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Nckβ Interacts with Tyrosine-Phosphorylated Disabled 1 and Redistributes in Reelin-Stimulated Neurons

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The tyrosine phosphorylation sites of the Disabled 1 (Dab1) docking protein are essential for the transmission of the Reelin signal, which regulates neuronal placement. Here we identify Nckβ as a phosphorylation-dependent, Dab1-interacting protein. The SH2 domain of Nckβ but not Nckα binds Dab1 phosphorylated on the Reelin-regulated site, Y220, or on Y232. Nckβ is coexpressed with Dab1 in the developing brain and in cultured neurons, where Reelin stimulation leads to the redistribution of Dab1 from the cell soma into neuronal processes. We found that tyrosine-phosphorylated Dab1 in synergy with Nckβ disrupts the actin cytoskeleton in transfected cells. In Drosophila melanogaster, exogenous expression of mouse Dab1 causes tyrosine phosphorylation site-dependent morphological changes in the compound eye. This phenotype is enhanced by overexpression of the Drosophila Nck protein Dock, suggesting a conserved interaction between the Disabled and Nck family members. We suggest a model in which Dab1 phosphorylation leads to the recruitment of Nckβ to the membrane, where it acts to remodel the actin cytoskeleton.

The Dab1 gene, which encodes a cytoplasmic docking protein, plays an essential, cell-autonomous role during brain development (15, 17, 26, 44, 51). Animals that lack a functional Dab1 gene have anomalies in the formation of neuronal laminae in the cerebral cortex, hippocampus, and cerebellum, as well as defects in the olfactory bulbs and spinal cord (26, 44, 45, 51, 52). In mammals, neuronal placement is regulated by a number of genes encoding transcription factors, extracellular matrix proteins, receptor molecules, kinases, and actin and tubulin binding proteins (16, 19, 36, 40, 42). Among these, three have been shown to affect Dab1 function through a linear signaling pathway, the Reelin gene and two genes encoding members of the low-density lipoprotein (LDL) receptor family, VLDLR and ApoER2 (19, 40).

The protein encoded by the Reelin gene, a secreted glycoprotein produced in discrete regions during the developing brain (10, 38), physically interacts with the extracellular domains of the VLDLR and ApoER2 transmembrane proteins expressed on migrating neurons (2, 9, 20). These partially redundant receptors bind to an N-terminal domain in Dab1, the PTB domain, through an N-P-X-Y peptide sequence in their cytoplasmic domains (29, 46, 47). The formation of the Reelin-receptor interaction is a requisite for Reelin-induced Dab1 tyrosine phosphorylation. This was shown by blocking the binding of Reelin to the receptors biochemically and, more recently, by Reelin stimulation experiments with neurons cultured from mice that lacked both receptors, where Dab1 phosphorylation did not occur (2, 9, 20). During brain development, the level of Dab1 tyrosine phosphorylation is lower in Reelin mutants than in wild-type animals (27). Taken together, these data point to Dab1 tyrosine phosphorylation as an outcome of Reelin action on neurons during the formation of the nervous system.

Five residues proximal to the Dab1 PTB domain account for all of the tyrosine phosphorylation sites utilized during brain development (28). Reelin induces phosphorylation of two of these, Y198 and Y220, in vitro (32). A third tyrosine, Y232, is not detectably phosphorylated in cultured neurons but is phosphorylated in embryonic brain (B. Howell, unpublished results) and is phosphorylated in transfected cells in the presence of the Src kinase (32). The phosphorylation sites are required to rescue the Dab1 null phenotype (28). An expression cassette that contains either a wild-type or mutant cDNA for Dab1 restores expression of the Dab1 protein when integrated into the dab1 gene locus. However, only the wild-type Dab1, not the tyrosine phosphorylation site-substituted Dab1 (Dab1-5F), is capable of restoring normal brain development. Understanding the role of the Dab1 phosphorylation sites is therefore central to the resolution of the downstream consequences of Reelin action.

