Doublecortin Association with Actin Filaments Is Regulated by Neurabin II*

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Mutations in the human Doublecortin (DCX) gene cause X-linked lissencephaly, a neuronal migration disorder affecting the neocortex and characterized by mental retardation and epilepsy. Because dynamic cellular asymmetries such as those seen in cell migration critically depend on a cooperation between the microtubule and actin cytoskeletal filament systems, we investigated whether Dcx, a microtubule-associated protein, is engaged in cytoskeletal cross-talk. We now demonstrate that Dcx co-sediments with actin filaments (F-actin), and using light and electron microscopy and spin down assays, we show that Dcx induces bundling and cross-linking of microtubules and F-actin in vitro. It has recently been shown that binding of Dcx to microtubules is negatively regulated by phosphorylation of the Dcx at Ser-47 or Ser-297. Although the phosphomimetic green fluorescent protein (GFP)-DcxS47E transfected into COS-7 cells had a reduced affinity for microtubules, we found that pseudophosphorylation was not sufficient to cause Dcx to bind to F-actin. When cells were co-transfected with neurabin II, a protein that binds F-actin as well as Dcx, GFP-Dcx and to an even greater extent GFP-DcxS47E became predominantly associated with filamentous actin. Thus Dcx phosphorylation and neurabin II combinatorially enhance Dcx binding to F-actin. Our findings raise the possibility that Dcx acts as a molecular link between microtubule and actin cytoskeletal filaments that is regulated by phosphorylation and neurabin II.

Both microtubule and actin filament networks were classically thought to fulfill distinct mechanical cellular functions such as bipolar spindle formation for microtubules and muscle contraction for actin filaments. However, a number of proteins have been discovered that mediate interactions between microtubules and actin filaments occurring in processes in which dynamic cellular asymmetries are established and maintained, including cell migration, growth cone guidance, cell division, and wound healing (1).

The cytoskeleton of neurons receives increasing attention as a model system to study regulatory and structural interactions between microtubules and actin filaments, because neurons have a unique morphology and migrate during development. Several human neuronal diseases are the result of neuronal migration defects. Doublecortin (DCX) located on the X chromosome encodes a microtubule-associated protein (MAP), which is required for neuronal migration during cortical development, and mutations in DCX cause lissencephaly (smooth brain) in males and double cortex syndrome in females (2, 3). Although genetic deletion of Dcx in mice failed to produce any obvious malformation in the neocortex (4), acute Dcx inactivation using RNAi in developing rat neocortex caused disruptions in radial neuronal migration (5).

DCX consists of two DCX domains located N-terminally and a C-terminal Ser/Pro-rich domain (6, 7). Structure-function studies have shown that the first DCX domain binds to microtubules, whereas the second one binds to microtubules and to unpolymerized tubulin (8). In conjunction with the well-characterized DCX function as a microtubule-polymerizing, -stabilizing, and -bundle binding protein (6, 9–11), it is thought that the second DCX domain brings tubulin dimers to the growing tips of the microtubules to accelerate microtubule growth, and microtubules are stabilized or bundled when both domains bind to microtubules. Recent cryo-electron microscopic studies have shown that DCX binds between the protofilaments from which microtubules are built and such a location of DCX binding sites is ideal for microtubule stabilization (12).

Of particular importance for the present study is the observation that the binding of Dcx to microtubules is negatively regulated by phosphorylation of serine residues 47 and 297, which are phosphorylated by protein kinase A, MAP1/microtubule affinity regulatory kinase, and cyclin-dependent kinase 5 (Cdk5) (13, 14). Dcx does not bind to all microtubules of a migrating neuron but is highly enriched in the leader process and in the growth cones of differentiating neurons (9, 13, 16, 17). Localized Dcx binding to microtubules is regulated by site-specific phosphorylation by kinases that are present in these regions (13). It has recently been found that Dcx phosphorylated by c-Jun N-terminal kinase is concentrated in the actin-rich region of growth cones (15). In addition to phosphorylation/dephosphorylation, other regulatory mechanisms mediating DCX function can be envisaged. For example, we have shown that the F-actin-binding protein neurabin II (also known as spinophilin) interacts with Dcx (18) raising the possibility that a Dcx-neurabin II protein complex could bridge microtubules and actin filaments. This would be reminiscent of the microtubule actin cross-linking factor, a member of the plakin protein family (19–21).

