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Influence of Ser/Pro-rich domain and kinase domain of double cortin-like protein kinase on microtubule-binding activity

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Tadashi Nagamine, Sachiko Shimomura, Noriyuki Sueyoshi and Isamu Kameshita*

Department of Life Sciences, Faculty of Agriculture, Kagawa University, Kagawa 761-0795, Japan

*Isamu Kameshita, Department of Life Sciences, Faculty of Agriculture, Kagawa University, Ikenobe 2393, Miki-cho, Kagawa 761-0795, Japan. Tel: +81 87 891 3120, Fax: +81 87 891 3120, email: kamesita@ag.kagawa-u.ac.jp

Doublecortin-like protein kinase (DCLK) is a Ser/Thr protein kinase predominantly expressed in brain. DCLK is composed of three functional domains; the N-terminal doublecortin-like (DC) domain, the C-terminal kinase domain and Ser/Pro-rich (SP) domain in between DC and kinase domains. Although the DC domain is known to mediate microtubule association, functional roles of the SP domain and the kinase domain on microtubule association is not known. In this study, we investigated the microtubule-binding activity of zebrafish DCLK (zDCLK) using various deletion mutants and chimeric proteins. The microtubule-binding activity of various mutants of zDCLK was assessed both by immunocytochemical analysis and by biochemical analysis using detergent extraction method. When the kinase domain was removed from zDCLK, the microtubule-binding activity was significantly enhanced. Although the zDCLK(DC + SP) mutant showed a strong microtubule-binding activity, the DC domain alone showed much lower microtubule-binding activity, indicating that the SP domain of zDCLK plays a role in enhancing microtubule-binding activity of the DC domain. These results suggest that both the kinase domain and the SP domain are involved in regulating the microtubule-binding activity of DCLK.

Keywords: doublecortin/doublecortin-like protein kinase/microtubule-associated protein/microtubule-binding activity/Ser/Pro-rich domain.

Abbreviations: DCLK, doublecortin-like protein kinase; DCX, doublecortin; SP domain, Ser/Pro-rich domain.

Doublecortin-like protein kinase (DCLK), one of the members of the doublecortin (DCX) family, is a Ser/Thr protein kinase expressed in both adult and embryonic brain (*1*). DCLK and DCX are known to bind to microtubules and promote polymerization/stabilization of microtubules (*2–6*). In human, mutation in the DCX leads to cortical lamination defects in the developing brain (*7*, *8*). In recent years, knockout mice of DCLK and DCX have been created to examine whether DCLK and DCX play a role in the developing brain (*9*, *10*). Although a single knockout mouse of DCLK or DCX does not have obvious cortical migration defects, double knockout mouse for both DCLK and DCX shows perinatal lethality, disorganized neocortical layering and profound hippocampal cytoarchitectural disorganization. These results strongly suggested that DCLK and DCX have genetically compensatory roles in neuronal migration (*9*, *10*).

The primary structure of DCLK is composed of three functional domains. The N-terminal doublecortin-like (DC) domain is highly homologous to DCX and believed to be involved in microtubule association. The middle part of DCLK is a Ser/Pro-rich (SP) domain and the C-terminal region is a Ser/Thr protein kinase domain, which is highly homologous to multifunctional Ca^{2+}/calmodulin-dependent protein kinases. However, functional roles of the SP domain and the kinase domain of DCLK are still unknown. Although microtubule binding of DCX is reported to be negatively regulated by phosphorylation with several protein kinases including cAMP-dependent protein kinase, JNK and Cdk5 (*11–13*), it is still unclear whether microtubule-binding activity of DCLK is regulated by phosphorylation with these protein kinases as in case of DCX. In the present study, we analysed the microtubule-binding activity of various mutants of DCLK to clarify the differences between DCLK and DCX on microtubule binding. In our previous study, we have cloned zebrafish DCLK (zDCLK) and examined whether or not the kinase activity of zDCLK is involved in its microtubule-binding activity using wild-type zDCLK and kinase-dead zDCLK(K449R). In these experiments, we revealed that the kinase activity had no effect on the binding of zDCLK to microtubules (*14*).

In this study, we carried out more detailed analysis concerning the functional roles of the SP domain and the kinase domain in the microtubule-binding activity of zDCLK. Here, we demonstrate the evidence to show that both the SP domain and the kinase domain have crucial roles in regulating the microtubule-binding activity of DCLK.

Materials and Methods

Materials

Cy3-labelled anti-mouse IgG, Cy5-labelled anti-rabbit IgG, anti-FLAG antibody and bovine serum albumin were purchased from Sigma Chemicals. Goat anti-mouse IgG and goat anti-rabbit IgG, conjugated with horseradish peroxidase, were from Pierce Biotechnology. Anti-GFP antibody was obtained from Roche Diagnostics.

For mammalian cells, the cDNA encoding zDCLK (Accession No. AB266175) and its deletion mutants, or mDCX (Accession No. BC056391) were inserted into a pcDNA3.1(+)/myc-HisB (Invitrogen)-backbone plasmid with the FLAG tag (pcFLAG) (*14*) or EGFP tag (pcEGFP). pcEGFP was generated by insertion of EGFP tag into *Nhe*I sites of a pcDNA3.1(+)/myc-HisB-backbone plasmid with an *Nhe*I site. The various mutants of zDCLK and mDCX having a *Hin*dIII site on the 5′-end and an *Xho*I site on the 3′-end were inserted into the pcFLAG or the pcEGFP vector.

Cell culture and transfection

COS7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Wako) containing 10% heat inactivated foetal calf serum. Cells were grown at 37°C in a humidified incubator with a 5% CO_2/95% air atmosphere. Transfection of COS7 cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. COS7 cells were plated at 2×10^5 in a 35-mm dish in 2 ml of DMEM containing 10% foetal calf serum. After 24 h of culture, cells were transfected with 4 μl of Lipofectamine 2000 and 2 μg of plasmid DNA by incubation for 24 h in 0.8 ml of DMEM containing 5% foetal calf serum.