The SH2 domain-containing proteins Src, Fyn, and Abl have previously been shown to bind Dab1 in a phosphorytrosine-dependent manner in vitro (25). Recently, it was revealed that the Src family kinases, including Src, Fyn, and Yes, are the predominant kinases required for the high stoichiometry of Dab1 phosphorylation observed during development and Reelin stimulation (1, 3). The activity of the Src, Fyn, and Yes kinases is increased by Reelin treatment of primary neurons in a manner that requires Dab1 for full activation (1, 3), and they therefore likely act upstream and downstream of Dab1 tyrosine phosphorylation.

Here we identify a novel phosphotyrosine-dependent Dab1-binding partner, the SH2-SH3 adaptor molecule Nckβ. The Dab1-Nckβ interaction required the Nckβ SH2 domain and Dab1 tyrosine phosphorylation sites. Reelin stimulation resulted in the redistribution of Nckβ into the processes of cul-
tured neurons. In addition, we show that overexpression of Nckβ in cultured fibroblasts alters the actin cytoskeleton when coexpressed with tyrosine-phosphorylated Dab1. In *Drosophila melanogaster*, the conserved Nck family member Dock is thought to regulate cytoskeletal dynamics required for axon guidance and target recognition (13, 14). We show that overexpression of the *Drosophila* Nck protein Dock enhances phenotypes caused by exogenous expression of mouse Dab1 in the *Drosophila* eye. This enhancement is dependent upon the Dab1 tyrosine phosphorylation sites. We propose that during mouse development, tyrosine-phosphorylated Dab1 recruits Nckβ to membrane compartments where this complex acts to remodel the actin cytoskeleton.

**Materials and Methods**

**Plasmids.** The vector pB TM116-Dab PTB-5Y (Src) was constructed by ligating the *SalI*-cut parental vector pB TM116 (Src) (gift from M. Lioubl [35]) with the *XhoI*-digested PCR product from the mouse Dab1 cDNA and primer pair BTMS (GCCG CTGAGGAGTAGTCACTGAGCAGA) and B TMSY (GCCGCT GCAGCT AACTCGAGGGTGGACATGTCTCC). This construct encodes the LexA DNA binding domain fused to residues 1 to 257 of Dab1, which includes the PTB domain and the tyrosine phosphorylation sites.

The GSTDab1-276 fusion proteins were constructed to express residues 1 to 276 of Dab1 by ligating BamHI- and EcoRI-digested PCR products of the primer pair DabBamATG (GCCG CTGACGAGTAGTCACTGAGCAGA) and DabStop276 (GCCGAATTCCTAGGACGACGGGAG) into the respective sites in pGex-2T (Pharmacia). The generation of the tyrosine to phenylalanine substitutions in Dab1 has been described elsewhere (28). The pDsDab1RFP and pDsDab1RFP-5F clones were generated by subcloning the mouse Dab1 cDNA from the plasmid pB SRA555 (25) or the Dab1-5F mutant, respectively (28), into the pDsRed2 vector (Clontech) between the *XhoI* and BamHI sites to produce a full-length fusion protein with the red fluorescent protein (RFP) C-terminal to Dab1 residues 1 to 555. The restriction sites were introduced by PCR with primers GCCGCTGAGGAGTAGTCACTGAGCAGA and GGGCAATTCCTAGGACGACGGGAG into the respective sites in the pGex-2T vector. The vector pB TM116-Dab PTB-5Y (Src) was constructed by ligating the *SalI*-cut parental vector pB TM116 (Src) (gift from M. Lioubl [35]) with the *XhoI*-digested PCR product from the mouse Dab1 cDNA and primer pair BTMS (GCCG CTGAGGAGTAGTCACTGAGCAGA) and B TMSY (GCCGCT GCAGCT AACTCGAGGGTGGACATGTCTCC). This construct encodes the LexA DNA binding domain fused to residues 1 to 257 of Dab1, which includes the PTB domain and the tyrosine phosphorylation sites.

