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Doublecortin Interacts with μ Subunits of Clathrin Adaptor Complexes in the Developing Nervous System

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Doublecortin is a microtubule-associated protein required for normal corticogenesis in the developing brain. We carried out a yeast two-hybrid screen to identify interacting proteins. One of the isolated clones encodes the μ1 subunit of the adaptor complex AP-1 involved in clathrin-dependent protein sorting. We found that Doublecortin also interacts in yeast with μ2 from the AP-2 complex. Mutagenesis and pull-down experiments showed that these interactions were mediated through a tyrosine-based sorting signal (YLPL) in the C-terminal part of Doublecortin. The functional relevance of these interactions was suggested by the coimmunoprecipitation of Doublecortin with AP-1 and AP-2 from mouse brain extracts. This interaction was further supported by RNA *in situ* hybridization and immunofluorescence studies. Taken together these data indicate that a certain proportion of Doublecortin interacts with AP-1 and/or AP-2 *in vivo* and are consistent with a potential involvement of Doublecortin in protein sorting or vesicular trafficking.

INTRODUCTION

During embryonic development of the mammalian brain, neuronal migration is one of the key processes that leads to the formation of the cerebral cortex. Neurons migrate long distances from the neuroepithelium, where they arise, to the cortical plate where they form the complex laminar structures of the cortex. Postmitotic neurons exit from the neuroepithelium in waves, then migrate tangentially, or radially, along glial fibres to the cortical plate. They settle in six layers, which are formed sequentially from the deepest to the most superficial ("inside-out") so that each new wave of neurons migrates past the previously formed ones (McConnell, 1995; Caviness *et al.,* 1997). The molecular events that drive migrating neuronal cells to their final destinations as well as the environmental cues that regulate their spatial position are not yet fully understood. However, the genetic analysis of mammalian neuronal migration disorders suggests the involvement of several pathways, which can be more and more considered as complementary.

Mutations in *LISI* (Reiner *et al.,* 1993), *doublecortin* (des Portes *et al.,* 1998; Gleeson *et al.,* 1998), *cdk5* (Oshima *et al.,* 1996), *p35* (Chae *et al.,* 1997), *filamin 1* (Fox *et al.,* 1998), *reelin* (D'Arcangelo *et al.,* 1995), *mdab1* (Howell *et al.,* 1997; Ware *et al.,* 1997; Sheldon *et al.,* 1997), *VLDLR* and *ApoER2* (Trommsdorff *et al.,* 1999) have been found to lead to specific phenotypes in which corticogenesis is disrupted. The strikingly similar phenotype of lissencephaly (Pinard *et al.,* 1994) between humans with *LIS1* mutations and those with *doublecortin* mutations along with findings demonstrating that both proteins are microtubule-associated proteins (MAPs) (Sapir *et al.,* 1997; Francis *et al.,* 1999; Gleeson *et al.,* 1999; Horesh *et al.,* 1999) suggest that LIS1 and Doublecortin may function through similar mechanisms in neuronal migration. Moreover, recent studies showed that *reelin, mDab1, ApoER2,* and *VLDLR* are potentially involved in the

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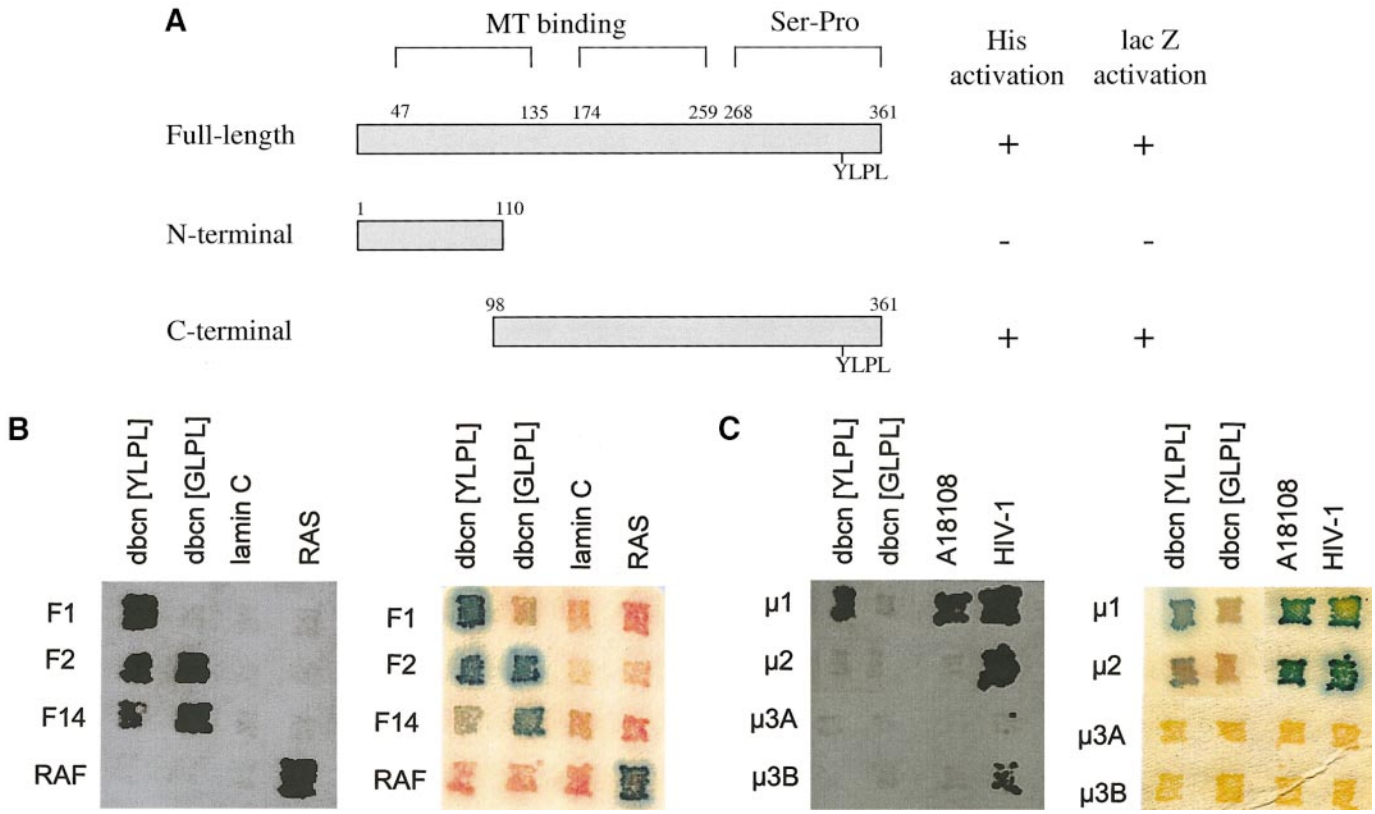

