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Site-specific dephosphorylation of *doublecortin* (DCX) by protein phosphatase 1 (PP1)

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Mutations in *doublecortin* (DCX) cause X-linked lissencephaly ("smooth brain") and double cortex syndrome in humans. DCX is highly phosphorylated in migrating neurons. Here, we demonstrate that dephosphorylation of specific sites phosphorylated by JNK is mediated by Neurabin II, which recruits the phosphatase PP1. During cortical development, the expression pattern of PP1 is widespread, while the expression of DCX and Neurabin II is dynamic, and they are coexpressed in migrating neurons. In vitro, DCX is site-specific dephosphorylated by PP1 without the presence of Neurabin II, this dephosphorylation requires an intact RVXF motif in DCX. Overexpression of the coiled-coil domain of Neurabin II, which is sufficient for interacting with DCX and recruiting the endogenous Neurabin II with PP1, induced dephosphorylation of DCX on one of the JNK-phosphorylated sites. We hypothesize that the transient recruitment of DCX to different scaffold proteins, JIP-1/2, which will regulate its phosphorylation by JNK, and Neurabin II, which will regulate its dephosphorylation by PP1, plays an important role in normal neuronal migration.

Keywords: Doublecortin; Lissencephaly; Phosphorylation; Protein phosphatase 1; Neurabin II; Spinophilin

Introduction

Mutations in *doublecortin* (DCX) cause X-linked lissencephaly ("smooth brain") and double cortex syndrome in humans (des Portes et al., 1998; Gleeson et al., 1998). In mice, in utero reduction of DCX's expression by RNAi has been shown to cause inhibition of neuronal migration (Bai et al., 2003). DCX is a microtubule-associated protein (MAP) that stabilizes microtubules (MTs) (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999). The interaction with MTs is via an evolutionarily conserved Doublecortin (DCX) domain (Kim et al., 2003; Sapir et al., 2000; Taylor et al., 2000), where most missense mutations cluster. The effect of several of these mutations and the interaction of DCX with MTs studied in vitro and in transfected cells (Sapir et al., 2000; Taylor et al., 2000) demonstrated variable results. While the overexpression of some mutant DCX proteins stabilized MTs, the overexpression of others resulted in less stable MTs, thus suggesting that the dynamic regulation of MTs is an important factor in regulation of neuronal migration. The expression and phosphorylation of DCX are regulated during brain development (Francis et al., 1999; Gleeson et al., 1999). The phosphorylation of DCX by at least three different kinases has been demonstrated: JNK (Gdalyahu et al., 2004), Cdk5 (Tanaka et al., 2004), Protein Kinase A (PKA) and/or the MARK/PAR-1 family of protein kinases (Schaar et al., 2004). JNK phosphorylated DCX on at least three different sites: T321, T331, and S334 (Gdalyahu et al., 2004). Cdk5 phosphorylated DCX on S297 (Tanaka et al., 2004), and a different study suggested that S28 and S339 (which is identical to the S334 site phosphorylated by JNK, numbers differ in two DCX isoforms) are the major Cdk5 sites, with additional minor sites (Graham et al., 2004). PKA and/or the MARK kinase phosphorylated DCX on several sites, with the most significant one being S47 (Schaar et al., 2004). Since addition of low concentrations of okadaic acid, which inhibits PP2A (but not PP1), increased DCX's phosphorylation, it has been suggested that this is the main phosphatase acting on DCX (Schaar et al., 2004). In vitro analysis indicated that DCX's phosphorylation by Cdk5, PKA, and MARK reduced the affinity of DCX to MTs (Tanaka et al., 2004; Schaar et al., 2004). DCX is found in high concentrations in the cell soma, tips of neurites, and in growth cones of neurons (Friocourt et al., 2003; Gdalyahu et al., 2004; Schaar et al., 2004; Tanaka et al., 2004). Phosphorylation by Cdk5 apparently controls and localizes DCX to fine perinuclear MTs, but not to MT bundles in proximal processes (Tanaka et al., 2004). This specific localization suggested a unique role for

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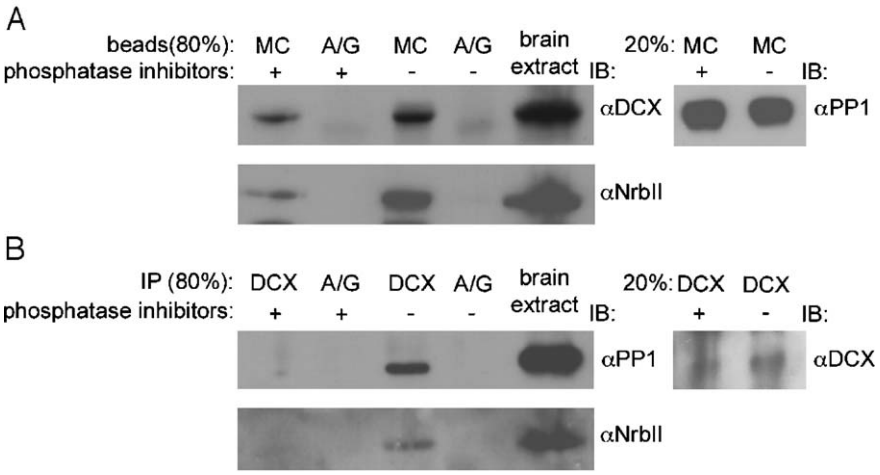