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**Western Blotting.** Yeast cell lysates were tested for the expression of Src by Western blotting with various antibodies. A single positive clone was selected and the plasmid was purified and sequenced with an ABI Prism sequencer with both the Gad GH 5′- and 3′-flanking sequences.

**Northern Blotting.** Yeast cells were transformed with the *GAL4-*inducible vector pBD, which expresses proteins as fusions with the *GAL4* activation domain. The pBD library (29) in the Clontech pGADGH vector, which expresses proteins as fusions with the *GAL4* activation domain, was transformed into the *S. cerevisiae* strain L40. Colonies were tested for the expression of Src by Western blotting yeast cell lysates with the antiphosphotyrosine antibody 4G10 (Upstate Biotechnology). A single positive clone was selected and the plasmid was purified and sequenced with an ABI Prism sequencer with both the Gad GH 5′- and 3′-flanking sequences. The plasmid was transformed into the *S. cerevisiae* strain L40. Colonies were tested for the expression of Src by Western blotting yeast cell lysates with the antiphosphotyrosine antibody 4G10 (Upstate Biotechnology). A single positive clone was selected and the plasmid was purified and sequenced with an ABI Prism sequencer with both the Gad GH 5′- and 3′-flanking sequences. The plasmid was transformed into the *S. cerevisiae* strain L40. Colonies were tested for the expression of Src by Western blotting yeast cell lysates with the antiphosphotyrosine antibody 4G10 (Upstate Biotechnology). A single positive clone was selected and the plasmid was purified and sequenced with an ABI Prism sequencer with both the Gad GH 5′- and 3′-flanking sequences.
Drosophila stocks and culture. All fly culture genetics and production of the transgenics were done according to standard protocols. The w1118 strain was used to generate the transgenic lines. Multiple independent transformant lines were established and analyzed by Western blotting and fluorescence microscopy to assay protein expression. The UAS-Dock transgenic flies were a gift from L. Zipursky. Lines homozygous for UAS-RFP, UAS-Dab1-RFP, UAS-Dab1-5F, UAS-Dock, UAS-Dock; UAS-Dab1-RFP, and UAS-Dock; UAS-Dab1-5F were generated and crossed with flies homozygous for GMR-GAL4 to generate the flies shown in Fig. 10.

Scanning electron microscopy. Adult flies were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C. Flies were washed with 0.1 M cacodylate buffer and dehydrated through a graded series of ethanol washes. Next, flies were incubated in hexamethyldisilazane (HMDS; Electron Microscopy Sciences [EMS]) twice. Samples were air dried under low vacuum, mounted with silver paint conducting paste (EMS), sputter coated with Pd-Au wire in a Denton vacuum evaporator, and visualized with a Hitachi S570 scanning electron microscope.

RESULTS

Identification of Nckβ as a Dab1 binding protein. Since the Dab1 tyrosine phosphorylation sites are critical for Dab1 function, we sought to identify binding partners of Dab1 dependent on these sites. We did this with a modified yeast two-hybrid screen, in which expression of the tyrosine kinase Src ensured Dab1 tyrosine phosphorylation (31). This screen was predicted to identify proteins that interact with Dab1 in both a phosphotyrosine-dependent and -independent manner.

The PTB domain and downstream tyrosine phosphorylation sites were fused with the LexA DNA binding site and used to screen a library of neonatal mouse brain cDNAs expressed as fusions with the Gal4 transactivator. Three interacting proteins were identified. Two of these were members of the amyloid precursor protein (APP) family and have been identified from...
other Dab1 interaction screens (23, 29, 46). The cytoplasmic domains of APP and APLP1 have been shown to interact with the isolated Dab1 PTB domain in the absence of tyrosine phosphorylation (23, 29, 46). The third clone, Nck/H9252, has not previously been shown to interact with Dab1. This Nck/H9252 isolate contains residues 201 through the termination codon, including the third SH3 domain as well as the sole SH2 domain.