FIG. 1. Two-hybrid analysis. (A) Schematic representation of different parts of Doublecortin tested in the two-hybrid system. The domain implicated in the Doublecortin-μ1 interaction was found to reside within the C-terminus of Doublecortin. Numbers correspond to amino acid positions in Doublecortin sequence. MT, microtubule; Ser-Pro, serine–proline-rich region. (B) Effects of the site-directed mutagenesis on the Doublecortin interaction with μ1. Mating was performed between L40 transformants (indicated above the plates) and AMR70 transformants (indicated on left-side). Replica plating was carried out on plates lacking tryptophan, leucine and histidine. Only the transformants expressing interacting constructs were able to grow without histidine. F1, F2, and F14 are the 3 clones isolated in the two-hybrid screen. F1 corresponds to human μ1 not containing the first 20 amino acids of the protein. RAS-RAF: positive interaction controls. dbcn [YLPL]: wild-type Doublecortin. dbcn [GLPL], mutated Doublecortin. Wild type but not mutated Doublecortin interacts with μ1. (C) Study of homologous proteins. μ1, μ2, μ3A, and μ3B were tested in comparison to F1. μ1, but not μ3A and μ3B, was found to clearly interact with Doublecortin. HIV-1 was used as a control, known to interact with μ1 and μ2 (Berlioz-Torrent *et al.*, 1999).

control of the final position of neurons and function through a linear pathway (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Trommsdorff *et al.*, 1999). Thus, further studies are required to investigate the links between this pathway, the MAPs and the other molecules involved in neuronal migration.

Toward this aim, we carried out a yeast two-hybrid screen to identify proteins interacting with Doublecortin. One of the clones isolated encodes the μ1 subunit of the adaptor complex AP-1 implicated in clathrin-dependent protein sorting from the *trans*-Golgi network (TGN) to the endosomes and lysosomes. Three other adaptor complexes, AP-2, AP-3, and AP-4, have also been characterized, and additional complexes may similarly exist. AP-2 is involved in clathrin-mediated endocytosis and receptor recycling at the plasma membrane (reviewed by Hirst *et al.*, 1998), whereas AP-3 and AP-4 seem to play a role in the endosome-lysosome or TGN-lysosome pathways (Simpson *et al.*, 1996; Dell'Angelica *et al.*, 1997, 1999; Hirst *et al.*, 1999). Very few studies have been performed to assess the roles of these adaptor complexes in neurons of the developing nervous system, although both AP-2 and AP-3 have been implicated in synaptic vesicle recycling in mature neurons (Gonzalez-Gaitan *et al.*, 1997; Shi *et al.*, 1998; Faundez *et al.*, 1998). Each complex is a heterotetramer composed of two large subunits or adaptins, *i.e.*, γ and β1 (AP-1), α and β (AP-2), δ and β3 (AP-3), and ε and β4 (AP-4), a medium-sized subunit (μ1, μ2, μ3, or μ4) and a small subunit (σ1, σ2, σ3, or σ4). The μ chains have been shown to mediate the capture of specific proteins (often

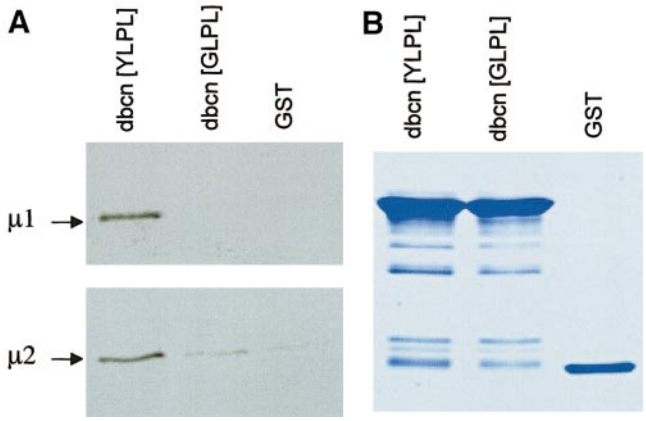

FIG. 2. *In vitro* binding assay. ^{35}S-labeled *in vitro*-translated μ1 and μ2 were incubated with 10 μg of the indicated GST fusion proteins. Wild-type Doublecortin (dbcn [YLPL]) was compared with Doublecortin mutated at the tyrosine-based sorting signal (dbcn [GLPL]) and GST. (A) The autoradiography shows binding of μ1 specifically to wild-type Doublecortin and a significant difference between μ2/wild-type Doublecortin and μ2/mutated Doublecortin, which is in agreement with an interaction between μ1/μ2 and Doublecortin. (B) A Coomassie blue-stained gel showed that similar amounts of wild type and mutated Doublecortin were used in this assay.

integral membrane proteins) in clathrin-coated vesicles by binding to tyrosine-based sorting signals and possibly dileucine signals present in the protein (Ohno *et al.*, 1998). Our data described here show that Doublecortin interacts with both μ1 and μ2 subunits in the two-hybrid system and in *in vitro* binding studies, through a tyrosine-based sorting signal present in its C-terminus. RNA *in situ* hybridizations show a strong expression of both *μ1* and *μ2* in the developing nervous system where *doublecortin* is expressed. In migrating and differentiating neurons, Doublecortin immunofluorescence staining partially overlapped with both γ (AP-1) and α (AP-2). In addition, both γ and α subunits were coimmunoprecipitated from embryonic brain extracts using anti-Doublecortin antibodies. These observations strongly suggest an association with the AP-1 and AP-2 complexes *in vivo.*

RESULTS

Doublecortin Interacts with the μ1 Subunit of the AP-1 Complex in Yeast

Using the full-length cDNA of Doublecortin as bait, we screened a human foetal brain cDNA library using the two-hybrid system (Fields *et al.*, 1989). Seventeen clones, of 10^6 screened, scored positive for both reporter genes as they grew on selective medium without histidine, and were positive in the lacZ test. To assess the specificity of the interaction we used RAS, RAF, and lamin C proteins as controls. Only 3 of the 17 clones showed a consistent specific interaction with Doublecortin. Clone F1 shared 90% nucleotide identity with a mouse cDNA encoding the μ1 subunit of the adaptor complex AP-1 (Genbank Accession No. M62419) and is thus likely to be the human ortholog. Sequencing showed that the first 20 amino acids of the protein were not contained in this clone.

In order to determine which region of Doublecortin interacts with μ1, we tested two different fusion proteins containing either the N-terminal part (amino acids 1 to 110) or the last two-thirds (amino acids 98 to 361) of Doublecortin, respectively. Using a two-hybrid assay, a specific interaction with μ1 was detected only with the fusion protein containing the C-terminal part of Doublecortin, which indicates that the interacting domain resides within this region (Fig. 1A).

Recent studies have reported that the μ subunits of adaptor complexes interact with either a tyrosine-based sorting signal, YXXΦ (where Φ is an amino acid with a bulky hydrophobic side-chain like leucine, isoleucine, phenylalanine, methionine and valine (Ohno *et al.*, 1998)), and/or a dileucine signal present in their various target proteins. This prompted us to search for these motifs in the sequence of Doublecortin: a YLPL motif (amino acids 345 to 348) was found in its C-terminal end. To further confirm the involvement of this potential tyrosine-based sorting signal in the interaction with μ1, site-directed mutagenesis of the tyrosine to a glycine residue was performed and the mutated full-length Doublecortin was assessed in a two-hybrid assay. Lack of growth in histidine-free medium as well as a negative lacZ test indicated a loss of interaction between μ1 and mutated Doublecortin, whereas the mutation did not affect the interaction with two different clones F2 and F14 isolated in the two-hybrid screen (Fig. 1B). These data strongly suggest that μ1 interacts specifically with Doublecortin via its tyrosine-based sorting signal.