Immunocytochemistry of zDCLKs

Transfected cells were cultured on 0.1 mg/ml poly-L-lysine coated cover glass and treated with cold methanol for 10 min or 10% formalin in phosphate-buffered saline (PBS) for 20 min. After being rinsed with PBS, formalin-fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Alternatively, transfected cells on cover glass were permeabilized first with 0.5% Triton X-100 in MES buffer [50 mM MES-KOH (pH 6.8), 2.5 mM EGTA and 2.5 mM $MgCl_2$] containing 10 μg/ml protease inhibitors (antipain, pepstatin, leupeptin and chymostatin) and 1% phosphatase inhibitor cocktail (EDTA free, Nacalai tesque) for 3 min at room temperature. The permiabilized cells by Triton X-100 were treated with 10% formalin in PBS for 20 min. The cells were washed with PBS, treated with 1% bovine serum albumin in PBS, and then incubated with anti-myc antibody (mouse IgG) diluted 1 : 1000 or anti-tubulin α antibody (rabbit IgG, Epitomics) diluted 1 : 200 (formalin-fixation) or 1 : 25 (methanol-fixation) with 1% bovine serum albumin in PBS at room temperature for 2 h. The cell samples were then incubated with Cy3-labelled anti-mouse IgG or Cy5-labelled anti-rabbit IgG, respectively, at room temperature for 2 h, and observed by a confocal laser-scanning microscope (FV1000-D, OLYMPUS).

Detergent extraction analysis

At 24 h after transfection, adherent cells were washed by PBS and MES buffer and extracted for 3 min at room temperature with 500 μl of MES buffer containing 0.5% Triton X-100, 10 μg/ml protease inhibitors and 1% phosphatase inhibitor cocktail. The supernatant was transferred to 2-ml tubes and centrifuged at 16,000*g* for 2 min and the proteins in the supernatant were precipitated by 1 ml of ethanol at −30°C overnight. Then, centrifuged at 16,000*g* for 10 min and the pellet was resolved in 150 μl of SDS sample buffer and used as a soluble protein fraction. The detergent-insoluble pellet was washed three times with MES buffer containing 10 μg/ml protease inhibitors and 1% phosphatase inhibitor cocktail, and then dissolved in 150 μl of 2× SDS sample buffer (insoluble proteins). Equal volumes of soluble and insoluble samples were subjected to SDS–PAGE and analysed by western blotting.

SDS–PAGE and western blotting

SDS–PAGE was performed on slab gels consisting of a 10% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare) and immunoreactive protein bands were detected essentially according to the method described previously (*15*).

After western blotting experiments, densitometric analysis of each band was performed under appropriate conditions. Quantitative analysis by the Image J software was carried out on the basis of standard curve obtained using recombinant zDCLK of known concentrations. The percentage of proteins in the soluble and insoluble fractions was calculated from three independent experiments.

Other methods

Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard (*16*). 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

Proteolytic processing of zDCLK by endogenous protease

DCLK is thought to be associated with microtubules through its N-terminal DC domain (*1*, *2*), and may play important roles in neuronal migration by controlling microtubule polymerization/stabilization. In earlier studies, cDNA clones for DCLK1 (*1–3*) and DCLK2 (*14*, *17*, *18*) were isolated and characterized. Among them, DCLK1 is known to be cleaved by proteases such as calpain and caspase at the C-terminal side in the SP domain to release the kinase domain from its full length protein (Fig. 1A) (*19*, *20*). However, the cleavage site present in DCLK1 is not conserved in DCLK2, and proteolytic processing of DCLK2 has not been reported so far. In a previous article, we reported that zDCLK, a zebrafish homolog of DCLK2, plays crucial roles in the central nervous systems during the early stages of zebrafish embryogenesis (*14*). To examine whether zDCLK is cleaved by endogenous protease(s), three expression vectors for zDCLK; zDCLK(WT), zDCLK(DC + SP) and zDCLK(Kinase), were constructed (Fig. 1B). When zDCLK(WT) was transiently transfected to COS7 cells, not only a full-length protein (112 kDa) but also an N-terminal fragment (48 kDa) of zDCLK were detected by western blotting using anti-FLAG antibody (Fig. 1C, lane 2). In addition, a C-terminal fragment (65 kDa) was also detected by anti-myc antibody (Fig. 1C, lane 5). The migration positions of the N-terminal and the C-terminal proteolytic fragments were quite similar to those of zDCLK(DC + SP) and zDCLK(Kinase), respectively (Fig. 1C, lanes 3 and 6). These results suggest that zDCLK was cleaved into the N-terminal DC + SP domain and the C-terminal kinase domain by endogenous protease(s) having the cleavage site between the SP domain and the kinase domain (Fig. 1D).