**FIG. 2.** Nckβ coimmunoprecipitates with tyrosine-phosphorylated Dab1 but not the unphosphorylated Dab1-5F mutant. Immunoprecipitations with anti-Dab1 or anti-HA antibody, as indicated to the left of the panels, were done from lysates of HEK293T cells transfected with SrcY527F (lanes 1 to 5) and combinations of Nckβ (lanes 1, 3, and 5) and Dab1 wild-type (lanes 2 and 3) or Dab1-5F (lanes 4 and 5). Immunoprecipitation of Dab1 with an anti-C-terminal antibody followed by Western blotting with anti-Dab1 (B3) (top panel) shows that transfected cells express at least as much mutant Dab1-5F as wild-type Dab1, but immunoblotting with antiphosphotyrosine antibody (4G10; second panel) shows that only the wild-type Dab1 was tyrosine phosphorylated. Equal amounts of Nckβ were immunoprecipitated from all HA-Nckβ-transfected cells (third panel, lanes 1, 3, and 5), yet Dab1 was only detected in anti-HA immunocomplexes (bottom panel) from cells expressing both Nckβ and wild-type Dab1 (lane 3).

**FIG. 3.** Nckβ is expressed in Dab1-expressing Reelin-responsive cells. (A) The Nckβ protein is detected in the cell bodies of Purkinje cells, which also express Dab1 (B) in the brains of adult mice. (C) Both Dab1 and Nckβ are absent from the internal granule cell layer, which is apparent below the Purkinje cells with the nuclear indicator DAPI. Bar, 10 μm.

**FIG. 4.** Nckβ is redistributed into neuronal processes after Reelin stimulation. (A and B) In cultures treated with control conditioned medium for 30 min, Nckβ (A; green) is found predominantly in the cell soma. (C and D) After Reelin treatment, Nckβ (C; green) is apparent in the majority of processes. Processes were detected (B and D) with fluorescently labeled phalloidin to visualize filamentous actin (red), and nuclei were detected with DAPI. Bar, 20 μm.

**FIG. 4.** Nckβ is redistributed into neuronal processes after Reelin stimulation. (A and B) In cultures treated with control conditioned medium for 30 min, Nckβ (A; green) is found predominantly in the cell soma. (C and D) After Reelin treatment, Nckβ (C; green) is apparent in the majority of processes. Processes were detected (B and D) with fluorescently labeled phalloidin to visualize filamentous actin (red), and nuclei were detected with DAPI. Bar, 20 μm.

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fusion with Dab1 that expresses the same region used in the two-hybrid screen, residues 1 to 257 of Dab1, was used either unphosphorylated or after tyrosine phosphorylation by Abl kinase to test the binding of Nckβ/H9252 from lysates of transfected HEK293T cells. The same strategy was used to test binding of the closely related adaptor molecule Nckα/H9251. The GST-Dab1 fusion protein was immobilized on Sepharose and incubated with lysates from HEK293T cells transfected with HA-tagged versions of either Nckα, Nckβ, Nckβ-R312K, that has a defective SH2 domain, or Nckβ-W39,149,235K, which has three defective SH3 domains (Fig. 1).