Because of the high similarity between μ1, μ2, and μ3 subunits, we also tested for a possible interaction between Doublecortin and μ2 and μ3. Two forms of μ3, μ3A, and μ3B, were tested, which represent ubiquitous and neuronal-specific forms, respectively. The results presented in Fig. 1C show an interaction between Doublecortin and μ1, but none with either μ3A or μ3B. This suggests a specific interaction, and not simply a nonspecific binding of the tyrosine-based sorting signal. Cotransformation of Doublecortin and μ2 showed a

very small amount of growth on histidine-lacking medium and the LacZ gene was activated, which may suggest a faint interaction (Fig. 1C). The cytoplasmic domain of the transmembrane envelope protein of the human immunodeficiency virus type 1 (HIV-1), used as a control, interacted strongly with μ1 and μ2 and more faintly with μ3B (Berlioz-Torrent *et al.*, 1999).

Doublecortin is homologous to another gene KIAA0369 (Omori *et al.*, 1998) that has numerous isoforms produced by alternative splicing. One mouse transcript, A18108, shares 75% amino-acid identity to mouse Doublecortin and has a similar gene structure (F. Francis and J. Chelly, unpublished observations). It also contains a conserved tyrosine-based sorting signal in its C-terminus: YRPL (amino acids 348 to 351). We thus tested this protein with the μ subunits of AP-1, AP-2, and AP-3 using a two-hybrid assay (Fig. 1C). The results were similar to those obtained with Doublecortin, *i.e.*, A18108 interacts strongly with μ1, but not with μ3A or μ3B in the yeast system. The results of the lacZ test suggest that μ2 interacts more strongly with A18108 than with Doublecortin.

In Vitro Binding Assay

To confirm the two-hybrid results concerning a direct interaction between the YLPL motif in Doublecortin and the μ subunits, an *in vitro* binding assay was performed. μ1 and μ2 proteins were translated and labeled with [^{35}S]methionine using a rabbit reticulocyte lysate system. The labeled proteins were incubated with GST-Doublecortin, mutated GST-Doublecortin (GLPL) and GST control, and bound proteins were purified using glutathione-sepharose 4B. Figure 2 shows an interaction between Doublecortin and μ1, which is lost when the tyrosine-based sorting signal of Doublecortin is mutated. An interaction between Doublecortin and μ2 is also suggested by the significant difference between the signals corresponding to wild-type and mutated Doublecortin. There is still a faint band with mutated Doublecortin but it is clear that the mutation strongly decreases the affinity of μ2 for Doublecortin. These results thus suggest that the YLPL signal of Doublecortin is required for an interaction with both the μ1 and μ2 subunits.

Expression of AP-1, AP-2, and Doublecortin

In order to compare the patterns of expression of Doublecortin and the AP-1 and AP-2 complexes, we first performed *in situ* hybridization experiments on mouse sections at different stages using μ*1* and μ*2* RNA probes. In every section examined, μ*1* and μ*2* showed similar expression patterns (Fig. 3). The results are likely to be specific since the sense probes gave no labelling. μ*1* and μ*2* were ubiquitous at early stages (E10.5), but at E12.5 and E15.5, mRNA appeared more abundant in the developing nervous system (Figs. 3A–3D). This was obvious in the developing brain (Figs. 3I–3N), the eye, the developing spinal cord (Figs. 3Q and 3R), the dorsal root ganglia (Figs. 3O and 3P) and the trigeminal and inferior glossopharyngeal ganglia. We focused on the lateral ventricle where the cortex begins to form between E11 and E17; μ*1* and μ*2* were expressed in the ventricular zone, a region of cell proliferation, in the migrating neurons of the intermediate zone and in the cortical plate where the neurons settle (Figs. 3I–3N). In neonatal brain, both μ*1* and μ*2* were still widely expressed in every region (Figs. 3F and 3G), whereas in adult brain they displayed more restricted expression patterns (Fig. 3S): they showed labelling of higher intensity in the cerebellum (grey matter), the olfactory bulb and trunk and the dentate gyrus in the hippocampus. These data were compared to the expression of *doublecortin*, which has previously been thoroughly studied in our laboratory (Francis *et al.*, 1999). At E14, *in situ* hybridization shows a strong expression of *doublecortin* in all brain regions, the developing spinal cord, the dorsal root ganglia and the retina (Fig. 3E). In the developing cortex, although *doublecortin* is not present in the cells of the ventricular zone, it is highly expressed in neurons of the intermediate zone and in the cortical plate. Thus, there is a large overlap in expression during development with both μ*1* and μ*2* most strongly expressed in regions containing *doublecortin*.

To examine the subcellular distribution of AP-1, AP-2, and Doublecortin, we analyzed their expression in primary neuronal cultures from fetal mouse brain by immunofluorescence (Fig. 4). As previously observed (Francis *et al.*, 1999), Doublecortin appeared more abundant at the end of certain neurites and in the proximal part of growth cones (Figs. 4A, 4D, and 4J). In order to detect AP-1 and AP-2 complexes, we used anti-γ and anti-α, respectively, since anti-μ1 and anti-μ2 antibodies do not work in immunofluorescence experiments. Anti-γ showed a punctate labeling around the nucleus, which is consistent with its known localization in the TGN and endosomes (Fig. 4E). In most neurons, the staining was polarised and seemed to enter preferentially into one neurite (Fig. 4B). Nevertheless, a light punctate staining was observed along the length of some neurites as well as in growth cones. The pattern of γ-adaptin staining in growth cones was usually distal to

