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Identification of an endogenous substrate of zebrafish doublecortin-like protein kinase using a highly active truncation mutant*

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*The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank under accession number AB512277 (zebrafish synapsin II)

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Doublecortin-like protein kinase (DCLK), a Ser/Thr protein kinase predominantly expressed in brain and eyes, is believed to play crucial roles in neuronal functions. However, the regulatory mechanisms for DCLK activation and its physiological targets are still unknown. In the present study, we found that a deletion mutant consisting of the catalytic domain of zebrafish DCLK, zDCLK(377–677), exhibited the highest activity among various mutants. Since fully active zDCLK (377–677) showed essentially the same substrate specificity as wild-type zDCLK, we used it to search for physiological substrates of zDCLK. When a zebrafish brain extract was resolved by isoelectric focusing and then phosphorylated by zDCLK(377–677), a highly basic protein with a molecular mass of ~90 kDa was detected. This protein was identified as synapsin II by mass spectrometric analysis. Synapsin II was found to interact with the catalytic domain of zDCLK and was phosphorylated at Ser-9 and Ser-58. When synaptosomes were isolated from zebrafish brain, both synapsin II and zDCLK were found to coexist in this preparation. Furthermore, synapsin II in the synaptosomes was efficiently phosphorylated by zDCLK. These results suggest that zDCLK mediates its neuronal functions through phosphorylation of physiological substrates such as synapsin II.

Keywords: autoinhibitory domain/catalytic domain/doublecortin-like protein kinase/synapsin II/synaptosomes.

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CREB, cyclic AMP-responsive element-binding protein; DCLK, doublecortin-like protein kinase; DCX, doublecortin; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein.

Doublecortin-like protein kinase (DCLK) is a Ser/Thr protein kinase that is specifically expressed in brain and eyes (*1*, *2*). The N-terminal region of DCLK is highly homologous to doublecortin (DCX) and capable of interacting with microtubules. The C-terminal region of DCLK consists of a catalytic domain and shows homology with multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) (*1*, *2*). Mammalian DCX is localized with microtubules and involved in their stability as well as neurite outgrowth (*3*). Mutations of this protein are known to cause a severe disorder of neuronal migration during cerebral cortex development (*4*). DCLK as well as DCX are localized with microtubules, and are involved in tubulin polymerization and microtubule stability (*5–7*). CaMKI, which is one of the multifunctional CaMKs (*8*), shows ~47% identity with the C-terminal region of DCLK, and is known to be activated by an upstream kinase, CaMK kinase (*9–12*), and deactivated by protein phosphatases such as protein phosphatase 2A and CaMK phosphatase (*13*). However, the regulatory mechanisms for the activation of DCLK and the physiological significance of its kinase activity are still unknown.

There are several splice variants of DCLK, which show autophosphorylation activity and phosphorylate myelin basic protein (MBP) *in vitro* (*14*, *15*). The C-terminal region of mouse DCLK (mDCLK) serves as an autoinhibitory domain, and deletion of the C-terminal domain results in a 6-fold stimulation of its kinase activity (*16*). In a previous study, we cloned zebrafish DCLK (zDCLK) and demonstrated that it is regulated by both autophosphorylation and phosphorylation by an unknown upstream kinase (*17*). However, in contrast to the CaMK and MAPK cascades that have been studied in detail (*18*, *19*), the molecular mechanisms by which DCLK is regulated remain unclear.

In the present study, we investigated the activation mechanisms of zDCLK, and attempted to search for physiological substrates of zDCLK. First, we prepared various deletion mutants of zDCLK and examined their catalytic properties. The deletion mutant zDCLK(377–677), which was designed on the basis of the cleavage site of the constitutively active 30-kDa fragment of CaMKII (*20*, *21*), showed the highest activity among the mutants. Using this highly active truncation mutant of zDCLK, we identified synapsin II is one of the potential physiological substrates of zDCLK in the zebrafish brain.

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Materials and Methods

Materials

Adenosine triphosphate (ATP), bovine serum albumin, histone type IIA from calf thymus, α-casein from bovine milk and MBP from bovine brain were purchased from Sigma Chemicals. Goat anti-mouse IgG and goat anti-rabbit IgG, conjugated with horseradish peroxidase, were purchased from ICN Pharmaceuticals. An anti-synapsin antibody that could detect both synapsin I and synapsin II was obtained from Synaptic Systems. An anti-phospho-Ser9 synapsin I antibody was purchased from PhosphoSolutions. [γ-^{32}P]ATP (111 TBq/mmol) was obtained from PerkinElmer. Myosin light chain was prepared as described previously (*22*). Recombinant mouse CaMK kinase α (*23*) and rat CaMKIα (*24*) were expressed in *Escherichia coli* and purified as described previously. An anti-zDCLK antibody was prepared as described previously (*17*).

Construction of plasmids

pGEXzDCLK and pGEXzDCLK (377–810) expression vectors for GST-fused zDCLK proteins with a His_6 tag at their C-terminal ends were prepared as described previously (*17*). To generate GST-zDCLK C-terminal truncated mutants [(377–726), (377–706), (377–694), (377–677) and (377–652)], a ΔDC-upstream primer (5′-GGATCCATTCCGACACATCACTCCTCCG-3′) and various 3′-downstream primers [5′-CTCGAGCTGGATAGTTTCCTTATC TAGAGCTG-3′ for zDCLK(377–726); 5′-CTCGAGGCTCTGTTT CTGCACAGAGTTAAA-3′ for zDCLK(377–706); 5′-CTCGAGT TTACCTGCCACCTCCATCTTCA-3′ for zDCLK(377–694); 5′-CTCGAGGACCCAGGGGTGAGAGAGCACA-3′ for zDCLK(377–677); and 5′-CTCGAGCTCTTTAGCAGAATCAG AGATATTG-3′ for zDCLK(377–652)] were used for PCR with pGEXzDCLK as a template. The PCR products were digested with BamHI (underlined) and XhoI (double-underlined), and inserted into the BamHI–XhoI sites of pGEX-4T-1 (GE Healthcare Bio-Sciences). pGEXzDCLK(678–810) was generated by an inverse PCR method (*25*) with a sense primer (5′-ACAGAG GATGCAGCAATGGAAA-3′) and an anti-sense primer (5′-GGAT CCACGCGGAACCAGAT-3′) using pGEXzDCLK as a template.

In the case of zebrafish synapsin II, a sense primer (5′-AGCGGA GATTCCTGGAAAGC-3′) and an anti-sense primer (5′-GGAGAG GTTGAATGTACACGGC-3′) were designed from the outside sequences of the open reading frame. A full-length cDNA was prepared by PCR using these primers and a 3′-RACE ready cDNA library as a template with Pyrobest DNA polymerase (TaKaRa Bio). The PCR product was cloned into pGEM-T Easy, and 12 independent clones (pGEMzSynapsin II-1 to 12) were sequenced. To construct pETzSynapsin II, the following primers were used for PCR with pGEMzSynapsin II-1 as a template: 5′-upstream primer (5′-GCTAGCATGAACTTCCTGCGTCGTC-3′) and 3′-downstream primer (5′-GTCGACGTCAGAGAACAGGCTTG CAAAAG-3′). The NheI (underlined)-SalI (double-underlined) fragment was inserted into the NheI–SalI sites of pET-23a(+) (Novagen), thereby generating the plasmid pETzSynapsin II. To construct pETzSynapsin Ia, the following primers were used for PCR with a 3′-RACE ready cDNA library as a template: 5′-upstream primer (5′-GGATCCATGAATTACCTGCGACGTC GACT-3′) and 3′-downstream primer (5′-CTCGAGTTCCACGGA GAAGAGGCTTGC-3′). The BamHI (underlined)-XhoI (double-underlined) fragment was inserted into the BamHI–XhoI sites of pET-23a(+), thereby generating the plasmid pETzSynapsin Ia. Mutagenesis of Ser-9 and Ser-58 was performed by the inverse PCR method with sense primers (5′-GACAGCAGCTTCATCGCT AATCTG-3′ for S9A; and 5′-CCGGCTCCAGAGCGGCGTCC-3′ for S58A) and anti-sense primers (5′-AGCGAGACGACGACGCA GGAAGTTC-3′ for S9A; 5′-AGATGAAGCCGGAGACCTGTTT TCTGC-3′ for S58A; the mutation sites are underlined) using pETzSynapsin II as a template. The 5′-ends of the PCR fragments were phosphorylated by T4 polynucleotide kinase (Nippon Gene) and self-ligated by T4 DNA ligase (Nippon Gene), and the recombinant plasmids obtained were designated pETzSynapsin II(S9A) and pETzSynapsin II(S58A), respectively. pETzSynapsin II(S9/58A) was generated by an inverse PCR method with a sense primer (5′-CCGGCTCCAGAGCGGCGTCC-3′) and an anti-sense primer (5′-AGATGAAGCCGGAGACCTGTTTTCTGC-3′; the mutation site is underlined) using pETzSynapsin II(S9A) as a template.