Nckβ bound to the tyrosine-phosphorylated Dab1 fusion but not the unphosphorylated fusion. Interestingly, Nckα did not bind to the Dab1 fusion protein regardless of its phosphorylation. We sequenced the HA-Nckα-expressing vector to ensure that the SH2 domain was intact and found it to be free of errors. Mutation of the SH2 domain of Nckβ prevented binding, but mutation of all three SH3 domains of Nckβ had little or no effect on the interaction with tyrosine-phosphorylated Dab1. Nckβ bound well to tyrosine-phosphorylated GST-Dab1 fusions with substitutions of tyrosines to phenylalanine at positions 185, 198 and 200, 220, or 232, suggesting that the interaction was not mediated by a single phosphorylation site on Dab1. We found, however, that the Dabl Y220,232F double mutant did not support binding to Nckβ. All GST-Dab1 fusions, including the Y220,232F mutant, were tyrosine phosphorylated by Abl kinase (Fig. 1). This shows that phosphorylation of either Y220 or Y232 was sufficient for the interaction.

To determine if the affinity of the Dab1 and Nckβ interaction was high enough to support binding in vivo, we assayed for coimmunoprecipitation of Dab1 and Nckβ from transfected cells. This assay would also help determine if the C-terminal sequences absent from both the yeast two-hybrid screen and the in vitro binding assay might support binding to Dab1 in a phosphotyrosine-independent manner. To maximize Dab1 tyrosine phosphorylation, we expressed Dab1 in HEK293T cells in the presence of the activated Src mutant SrcY527F. Dab1 expression and tyrosine phosphorylation were examined by immunoprecipitating Dab1 and Western blotting with anti-Dab1 and anti-phosphotyrosine antibodies. Equal levels of Dab1 were observed in the Dab1 immunoprecipitates from cells transfected with cDNAs for Dab1-wt or Dab1-5F, but only Dab1-wt was tyrosine phosphorylated (Fig. 2), consistent with previous studies (28, 32).

Equal amounts of HA-tagged Nckβ (HA-Nckβ) were recovered from all HA-Nckβ-transfected cells by immunoprecipitation with the anti-HA antibody (Fig. 2). Dab1, however, was only detected in the anti-HA immunoprecipitate from cell lysates having both wild-type, tyrosine-phosphorylated Dab1 and Nckβ. The unphosphorylated Dab1-5F protein was not detected in anti-HA immunoprecipitates, suggesting that Dab1 tyrosine phosphorylation sites are required for the interaction. Dab1 was not detected in immunocomplexes from cells not expressing HA-Nckβ, suggesting that Dab1 does not precipitate nonspecifically under the assay conditions. We were unable to detect HA-Nckβ in the Dab1 immunoprecipitates. This may suggest that only a small fraction of HA-Nckβ interacts with Dab1 under these conditions.

Other phosphoproteins may compete with Dab1 for binding to the Nckβ SH2 domain. The requirement of the tyrosine phosphorylation sites for the coimmunoprecipitation supports the evidence from the in vitro study that the SH2 domain, and not the SH3 domains, of Nckβ is sufficient for binding to Dab1.

![Figure 5](http://mcb.asm.org/)

**FIG. 5.** Nckβ colocalizes with Dab1 in process termini after Reelin stimulation. (A to C) Nckβ (green; A and C) is predominantly found in the soma of primary neurons from Reelin mutant animals fixed after 10-min treatments with control conditioned medium, while Dab1 (red; B and C) was found throughout the neuron. Colocalization between Nckβ and Dab1 was observed in the cell soma but not in the processes (yellow; C). (D to F) After treatment with Reelin-conditioned medium for 10 min prior to fixation, Nckβ (D and F) was detected in processes with Dab1 (E and F), and colocalization (F) was apparent near the process termini. Bar, 20 μm.
In addition, the SH3 domains of Nckβ did not support a high-affinity interaction with C-terminal residues of Dab1 absent from the yeast two-hybrid screen or the GST-Dab1 binding assay.