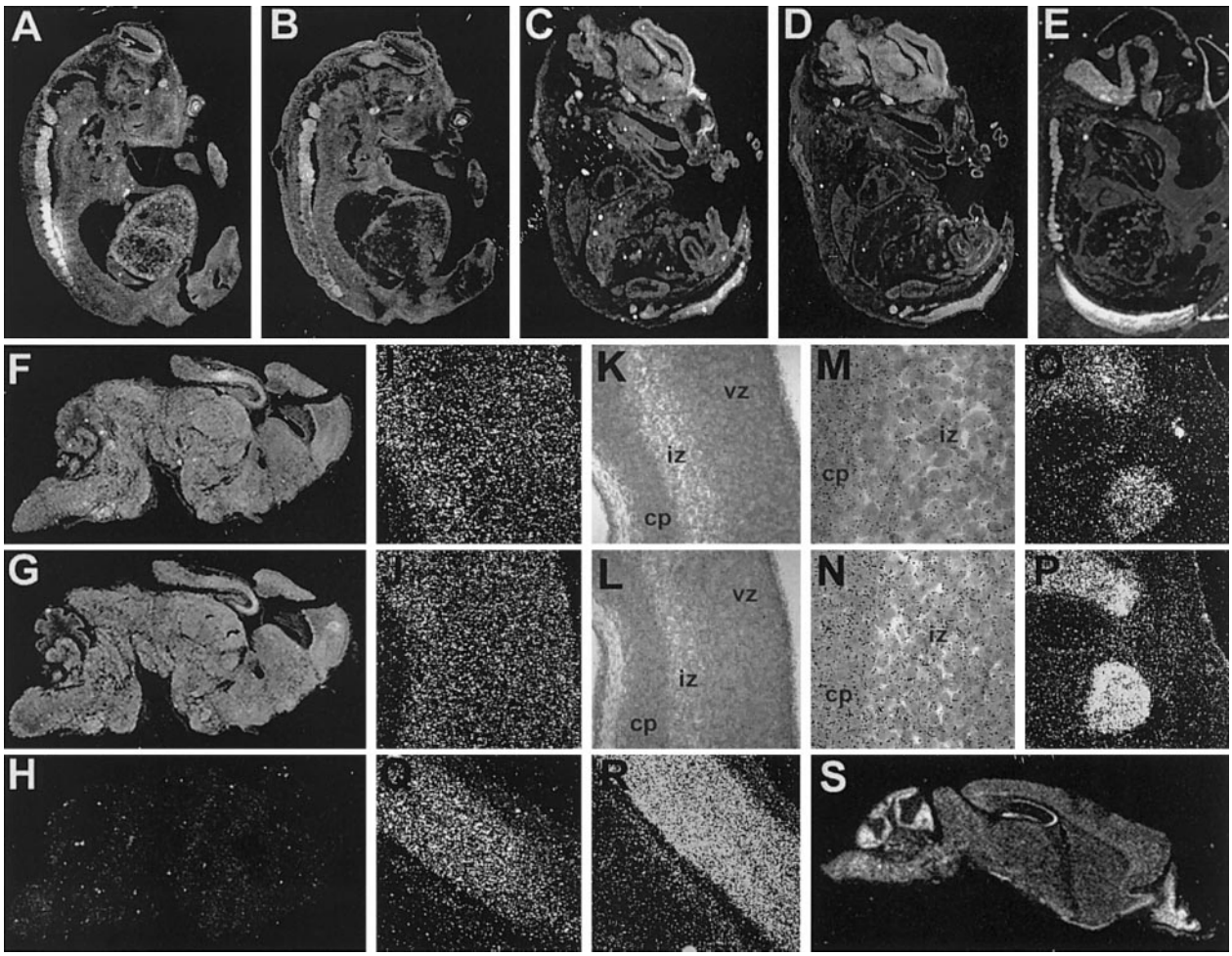

FIG. 3. Localization of μ1 and μ2 in mouse sections. Sagittal sections from mouse were hybridised with μ1 RNA (A, C, F, I, K, M, O, Q, and S) or with μ2 RNA (B, D, G, J, L, N, P, and R) probes. Their localization was compared with *doublecortin* (E). Different stages were studied: E12.5 (A, B), E14 (E), E15.5 (C, D), neonatal brain (F–H, H, sense probe), and adult brain (S). At E15.5, μ1 and μ2 showed higher expression in the developing cortex (I, J: dark field; K–N, bright field), the dorsal root ganglia (O, P) and in the developing spinal cord (Q, R). In bright field, the labeling is shown as deposited small black grains. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

Doublecortin, but with some partial overlap (Fig. 4C). Anti-α-adaptin gave a punctate labelling of the plasma membrane of both the soma and the neurites as seen with confocal microscopy (Fig 4K). This latter staining likely corresponds to endosomes and is consistent with the role of AP-2 in clathrin-mediated endocytosis. Thus, double-labeling experiments show that doublecortin and the AP complexes are expressed in some common regions of differentiating neurons (Figs. 4C, 4F, and 4L).

To determine whether the Doublecortin-AP-1 interaction depends on the ARF GTPase, we treated neurons in culture with brefeldin A. This drug is known to block the exchange of GTP to GDP, which results in the constitutive activation of the ARF GTPase (Robinson *et al.*, 1992), and under these conditions, AP-1 is dissociated from the membrane. Immunofluorescence data showed that, whereas γ staining became more diffuse, Doublecortin labelling was identical to the one obtained in untreated cells: this observation suggests that the Doublecortin localisation is not ARF-dependent (Figs. 4G and 4H). We also treated the cells with taxol or nocodazole. Whereas the taxol did not seem to have any major effect on γ or α localisation, the γ signal appeared less polarised in the presence of nocodazole (Fig. 4I). This latter observation highlights the relationship between the AP-1 complex and microtubules.