Influence of the kinase domain on microtubule binding

To examine the effect of deletion of the kinase domain on microtubule-binding activity of zDCLK, subcellular localization of zDCLK(WT) and zDCLK(DC + SP) mutant in COS7 cells was observed by indirect immunocytochemistry (Fig. 2A). When the cells were fixed by formalin, zDCLK(WT) was detected not only on microtubules but also in cytosol (Fig. 2A, panel a). On the other hand, zDCLK(DC + SP) mutant was mainly localized on microtubules (Fig. 2A, panel c). Furthermore, the cells expressing zDCLK(DC + SP) showed more tightly bundled microtubules as compared with untransfected cells in the same field (Fig. 2A, panel d, arrowheads). When the cells were permeabilized with Triton X-100 before formalin fixation, localization of zDCLK(WT) showed somewhat

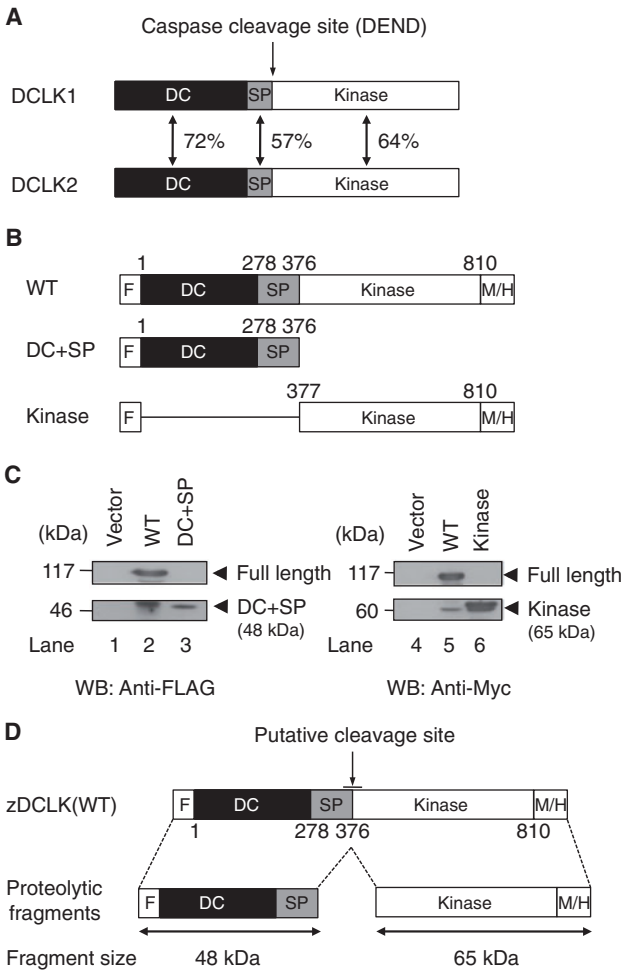

Fig. 1 Proteolytic processing of zDCLK expressed in COS7 cells. (A) Schematic illustration of mouse DCLK1 (Accession No. AF155819) and mouse DCLK2 (Accession No. AB198721). DC domain, SP domain and kinase domain are shown by black, grey and white boxes, respectively. The predicted cleavage site of DCLK1 by caspase is indicated above the figure. Amino acid identities between indicated domains are also shown. (B) Schematic illustration of FLAG/myc/His_6-tagged zDCLK(WT), zDCLK(DC + SP) and zDCLK(Kinase). FLAG tag (F) and myc/His_6 tag (M/H) were fused on the N-terminal and C-terminal ends of zDCLK, respectively. (C) Proteolytic processing of zDCLK by endogenous protease(s) in COS7 cells. Expression vectors (2 μg) for zDCLK(WT), zDCLK(DC + SP) and zDCLK(Kinase) were transfected to COS7 cells by Lipofectamine 2000. After 24 h of culture, the cell lysates (10 μg) were subjected to SDS–PAGE and then analysed by western blotting with anti-FLAG (left panel) and anti-myc (right panel) antibodies. (D) Schematic illustration of zDCLK(WT) and its proteolytic fragments. Putative cleavage site by endogenous protease(s) was deduced from the molecular sizes of proteolytic fragments as shown in panel (C).

different images from those of formalin-fixation without Triton X-100 pretreatment (Fig. 2A, panels a and e). These images may be caused by a loss of cytosolic zDCLK by pretreatment with Triton X-100, while microtubule-associated zDCLK still exist as tightly bound forms. In contrast, clear colocalization of zDCLK(DC + SP) and microtubules was observed in the zDCLK(DC + SP)-expressing cells either with or without Triton X-100 treatment (Fig. 2A, panels c and g). These results implicated that microtubule-binding and microtubule-bundling activity of zDCLK(DC + SP) mutant was enhanced by removal of the kinase domain from zDCLK. When the cells were fixed with methanol, both zDCLK(WT) and zDCLK(DC + SP) mutant appeared to be localized on microtubules (Fig. 2A, panels i and k). Thus, these data suggest that only zDCLK associated with microtubules could be detected when the cells were fixed with methanol, indicating that the soluble proteins had been washed out of the cells during the process of methanol fixation. Therefore, methanol fixation is not suitable for observation of subcellular localization of DCLK and DCX.

To define the intracellular localization of microtubule-associated proteins, detergent extraction assay, a gentle extraction protocol to remove soluble proteins from cytoskeleton framework, has been employed in the previous studies (*6*, *21*, *22*). In this study, we employed this technique to examine interaction between microtubules and various zDCLK mutants. Expression plasmids for zDCLK(WT), zDCLK(DC + SP) and zDCLK(Kinase) were transfected to COS7 cells, and the cells were homogenized with a buffer containing 0.5% Triton X-100 and fractionated into cytosolic (S; soluble) and cytoskeletal (P; insoluble pellet) fractions. In a typical experiment, zDCLK(WT) was detected mainly in insoluble fraction and partly in soluble fraction by anti-myc antibody, which recognizes myc tag on the C-terminal end of zDCLK (Fig. 2B). On the other hand, zDCLK(DC + SP), which is devoid of kinase domain, is almost exclusively detected in an insoluble fraction. The most part of the zDCLK(Kinase) mutant was observed in soluble fraction (Fig. 2B, upper panel), since this mutant lacks DC domain necessary for microtubule association. These results were in good agreement with the subcellular localization observed in the formalin-fixed cells (Fig. 2A, panels a and c) and indicated that deletion of the kinase domain increased the microtubule-binding activity of DCLK. Under the conditions, distribution of tubulin α in the soluble and insoluble fractions was not changed by the expression of DCLK mutants (Fig. 2C). In our previous study, we showed that the kinase activity of zDCLK did not affect the association of zDCLK with microtubules (*14*). Moreover, the kinase activity of zDCLK(Kinase) mutant expressed in *Escherichia coli* was significantly higher than that of zDCLK(WT) (*14*, *18*). Taken together, the present data suggested that proteolysis of zDCLK in the SP domain could enhance both the microtubule-binding activity of the N-terminal DC domain and the catalytic activity of the C-terminal kinase domain.