Nckβ is expressed in embryonic neurons. As a minimum requirement for Nckβ to collaborate with Dab1 in Reelin signa
tion transduction, it must be expressed in Reelin-responsive
cells in the developing brain. We therefore examined Nckβ
protein levels in mouse brain by Western blot at E16.5, a time
when Dab1 phosphorylation levels have been shown to be
Reelin dependent (27). A single band corresponding to Nckβ
was detected in total brain lysates of animals wild-type, het-
erozygous, or homozygous for mutations in the Reelin gene
data not shown). The protein level did not vary between
samples, suggesting that Nckβ protein levels are not down-
regulated by Reelin signaling, unlike Dab1 (41). The Nckβ
antibody (Upstate) was raised to peptide sequences not found
in Nckα, and it did not recognize anti-HA immunoreactive
HA-Nckα in Western blots (data not shown). This demon-
strates that Nckβ is expressed in the embryonic brain at times
when the Reelin signaling pathway is regulating neuronal po-
tioning and that the antibody is specific.

We detected Dab1 expressed broadly in the cerebral cortex
of embryonic animals at E15.5 (data not shown), suggesting
that it is expressed in the correct cell populations to play a role
in Reelin signaling. In addition, we detect Nckβ expression
in the adult cerebellum, where it is restricted to Purkinje cells
(Fig. 3). Purkinje cells represent another Dab1-expressing,
Reelin-responsive cell type that is not appropriately positioned
in Dab1 or Reeler mutant animals.

Reelin stimulation induces Nckβ redistribution. A tyrosine
kinase-based signaling cascade has previously been shown to
cause a redistribution of Nckβ (8). Since regulated subcellular
compartmentalization is a critical means of regulating protein
function, we examined the localization of Nckβ before and
after Reelin stimulation. In the absence of the Reelin signal,
the majority of Nckβ was localized to the cell soma of primary
forebrain neurons grown 1 day in vitro (Fig. 4). The majority of
neurites expressed very little Nckβ throughout their length,
and the leading edges of the processes were devoid of this
adaptor. After 30 min of Reelin stimulation, however, the
Nckβ distribution was changed. Neurites of Reelin-treated
cells contained Nckβ, and the fluorescence was less intense in
the cell soma compared to control-treated neurons (Fig. 4).
Nckβ was observed to be concentrated at the leading edge of
processes, at levels comparable to that observed in the cell soma,
in approximately 5% of Reelin-treated neurons. The translo-
cation was very rapid, with Nckβ-enriched regions
found at the tip of processes 10 to 15 μm away from the soma
after 10 min of stimulation with Reelin-enriched medium.
Typically, only one process per cell was observed to have a Nckβ-
enriched region, and Dab1 was found to colocalize with Nckβ
in these distal sites in Reelin-treated cultures (Fig. 5).
Nckβ did not colocalize with Dab1 at the leading edge or
growth cones of control-treated cells (Fig. 5). With longer
times of stimulation, the percentage of neurons expressing the
Nckβ-enriched regions was reduced (data not shown). In con-
trast, the process termini of cultured neurons treated with
control-conditioned medium showed low levels of Nckβ (Fig.
5). Reelin stimulation of neurons grown for 7 days in vitro led

![Fig. 6. Mouse Dab1RFP fusion is tyrosine-phosphorylated when expressed in Rat-2 cells and fly eyes. (A) Cell lysates from Rat-2
fibroblasts transfected with Dab1GFP (lanes 1 and 4), Dab1-wt (lanes
2 and 5), or Dab1RFP (lanes 3 and 6) were immunoprecipitated with
anti-Dab1 C-terminal antibody. Western blotting with anti-Dab1 anti-
body (lanes 1 to 3) showed that comparable levels of Dab1 were
expressed, but in the antiphosphotyrosine Western blot (lanes 3 to 6),
only Dab1RFP (lane 6) was detected. (B) Expression of Dab1RFP and
Dab1RFP-5F was detected in total cell lysates of flies with the geno-
types UAS-Dab1RFP/GMR-GAL4 (lane 1), UAS-Dab1RFP-5F/GMR-
GAL4 (lane 2), and UAS-Dab1RFP-5F/UAS-Dab1RFP-5F/GMR-
GAL4 (lane 3) to compare the levels of expression of the wild-type and
Dab1-5F mutant flies as adults. Expression of both of these protein
products was also observed by Western blot in wandering third-instar
larvae and pupae (not shown). Lysates from UAS-Dab1RFP/GMR-
GAL4 (lane 4) and UAS-Dab1RFP-5F/GMR-GAL4 (lane 5) flies were
immunoprecipitated with anti-Dab1 antibody (Chemicon) and West-
ern blotted for antiphosphotyrosine (4G10; Upstate Biotechnology),
showing that the Dab1RFP fusion but not the Dab1RFP-5F protein is
tyrosine phosphorylated when expressed in the adult fly. The same was
observed for wandering third-instar larvae and pupae (not shown).]
to the redistribution of Nckβ away from the cell soma into the processes, but we did not detect the Nckβ-enriched regions with these older cultures (data not shown).