For mammalian cells, the cDNAs encoding zDCLK and its mutants were inserted into a pcDNA3.1(+)/*myc*-HisB (Invitrogen)-backbone plasmid with a FLAG epitope (pcFLAG) (*17*). pcFLAGzDCLK was prepared by PCR with primers (5′-AAGCT TGTTATGTCCAGCAGAAACATCGAGT-3′ and 5′-TAACTCG AGTTATTCTTCCTTCAGGAGGTCTTTACT-3′) using pGEXzDCLK as a template. pcFLAGzDCLK(1–376) was prepared by PCR with primers (5′-AAGCTTGTTATGTCCAGCAGAAAC ATCGAGT-3′ and 5′-TAACTCGAGTTACTTGAAGTTGCGCA TACTGCG-3′) using pGEXzDCLK as a template. pcFLAGz DCLK(377–810) and pcFLAGzDCLK(377–810)K449R were prepared by PCR with primers (5′-AAGCTTGTTATGATTCCGAC ACATCACTCCTCC-3′ and 5′-TAACTCGAGTTATTCTTCCTT CAGGAGGTCTTTACT-3′) using pGEXzDCLK(377–810) and pGEXzDCLK(377–810)K449R as templates, respectively. pcFLAGzDCLK(377–677) was prepared by PCR with primers (5′-AAGCTTGTTATGATTCCGACACATCACTCCTCC-3′ and 5′-TAACTCGAGTTAGACCCAGGGGTGAGAGAGCA-3′) using pGEXzDCLK as a template. The HindIII (underlined)-XhoI (double-underlined) fragments were inserted into the HindIII- XhoI sites of pcFLAG. pcFLAGzDCLK(377–677)K449A was prepared by the inverse PCR method with a sense primer (5′-GCTATCATCG ATAAAAAACAAATGCAGAGG-3′; the mutation site is underlined) and an anti-sense primer (5′-CAGTGCAAACTCCTTTCCA GTGG-3′) using pcFLAGzDCLK(377–677) as a template. In the case of pcDNAzSynapsin II, the following primers were used for PCR with pGEMzSynapsin II as a template: 5′-upstream primer (5′-AAAGCTAGCATGAACTTCCTGCGTCGTCGT-3′) and 3′-downstream primer (5′-AAAGCGGCCGCTGTCAGAGAACA GGCTTGCAAAAG-3′). The NheI (underlined)-NotI (double-underlined) fragment was inserted into the NheI–NotI sites of a pcDNA3.1(+)/*myc*-HisB-backbone plasmid with an NheI site.

Expression and purification of recombinant proteins

pGEXzDCLKs were introduced into *E. coli* strain Rosetta (DE3) (Novagen). The transformed bacteria were grown at 37°C to an A_{600} of 1.0, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After incubation for 8 h at 15°C, the bacteria were harvested by centrifugation and suspended in buffer A [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 40]. In the case of CREB, pET-CREB (*17*) was introduced into *E. coli* strain BL21(DE3). The transformed bacteria were grown at 25°C for 24 h, harvested by centrifugation and suspended in buffer A. In the case of zebrafish synapsin I and synapsin II, pETzSynapsin plasmids were introduced into *E. coli* strain Rosetta (DE3). The transformed bacteria were grown at 37°C to an A_{600} of 1.0, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After incubation for 8 h at 18°C, the bacteria were harvested by centrifugation and suspended in buffer A. After sonication, cell debris was removed by centrifugation (20,000*g*) at 4°C for 10 min, and the obtained supernatant was loaded on a HiTrap Chelating HP column (GE Healthcare Bio-Sciences) pre-equilibrated with buffer A. The column was sequentially washed with buffer A, buffer A containing 20 mM imidazole and buffer A containing 50 mM imidazole, before being eluted with buffer A containing 200 mM imidazole. The purified fractions were pooled, dialysed against 20 mM Tris–HCl (pH 7.5) containing 0.05% Tween 40 and 1 mM 2-mercaptoethanol, and used for kinase assays.

Isolation and identification of endogenous substrates of zebrafish DCLK

Brains from adult zebrafish were homogenized by sonication with a tissue lysis buffer [100 mM Tris–HCl (pH 6.8), 100 mM NaCl, 0.2% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml protease inhibitors (antipain, pepstatin, leupeptin and chymostatin)]. The homogenate was centrifuged at 20,000*g* for 20 min, and the supernatant was collected. The protein extract (2.5 mg) was dissolved in 2.5 ml of IEF buffer [5 mM dithiothreitol, 4% (w/v) Chaps, 5% (v/v) glycerol, 2% (v/v) Pharmalyte, pH 3-10], applied to a MicroRotofor (Bio-Rad Laboratories) and electrophoresed according to the manufacturer's instructions for 2.5 h at a constant power of 1 W at 4°C. After the electrophoresis, the protein fractions from each compartment (200 μl) were harvested (*26*). The proteins phosphorylated by zDCLK(377–677) were resolved by SDS-PAGE and

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detected by autoradiography. A 90-kDa protein band corresponding to a phosphorylated protein was excised, digested in-gel with 10 µg/ml trypsin (Promega) and analysed by LC-MS/MS as described previously (*27*).

Cell culture and transfection

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% heat-inactivated fetal calf serum. Cells were grown at 37°C in a humidified incubator under a 5% CO_2/95% air atmosphere. Transfection of COS7 cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, COS7 cells (2×10^5) were plated in 35-mm dishes in 2 ml of DMEM containing 10% fetal calf serum. After 24 h of culture, the cells were transfected by incubation for 24 h in 1 ml of DMEM containing 5% fetal calf serum, 5 µl of Lipofectamine 2000 and 2.5 µg of plasmid DNA.

Immunoprecipitation

Cells were collected at 30 h after transfection and homogenized with IP buffer [10 mM Tris–HCl (pH 8.0), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml protease inhibitors (antipain, pepstatin, leupeptin and chymostatin)]. The homogenates were centrifuged at 20,000*g* for 20 min, and the supernatants were mixed with antibodies (0.5 µg of anti-FLAG or 5 µl of anti-DCLK). After rotation for 1.5 h at 4°C, 20 µl of protein G-Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences) was added to the samples, and incubated for 1.5 h at 4°C. The obtained complexes were washed three times with IP buffer, and eluted with SDS-PAGE sample buffer.