We have embarked on in vitro binding studies to investigate the idea that neurabin II and Dcx cooperate to link microtubules and actin filaments and thereby contribute to neuronal migration. Sedimentation assays unexpectedly revealed that Dcx binds to F-actin. Fluorescence and electron microscopy was used to demonstrate that Dcx bundles and cross-links microtubules and actin filaments. Using transient transfection as-
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DNA Constructs and Recombinant Protein—The Dcx coding region was subcloned into pEGFP-C3 (Clontech) and pQEX1 (Qiagen). PCR was utilized to isolate full-length neurabin II from mouse cDNA and was cloned into pCMV-Tag 2A. Site-directed mutagenesis was performed using the Stratagene QuikChange™ site-directed mutagenesis kit. Recombinant His-Dcx was expressed in Escherichia coli XLI-Blue and purified with nickel nitritrocetic acid-agarose beads (Qiagen). Standard buffer conditions were used for purification of His-Dcx except that purified proteins were eluted in PEM buffer (80 mM Na-PiPES, pH 6.9, 60 mM EGTA, 2.5 mM MgCl2, and 10 mM imidazole) containing 250 mM imidazole. Proteins were stored at 4 °C, and the concentration of purified His-Dcx was determined using a Bradford protein assay (Ref. 22, Bio-Rad).

In Vitro Co-sedimentation Assays—F-actin co-sedimentation assays were performed with the non-muscle actin-binding protein spin-down biochem kit (Cytoskeleton, Inc.). For this, His-Dcx eluted from nickel nitritrocetic acid-agarose beads in general actin buffer containing 250 mM imidazole was used. The protein solution was adjusted to 1 mM dithiothreitol, 5% glycerol, and 1% Triton X-100 and was centrifuged for 1 h at 150,000 × g at 4 °C to remove aggregated protein. An aliquot of the supernatant was incubated with 0.4 mg/ml F-actin for 30 min at room temperature (RT). After centrifugation for 90 min at 150,000 × g at RT, supernatant and pellet fractions were separated and subjected to SDS-PAGE followed by protein staining with Coomassie Blue. To measure the binding affinity of Dcx for actin, a constant amount of actin filaments (2.1 μm) was incubated with various amounts of Dcx (0.2–8 μM) in a co-sedimentation assay. Coomassie Blue-stained gels were digitally photographed (Nikon Coolpix 995), and bands were quantified using NIH Image software.

Low Speed Pelleting Assays—Various amounts of His-Dcx eluted in general actin buffer containing 250 mM imidazole were added to preassembled actin filaments (4.2 μM) in 50-μl reaction mixtures that were then incubated for 20 min at RT. After centrifugation for 3 min at 13,000 × g at RT, the supernatant and pellet were separated and analyzed by SDS-PAGE, and protein was detected by Coomassie Blue staining. Under these centrifugation conditions, actin filaments remain in the supernatant, whereas F-actin bundles are found in the pellet (23). For F-actin and microtubule low speed pelleting assays, polymerized actin (4.2 μM) and/or taxol-stabilized microtubules (4.7 μM) were incubated with His-Dcx for 20 min at RT in 50-μl reaction mixtures (in PEM containing 1 mM ATP, 1 mM GTP, and 20 μM taxol). After centrifugation for 3 min at 2000 × g at RT, the supernatant and pellet were separated and analyzed by SDS-PAGE and Coomassie Blue staining. Actin bundles remained in the supernatant under these centrifugation conditions, whereas microtubule bundles were found in the pellet.