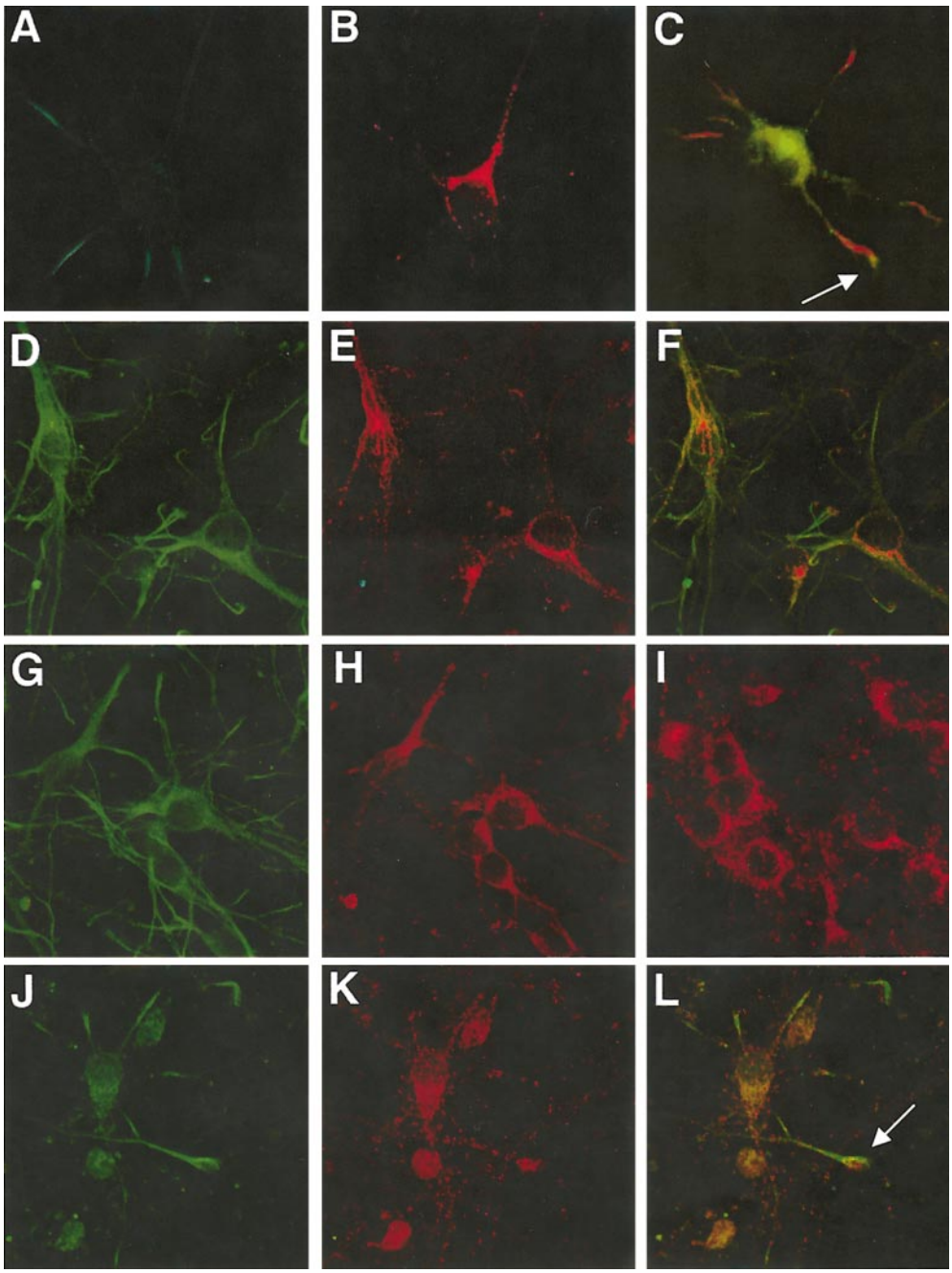

FIG. 4. Localization of AP-1 and AP-2 in primary cultures of neurons. Expression in neurons was detected with anti-dbcn pep (A, J) or purified anti-N-term (Doublecortin) (C, D, G) and monoclonal anti-γ-adaptin (B, C, E, H, I) or anti-α-adaptin (K). Doublecortin is labeled in green and the adaptins in red, except for C, in which Doublecortin is in red and γ in green. Double-labeling experiments show a partial colocalization between Doublecortin and γ (C, F) and Doublecortin and α (L). The treatment with brefeldin A induced a relocalization of γ (H) but had no effect on Doublecortin labeling (G), which suggests that Doublecortin-γ interaction is ARF-independent. In the presence of nocodazole, γ appeared more diffuse (I).

To study AP-1 and AP-2 localization in migrating neurons, we performed immunohistochemistry using mouse E15.5 sections. To detect AP-1 and AP-2 complexes we used anti-γ and anti-α or anti-β2, respectively. The regional distributions of γ and α or β2 examined in this way were found to be very similar to

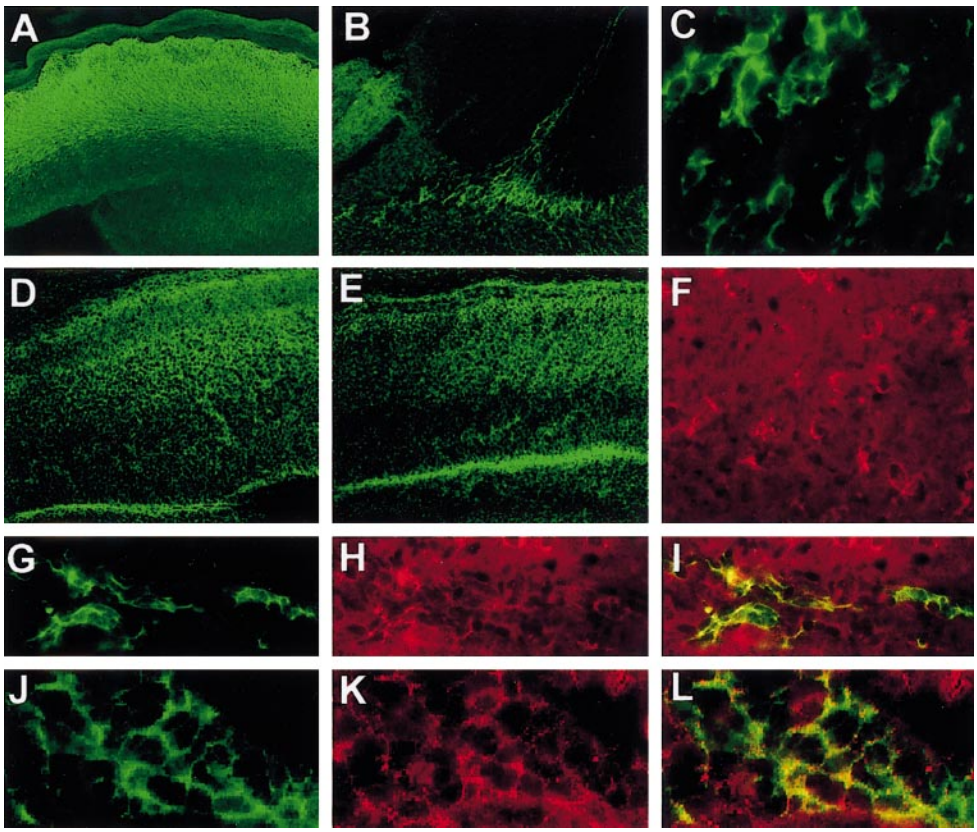

FIG. 5. Localization of AP-1 and AP-2 in migrating neurons. E15.5 mouse (A, D, E) and adult brain sections (B, C, F–L) were hybridized with anti-N-term (A–C, G, J), anti-β2 (D), polyclonal anti-γ-adaptin (E), or monoclonal anti-γ-adaptin (F, H, K). In adult brain, Doublecortin is only expressed in the cells of the rostral migratory stream (B, C, G, J). γ staining seemed less polarized in migrating neurons (H, K) than in mature neurons (F). In these cells, Doublecortin and γ staining partially overlaps.

the localization of *μ1* and *μ2* transcripts revealed by *in situ* hybridization (Fig. 5). In the developing cortex, γ (AP-1) and β2 (AP-2) were expressed in cells of the ventricular zone, the intermediate zone and the cortical plate (Figs. 5D and 5E). Their subcellular localisation in comparison to Doublecortin was difficult to observe because of the large number of cells stained (data not shown). Double-labeling experiments were thus performed using brain sections from adult mouse, where Doublecortin is only expressed in a small population of neurons migrating to the olfactory bulb: the rostral migratory stream (Figs. 5B, 5C, 5G, and 5J). In these cells, Doublecortin labeling is distributed in all intracellular regions. Interestingly, we found that γ staining was slightly different in cortical mature neurons (Fig. 5F) than in migrating neurons (Figs. 5H and 5K). It appeared more punctate and evenly distributed in the cells that express Doublecortin than in mature neurons, where the labeling was more compact and polarized. The double-labeling results hence showed a partial colocalization between Doublecortin and γ-adaptin in some regions of migrating cells (Figs. 5G–5L). Together these results suggest a possible colocalization between Doublecortin and γ-adaptin in migrating neurons.