Fractionation of synaptosomes from zebrafish brain

Synaptosomes were prepared from zebrafish brains as described previously (*28*). Briefly, zebrafish brains were washed in buffered sucrose [4 mM HEPES-NaOH (pH 7.4), 320 mM sucrose] and homogenized in the same buffer with 12 up-and-down strokes in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 1,100*g*. The resulting supernatant was centrifuged for 15 min at 9,200*g*, and the pellet was resuspended in the same buffer and centrifuged for 15 min at 10,500*g*. The resulting pellet was resuspended in buffered sucrose, and used as a synaptosome preparation.

SDS-PAGE and western blotting

SDS-PAGE was performed essentially according to the method of Laemmli (*29*) using slab gels consisting of 10% and 12% acrylamide separation gels and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Protran BA85; Schleicher & Schuell) and immunoreactive protein bands were detected as described previously (*30*).

Protein kinase assay

The protein kinase activities of zDCLKs were determined as described previously (*17*). Phosphorylation of proteins was carried out at 30°C in a standard reaction mixture (10 µl) consisting of 40 mM HEPES-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 100 µM [γ-^{32}P]ATP, 1 µg of protein substrate and various amounts of zDCLK or its mutants. After the incubation, the reaction was stopped by the addition of 10 µl of 2× SDS-PAGE sample buffer. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. Phosphorylation of syntide-2 was carried out at 30°C in a final volume of 20 µl in a standard reaction mixture consisting of 40 mM HEPES-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 40 µM syntide-2, 100 µM [γ-^{32}P]ATP and 0.05 µg of zDCLK or its mutants for 10 min. After the incubation, a 10-µl aliquot of each mixture was withdrawn, spotted onto a 2-cm square of phosphocellulose paper P81 (Whatman) and immediately placed in 75 mM phosphoric acid. The [^{32}P]phosphate incorporation into syntide-2 was measured as described previously (*31*).

Other methods

Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard (*32*). 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

Catalytic properties of deletion mutants of zDCLK

The C-terminal regions of various protein kinases have been reported to function as autoinhibitory domains. Consequently, deletion of these regions often results in stimulation of the kinase activity. To examine whether this is also the case for zDCLK, we prepared a series of successive C-terminal truncation mutants (Fig. 1B). The deletion mutant zDCLK(377–677) retained all the highly conserved subdomains specific to the catalytic domains in protein kinases. However, in case of the C-terminal truncation mutant zDCLK(377–652), part of the subdomain XI sequence essential for the kinase activity was deleted (Fig. 1A and B). To examine the catalytic properties of these deletion mutants, recombinant enzymes were expressed in *E. coli* and purified (Fig. 1C, upper panel). When the autophosphorylation activities and kinase activities towards MBP were examined, the protein kinase activity increased depending on the size of the C-terminal region deleted (Fig. 1C). The protein kinase activities of the deletion mutants were quantitatively determined using MBP and syntide-2 as substrates (Fig. 1D and E). In both cases, the kinase activity gradually increased as the C-terminal region was removed, and the highest activity were observed for the deletion mutant zDCLK(377–677), which exhibited 50-fold higher activity compared with zDCLK(377–810) (Fig. 1D and E). In contrast, the deletion mutant zDCLK(377–652), which lacked part of an important sequence in the catalytic domain, did not show any activities toward these two substrates (Fig. 1D and E).

The C-terminal region of zDCLK is an autoinhibitory domain

The fact that deletion of the C-terminal region of zDCLK stimulated its kinase activity suggested the possibility that the C-terminal region may serve as an autoinhibitory domain for the enzyme. To confirm this possibility, we examined the effect of an antibody raised against a C-terminal peptide of 15 amino acids (Fig. 1A, double-underlined region) on the zDCLK activity. When the anti-zDCLK(C-term) antibody was added to zDCLK(377–810), which possessed the C-terminal antigenic sequence, the kinase activity was significantly increased (Fig. 2A, left panel, lane 6). Conversely, addition of the anti-zDCLK(C-term) antibody to zDCLK(377–677), which lacked the antigenic sequence, had no effect on the kinase activity (Fig. 2A, right panel, lane 12). These results suggest that the binding of the anti-zDCLK(C-term) antibody to the C-terminal sequence of zDCLK(377–810) stimulated the kinase activity by dissociating the autoinhibitory domain from the kinase active site.

Next, we examined the effect of the C-terminal polypeptide, which was supposed to contain an autoinhibitory domain, on the zDCLK activity.

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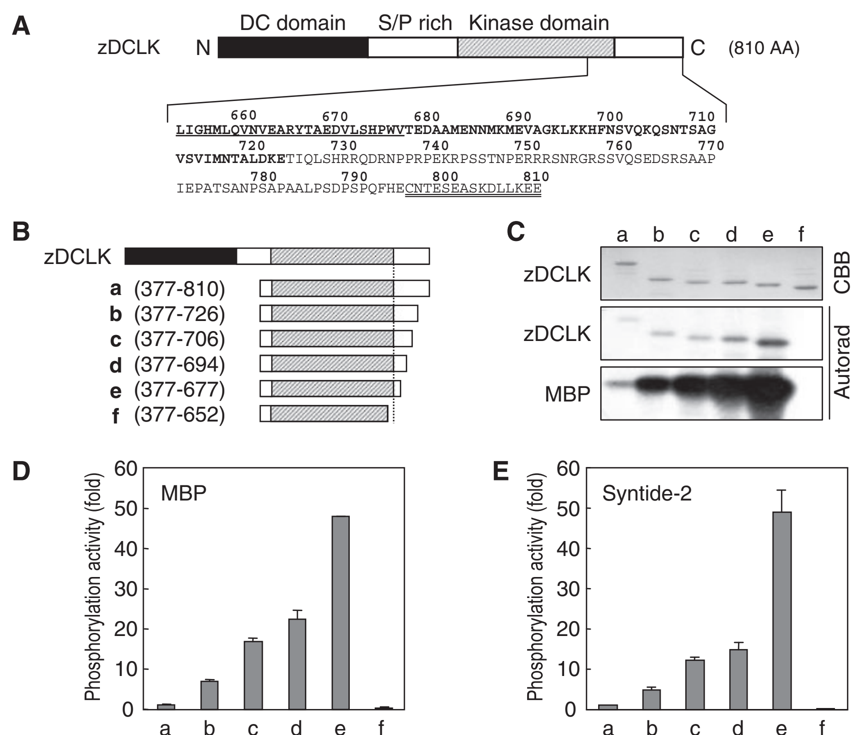

Fig. 1 Protein kinase activities of deletion mutants of zDCLK. (A) Schematic illustration of zDCLK. The black and hatched boxes indicate the doublecortin domain and kinase domain, respectively. The amino acid sequence from positions 652–810 is also shown. The highly conserved region between DCLKs is denoted by bold characters. The kinase domain and peptide used for antibody production are underlined and double-underlined, respectively. (B) Construction of expression vectors for the deletion mutants. zDCLK(377–652) lacks part of the kinase domain. (C) Kinase activities of the deletion mutants. Recombinant zDCLK proteins (0.2 µg) were incubated with (lower panel) or without (middle panel) MBP (1 µg) in a standard phosphorylation mixture (10 µl) containing 100 µM [γ-^{32}P]ATP. After incubation at 30°C for 10 min, the reactions were stopped by the addition of 10 µl of 2 × SDS-PAGE sample buffer. Phosphorylated proteins were resolved by SDS-PAGE using a 12% gel, followed by protein staining (upper panel) or autoradiography (middle and lower panels). (D) Phosphorylation of MBP by various deletion mutants of zDCLK. MBP was phosphorylated by zDCLKs as described for (C), and the radioactivity incorporated into MBP was quantified using a BAS1800 (Fuji Film). Each data point represents the mean ± SE ($n = 3$) of separate experiments. (E) Phosphorylation of syntide-2 by deletion mutants of zDCLK. Syntide-2 (40 µM) was incubated with 0.05 µg of zDCLKs in a standard phosphorylation mixture (20 µl) containing 100 µM [γ-^{32}P]ATP. After incubation at 30°C for 10 min, a 5-µl aliquot of each mixture was withdrawn, spotted onto a 2-cm square of phosphocellulose paper P81 and immediately placed in 75 mM phosphoric acid. The radioactivity incorporated into syntide-2 was quantified using a liquid scintillation counter. Each data point represents the mean ± SE ($n = 3$) of separate experiments. The phosphorylation activities of zDCLK(377–810) against MBP or syntide-2 were arbitrarily set to 1.0.

