. It is noteworthy, however, CDKL5(R175S) exhibited similar autophosphorylation activity as in case of wild type CDKL5 (Fig. 7(B)). These results showed that pathogenic mutations of CDKL5 resulted in the loss of kinase activity toward its substrates, but not necessarily autophosphorylation, suggesting that the endogenous target of CDKL5, possibly Amph1, mediates the pathogenesis of neurodevelopmental disorders originated from mutation in *CDKL5* gene.

Discussion

CDKL5 is highly expressed in neurons but undetectable in glial cells [3], indicating that this kinase plays important roles in neuronal functions. However, there have been very few reports regarding endogenous substrates for CDKL5 to date. To the best of our knowledge, MeCP2 [6,8] and Dnmt1 [13] are the only substrates shown to be phosphorylated by CDKL5 *in vitro*. Both MeCP2 and Dnmt1 are well-known DNA-binding proteins that may function in the nucleus. It is still unclear whether or not these proteins are direct target of CDKL5 in neurons, since the phosphorylation of these proteins is rather weak as the endogenous substrates. CDKL5 is known to be localized not only in the nucleus but also in the cytoplasm. Cellular localization of CDKL5 was reported to change during the process of development; mainly expressed in the cytoplasm in the early stages and gradually increased in the nucleus thereafter [3]. Therefore, it is important to identify the endogenous substrates for CDKL5 in the early developing brain in relation to neurodevelopmental disorders resulted from mutations in *CDKL5* gene.

Protein kinases such as cAMP-dependent protein kinase, protein kinase C, MAP kinases and CaMKs phosphorylate various proteins including MBP as non-physiological substrates, suggesting that these protein kinases have broad substrate specificities. In contrast,