Fig. 1. DCX is found in a protein complex with Neurabin II and with PP1. (A) Brain extract (P6) was subjected to precipitation of PP1 using microcystin (MC) beads, which specifically binds the catalytic site of PP1 and inhibits enzymatic activity. A/G beads with anti-myc antibodies were used as a control. A cocktail of phosphatase inhibitors was added (+) or not (−) to the reaction samples. All samples were subjected to SDS-PAGE and Western blotted with the indicated antibodies. DCX and Neurabin II (NrbII) were detected in the MC precipitate only when phosphatase inhibitors were not included in the reaction. PP1 was detected in MC precipitates with and without phosphatase inhibitors. (B) Brain extract (P6) was subjected to immunoprecipitation of DCX using anti-DCX antibodies or A/G beads together with rabbit anti-mouse IgM as a control. PP1 and Neurabin II (NrbII) were detected in the DCX immunoprecipitate lacking phosphatase inhibitors. DCX was immunoprecipitated in the presence or absence of phosphatase inhibitors.

DCX phosphorylated by Cdk5 during somal translocation. Overexpression of DCX in cerebellar neurons resulted in faster migration, while a point mutation in S297 (the site phosphorylated by Cdk5) or pharmacological inhibition of Cdk5 abolished this effect. The position of DCX in the growth cones, colocalizing with filamentous actin, required phosphorylation by JNK (Gdalyahu et al., 2004). This localization suggested that DCX's phosphorylation by JNK might play an important role in neurite outgrowth, which is an important step in all modes of migration. Indeed, phospho- and unphospho-mimicry mutants of DCX (for the sites phosphorylated by JNK) exhibited a biological effect. Neurons overexpressing the phospho-mimicry

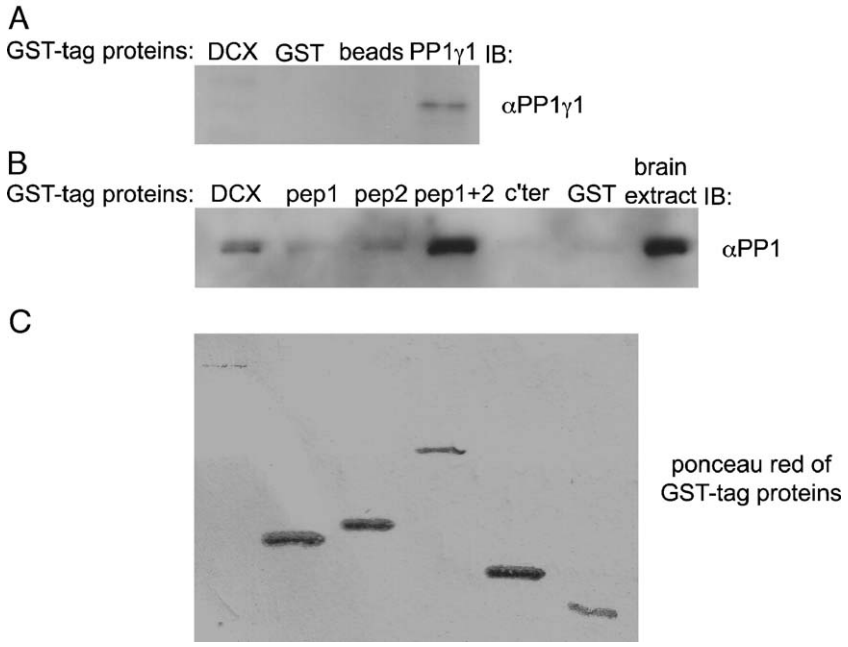

Fig. 2. PP1–DCX interaction required cellular mediators and an intact tandem DCX domain. (A) GST-DCX, GST, or glutathione agarose beads were incubated with recombinant PP1γ1; following incubation, the beads were washed, loaded on SDS-PAGE, and Western blotted. The blot was reacted with anti-PP1 antibodies, which detected the protein only in the control lane. (B) GST-tagged proteins DCX, pep1, pep2, pep1 + 2, C-terminus of DCX (c-ter), or GST was used to pull down PP1 from P3 brain extract. PP1 present in the extract (right lane) was pulled down by the full-length DCX and by the tandem DCX domain (pep1 + 2). (C) The recombinant proteins used in A and B were blotted and stained with Ponceau red, demonstrating the relative amounts of the proteins used in these assays, the amount of GST-DCX and GST-pep1 + 2 was slightly reduced in comparison with the other proteins.