When GST-zDCLK(678–810) was added to the phosphorylation mixture, the kinase activities of both zDCLK(377–810) and zDCLK(377–677) against MBP were significantly inhibited (Fig. 2B). Unlike GST-zDCLK(678–810), addition of GST alone had no effect on the kinase activity of zDCLK (Fig. 2B). This inhibitory polypeptide, zDCLK(678–810), showed weak inhibition of CaMKI, but had essentially no effect on cAMP-dependent protein kinase (Fig. 2B).

Substrate specificity of C-terminal deletion mutants

The C-terminal deletion mutant zDCLK(377–677), which lacked the autoinhibitory domain, was found to be a highly activated form of zDCLK (Fig. 1). In the next experiment, we examined whether the substrate specificity of zDCLK(377–677) was altered compared with that of wild-type zDCLK. The protein kinase activities of zDCLK(377–677) were compared with those of wild-type zDCLK and zDCLK(377–810) using MBP, myosin light chain, casein, histones and CREB as substrates. The substrate specificities were also compared with those of CaMKI, the kinase domain of which is highly homologous to that of zDCLK. Zebrafish DCLK(377–677) efficiently phosphorylated MBP and histones (Fig. 3A), similar to the case for zDCLK(WT) and zDCLK(377–810) (Fig. 3B). Myosin light chain, casein and CREB served as poor substrates for zDCLK(WT) and the C-terminal deletion mutants, while both myosin light chain and CREB served as good substrates for CaMKI (Fig. 3B). Three recombinant zDCLKs, zDCLK(WT), zDCLK(377–810) and zDCLK(377–677), showed essentially the same substrate specificities, even though their kinase activities were quite different (Fig. 3), suggesting that the substrate specificities of zDCLK may be mainly determined by the catalytic domain.

Search for endogenous substrates for zDCLK using the highly active zDCLK(377-677) as an enzyme source

Since the highly active zDCLK(377–677) showed essentially the same substrate specificity as zDCLK(WT), it can be used as a powerful tool in searches for endogenous substrates for zDCLK. Therefore, we used this mutant to search for endogenous substrates present in a zebrafish brain extract, in

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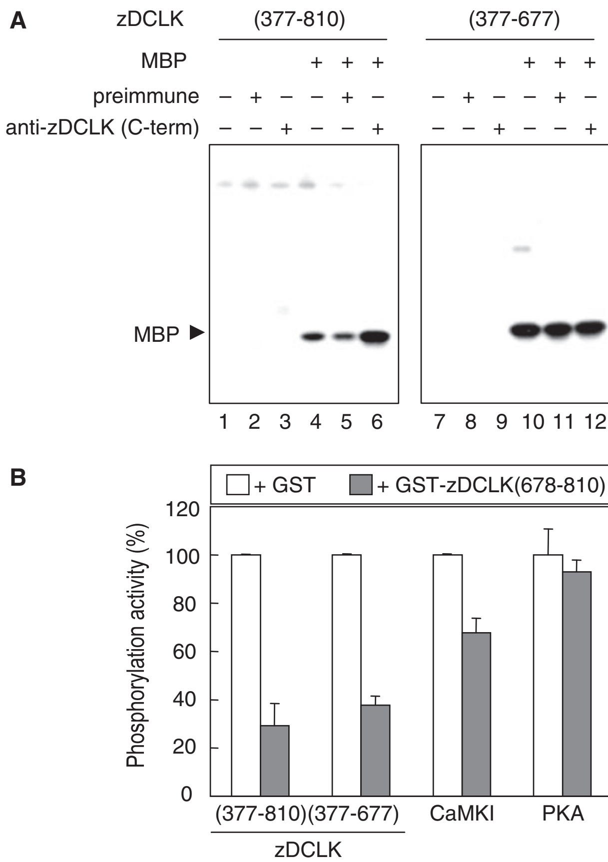

Fig. 2 Inhibitory effect of the C-terminal region on the kinase activity. (A) Effect of the anti-zDCLK antibody on zDCLK activity. Recombinant zDCLKs (0.2 µg) and MBP (1 µg) were incubated with an antibody against the C-terminal peptide (C-term) of zDCLK or preimmune serum in a standard phosphorylation mixture (10 µl) containing 100 µM [γ-^{32}P]ATP. After incubation at 30°C for 10 min, the reactions were stopped by the addition of 10 µl of 2 × SDS-PAGE sample buffer. The phosphorylated proteins were resolved by SDS-PAGE using a 12% gel and detected by autoradiography. (B) Inhibitory effect of the C-terminal polypeptide of zDCLK(678–810) on zDCLKs. Recombinant zDCLKs (0.2 µg) and MBP (1 µg) were incubated with GST (2.2 µg) or GST-zDCLK(678–810) (3.5 µg) in a standard phosphorylation mixture (10 µl) containing 100 µM [γ-^{32}P]ATP. After incubation at 30°C for 10 min, the phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. [^{32}P]phosphate incorporated into MBP was quantified by densitometric scanning using Scion Image and phosphorylation activity in the presence of GST was taken as 100%. Each data point represents the mean ± SE ($n = 3$) of separate experiments.