Actin/Microtubule Polymerization/ Bundling Assays—10 μg of rhoadmine non-muscle actin (Cytoskeleton, Inc.) was thawed and reconstituted in 20 μl of general actin buffer (5 mM Tris-Cl, pH 8.0, 0.2 mM CaCl2) by incubation for 30 min on ice. 0.1 volume of actin polymerization buffer (200 mM KCl, 20 mM MgCl2, 20 mM ATP) was added to the monomeric actin and incubated for 1 h at RT. This yielded an F-actin stock solution of 11.5 μM. Microtubules were prepared by polymerizing fluorescein-labeled tubulin (Cytoskeleton, Inc.) 20 μg of fluorescein-labeled tubulin was thawed and reconstituted in 12 μl of general tubulin buffer (PEM buffer plus 1 mM GTP and 5% glycerol) by incubating for 2–3 min at 37 °C. The addition of 1 μl of Me2SO and incubation for additional 20–30 min at 37 °C induced microtubule polymerization. The addition of 2.3 μl of 200 μM taxol yielded stable microtubules equivalent to a 23.5 μM tubulin monomer. Microtubules and actin filaments were examined by fluorescence microscopy after 1/50 dilution in either PEM containing 1 mM GTP and 20 μM taxol for microtubules or in general actin buffer for actin filaments. Bundling assays were carried out by mixing polymerized actin and/or taxol-stabilized micro-

tubes and His-Dcx for 2–10 min at RT. For co-incubation of microtubules and F-actin, both filaments were diluted in PEM containing 1 mM GTP and 20 μM taxol. The samples were spotted on a slide and examined under the Leica DMR fluorescence microscope.

Electron Microscopy—Samples were prepared as described for actin/ microtubule polymerization/bundling assays except that native tubulin and actin (from bovine brain and human platelet non-muscle) were used. A 1/20 dilution of sample solution yielded a final concentration of 575 nM for actin monomer, 1175 nM for tubulin monomer, and 1500 nM for His-Dcx. The samples were adsorbed to carbon film and then negatively stained with 2% uranyl acetate. The carbon films were applied to grids and examined using a Philips 301 electron microscope.

Cell Culture and Transfection—COS-7 cells (ECACC, UK) were cultured under standard condition as described by Sweeney et al. (24). For transient co-transfection, 25,000 cells were plated on glass coverslips (12-mm diameter, Marienfeld) in 4-well culture dishes (NUNC). On the next day, the cells were transfected with Lipofectamine Plus (Invitro-
gen) according to the manufacturer's instructions and incubated at 37 °C for 1 day prior to immunofluorescence analysis.

Immunofluorescence Staining and Quantitative Image Analysis—

Immunofluorescence staining of cells on the coverslips was carried out as described by Rivas and Hatten (25). A guinea pig polyclonal anti-Dcx antibody (Chemicon International) was used at 1:100 dilution. A mouse monoclonal anti-tubulin antibody (Sigma) was used at 1:1000 dilution. Neurabin II was detected with a mouse monoclonal anti-neurabin II antibody at 1:1 dilution. Specimens were examined with a ZEISS Axi

DcxWT and 13 cells expressing GFP-DcxS47R were chosen for this measurement, and cells were selected that had similar overall fluorescence intensity. Each cell contained on average 10 Dcx signal peaks. The mean value of average pixel intensities for 14 (or 13) cells was evaluated statistically by the two-sided Student's t test. The mean difference can be considered to be significant when p < 0.05.

RESULTS

Dcx Binds to and Bundles Actin Filaments—To investigate interactions of Dcx with the cytoskeleton, we first asked whether Dcx directly binds to F-actin. Co-sedimentation assays with purified His-tagged Dcx (Fig. 1A) and actin filaments showed that Dcx co-sedimented with F-actin (Fig. 1B). To determine the stoichiometry of binding at saturation, we incubated a constant amount of actin filaments (2.1 μM) with increasing amounts of Dcx (0.2–8 μM) in a co-sedimentation assay. The molar ratio at saturation between Dcx and the actin monomer was close to 1 (data not shown), and the Kd value was ~400 nM.

Next, we have carried out in vitro actin/microtubule bundling assays by fluorescence microscopy to explore the effect of Dcx on fluorochrome-labeled microtubules and F-actin. When purified His-tagged Dcx (Fig. 1A) was added to preassembled microtubules (the final concentration was 470 nM), Dcx induced, within minutes, the formation of long microtubule bundles (Fig. 1C, + Dcx 600 nM), consistent with previous reports (6, 10, 11). Unexpectedly, Dcx was also capable of bundling actin filaments (final actin concentration was 230 nM, Fig. 1C). Actin filaments were significantly longer and brighter than in the absence of Dcx even when the Dcx concentration was as low as 60 nM (Fig. 1C). When BSA at a concentration of 1 μM was added to either type of filament, no bundling activity was observed (Fig. 1C). Taken together, these results suggest that Dcx binds to F-actin and assembles it into bundles.