Comparative study of the microtubule-binding activity of zDCLK and mDCX

Although both DCLK and DCX have been reported to be microtubule-associated proteins, any comparative study concerning microtubule-binding activities and microtubule-stabilities of DCLK and DCX have not been documented to date. Alignment of the primary structures of DCLK and DCX indicated that the DC domains of zDCLK(DC + SP) mutant and mDCX show 71% sequence identity (Fig. 3A). Therefore,

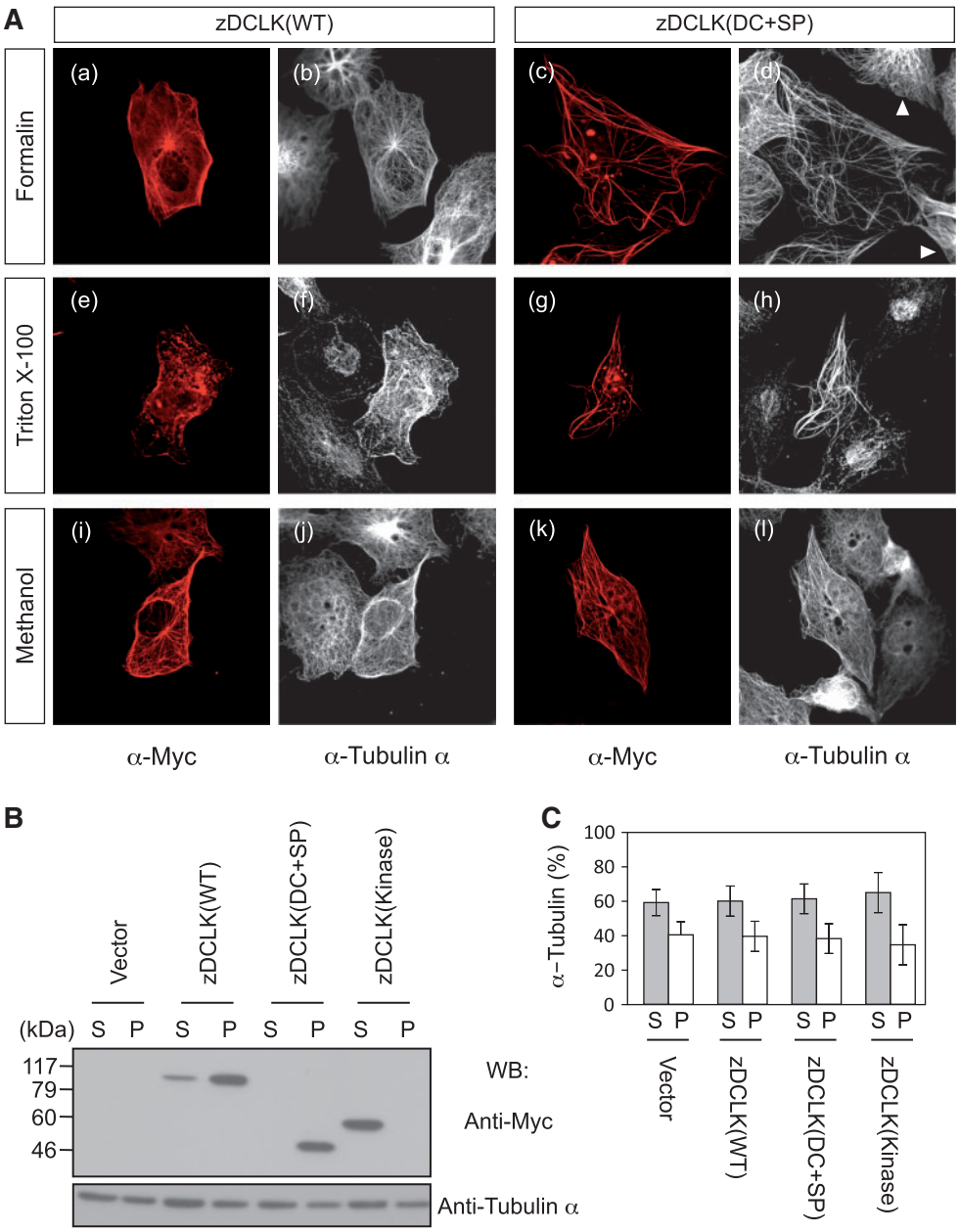

Fig. 2 Influence of the kinase domain on microtubule binding. (A) Subcellular localization of zDCLK(WT) and zDCLK(DC + SP) in COS7 cells. COS7 cells were transfected with 0.5 μg of plasmid DNAs [pcFLAGzDCLK and pcFLAGzDCLK(DC + SP)] using Lipofectamine 2000. After 24 h, cells were fixed by formalin and permeabilized with Triton X-100 (panels a–d) or treated by Triton X-100 before formalin-fixation (panels e–h), or fixed by methanol (panels i–l). Then, the cells were stained by means of indirect immunofluorescence with anti-myc (panels a, c, e, g, i and k) or anti-tubulin α (panels b, d, f, h, j and l) antibodies, and visualized by a confocal microscopy. Arrowheads show the untransfected cells. (B) Microtubule-binding activity of various zDCLKs as examined by detergent extraction analysis of transfected cells. COS7 cells were transfected with 2 μg of plasmid DNAs [pcFLAGzDCLK, pcFLAGzDCLK(DC + SP), pcFLAGzDCLK(Kinase) and an empty vector] and cell extracts were fractionated by detergent extraction method. Soluble (S) and insoluble (P) proteins from 5 μg of cell extract were resolved on SDS–PAGE and detected by western blotting with anti-myc antibody (upper panel). Tubulin α in each fraction was determined by western blotting with anti-tubulin α antibody (lower panel). (C) Tubulin α in Fig. 2B was quantitated by densitometric analysis using the Image J software from three independent experiments.