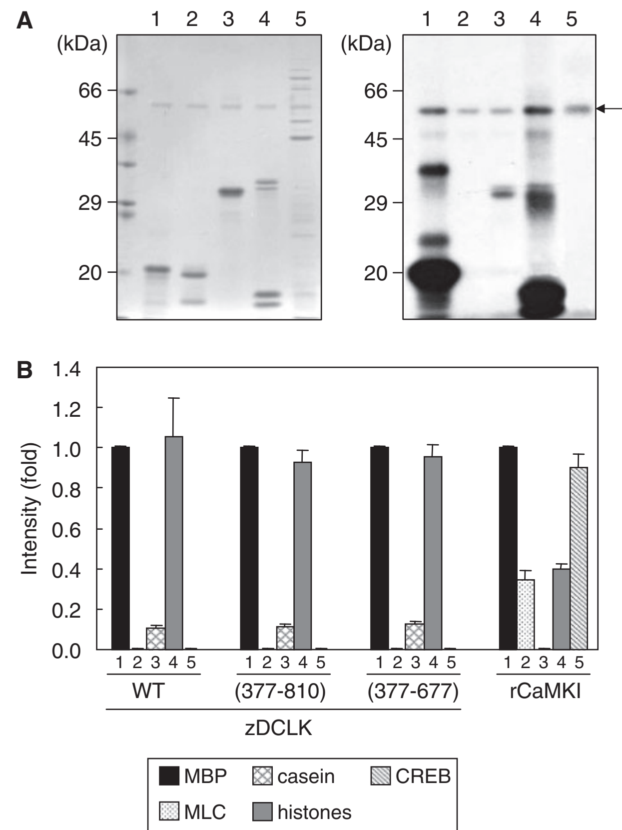

Fig. 3 Substrate specificities of zDCLKs and rat CaMKI. (A) Phosphorylation of various proteins by zDCLK(377–677). Approximately 1 µg of MBP (lane 1), myosin light chain (lane 2), α-casein (lane 3), histones (lane 4) and CREB (lane 5) were incubated with zDCLK(377–677) (0.025 µg) in a standard phosphorylation mixture (10 µl) containing 100 µM [γ-^{32}P]ATP. The phosphorylated proteins were analyzed by SDS-PAGE using 12% gel followed by protein staining with Coomassie brilliant blue (left panel) or autoradiography (right panel). The arrow indicates the autophosphorylated protein bands of zDCLK(377–677). (B) Phosphorylation of various protein substrates by recombinant zDCLKs or CaMKI. Prior to use, rat CaMKI (1 µg) was phosphorylated with CaMK kinase (0.5 µg) in the presence of 1 µM calmodulin and 0.5 mM $CaCl_2$ in a standard phosphorylation mixture (100 µl) containing 100 µM ATP at 30°C for 30 min. MBP, myosin light chain, α-casein, histones and CREB (1 µg each) were incubated with zDCLK(WT) (0.2 µg), zDCLK(377–810) (0.2 µg), zDCLK(377–677) (0.025 µg) or rat CaMKI (0.01 µg) in a standard phosphorylation mixture (10 µl) containing 100 µM [γ-^{32}P]ATP. After incubation at 30°C for 10 min, the reactions were terminated by the addition of 10 µl of 2× SDS-PAGE sample buffer. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. [^{32}P]Phosphate incorporation into the radioactive protein bands was quantified with Scion Image. The phosphorylation activity against MBP was arbitrarily set to 1.0 and the activities against the other proteins were expressed as relative values.

which DCLK is supposed to be abundantly expressed. The zebrafish brain extract was first heat-treated to inactivate endogenous protein kinases, and the resulting protein mixture was used as a substrate preparation for zDCLK(377–677). When the mixture was phosphorylated with zDCLK(377–677) in the presence of [^{32}P]ATP, several radioactive protein bands were observed (Fig. 4A, lane 2). In contrast, no radioactive protein bands were detected in the absence of zDCLK(377–677) (Fig. 4A, lane 3). To further separate the substrate proteins, the zebrafish brain extract was subjected to isoelectric focusing using a MicroRotofor (Fig. 4B). When the protein solutions in the separated fractions were phosphorylated by zDCLK(377–677), phosphorylated protein bands were detected in some fractions in addition to the major bands of autophosphorylated zDCLK(377–677) (Fig. 4B, right panel). In fraction 9, a major phosphorylated protein band of ~90 kDa was also observed as a silver-stained band with the same mobility in a SDS-polyacrylamide gel (Fig. 4B). Therefore, we attempted to identify this basic protein. The 90-kDa protein band was excised from the SDS-polyacrylamide gel, digested with trypsin in-gel and

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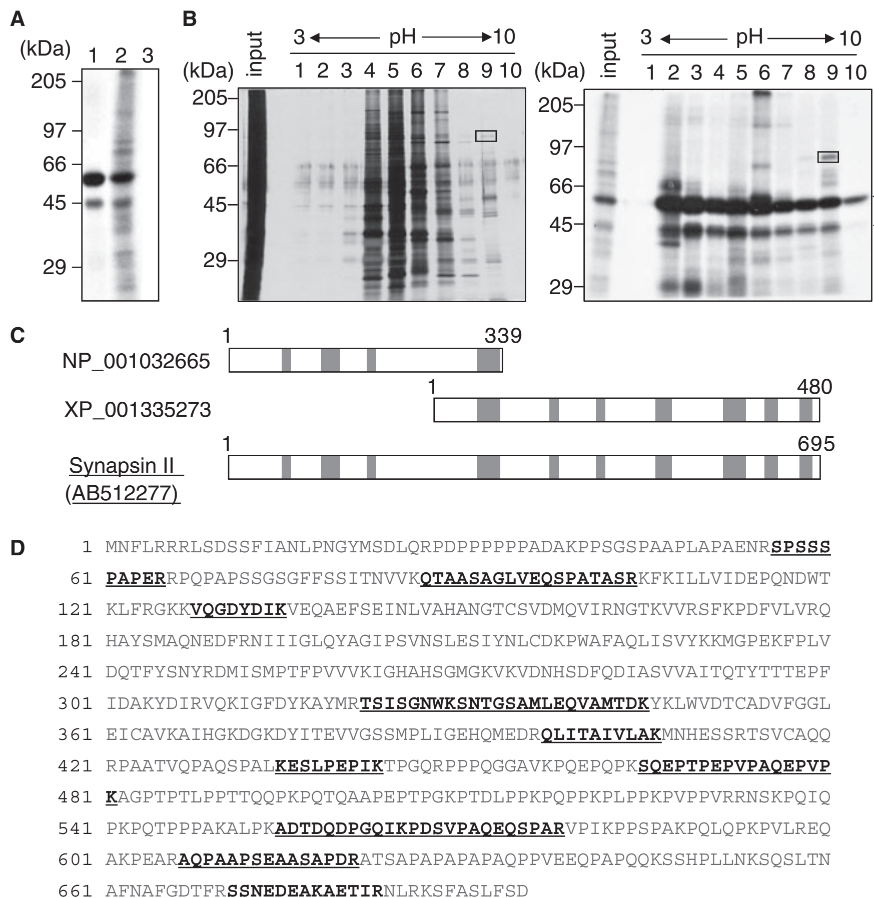

Fig. 4 Identification of an endogenous target for zDCLK. (A) Phosphorylation of a zebrafish brain extract by zDCLK(377–677). A heat-treated extract (40 µg) of adult zebrafish brain was incubated in the presence (lane 2) or absence (lane 3) of zDCLK(377–677) (0.4 µg) in a standard phosphorylation mixture (20 µl) containing 100 µM [γ-^{32}P]ATP at 30°C for 30 min. Zebrafish DCLK(377–677) was also incubated without the brain extract (lane 1). The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. (B) A crude extract (2.5 mg) prepared from adult zebrafish brain was loaded into a MicroRotofor and separated into 10 fractions on the basis of the pI values of the endogenous proteins. Each fraction (5 µl) was separated by SDS-PAGE using a 10% gel and visualized by silver staining (left panel). The resolved proteins in each 5-µl fraction were incubated with zDCLK(377–677) (0.2 µg) in the presence of 100 µM [γ-^{32}P]ATP at 30°C for 30 min. Each sample was separated by SDS-PAGE using a 10% gel and the [^{32}P]phosphate incorporation into the endogenous proteins was detected by autoradiography (right). The significant phosphorylated band indicated by the square was excised and analysed by LC-MS/MS. (C) Schematic illustrations of two proteins, similar to zebrafish synapsin IIb (Accession No. XP_001335273) and hypothetical protein (Accession No. NP_001032665), deduced by the LC-MS/MS analysis, and the zebrafish synapsin II homologue isolated in this study (Accession No. AB512277). The peptide fragments identified by LC-MS/MS are shown by shaded boxes. (D) Amino acid sequence of zebrafish synapsin II (Accession No. AB512277) deduced from the cDNA isolated in this study. The amino acid sequences indicated by the underlines are the peptides determined by the LC-MS/MS analysis.

subjected to LC-MS/MS analysis. As a result, two putative proteins (NP_001032665 and XP_001335273) were obtained from the zebrafish database with high scores. The expected proteins corresponding to these clones were much smaller than 90 kDa, indicating that these clones were not the genes for the 90-kDa protein. However, when the amino acid sequences of these clones were aligned, the N-terminal sequence of XP_001335273 was found to be exactly the same as the C-terminal sequence of NP_001032665 (Fig. 4C). Therefore, we supposed that these two clones were not different genes, but instead may be different parts of the same gene for the 90-kDa protein. On the basis of the sequence data of NP_001032665 and XP_001335273, we prepared primers and obtained a full-length cDNA from a zebrafish cDNA library using a PCR method. The open reading frame of the obtained gene consisted of 2,085 bp and encoded a protein of 695 amino acids with a predicted molecular weight of 75,365. This protein was identified as zebrafish synapsin II on the basis of homology searches in the database. However, since this clone had not yet been registered, the nucleotide sequence of the gene was submitted to the database (Accession No. AB512277). All of the peptide sequences obtained by the LC-MS/MS analysis were detected in the amino acid sequence of this protein (Fig. 4D), indicating that the 90-kDa protein substrate detected in fraction 9 is zebrafish synapsin II.