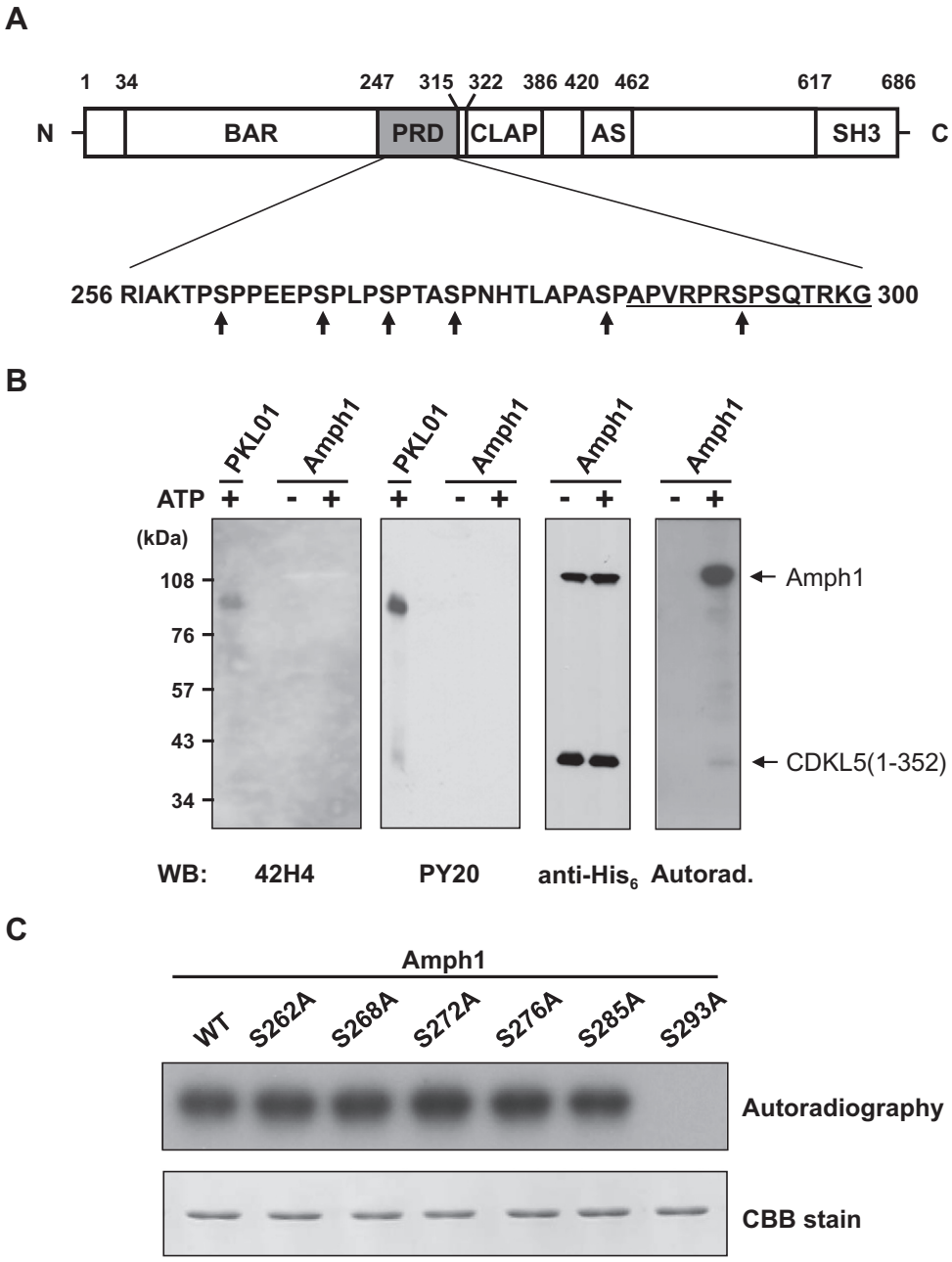


Fig. 4. Determination of phosphorylation site(s) of Amph1 by CDKL5. (A) Schematic illustration of the primary structure of Amph1. Amino acid sequence of the PRD region was shown below. Possible phosphorylation sites by proline-directed protein kinases are indicated by arrows. (B) Amph1 (100 ng) was phosphorylated by CDKL5(1-352) (100 ng) in the standard reaction mixture either in the presence (+) or absence (−) of 100 μM ATP at 30 °C for 60 min. The reaction was stopped by adding an equal volume of 2× SDS sample buffer, electrophoresed on SDS–PAGE and analyzed by Western blotting using anti-phosphothreonine antibody (42H4), anti-phosphotyrosine antibody (PY20) and anti-His_6 antibody. Autoradiogram after phosphorylation with [γ-^{32}P]ATP was also shown. In case of Western blotting with 42H4 and PY20, autophosphorylated GST-PKL01 (50 ng) was used as a positive control. (C) Point mutants of Amph1, whose possible phosphorylation sites were replaced by Ala, were expressed in *E. coli* and purified by HiTrap Chelating column (shown in the lower panel). Amph1(WT) and six Amph1 mutants (250 ng), Amph1(S262A), (S268A), (S272A), (S276A), (S285A) and (S293A), were incubated in the standard reaction mixture containing CDKL5(1-352) (100 ng) and 100 μM [γ-^{32}P]ATP. After 60-min incubation at 30 °C, reaction was stopped by adding an equal volume of 2× SDS sample buffer, electrophoresed on SDS–PAGE and phosphorylated proteins were detected by autoradiography (upper panel). Protein staining pattern by Coomassie brilliant blue was shown in the lower panel.

CDKL5 did not phosphorylate any well-known protein substrates such as histones, casein or MBP, indicating that this kinase has very strict substrate specificity. In the present study, therefore, we attempted to search for the endogenous substrates in mouse brain that can be phosphorylated efficiently by CDKL5. Recently, we developed a unique method to detect the endogenous substrates for protein kinases in crude extracts after fractionating samples by a liquid-phase isoelectric focusing, MicroRotofor [14]. This method is useful because endogenous inhibitors of protein kinases present in the crude extracts could be removed by separation with the isoelectric focusing, and this fractionation made it possible to detect novel substrates that had not been detected previously in the crude preparation. Using this procedure, we identified synapsin II as a possible substrate for doublecortin-like protein kinase [31].