This conclusion is further supported by a low speed pelleting assay (23) and an ultrastructural analysis by electron microscopy. In the case of the low speed pelleting assay, various amounts of Dcx were added to preassembled actin filaments (4.2 μM). Neither F-actin (Fig. 2A) nor Dcx alone (data not
shown) pelleted after centrifugation at 13,000 \times g. However, addition of increasing amounts of Dcx increased the amount of F-actin present in the pellet (Fig. 2A). This titration experiment further supports the possibility that Dcx assembles F-actin into bundles, which sediment at a low g force.

For ultrastructural analysis, purified actin was polymerized and recombinant His-Dcx was added. An aliquot of the incubation mixture was negatively stained with uranyl acetate and viewed under the electron microscope. We observed that adding Dcx to F-actin induced the formation of long actin filament bundles (Fig. 2B), which are likely to correspond to the bundles seen by fluorescence microscopy (Fig. 1C). Of note, Dcx also bundled microtubules (Fig. 2B, right panels, 10 and 11).

**Dcx Cross-links Actin and Microtubules**—The finding that the microtubule-associated protein Dcx binds to and assembles actin filaments into bundles raises the possibility that Dcx is capable of cross-linking microtubules and F-actin. When Dcx was added to a mixture of F-actin and microtubules, F-actin bundles co-localized with microtubule bundles (Fig. 3A, +Dcx 600 nM). The addition of bovine serum albumin induced neither bundling of filaments nor co-localization of the filaments. Purified α-actinin, a well characterized actin-bundling protein (28), induced the formation of actin filament aggregates, but there was, as expected, no bundling of microtubules. Next, we examined the structure of the Dcx-F-actin-microtubule complexes at the ultrastructural level using electron microscopy. In the absence of Dcx, microtubules and actin filaments appeared as individual filaments, which did not interact with each other (Fig. 3B). However, in the presence of Dcx, microtubule bundles were formed and actin filaments were often seen in close proximity to these bundles (Fig. 3B, arrows). It is likely that these large bundles contain microtubules and actin filaments, i.e., Dcx cross-links microtubules and F-actin, because individual microtubules and actin filaments were depleted from the area surrounding the large bundles. To further investigate this possibility, the molar ratio of actin to tubulin was changed from 1:2.
F-actin

glob filaments in the absence of Dcx (F-tron microscopy. F-actin appeared as sin-

bundling activity of Dcx examined by electron microscopy. F-actin induced that actin filaments remain in the super-

natant, whereas F-actin bundles induced microtubules. Various amounts of Dcx were mixed with F-actin (final Dcx con-

centrations are indicated), incubated for 20 min at RT, and then centrifuged for 3 min at 13,000 × g. Samples were applied 
to 15% SDS-PAGE gel and Coomassie-stained (s, supernatant; p, pellet). Note that actin filaments remain in the super-
natant, whereas F-actin bundles induced by Dcx end up in the pellet. B, F-actin-bundling activity of Dcx examined by elec-
tron microscopy. F-actin appeared as single filaments in the absence of Dcx (F-actin) but was bundled by addition of Dcx 
(F-actin + Dcx). The two small panels on the right show the previously reported microtubule (MTs)-bundling activity of 
Dcx examined by electron microscopy. Scale bar, 250 nm.