the microtubule-binding activity and microtubule-stabilities of mDCX were predicted to be comparable to zDCLK(DC + SP) mutant. To compare the microtubule-binding activity and microtubule-stability of zDCLK with that of mDCX, we constructed an expression plasmid for mDCX (Accession No. BC056391) carrying FLAG tag and myc/His_6 tag (Fig. 3A). Unexpectedly, however, immunostaining pattern of mDCX in formalin-fixed COS7 cells showed widespread distribution, which was quite different from that of zDCLK(DC + SP) mutant (Fig. 3B, panel a; Fig. 2A, panel c). When the cells were fixed with methanol, mDCX appeared to be localized on microtubules as in cases of DCLK(WT) and DCLK(DC + SP) (Fig. 3B, panel c; Fig. 2A, panels i and k). To further investigate cellular localization of

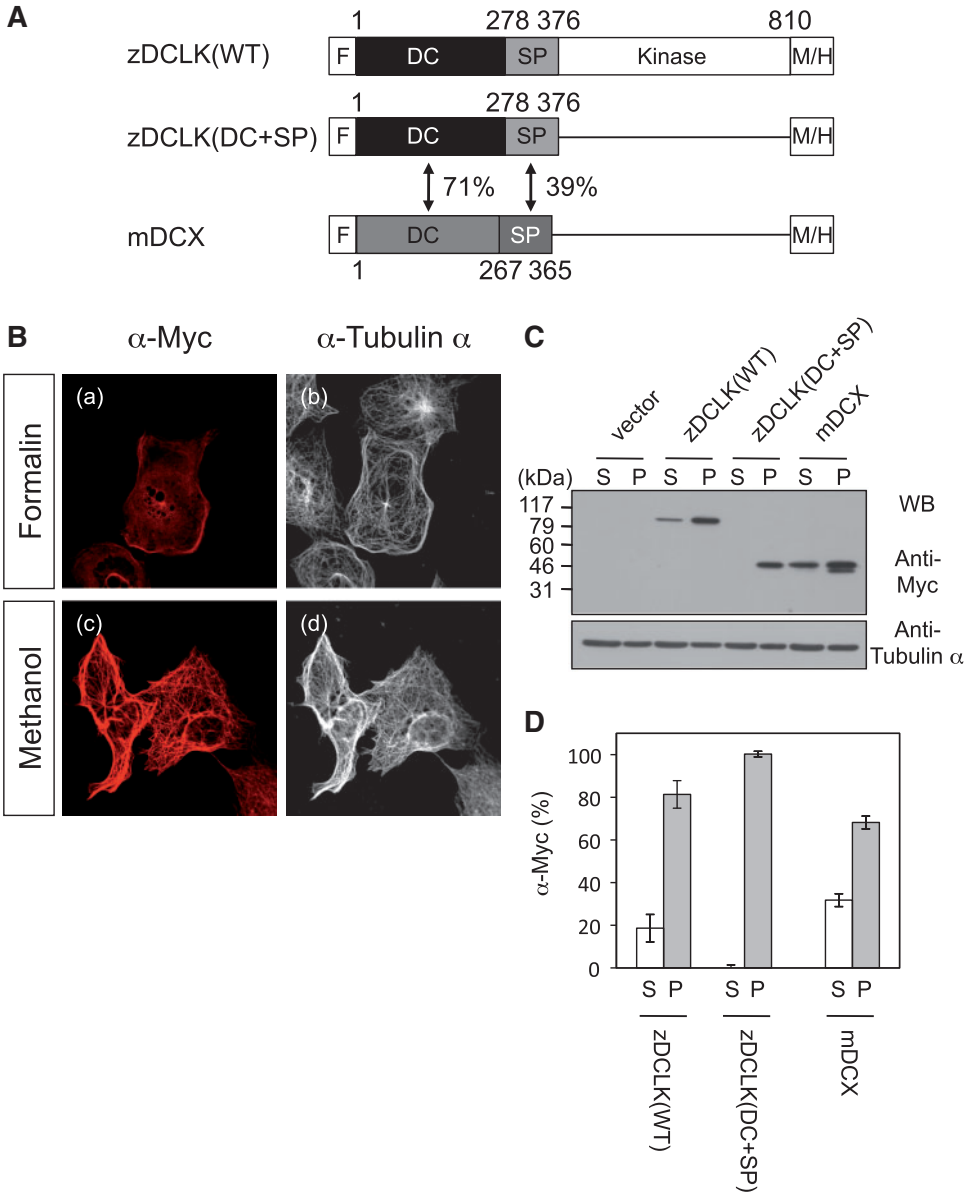

Fig. 3 Microtubule-binding of zDCLK and mDCX. (A) Schematic diagrams of the primary structures of zDCLK(WT), zDCLK(DC + SP) and mDCX. Amino acid identities between the DC domains and SP domains are indicated. FLAG tag (F) and Myc/His_6 tag (M/H) were fused on the N-terminal and C-terminal ends, respectively. (B) Subcellular localization of mDCX in COS7 cells. COS7 cells were transfected with 0.5 μg of pcFLAGmDCX using Lipofectamine 2000. After 24 h, cells were fixed by formalin and permiabilized with Triton X-100 (panels a and b) or fixed by methanol (panels c and d) and stained by means of indirect immunofluorescence with anti-myc (panels a and c) or anti-tubulin α (panels b and d) antibodies, and visualized by a confocal microscopy. (C) Microtubule-binding activity of zDCLK(WT), zDCLK(DC + SP) and mDCX as examined by detergent extraction analysis of transfected cells. COS7 cells were transiently transfected with 2 μg of plasmid DNAs [pcFLAGzDCLK, pcFLAGzDCLK(DC + SP), pcFLAGmDCX and an empty vector] and cell extracts were fractionated by detergent extraction method. Soluble (S) and insoluble (P) proteins from 5 μg of cell extract were analysed as in Fig. 2B. (D) The percentage of zDCLK and mDCX in each fraction was calculated by the Image J software. Each data point represents the mean ± SE ($n = 3$) of separate experiments.

mDCX, we carried out detergent extraction analysis (Fig. 3C). Localization of proteins was analysed by densitometric analysis using the Image J software (Fig. 3D). Although 81 and 100% of zDCLK(WT) and zDCLK(DC + SP), respectively, were found in insoluble fractions, 32% of mDCX was observed in a soluble fraction. Therefore, immunocytochemical observations of formalin-fixed cells as well as detergent extraction analysis suggest that microtubule-binding activity of mDCX was significantly lower than that of zDCLK(DC + SP) mutant.