zDCLK phosphorylates synapsin II

The amino acid sequence of zebrafish synapsin II was aligned with those of mouse synapsin II (Accession No. Q64332), zebrafish synapsin I (Accession No. XP_699270) and mouse synapsin I (Accession No. O88935) (Fig. 5A). Mammalian synapsins are known

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Fig. 5 Phosphorylation of zebrafish synapsin II by zDCLK. (A) Alignment of the deduced amino acid sequence of zebrafish synapsin II (Accession No. AB512277) with the amino acid sequences of mouse synapsin II (Accession No. Q64332), zebrafish synapsin I (Accession No. XP_699270) and mouse synapsin I (Accession No. O88935). Identical amino acids are shaded, and the predicted phosphorylation sites by DCLK in zebrafish synapsin II are shown by asterisks. (B) Time courses of the phosphorylation of zebrafish synapsins. Recombinant synapsin Ia (0.5 μg, closed squares) and synapsin II (0.5 μg, open circles) were incubated with zDCLK(377–677) (0.025 μg) in a standard phosphorylation mixture (20 μl) containing 100 μM [γ-^{32}P]ATP at 30°C for the indicated periods of time. Phosphorylated synapsins were subjected to SDS-PAGE using a 10% gel followed by autoradiography. [^{32}P]Phosphate incorporation into the synapsins was quantified using a BAS1800. (C) Synapsin Ia (0.5 μg, lanes 1 and 3) and synapsin II (0.5 μg, lanes 2 and 4) were phosphorylated by zDCLK(377–677) (0.025 μg) in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 100 μM ATP at 30°C for 30 min. The samples were separated by SDS-PAGE using a 10% gel and probed with an anti-synapsin antibody (upper panel) or an anti-phospho-Ser9 (pS9) synapsin I antibody (lower panel). (D) Point mutants of zebrafish synapsin II (0.5 μg) were phosphorylated by zDCLK(377–677) (0.025 μg) in a standard phosphorylation mixture (20 μl) containing 100 μM [γ-^{32}P]ATP at 30°C for 30 min. The phosphorylated proteins were resolved by SDS-PAGE using a 10% gel and detected by autoradiography. The radioactive signals were quantified with Scion Image. Each data point represents the mean ± SE ($n = 3$) of separate experiments.

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to be localized in synaptic terminals and are phosphorylated by different protein kinases (*33–36*). Phosphorylation of synapsins has been reported to be correlated with changes in their localizations and functions (*33–35*). Ser-9 is a conserved phosphorylation site, known as site 1, in synapsin I and synapsin II for cAMP-dependent protein kinase, CaMKI and CaMKIV, and phosphorylation at this site decreases the affinities of these proteins for synaptic vesicles (*33*, *34*). Using synthetic peptide substrates, substrate recognition sequence for rat DCLK was identified as Arg-X-X-Ser/Thr (*16*). There are three possible sites with consensus phosphorylation sequence by DCLK in zebrafish synapsin II; Ser-9, Ser-58, and Thr-165. Among these Ser-9 is highly conserved residue throughout the synapsin family proteins, while others are not (Fig. 5A, asterisks).

To examine whether zDCLK directly phosphorylates synapsin family proteins, we produced zebrafish synapsin I and synapsin II using an *E. coli* expression system and purified the resulting proteins. When synapsin I and synapsin II were incubated with zDCLK(377–677) under phosphorylation conditions, time-dependent phosphate incorporation into both synapsin I and synapsin II was observed. Under the conditions, 0.45 mol phosphate was incorporated into 1 mol of synapsin II (data not shown), and the phosphate incorporation into synapsin I was ~50% lower than that into synapsin II (Fig. 5B). When detected by a phospho-specific antibody against Ser-9, both synapsin I and synapsin II were found to be phosphorylated at Ser-9 (Fig. 5C).

We investigated other phosphorylation sites than Ser-9 in zebrafish synapsin II. When phosphorylated synapsin II by zDCLK(377–677) was analyzed by Western blotting with anti-phospho-Thr antibody, no phospho-Thr was detected (data not shown), suggesting that phosphorylated residues in zebrafish synapsin II are Ser but not Thr. Therefore, we prepared point mutants in which Ser-9 or Ser-58 was replaced with Ala. When these point mutants were phosphorylated with zDCLK(377–677), phosphate incorporation into S9A and S58A were decreased to 60% and 31%, respectively, of the incorporation into wild-type synapsin II (Fig. 5D). Furthermore, phosphate incorporation into the double-mutant S9/58A was reduced to <15% of the incorporation into wild-type synapsin II (Fig. 5D), suggesting that the major phosphorylation sites in zebrafish synapsin II for zDCLK was Ser-9 and Ser-58. Phosphorylation at these two sites by zDCLK was also confirmed by LC-MS/MS analysis (data not shown).

Synapsin II can bind to the catalytic domain of zDCLK

To investigate the interaction between synapsin II and zDCLK, we carried out immunoprecipitation experiments using various deletion mutants of zDCLK (Fig. 6A). The various FLAG-zDCLK mutants and synapsin II-Myc were cotransfected into COS7 cells, and immunoprecipitated with an anti-FLAG antibody. Zebrafish synapsin II was coprecipitated with zDCLK(WT), zDCLK(377–810) and zDCLK(377–677), but was especially strongly bound

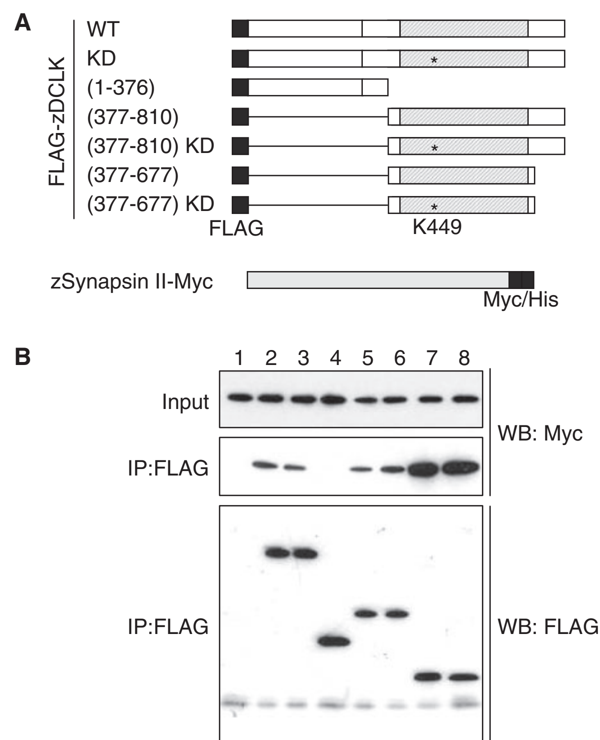

Fig. 6 Analysis of the interactions between synapsin II and deletion mutants of zDCLK by coimmunoprecipitation. (A) Schematic representations of the deletion mutants of zDCLK and synapsin II constructs used in this experiment. The asterisk indicates the site of mutation in the kinase-dead (KD) zDCLK. (B) Interactions between synapsin II and various mutants of zDCLK. COS7 cells were transfected with synapsin II and empty vector (lane 1) or various zDCLK constructs: zDCLK(WT) (lane 2), zDCLK(KD) (lane 3), zDCLK(1–376) (lane 4), zDCLK(377–810) (lane 5), zDCLK(377–810/KD) (lane 6), zDCLK(377–677) (lane 7) and zDCLK(377–677/KD) (lane 8). Immunoprecipitation was performed using an anti-FLAG antibody and the coimmunoprecipitated synapsin II was detected by western blotting with an anti-Myc antibody.