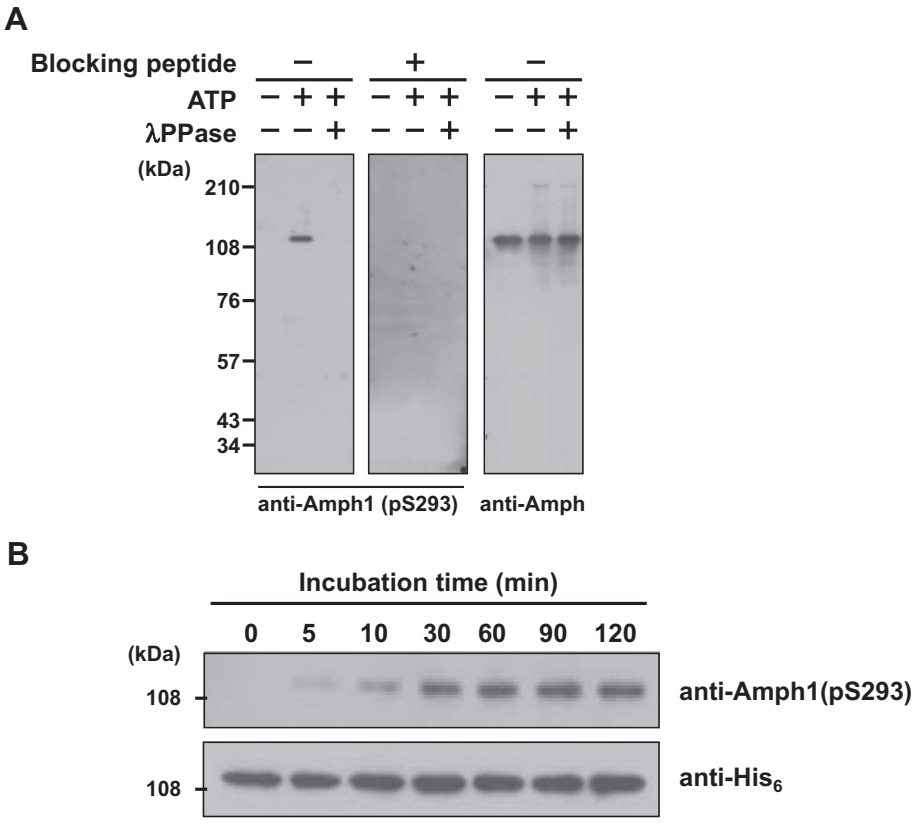


Fig. 5. Detection of phosphorylation at Ser-293 by phosphospecific antibody, anti-Amph1(pS293). (A) Amph1 (2.5 μg) was incubated with CDKL5(1-352) (500 ng) in the reaction mixture (100 μl) in the presence (+) or absence (−) of 100 μM ATP at 30 °C for 60 min. After phosphorylation of Amph1 with ATP, an aliquot of the reaction mixture (20 μl) was transferred to another tube for λPPase (5 ng) treatment in a final volume of 50 μl. The reaction mixture was incubated at 30 °C for another 60 min. Then, the mixtures were electrophoresed on SDS–PAGE and analyzed by Western blotting with anti-Amph1(pS293) antibody or anti-Amph1 antibody. Alternatively, Western blotting was carried out using anti-Amph1(pS293) antibody which had been pre-treated with antigenic phosphopeptide (2.5 mg/ml) for blocking experiment (Blocking peptide (+)). (B) Amph1 (2.5 μg) was incubated in the standard reaction mixture (100 μl) containing CDKL5(1-352) (1 μg) and 100 μM ATP at 30 °C. At the indicated times, an aliquot (10 μl) was withdrawn and stopped the reaction by adding 2× SDS sample buffer. After SDS–PAGE, phosphorylation of Amph1 was analyzed by Western blotting using anti-Amph1(pS293) antibody (upper panel) or anti-His_6 antibody (lower panel).

In the present study, we explored the endogenous substrates for CDKL5 in mouse brain using this method, and found that Amph1 was an efficient and highly specific substrate for CDKL5. Phosphorylation of Amph1 was much more significant than those of MeCP2 and Dmnt1, reported previously. Amph1 is abundantly present in the cytoplasm and plays crucial roles in neuronal functions. CDKL5 is localized not only in the nucleus but also in the cytoplasm, especially highly expressed in cytoplasm in the early developmental stages [3]. Therefore, it is plausible that phosphorylation of Amph1 in the cytoplasm by CDKL5 plays a crucial role during the early stage of neuronal development.