Coimmunoprecipitation of Doublecortin, AP-1, and AP-2

To check whether the interaction between Doublecortin and AP-1 and/or AP-2 adaptor complexes exists *in vivo*, we performed coimmunoprecipitation experiments using E15 mouse embryos (Fig. 6). By immunoprecipitation with anti-Doublecortin antibodies, γ- and α-adaptin subunits were detected, but not δ-adaptin (AP-3 complex), which seems to be less expressed than γ and α in embryos at this stage. An anti-GST antibody was used as a negative control and no immunoprecipitation of either Doublecortin or the AP complexes was observed. We also attempted to coimmunoprecipitate Doublecortin with anti-γ and anti-α antibodies, but we

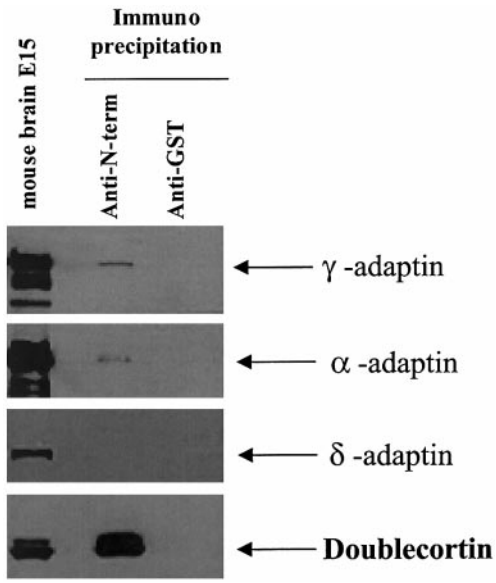

FIG. 6. Coimmunoprecipitation experiments. Extracts from E15 mouse embryos were immunoprecipitated with either anti-N-term or anti-GST antibodies. After transfer to nitro cellulose membrane and protein immunoblotting, blots were detected with anti-N-term (Doublecortin), anti-γ (AP-1), anti-α (AP-2), and anti-δ (AP-3). γ and α subunits were found to coimmunoprecipitate with Doublecortin. The AP-3 complex seemed to be less abundant than AP-1 and AP-2 in E15 mouse embryos.

were unable to detect convincing bands by Western blot analysis. This may be due to steric hindrance or to the involvement in this interaction of only a small percentage of each of the adaptor complexes. Nevertheless, our immunoprecipitation data using anti-Doublecortin antibodies confirm the results obtained using the two-hybrid system and *in vitro* binding assays and suggest that the Doublecortin–adaptor complex interaction can occur *in vivo*.

DISCUSSION

In this study, we searched for proteins interacting with Doublecortin using the two-hybrid system to better understand its role in neuronal migration. One clone was found to encode the human μ1 subunit of the adaptor complex AP-1, known to play a role in clathrin-dependent protein sorting. We characterized the Doublecortin–μ1 interaction and further tested for an interaction with the similar μ2 and μ3 subunits of the AP-2 and AP-3 complexes. These data showed a potential interaction between Doublecortin and μ1, and possibly μ2, but we found no evidence of an equivalent interaction with μ3. The spatial and temporal expression patterns of Doublecortin were compared with those of AP-1 and AP-2 adaptor complexes, including an examination of their subcellular localizations in primary cultures of neurons as well as in neurons of the developing cortex and in adult brain. Coimmunoprecipitation experiments from embryonic mouse brain were also used to verify the interactions. These combined data support the existence of an association *in vivo* between Doublecortin and μ1 and μ2. The interaction between A18108 and μ1/μ2 observed in the two-hybrid system is another interesting aspect. Indeed, this protein shares 75% homology with Doublecortin and contains a tyrosine-based sorting signal. It is also associated with microtubules but has a less restrictive pattern of expression than Doublecortin as it is also expressed in glial cells and in mature neurons. Thus, it is possible that A18108 replaces Doublecortin function in these cells. We are generating antibodies against this protein to better address these issues.

We previously demonstrated that Doublecortin is widely expressed in the developing nervous system from mouse embryonic day 11 until neonatal stages; but it is greatly down regulated after birth and is only expressed in the rostral migratory stream in the adult brain. In order to check that μ1 and μ2 were expressed in the same cells during development, we first performed *in situ* hybridization experiments using antisense RNA probes. Interestingly, although *μ1* and *μ2* were largely ubiquitous, a more prominent expression of both genes in the developing nervous system was obvious, and overlapped with Doublecortin expression. This may indicate that higher levels of AP-1 and AP-2 are required in young migrating and differentiating neurons over certain stages of development. Indeed, since neurons develop long leading processes and axons and dendrites, it may be expected that protein transport mechanisms play an important role within the cell. An enrichment of μ2 in the Drosophila developing nervous system has been previously documented (Zhang *et al.*, 1999); moreover, AP-2 and AP-3 complexes are known to be involved in mature nerve cell function (Gonzalez-Gaitan *et al.*, 1997; Shi *et al.*, 1998; Faundez *et al.*, 1998). Nevertheless, to our knowledge, there has been no previous report concerning AP-1 enrichment in the developing nervous system.

We performed immunofluorescence experiments to further study protein localisations in migrating and differentiating neurons of the developing and adult mouse brain. In agreement with *in situ* hybridization results, AP-1 and AP-2 displayed ubiquitous expression

patterns including the cells expressing Doublecortin. In primary neuronal cultures, Doublecortin and γ labelling partially overlapped in the proximal regions of growth cones. The growth cone is known to be an important region for endocytosis and recycling of cell adhesion molecules, as shown for L1 (Kamiguchi *et al.*, 1998, 2000). Altogether, these data suggest that the cellular and subcellular compartments where Doublecortin is present, *i.e.*, migrating neuronal cells and parts of the growth cones of differentiating neurons, are places where highly dynamic vesicular trafficking occurs.

It is known that there is a close relationship between microtubules and the Golgi network (reviewed by Thyberg *et al.*, 1999). Since Doublecortin is a MAP, an interaction with μ1 and/or μ2 may indicate a microtubule-dependent role in vesicle or tubule transport (Fig. 7). These data are consistent with a recent study that showed a direct interaction between the kinesin KIF13A and the AP-1 complex, which mediates the transport of mannose-6-phosphate receptors from the TGN to the plasma membrane. These results demonstrate that the AP complexes can function as motor adaptors in vesicular transport (Nakagawa *et al.*, 2000) and may further support an interaction between the AP complexes and other MAPs.

Both two-hybrid experiments and *in vitro* binding assays showed the importance of the C-terminus of Doublecortin for the interaction. This result may be in agreement with a microtubule-associated role of the Doublecortin-μ interaction since several reports have highlighted the important part played by the N-terminal part of Doublecortin in the interaction with microtubules (Horesh *et al.*, 1999; Sapir *et al.*, 2000; Taylor *et al.*, 2000). Tyrosine-based sorting signals, recognised by μ subunits, have previously been reported to be important for the recruitment of integral membrane proteins into clathrin-coated vesicles (Ohno *et al.*, 1995). We identified such a motif in the Doublecortin sequence and mutagenesis experiments confirmed its involvement in the interaction with both μ1 and μ2. Nevertheless, Doublecortin is not an integral membrane protein, which rules out that it is recruited into vesicle membranes in this way. However, several cytosolic proteins have been shown to act as connector molecules between adaptor complexes and receptors, and hence to influence endocytic and exocytic processes. Two well-characterized examples are Nef (Le Gall *et al.*, 1998; Piguet *et al.*, 1998) and the β-arrestins (Laporte *et al.*, 1999). One thus could assume that Doublecortin is such a connector molecule, recruited from the cytosol or attached to microtubules, which influences the trafficking of other integral membrane proteins by its interaction with either μ1 or μ2.