to zDCLK(377–677) (Fig. 6B, middle panel). Coprecipitation was also observed when kinase-dead mutants of zDCLK were used, indicating that the protein kinase activity was not necessary for binding to synapsin II. Taken together, these results suggest that zebrafish synapsin II can bind to zDCLK through its kinase domain.

zDCLK colocalizes with synapsin II in synaptosomes

Synapsin family proteins are localized on the synaptic vesicle membrane (*37*, *38*) and are correlated with neurotransmitter release from presynaptic terminals (*34*). Although zDCLK is known as a microtubule-binding protein, it is not clear whether zDCLK has certain functions within nerve terminals. Therefore, we prepared crude synaptosomes from zebrafish brain by centrifugation and examined whether zDCLK was present in this fraction. As shown in Fig. 7A, zDCLK and synapsin proteins were detected in the crude synaptosome preparation. The synapsin proteins were observed as multiple bands, and the immunoreactive band with the slowest mobility detected by western blotting with an anti-synapsin antibody was found to be synapsin II (Fig. 7A, upper panel, arrow).

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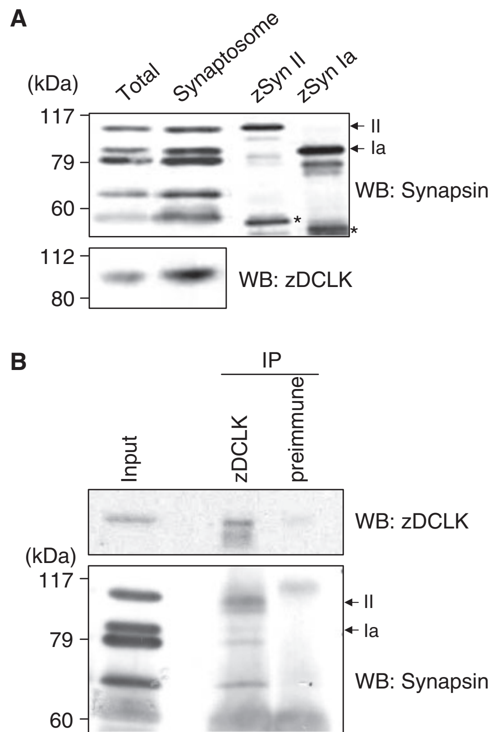

Fig. 7 Coimmunoprecipitation of zDCLK and synapsins from a synaptosome preparation. (A) Fractionation of synaptosomes from zebrafish brain by centrifugation. The total lysate (20 μg), synaptosome fraction (20 μg), recombinant synapsin II and synapsin Ia (0.1 μg) were analysed by western blotting with the indicated antibodies. The asterisks indicate the proteolytic fragments of synapsins. (B) Coimmunoprecipitation of synapsin II with zDCLK. Immunoprecipitation from the synaptosome preparation (200 μg) was performed using an anti-zDCLK antibody or pre-immune serum. Proteins were detected by western blotting with the indicated antibodies. The arrows show the migration positions of synapsin II and synapsin Ia.

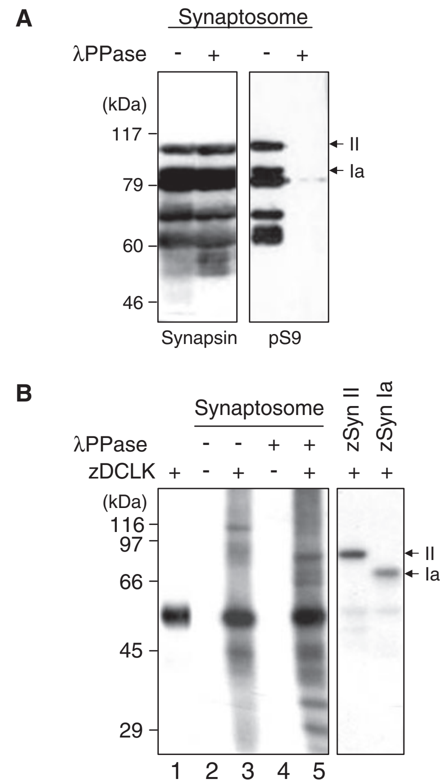

Fig. 8 Phosphorylation of endogenous synapsins by zDCLK. (A) Dephosphorylation of endogenous synapsins by λ-phosphatase (λPPase). Synaptosomes from zebrafish brain (20 μg for the anti-synapsin antibody; 50 μg for the anti-phospho-Ser9 synapsin I antibody) were incubated with or without λ-phosphatase at 30°C for 30 min. The protein samples were then subjected to western blot analysis using the anti-synapsin or anti-phospho-Ser9 synapsin I antibodies. The arrow indicates the migration position of synapsin II estimated from the position of the recombinant protein.
(B) Phosphorylation of endogenous synapsin II by zDCLK. Heat-inactivated synaptosomes (20 μg) which had been pre-treated with or without λ-phosphatase were phosphorylated by zDCLK(377–677) (0.1 μg) in a standard phosphorylation mixture (20 μl) containing 100 μM [γ-^{32}P]ATP at 30°C for 1 h (left). Alternatively, recombinant synapsins (500 ng) were phosphorylated as above (right). The phosphorylated proteins were resolved by SDS-PAGE using a 10% gel and detected by autoradiography. The arrows indicate the migration positions of synapsin II and synapsin Ia.

When crude synaptosomes were subjected to immunoprecipitation experiments using the anti-zDCLK(C-term) antibody, multiple protein bands that were immunoreactive for the anti-synapsin antibody was observed. Among these, the 90-kDa protein corresponding to synapsin II was most efficiently immunoprecipitated (Fig. 7B, lower panel, arrow). These results suggest that zDCLK can bind to multiple synapsin proteins and that, among the synapsin family members, zDCLK binds most strongly to synapsin II.

zDCLK efficiently phosphorylates synapsin II in synaptosomes

Next, the phosphorylation of synapsins in the synaptosomes prepared from zebrafish brain was examined. When the synaptosomes were analysed by western blotting with both an anti-synapsin antibody and a phospho-specific antibody against Ser-9 of synapsin I, all the synapsin family proteins detected was also immunoreactive for the phospho-specific antibody against Ser-9 (Fig. 8A). After extensive treatment of the synaptosomes with λ-phosphatase, the immunoreactive protein bands detected by the phospho-specific antibody against Ser-9 disappeared (Fig. 8A).

Next, we examined the phosphorylation of synapsins using dephosphorylated synaptosomes. Heat-treated synaptosomes did not show any radioactive bands when incubated with [^{32}P]ATP, since the endogenous protein kinases had been inactivated. However, several radioactive bands appeared when this fraction was incubated with zDCLK(377–677) (Fig. 8B, lanes 1 and 3). Furthermore, when zDCLK(377–677) was added to dephosphorylated synaptosomes that had been heat-treated, significant incorporation of [^{32}P]phosphate into the protein bands corresponding to synapsin II and synapsin I was observed (Fig. 8B, lane 5). These results indicate that synapsins are partially phosphorylated in the neuronal cells, and that zebrafish synapsins can serve as potential physiological substrates for zDCLK.