The SP domain of DCLK stimulates the microtubule association

Although the DC domains of DCLK and DCX are highly conserved (71%), identities of amino acid sequences of the SP domains of DCLK and DCX are relatively low (39%) (Fig. 3A). Therefore, we speculated that the SP domains may play specific roles in regulating microtubule association of the DC domains. To examine the functional roles of the SP domains, we prepared various deletion mutants and chimeric proteins of DCLK and DCX; zDCLK(DC + SP), zDCLK(DC),

mDCX(WT), mDCX(DC), zDCLK(DC) + mDCX(SP) and mDCX(DC) + zDCLK(SP), with FLAG tags on their N-terminal ends (Fig. 4A). These constructs were transfected to COS7 cells, and microtubule-binding activity was assessed by detergent extraction analysis. As shown in Fig. 4B and C, zDCLK(DC) + mDCX(SP) chimeric protein, in which SP domain of zDCLK was replaced with that of mDCX, showed weaker binding activity (60%) as compared to zDCLK(DC + SP) (96%). By contrast, chimeric protein of mDCX(DC) + zDCLK(SP) had a strong microtubule-binding activity (91%) as in case of zDCLK(DC + SP) (96%). The zDCLK(DC) mutant showed somewhat stronger binding activity (85%) as compared to mDCX(DC) (63%). Wild-type DCX, however, showed lower microtubule-binding activity (48%) than that of the mDCX(DC) mutant (63%). These results suggest that the microtubule-binding activity of the DC domain is increased by the SP domain of zDCLK but decreased by the SP domain of mDCX.

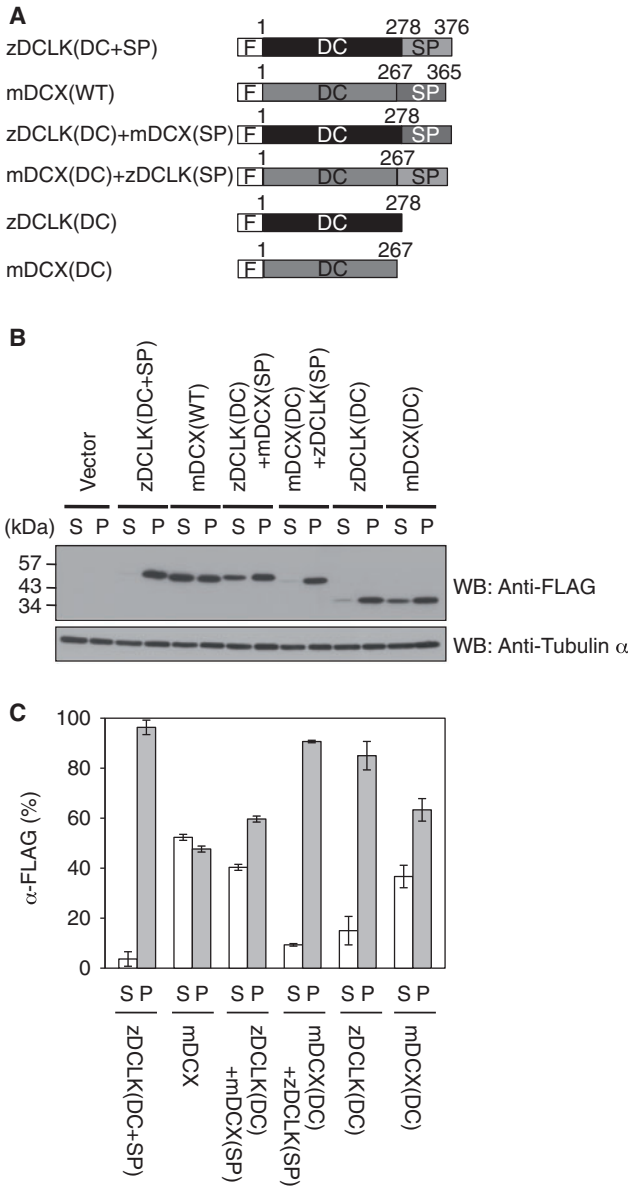

Fig. 4 Effects of SP domains of DCLK and DCX on the microtubule-binding activity of DC domains. (A) Schematic illustration of the primary structures of zDCLK and mDCX mutants. A FLAG tag (F) was fused on the N-terminal ends of each mutant. (B) Microtubule-binding activity of various zDCLK and mDCX mutants as examined by detergent extraction analysis of transfected cells. COS7 cells were transiently transfected with 2 μg of plasmid DNAs and cell extracts were fractionated by detergent extraction method. Soluble (S) and insoluble (P) proteins from 3 μg of cell extracts were resolved by SDS–PAGE and detected by western blotting with anti-FLAG antibody (upper panel) and anti-tubulin α antibody (lower panel). (C) The percentage of zDCLK and mDCX mutants in each fraction was calculated by the Image J software. Each data point represents the mean ± SE ($n = 3$) of separate experiments.