The importance of VLDLR, APOE-R2, cadherin-related neuronal receptors (CNR) and α3β1 integrin receptor for normal corticogenesis in the mouse has been previously highlighted (Trommsdorff *et al.*, 1999; D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Senzaki *et al.*, 1999; Dulabon *et al.*, 2000). In addition, another protein involved in neuronal migration, DabI has been shown to interact with a sorting signal NPxY present in the cytoplasmic domains of the VLDLR and APOE-R2 receptors (Trommsdorff *et al.*, 1999). This motif is also implicated in the internalisation and recycling of VLDLR and APOE-R2 via an interaction with either μ1 or μ2. Indeed, we have shown an interaction between μ1 and μ2 and both receptors using the two-hybrid system (unpublished data). Doublecortin may in some way influence this process by competing with the receptors for an interaction with the μ subunits (Fig. 7). It is also of interest that AP-2 complexes have been suggested to work as dimers (Owen *et al.*, 1998), which might allow simultaneous binding to different partners. These and other hypotheses remain to be elaborated, but may provide a link between the Reelin signalling pathway, vesicle transport and the microtubular network. Interestingly, LIS-1 has also been shown to interact with dynein, which is involved in cargo transport from the endoplasmic reticulum to the Golgi, further emphasising a role for vesicular trafficking in neuronal migration (Smith *et al.*, 2000; Liu *et al.*, 2000; Faulkner *et al.*, 2000).

Besides its known function as a MAP, the data described here point to a novel biological role for Doublecortin in protein transport or receptor recycling within migrating neurons. These findings provide new insights into understanding the physiopathology underlying cortical dysgenesis associated with the loss of function of Doublecortin.

EXPERIMENTAL METHODS

Yeast Cultures and Two-Hybrid Analysis

All the cloning procedures were carried out as described by Sambrook *et al.* (1989). The full-length *doublecortin* cDNA was cloned in pBTM116 downstream of the LexA binding domain (Bartel *et al.*, 1993). The human foetal brain cDNA library cloned in pACT2, was purchased from CLONTECH laboratories (MATCHMAKER) and screened according to manufacturer's instructions. This library was prepared from 19- to 22-week old embryos by a combination of oligo-dT and

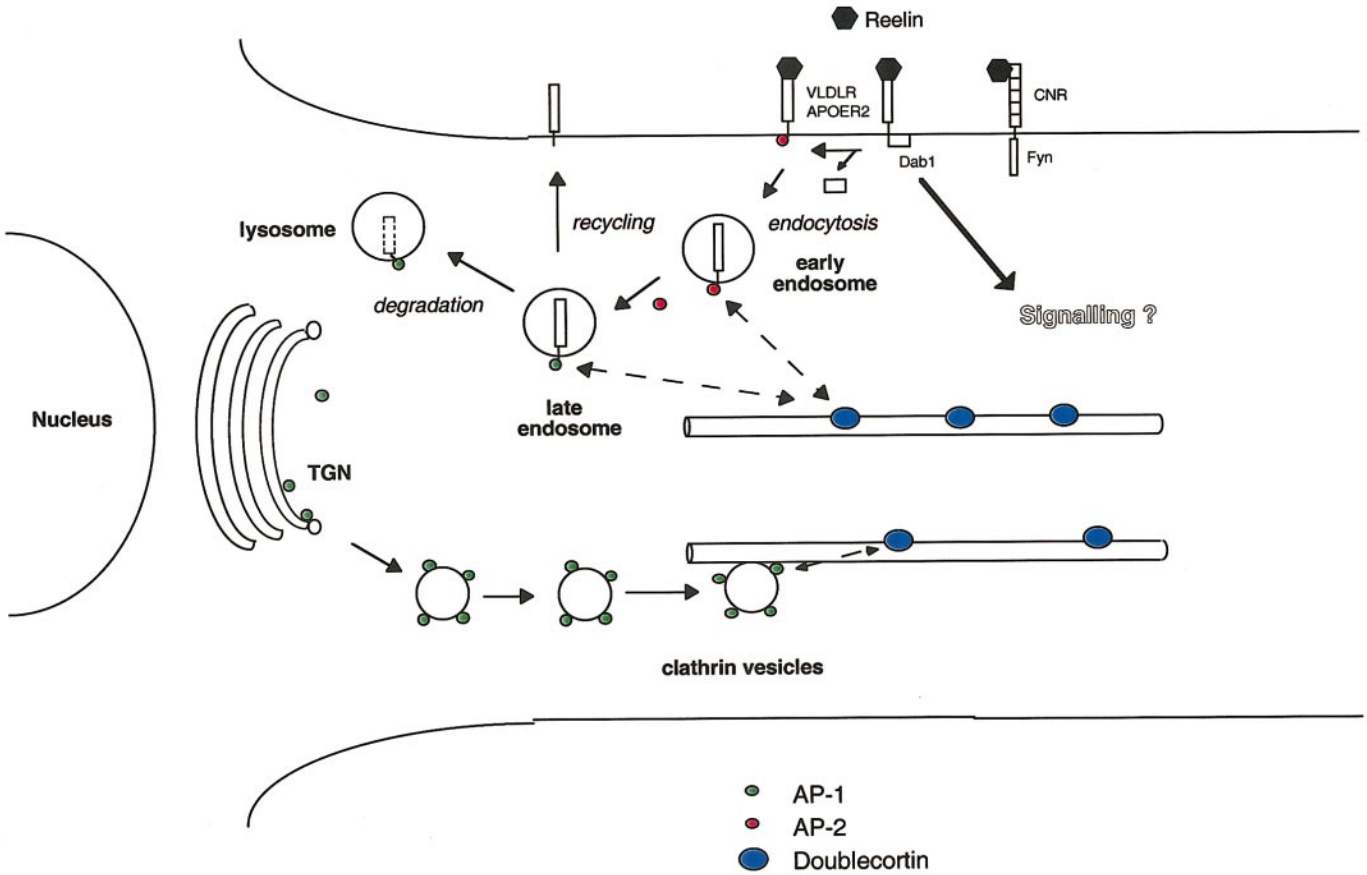

FIG. 7. A model for the role of the Doublecortin–AP interaction. Doublecortin may regulate the Reelin–Dab1 signalling pathway by influencing the internalisation or the recycling of VLDLR and APOER2 receptors. Alternatively, although Doublecortin is not a motor protein, interaction between AP-1 and Doublecortin may occur in vesicular trafficking involving microtubules.

random-priming, followed by orientated cloning. The *Saccharomyces cerevisiae* L40 strain (*MATa, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-lacZ*) was cotransformed with the plasmids using the lithium acetate procedure (Bartel *et al.*, 1995). Transformants were grown at 30°C on plates lacking tryptophan, leucine and histidine. His^+ colonies were screened for activation of the lacZ reporter gene by testing β-galactosidase activity using replica plating (lacZ test). Library plasmids from positive clones were rescued in *Escherichia coli* HB101 by plating on leucine-free medium. Each positive clone was then sequenced using an ABI 373 machine and a DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) with vector specific primers.