Amph1 is highly expressed in neurons and plays important roles in neuronal transmission and neuronal development through clathrin-mediated endocytosis [32]. Amph1 is also known as a phosphorylated protein *in vivo* and is dephosphorylated by calcineurin upon neuronal depolarization [33,34]. Previous mass spectrometric analysis revealed that Amph1 was phosphorylated at multiple phosphorylation sites; Ser-250, 252, 262, 268, 272, 276, 285, 293, 496, 514, 539, 626 and Thr-310 [25]. Among these sites, Thr-310 and Ser-293 are the two major phosphorylation sites and responsive sites for depolarization [25]. Up to date, four protein kinases have been reported to phosphorylate Amph1 [26–30]. MAP kinase phosphorylated Ser-285 and Ser-293 [26] and Cdk5 phosphorylated Ser-272, 276, 285 and some additional sites [27,28]. Dyrk1A phosphorylated Ser-293, 295 and Thr-310 in the PRD, but the most major site being Ser-293 Amph1(WT) [29]. CK2 phosphorylated Thr-350, 387 and some other unidentified sites, and this phosphorylation reduced the binding affinity of Amph1 to clathrin [30]. Among these kinases, MAP kinase, Cdk5 and Dyrk1A are proline-directed kinases and these kinases phosphorylated multiple Ser and Thr residues in the PRD. Here, we demonstrated that CDKL5, possessing very strict substrate specificity, phosphorylated Amph1 exclusively at Ser-293. The PRD region in Amph1 is known to be involved in the interaction with endophilin, and phosphorylation of Ser/Thr residues in the PRD caused reduction in the affinity of Amph1 to endophilin [29]. In the present study, to examine the effect of single phosphorylation at Ser-293, we prepared Amph1 mutants, Amph1(S293E) and Amph1(S293D), mimicking constitutive phosphorylation. By pull-down experiments, these phospho-mimic mutants of Amph1 exhibited significantly reduced binding affinity to endophilin as compared to wild type and S293A mutant of Amph1. These results suggest that phosphorylation at Ser-293 alone reduced the affinity of Amph1 for the interacting proteins such as endophilin with the PRD region and may influence the function of Amph1. However, correlation between phosphorylation of Amph1 at Ser-293 by CDKL5 and phosphorylation of other sites in the PRD by other protein kinases still remains to be elucidated.

Some mutations in the primary structure of CDKL5 have been reported to be closely associated with the pathogenesis of neurodevelopmental disorders such as X-linked West syndrome and RTT-like syndrome [3–9]. These mutations were found in all over the coding region of CDKL5, but missense mutations were found almost exclusively within the kinase domain [4,5,7,8]. CDKL5 is