FIG. 2. F-actin-bundling activity of Dcx. A, low speed pelleting assay of Dcx-
F-actin bundles. Various amounts of Dcx were mixed with F-actin (final Dcx con-

centrations are indicated), incubated for 20 min at RT, and then centrifuged for 3 

min at 13,000 × g. Samples were applied to 15% SDS-PAGE gel and Coomassie-

stained (s, supernatant; p, pellet). Note that actin filaments remain in the super-

natant, whereas F-actin bundles induced by Dcx end up in the pellet. B, F-actin-

bundling activity of Dcx examined by electron microscopy. F-actin appeared as sin-

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bar, 250 nm.

to 2:1, 6:1, and 18:1, respectively, so that an excess amount of F-actin was present in the reaction mixture. The concentration of Dcx was kept constant. As is clearly shown in Fig. 3C with a molar ratio of 18:1, single microtubules were decorated with numerous actin filaments. This suggests that Dcx cross-links actin filaments and microtubules.

The effects of Dcx on F-actin and microtubules were further analyzed by low speed pelleting assays. After incubating F-actin and/or microtubules with or without Dcx, the reaction mixtures were centrifuged for 3 min at 2000 × g. Under this centrifugation condition, small amounts of sedimented tubulin were observed in reactions that contained microtubules alone or microtubules and F-actin (Fig. 3D, lanes 2 and 10). Dcx-induced microtubule bundles are heavier than single microtubules and were thus abundant in the pellet fraction (Fig. 3D, lane 4). As expected, neither F-actin alone nor Dcx-induced F-actin bundles sedimented at this speed (Fig. 3D, lanes 6 and 8). However, in the presence of Dcx, massive amounts of actin filaments and microtubules co-sedimented (Fig. 3D, lane 12), which is consistent with the structure of the Dcx-F-actin-microtubule complexes observed by microscopy (Fig. 3A–C). Taken together, experiments using a broad spectrum of in vitro methods all support the notion that Dcx can interact with F-actin and, furthermore, cross-links microtubules and F-actin.

GFP-DcxS47E Associates with F-actin in the Presence of Neurabin II in COS-7 Cells—We next investigated whether the interaction between Dcx and F-actin can be demonstrated in mammalian cells. Cultured murine hippocampal neurons highly expressed Dcx with Dcx staining being most prominent in close proximity to F-actin especially in the actin-rich growth cones (9, 16, 17). Thus in the distal portion of neurites Dcx has the opportunity to directly interact with F-actin.

Others have shown that the interaction of microtubules and Dcx is negatively regulated by phosphorylation of serines 47 and/or 297 (see Introduction). We examined whether phosphorylation of Dcx would affect binding of Dcx to F-actin. Hence we generated pseudophosphorylation mutations by introducing a glutamic acid residue (E) at serine 47 or serine 297 in a GFP-tagged Dcx fusion protein. COS-7 cells were transfected either with GFP-Dcx, GFP-DcxS47E, or GFP-DcxS297E. On the next day, cells were fixed and stained for tubulin whose distribution was compared with that of wild-type or mutant GFP-Dcx. Fig. 4A, 1–3, shows that GFP-Dcx co-localized with microtubules, as had previously been reported (11), but there was no co-localization with actin filaments (Fig. 4A, 4–6). Cells transfected with GFP-DcxS47E (Fig. 4A, 7–9) often showed a quantitative reduction of microtubule localization of GFP-DcxS47E (compare Fig. 4A, 1 and 3 with 7 and 9). This is consistent with previous reports that demonstrate this effect for DcxS297D (14). Importantly, GFP-DcxS47E does not co-localize with actin that is revealed by phalloidin staining (Fig. 4A, 10–12).