Sequenced plasmids were transformed into the yeast strain AMR70 (*MAT*α) to check the specificity of the interaction. The resulting cells were mated with L40 transformed with the original and other unrelated baits (lamin C and RAS) cloned in pBTM116. Mating was carried out by replica plating on YPD medium, then diploids were selected on medium lacking tryptophan and leucine. Histidine and lacZ tests were performed as above.

To compare interactions between Doublecortin, a homologous protein A18108 and the μ subunits, the full-length A18108 cDNA (lacking the first 12 amino acids) was cloned in pBTM116. pSG μ1, μ2 and pACT2 μ1, μ2 (mouse cDNA), μ3A and μ3B (rat cDNA) constructs were kindly provided by S. Benarous and were originally a gift from J. S. Bonifacino (Ohno *et al.*, 1995). The plasmid pLex-HIV-1 LAI construct was described by Berlioz-Torrent *et al.* (1999). RAS and RAF, respectively, cloned in pBTM116 and pGAD424 were used as controls.

Site-directed mutagenesis was performed using the Quik Change Mutagenesis kit (Stratagene).

In Vitro Binding Assay

GST fusion proteins were expressed in *E. coli* BL21 and purified according to the manufacturer's instructions. *In vitro* transcription and translation of PSG μ1 and μ2 were performed using the Promega TNT/T7 rabbit reticulocyte lysate system with [^{35}S]methionine for 90 min at 30°C. Eight microliters of radioactive labeled product were incubated with 10 μl of glutathione–Sepharose 4B beads coated with 10 μg of GST fusion protein in 200 μl of buffer (50 mM Tris–HCl pH 7.5, 350 mM NaCl, 1% Triton X-100) for 2 h at 4°C. The beads were washed five times with the same buffer and resuspended in 40 μl of SDS sample buffer. Samples were separated by SDS/PAGE followed by autoradiography.

In Situ Hybridization

μ1 and μ2 cDNA were cloned in pBluescript and sense and antisense probes were generated by *in vitro* transcription. Mouse embryos and adult brains were fixed in 2–4% paraformaldehyde, cryoprotected with 30% sucrose in phosphate buffer, frozen with isopentane, and cut into ~20-μm-thick sections. Slides were incubated with α^{35}S-labeled RNA probes in a 50% formamide solution at 65°C. Washes were performed in successively stringent SSC solutions, with a final wash at 0.1× SSC at 60°C. Slides were then dipped in diluted Kodak NTB2 emulsion and exposed for 24–48 h. Emulsions were developed and sections counterstained with toluidine blue, mounted in Eukitt and examined under light microscopy. *In situ* hybridizations for *doublecortin* RNA probes were performed as previously described (Francis *et al.*, 1999).

Antibodies

The production of polyclonal antibodies against Doublecortin has been reported elsewhere (Francis *et al.*, 1999). We used anti-dbcn pep produced from synthetic peptides and the affinity-purified anti-N-term antibody directed against the N-terminal part of the protein. Polyclonal antibodies against δ, γ, and β2 were kindly provided by M. S. Robinson. Monoclonal antibodies: anti-α-adaptin (AC1-M11) and anti-γ-adaptin (A36120) were respectively purchased from Alexis Corporation and Transduction Laboratories.

Cell Cultures and Immunofluorescence Staining

Primary neuronal cultures were prepared from foetal mouse brain at 15 days of gestation as previously described (Berwald-Netter *et al.*, 1981). In some experiments, neurons were treated for 15 min at 37°C with brefeldin A (10–40 μg/ml, Sigma) or for 4 h with taxol (10 μg/ml) or nocodazole (3.5 or 8 μM). Neurons grown on coverslips were fixed for 10 min at −20°C in methanol/acetone. After four washes in phosphate-buffered saline (PBS), the cells were prehybridized for 1 h with TBST (10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% Triton X-100) containing 2% goat serum, and then incubated overnight at 4°C with the primary antibody (anti-α, 1:100, monoclonal anti-γ, 1:100, anti-Doublecortin pep, 1:300, affinity-purified anti-N-term, 1:200). The cells were washed three times in TBST and incubated for 1 h at room temperature with affinity-purified secondary antibodies (FITC conjugated goat anti-rabbit Ig [Biosys., Compiègne, France] or Texas red-conjugated goat anti-mouse Ig [Jackson ImmunoResearch, West Grove, PA]) used at 1:200 dilution. After three washes, coverslips were mounted in Mowiol and examined with a confocal microscope MRC-1000 (Bio-Rad) equipped with epifluorescence illumination.

Immunohistochemistry

Mouse embryos and adult brains were cryoprotected with 30% sucrose in phosphate buffer, frozen with isopentane and sectioned (~15–20 μm thick). Sections were fixed for 10 min in methanol at −20°C and washed with phosphate buffered saline (PBS). They were prehybridized for 1 h on ice with TBST containing 2% goat serum, washed in TBST and incubated overnight at 4°C with the primary antibody (polyclonal anti-γ, 1:100, monoclonal anti-γ, 1:100, anti-α, 1:100, anti-β2, 1:100, affinity-purified anti-N-term, 1:200). The following steps were done as previously described for immunocytochemistry. Sections were examined with a Zeiss microscope equipped with epifluorescence illumination.

Coimmunoprecipitation

Mouse brains taken from E15 embryos were crushed in liquid nitrogen and resuspended in 10 volumes of IP buffer (50 mM Tris–HCl pH8, 150 mM NaCl, 1% NP-40 and protease inhibitors). After a centrifugation at 50,000*g* for 30 min at 4°C, 2-ml aliquots of supernatant were incubated with protein A-agarose (Sigma) for 2 h at 4°C and spun at 10,000*g* for 5 min at 4°C to eliminate the immunoglobulins. Aliquots of the resulting supernatant were incubated at 4°C with the antibodies of interest (affinity-purified anti-N-term or affinity-purified anti-GST, 1:10). Two hours later, protein A–agarose

was added and samples were incubated overnight at 4°C. Immunoprecipitates were washed approximately 10 times with IP buffer, then proteins were separated on SDS–polyacrylamide gels. Transfer to nitrocellulose membrane and protein immunoblotting were carried out following standard protocols (Sambrook *et al.*, 1989). For Western blot detection, polyclonal anti-γ-adaptin was used at 1:500, anti-α at 1:200, anti-δ at 1:200, and nonpurified anti-N-term at 1:5000.

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