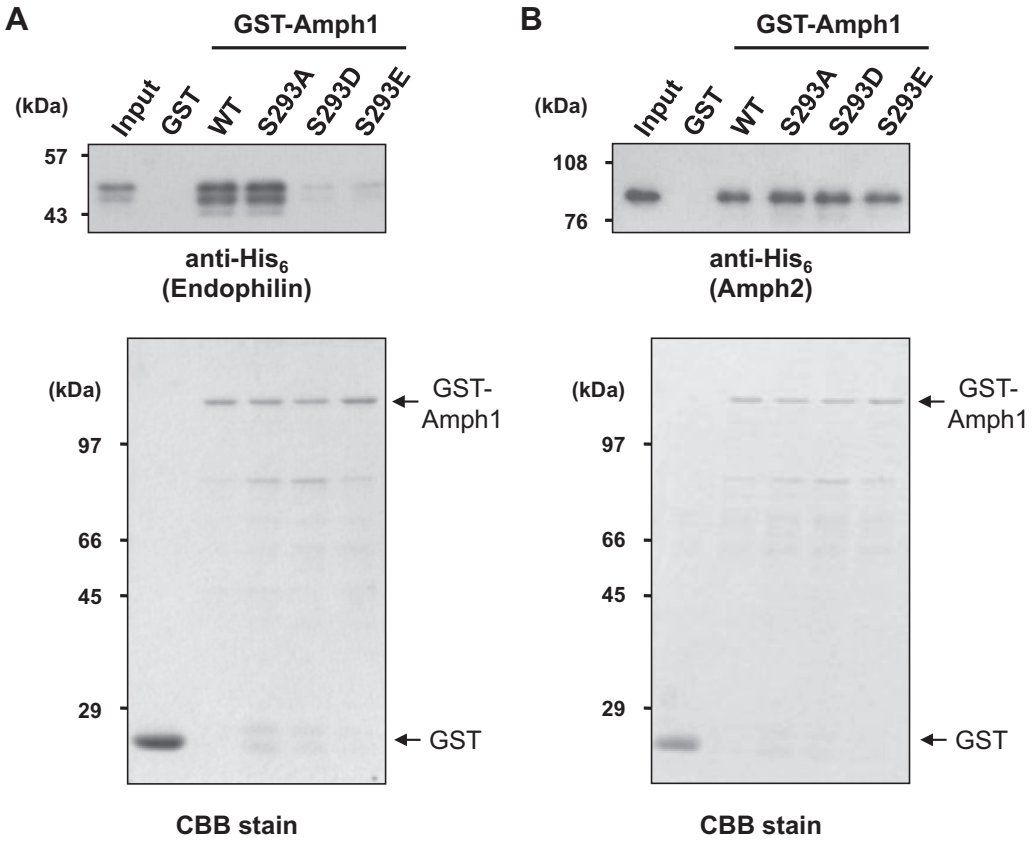


Fig. 6. Comparison of binding affinity of wild type and phosphorylation mimic mutants of Amph1 to endophilin and Amph2. GST, GST-Amph1, GST-Amph1(S293D) or GST-Amph1(S293E) (10 μg each) was incubated with glutathione Sepharose 4B (20 μl) at 4 °C for 2 h. The glutathione Sepharose was washed extensively with washing buffer, and then incubated with endophilin (10 μg) (A) or Amph2 (10 μg) (B) at 4 °C for 2 h. After the glutathione Sepharose was washed 5 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 on SDS–PAGE and analyzed by Western blotting with anti-His_6 antibody (upper panel) or protein staining with Coomassie brilliant blue (lower panel).

known to shuttle between the nucleus and the cytoplasm during neuronal development. Therefore, it is rational to speculate that a timely cellular translocation of CDKL5 and an appropriate targeting of the physiological substrate are important for normal development of the neuronal cells. To investigate the consequence of CDKL5 mutations that are found within the catalytic domain and that are related to the patients with X-linked neurodevelopmental disorders, we produced three mutants; CDKL5(C152F), CDKL5(R175S) and CDKL5(P180L) [4,5,8]. When the kinase activities of these point mutants of CDKL5 were examined, all of these mutant kinases showed no phosphorylating activity toward Amph1. In contrast to other point mutants, however, CDKL5(R175S) exhibited similar autophosphorylation activity as in case of wild type CDKL5. Amino acid residue Arg-175 lies within kinase subdomain VIII, which is known to play an important role in substrate recognition [35]. Exchange of a positively charged Arg with an uncharged Ser would likely influence substrate specificity, thereby producing an inactive kinase toward specific substrates. These results, taken together, suggest that phosphorylation of endogenous substrate by CDKL5, but not autophosphorylation of the kinase itself, is essential for normal development of neurons in the central nervous systems.

Recent finding from the CDKL5 knockout mice demonstrate the important role of CDKL5 in the neuronal functions [36]. However, physiological significance and molecular properties of this kinase remained largely uncharacterized. Previously, Chen et al. reported that CDKL5 colocalized and formed a complex with Rac1, a critical regulator of cytoskeletal reorganization and neuronal morphogenesis [37]. Furthermore, interaction of these proteins and subsequent formation of the protein complex were stimulated by growth factors such as brain-derived neurotrophic factor (BDNF). Additionally they reported that the activity of Rac1 was regulated by clathrin-mediated endocytosis [38]. On the other hand, reduced expression of BDNF after birth is thought to contribute to pathogenesis of RTT patients [39,40]. Therefore, concerning the Rac1 signaling pathway downstream of BDNF, it is important to identify a direct target of CDKL5 and clarify the effect of its phosphorylation on Rac1 signaling. Our present study demonstrated that Amph1 isolated from the brain is the most efficient and specific substrate for CDKL5 *in vitro*. Amph1 is a multifunctional adaptor molecule involved in neurotransmission and synaptic vesicle recycling through clathrin-mediated endocytosis [32], which can be potentially influenced by CDKL5 phosphorylation. Additionally, the finding that Amph1 serves as a much more efficient substrate than MeCP2 for CDKL5 suggest the different molecular mechanisms underlying the neurodevelopmental disorders related to *CDKL5* and *MeCP2* mutations. Taken together, Amph1 is a potential substrate for CDKL5, possibly a critical molecular component of pathogenesis underlying CDKL5-related neurodevelopmental disorders.