To quantify difference in microtubule binding between GFP-DcxWT and GFP-DcxS47E, we have compared average pixel intensities associated with microtubule in cells transfected with GFP-DcxWT or GFP-DcxS47E. As illustrated in Fig. 4B (right), we have determined the peak intensity of Dcx fluorescence in a small area defined by transecting lines passing through microtubules (see “Experimental Procedures”). As shown in the histogram of Fig. 4B, the average pixel intensity of DcxWT (left, black bar) was significantly higher than that of DcxS47E (right, black bar). By contrast, there was no significant difference in background signal intensity between the two proteins (white bars). Thus the visual impression of a signal reduction as a result of pseudophosphorylation of Dcx is confirmed by a more quantitative analysis.
This and previous studies (13, 14) indicate that phosphorylation negatively regulates Dcx binding to microtubules, but evidently it does not result in actin binding. One possibility is that for Dcx to bind to actin in a cell requires an additional protein. We have previously shown that neurabin II, an F-actin-binding protein (27), also interacts with Dcx (18). This prompted us to examine whether neurabin II regulates the affinity of Dcx to microtubules. We first co-transfected cells with GFP-Dcx and FLAG-neurabin II and found that GFP-Dcx partially co-localized with neurabin II and thus with actin filaments. In addition, we also observed in some cells co-localization of GFP-Dcx with whirls of microtubules. Thus in the presence of neurabin II, Dcx is to some extent associated with F-actin.
neurabin II and found a strong co-localization of GFP-DcxS47E and F-actin-associated FLAG-neurabin II (Fig. 4C 10–12). Consistent with these data, GFP-DcxS47E showed actin-like distribution (Fig. 4C, 13–15). The overlap of GFP-DcxS47E fluorescence and actin signal was more striking than that seen with GFP-DcxWT and actin (Fig. 4C, compare 6 with 15). In addition, we observed almost no co-localization of GFP-DcxS47E and microtubules (Fig. 4C, 16–18). The GFP-DcxS297E muta-
The binding of Dcx to microtubules is negatively regulated by phosphorylation (13, 14). Here we have extended this observation by showing that neurabin II drives pseudophosphorylated Dcx into a complex with F-actin.

**DISCUSSION**

Recent studies have shown that microtubules and actin cytoskeleton interact with each other to orchestrate cell migration and growth cone movement in neurons (28, 29). Several types of proteins have been isolated that mediate structural interactions between microtubules and actin (1). In this study, we showed for the first time that the microtubule-associated protein Dcx can bind to and bundle F-actin and moreover cross-links microtubules with F-actin.

Current models of the mechanism of action of DCX are based on its interaction with microtubules and with other DCX-interacting proteins. Interaction of DCX with µ1A subunit of the AP-1 adaptor complex involved in clathrin-dependent protein sorting suggests a role for DCX in protein transport in migrating neurons (16). DCX associated with phospho-FIGQY in the cytoplasmic domain of neurofascin, a human L1 cell-adhesion molecule could function as an adaptor between neurofascin and microtubules (30). Of particular relevance for the present study is the notion that DCX stabilizes and bundles microtubules in the leading process of migrating neurons and promotes microtubule growth (8, 31). Dcx as an F-actin-binding and -bundling protein sheds light on novel functions of Dcx. Recent studies have shown that in the cell body of migrating cells microtubules preferentially cross-link to and grow along focal adhesion-associated actin bundles (28) and that in neuronal growth cones filopodial F-actin bundles guide the assembly and transport of microtubules (29). Dynamic unbundled microtubules polymerize into the periphery along filopodia in the direction of growth cone extension (29). In the distal portion of neurites where Dcx is prominently localized, Dcx could promote microtubule growth into the actin-rich periphery by cross-linking to and growing along F-actin bundles. Our ultrastructural analysis of the Dcx/F-actin/microtubule complexes showed that microtubules bind to F-actin in parallel, which is consistent with the finding that microtubules grow along F-actin bundles.

Using transfected transfection in mammalian cells, we have shown that pseudophosphorylation mutant Dcx(S47E) reduces the binding of Dcx to microtubules and, in addition, increases binding to F-actin in the presence of neurabin II. Neurabin II, a previously isolated Dcx-binding protein (18) that also binds to MAP2c, is found to be associated with F-actin in the presence of neurabin II. The binding of Dcx to microtubules and F-actin could be regulated by site-specific phosphorylation and neurabin II would contribute to the ability of Dcx to respond to a wide range of guidance cues during cortical development.

Although it remains to be demonstrated that Dcx shuttles between microtubules and F-actin in growth cones and leading processes of living neurons, our in vitro and transient transfection data reveal Dcx as a promising candidate to play a major role in a cross-talk between the two types of cytoskeleton. It should be emphasized that serine residue 47 is not only critical for phosphoregulation (13) and for regulation through neurabin II but when mutated to arginine causes X-linked lissencephaly (3). This fact strongly suggests that the disturbance of a regulated cross-talk between the microtubules and the actin cytoskeleton is causative to this disease.
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