The SP domain and kinase domain of DCLK regulate microtubule-binding activity of the DC domain

To examine microtubule-binding activities of the SP domains, the SP domains of zDCLK and mDCX were created as the N-terminal EGFP fusion proteins (Fig. 5A). In contrast to zDCLK(DC + SP), both zDCLK(SP) and mDCX(SP) exhibited essentially no microtubule-binding activity as in case of zDCLK(Kinase) (Fig. 5B). To further examine the influence of the SP domain and the kinase domain of DCLK on the microtubule-binding activity, the deletion mutant of zDCLK devoid of the SP domain, zDCLK(DC + Kinase), was created (Fig. 6A). The microtubule-binding activity of zDCLK(DC + Kinase) mutant was compared with those of zDCLK(WT), zDCLK(DC) and zDCLK(DC + SP) using detergent extraction analysis (Fig. 6B and C).

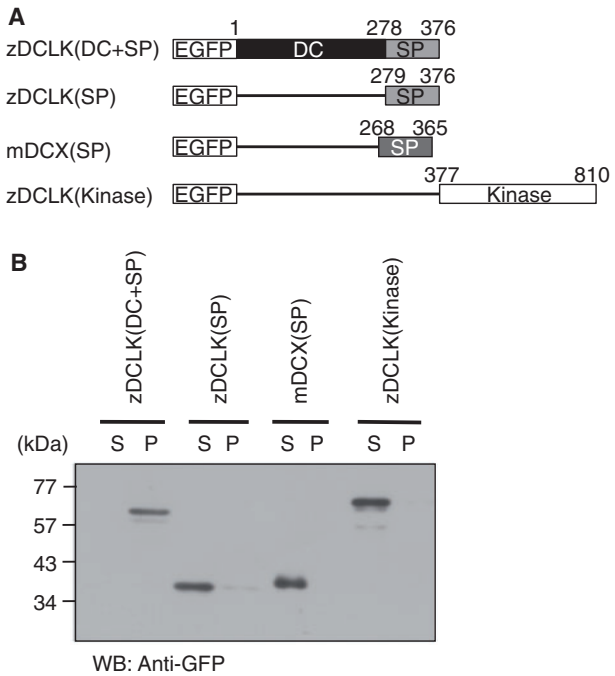

Fig. 5 Microtubule-binding activity of SP domains. (A) Schematic illustration of the primary structures of fusion proteins of SP domain and kinase domain of zDCLK. Each mutant was fused with EGFP on its N-terminus. (B) COS7 cells transiently expressed with mutants of zDCLK and mDCX were subjected to detergent extraction analysis, and soluble (S) and insoluble (P) proteins were analysed by western blotting with anti-GFP antibody.

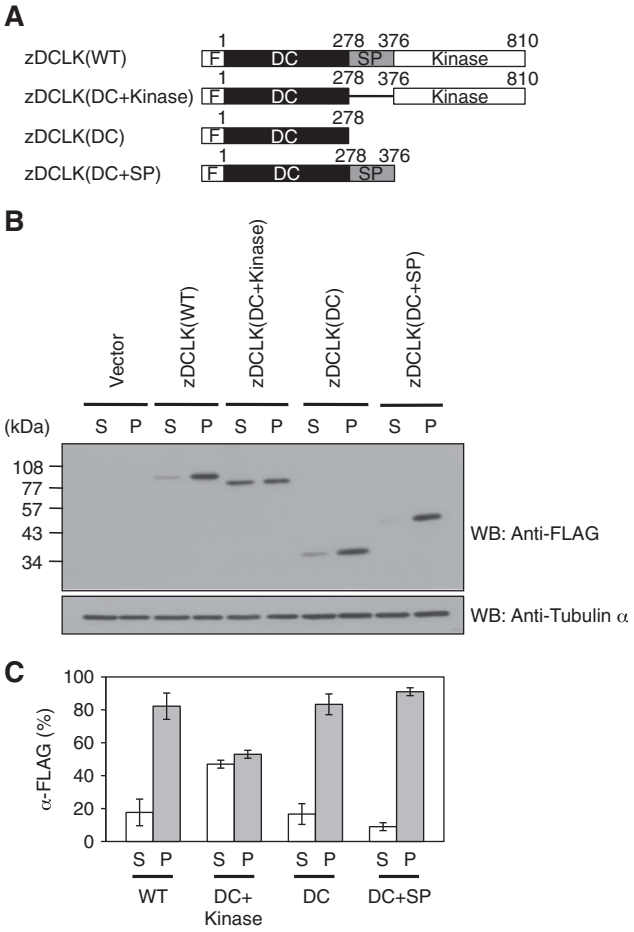

Fig. 6 Effect of SP domain and kinase domain of DCLK on microtubule-binding activity of the DC domain. (A) Schematic illustration of the primary structures of zDCLK mutants. A FLAG tag (F) was fused on the N-terminal ends of each mutant. (B) Microtubule-binding activity of various zDCLK mutants as examined by detergent extraction analysis of transfected cells. COS7 cells were transiently transfected with 2 μg of plasmid DNAs and cell extracts were fractionated by detergent extraction method. Soluble (S) and insoluble (P) proteins from 3 μg of cell extracts were resolved by SDS–PAGE and detected by western blotting with anti-FLAG antibody (upper panel) and anti-tubulin α antibody (lower panel). (C) The percentage of zDCLK mutants in each fraction was calculated by the Image J software. Each data point represents the mean ± SE ($n = 3$) of separate experiments.