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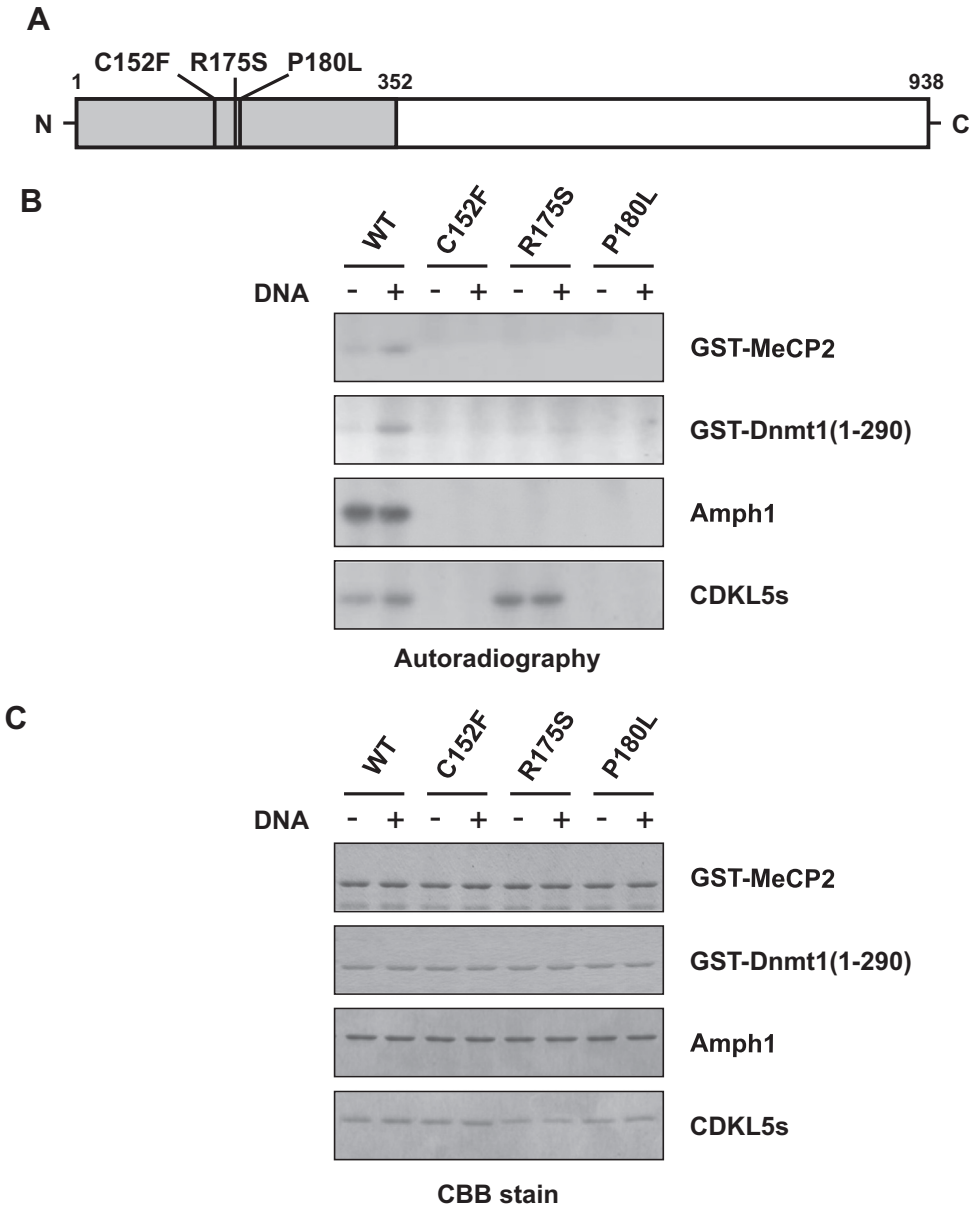

Fig. 7. Catalytic activity of point mutants of CDKL5 found in patients with RTT-like symptoms. (A) Positions of point mutations in CDKL5 which are closely associated with pathogenesis of RTT-like patients. (B) Point mutants of CDKL5(1-352) were produced in *E. coli* and subjected to the kinase assay using the substrate either GST-MeCP2 (400 ng), GST-Dnmt1(1-290) (400 ng) or Amph1 (400 ng) in the reaction mixture containing 100 μM [γ-^{32}P]ATP. The reaction was carried out either in the presence (+) or absence (−) of mouse genomic DNA (37 μg/ml). After incubation at 30 °C for 60 min, the reaction was stopped by adding an equal volume of 2× SDS sample buffer, and the mixtures were resolved on SDS–PAGE and analyzed by autoradiography. Phosphorylation of GST-MeCP2, GST-Dnmt1(1-290) and Amph1 was shown in the upper three panels. At the same time, autophosphorylation of CDKL5(1-352) was also shown in the lowest panel. (C) Protein staining patterns of the substrates and CDKL5(1-352) mutants with Coomassie brilliant blue were shown.

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