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Discussion

DCLK is a Ser/Thr protein kinase that is specifically expressed in the brain and binds to microtubules through its N-terminal domain. The N-terminal region of DCLK is highly homologous to DCX and involved in the stability of microtubules and migration of neuronal cells, similar to the case for DCX. Defects and mutations in DCLK as well as DCX are known to cause X-linked lissencephaly/double cortex syndrome, a severe developmental disorder of the cerebral cortex (*5–7*). Although DCLK has a protein kinase domain in its C-terminal domain, the physiological functions and endogenous targets of this kinase are still unknown.

To understand the functional roles of DCLK, it is necessary to elucidate the regulatory mechanisms for its kinase activity. CaMKs, which show sequence homology with DCLK, have autoinhibitory domains in their C-terminal regions, and are known to be activated upon binding of Ca^{2+}/calmodulin (*18*). In the case of CaMKII, the C-terminal truncated form (30-kDa CaMKII) exhibits full activity without the addition of Ca^{2+}/calmodulin (*20*). In a previous study, the C-terminal region of mouse DCLK was found to be an autoinhibitory domain, and removal of this domain resulted in the increase in the kinase activity against a peptide substrate (*16*). In the present study, we prepared the C-terminal deletion mutants of zDCLK; zDCLK(377–726), zDCLK(377–706) and zDCLK(377–694), on the basis of the deletion mutants of mouse DCLK reported previously. In addition, we produced another deletion mutant, zDCLK(377–677), whose C-terminal sequence was further deleted to a form like 30-kDa CaMKII. The C-terminal deletion mutant of mouse DCLK which corresponds to zDCLK(377–694) showed 6-fold increase in the kinase activity. On the basis of their results, Shang *et al.* speculated that autoinhibitory effect of the C-terminal domain of mouse DCLK may be due to the presence of pseudosubstrate-like sequence in the C-terminal region (*16*). However, we found that further deletion of the C-terminal sequence of zDCLK, as evidenced by zDCLK(377–677), resulted in 50-fold increase in the kinase activity. These results suggest that inhibitory effect of the C-terminal domain is not solely attributed to the pseudosubstrate-like sequence, but there may be additional inhibitory mechanisms still remain to be clarified.

The autoinhibitory effect of the C-terminal domain of zDCLK was further confirmed by direct inhibition of zDCLK(377–677) by addition of the C-terminal peptide and stimulation of zDCLK by addition of an antibody against the C-terminal peptide. It is noteworthy, however, that the inhibitory effect of the C-terminal domain on zDCLK(377–677) was only effective on the phosphorylation of exogenous substrates and not on its autophosphorylation (Fig. 2). These results suggest the possibility that the C-terminal region interacts with the recognition site for the substrates in the catalytic domain of zDCLK. This possibility was further supported by the fact that synapsin II was coprecipitated with zDCLK(377–677) more efficiently than with zDCLK(377–810) in our immunoprecipitation experiments using COS7 cells. Addition of GST-zDCLK(678–810) weakly inhibited the CaMKI activity, but not that of cAMP-dependent protein kinase. These results may be explained by the structural similarities between DCLK and CaMKI, since CaMKI shows 47% sequence identity with the catalytic domain of zDCLK (*39*).

There are three possible mechanisms for the conversion of DCLK from its inactive form to its active form *in vivo* as follows: (i) an activator binds to the autoinhibitory domain; (ii) a protease cleaves the C-terminal autoinhibitory domain; and (iii) a post-translational modification, such as phosphorylation, releases the autoinhibitory domain from the catalytic domain. In the case of CaMKs, conformational changes are induced by the binding of Ca^{2+}/calmodulin to the autoinhibitory domain, and consequently convert the enzymes to their active forms. However, DCLK is not activated by Ca^{2+}/calmodulin and no other activators have been reported to date. In a previous study, we demonstrated that zDCLK is activated by phosphorylation (*17*), but the precise mechanism of this activation is still unclear. The possibility of proteolytic processing of zDCLK at its C-terminal region and screening for binding proteins to the C-terminal domain of zDCLK are now under investigation in our laboratory.

We found that the highly active C-terminal truncation mutant zDCLK(377–677) exhibited essentially the same substrate specificity as wild-type zDCLK. These observations led us to employ this useful mutant as a tool to search for endogenous substrates of zDCLK. Using this highly active kinase, we found an endogenous protein substrate of zDCLK in the zebrafish brain. The 90-kDa basic protein found in the zebrafish brain was identified as synapsin II by LC-MS/MS analysis. Zebrafish synapsin II expressed in COS7 cells was found to interact with the catalytic domain of zDCLK. Wild-type zDCLK also phosphorylated synapsin II in a much slower rate than zDCLK(377–677). Under the conditions, relative phosphorylation rate of synapsin II to MBP by zDCLK(WT) was not changed from that by zDCLK(377–677) (data not shown). These results are consistent with the contention that the substrate specificity of wild-type zDCLK was highly conserved in the truncated mutant zDCLK(377–677). Taken together, these results indicate that zDCLK interacts with substrate proteins such as synapsin II through its catalytic domain.

Although both synapsin I and synapsin II could be phosphorylated by zDCLK(377–677) *in vitro*, phosphorylation of synapsin I appeared to be much weaker than that of synapsin II. This could be explained by the following reasons: Synapsin II has at least two phosphorylation sites including Ser-9 and Ser-58, while synapsin I does not possess phosphorylation site corresponding to Ser-58 in synapsin II. Therefore, extent of phosphate incorporation into synapsin I appeared to be lower than that of synapsin II (Fig. 5B). Furthermore, synapsin II could be coimmunoprecipitated with zDCLK much more

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efficiently than synapsin I (Fig. 7B). These results suggest that zDCLK binds to synapsin II more strongly and phosphorylates it more efficiently than synapsin I.

Synapsin I and synapsin II are neuronal proteins localized on the membrane of synaptic vesicles (*37*, *38*), and are believed to be correlated with neurotransmitter release (*33–35*). In particular, synapsin I has been reported to be phosphorylated by multiple protein kinases, leading to changes its localization and function in presynaptic terminals (*33*). The major phosphorylation sites in zebrafish synapsin II for zDCLK were identified as Ser-9 and Ser-58 in the present study. Ser-9 is a highly conserved phosphorylation site in synapsin I and synapsin II, and is known as phosphorylation site 1 for cAMP-dependent protein kinase, CaMKI and CaMKIV. Phosphorylation at this site lowers the affinity of synapsin I for synaptic vesicles (*34*). Ser-58 is another phosphorylation site unique to zebrafish synapsin II. In case of mouse synapsin I, Ser-62 is reported to be phosphorylated by MAPK and phosphorylation at this site lowers the binding capacity of synapsin I for actin filaments (*35*, *36*). The truncation mutant zDCLK(377–677) bound to synapsin II and phosphorylated both at Ser-9 and Ser-58. To elucidate physiological significance of kinase activity of DCLK, it is important to know whether or not DCLK really phosphorylates synapsin II *in vivo*. When synapsin II was transfected into COS7 cells, major part of Ser-9 of synapsin II was found to be phosphorylated either by cotransfection with or without zDCLK (data not shown), suggesting that certain protein kinases expressed in cells readily phosphorylated Ser-9 of synapsin II. Although phosphorylation of synapsins by zDCLK *in vivo* is still unclear at this moment, zDCLK has strong potential to phosphorylate synapsin II *in vitro*. In this study, we demonstrated that zDCLK bound to synapsin II efficiently in synaptosomes prepared from zebrafish brain and phosphorylated synapsin II more efficiently than synapsin I. Synapsins were reported to be correlated not only with neurotransmitter release but also with neurite outgrowth or axon transport (*40*, *41*). Furthermore, synapsins and DCLK are known to bind to microtubules, and DCLK stabilizes microtubules and may be correlated with neuronal growth (*5–7*). Therefore, we speculate that DCLK plays some unique roles in regulating neuronal functions through phosphorylation of physiological targets such as synapsin II.

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Conflict of interest

None declared.

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