About a half (53%) of zDCLK(DC + Kinase) mutant was observed in insoluble fraction, which was much lower value than that of zDCLK(WT) (82%) or zDCLK(DC) (83%). These data, taken together, suggest that the SP domain of zDCLK significantly stimulated the microtubule-binding activity of the DC domain when they were combined and that the kinase domain of DCLK decreased microtubule-binding activity of the DC domain.

Discussion

DCLK is known to be a microtubule-associated protein and has the kinase domain in its C-terminal region. However, functional roles of the kinase domain as well as the SP domain have not been clarified yet. In this study, we examined the roles of the SP domain and the kinase domain in the microtubule-binding activity of zDCLK. Removal of the kinase domain from zDCLK increased in the microtubule-binding activity of zDCLK and induced microtubule bundling, while deletion of SP domain from zDCLK significantly reduced the microtubule-binding activity. These results suggest that microtubule-binding activity of DCLK is either negatively or positively regulated by the different part of the C-terminal side of DC domain of this protein.

We also demonstrated that zDCLK was proteolytically cleaved into two functional fragments *in vivo*. These are 48-kDa zDCLK(DC + SP) fragment and 65-kDa kinase fragment. Furthermore, we previously reported that the kinase activity of zDCLK(Kinase) mutant expressed in *Escherichia coli* was much higher than that of zDCLK(WT) (*14*). Recently, we showed that the kinase domain of zDCLK interacted with substrate proteins such as synapsin II and the interaction between these proteins was enhanced when both the N-terminal (DC + SP) domain and the C-terminal autoinhibitory region (678–810) of zDCLK were deleted (*18*). Therefore, it is rational to speculate that DCLK serves as a precursor protein to supply two functional proteins at the same time; doublecortin-like protein with enhanced microtubule-binding activity and an activated form of Ser/Thr protein kinase that may function in cytosol. Especially, the N-terminal fragment of DCLK may promote polymerization/stabilization of microtubules and enhance microtubule bundling.

Although both DCLK and DCX are reported to be typical microtubule-associated proteins, our present results indicated that these proteins exist not only in microtubule-associated forms but also in soluble forms. These findings were supported by two different experiments; biochemical detergent extraction analysis and immunocytochemical studies using formalin-fixed cells. However, when the cells were fixed with methanol, which had been often used for fixation of cells, only microtubule-associated forms of DCLK and DCX were observed. These discrepancies may be attributed to the fact that cytosolic DCLK and DCX could not be efficiently fixed by methanol treatment and leaked out of the cells during washing steps. Therefore, when methanol-fixation of the cells was used for the assessment of cellular localization of proteins such as DCX and DCLK, it may lead to an incorrect conclusion that shows overestimation of microtubule-associated forms of these proteins.

There has been no paper that documented comparative studies concerning microtubule-binding activities of DCLK and DCX to date. Therefore, in this study, we compared the binding activities of various mutants of zDCLK and mDCX by means of detergent extraction analysis. Unexpectedly, we found that the microtubule-binding activity of mDCX was significantly lower than that of zDCLK(DC + SP) mutant. These differences could be attributed to the following reasons: (i) The DC domain of zDCLK shows somewhat stronger microtubule-binding activity than that of mDCX; (ii) The SP-domain of zDCLK significantly

increases microtubule-binding activity of the DC domain, while that of mDCX decreases microtubule-binding activity. It is especially noteworthy that the SP domains of homologous microtubule-associated proteins showed opposite effects on the microtubule-binding activity. Although the differences in the binding properties of these proteins may provide some important clues regarding the physiological significance of these microtubule-associated proteins, further studies are necessary to solve this problem.

In the present study, we investigated the interaction of various zDCLK mutants with microtubules by immunocytochemical analysis and by biochemical analysis using detergent extraction method. Although it is not clear whether the microtubule-binding activity of zDCLK mutants could properly be evaluated by the detergent extraction analysis, most of the results obtained in our present study coincided well with the results of the immunocytochemical analysis using formalin-fixed cells. Furthermore, when the detergent extraction assay was carried out to examine distribution of endogenous zDCLK using zebrafish brain extract, major population of zDCLK was observed in insoluble fractions as in case of transfected zDCLK in COS7 cells (data not shown). Availability of this method as binding assay would be further confirmed if the microtubule-binding activities of DCLK and DCX could be analysed by *in vitro* experiments using reconstituted microtubules and purified preparation of DCLK mutants. For that purpose, we have to prepare stable forms of zDCLK mutants such as zDCLK(DC + SP), which we have failed to obtain by conventional *E. coli* expression systems.

Microtubule-binding activity of DCLK and DCX may also be regulated by regulatory proteins interacting at the SP domain. For example, when an actin-binding protein Neurabin II binds to the SP domain of DCX, DCX colocalizes with actin filaments and takes part in depolymerization of F-actin (*23*, *24*). In addition, μ-subunits of clathrin adaptor complexes AP-1/2, which is known to play a role in protein sorting or vesicular trafficking, have been reported to interact with the SP domain of DCX (*25*). However, it is not known whether or not these proteins can also interact with the SP domain of DCLK, since amino acid sequence identity in this region is <40%. Therefore, exploration of regulatory proteins that interact with the SP domain of DCLK will be the next issue to be solved in the future studies.

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Conflict of interest
None declared.

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