**Nckβ overexpressed in the presence of phosphorylated Dab1 alters the actin cytoskeleton.** The Nck family of adaptor proteins plays a conserved role in linking extracellular signals to actin cytoskeletal remodeling (5, 33). To examine if tyrosine-phosphorylated Dab1 could substitute for an external signal and lead to Nckβ-dependent cytoskeletal rearrangement, we established a cell culture model with Rat-2 fibroblasts. These cells were chosen based on their flat morphology and the relative uniformity of their actin profiles.

To express tyrosine-phosphorylated Dab1 without an activated kinase, we used a Dab1 fusion with red fluorescent protein (Dab1RFP) that we have recently found to be tyrosine phosphorylated in cultured cells (Fig. 6A). Dab1-wt protein, expressed from the RFP vector with a stop codon introduced to prevent fusion to RFP, was not phosphorylated under these culture conditions. Dab1GFP was also not tyrosine-phosphorylated when expressed in Rat-2 cells (Fig. 6A). The tyrosine phosphorylation of Dab1RFP is likely a consequence of multimerization through fusion to the RFP tetramer (V. Strasser, D. Fasching, C. Hauser, H. Mayer, H. H. Bock, T. Hiesberger, J. Herz, E. J. Weeber, J. D. Sweatt, A. Pramatarova, B. Howell, W. J. Schneider, and J. Nimpf, submitted for publication).

Dab1RFP and Dab1RFP-5F were prominently distributed at the cell periphery when expressed in Rat-2 cells, while native Dab1-wt had a more diffuse cytoplasmic distribution (Fig. 7). When Nckβ was coexpressed with the tyrosine-phosphorylated Dab1RFP, it colocalized at the cell membrane (Fig. 7). In contrast, Nckβ expressed alone or in combination with native Dab1-wt was found throughout the cytoplasm with no enrichment at the cell membrane. Similarly, expression of Dab1RFP-5F was not capable of recruiting Nckβ to the cell periphery (Fig. 7). This colocalization at the membrane is suggestive of an in vivo association and maybe a mechanism through which Nckβ is functionally activated.

In the plane of the Rat-2 cell closest to the poly-L-lysine-coated cover glass, we observed a very fine mesh of actin. Overexpression of Nckβ by three- to fivefold did not detectably alter the actin cytoskeleton (Fig. 8A and B, and data not shown). Expression of Dab1RFP alone resulted in only a mod-
est loss of regularity of the array of actin (Fig. 8C and D) that may be due to an interaction with low levels of endogenous Nckβ. Overexpression of Nckβ and Dab1RFP, on the other hand, led to a dramatic loss of the actin mesh from the lower

FIG. 9. Nck-enriched regions in distal processes of Reelin-treated neurons have patterns of actin filaments not observed in control-treated neurons. (A and B) The growth cones of neurons at 1 day in vitro demonstrate intense actin filaments (arrow) detected by phalloidin staining (A and B; red and white) and were devoid of Nckβ (green; A). (C to F) Approximately 5% of neurons had Nckβ enrichments in distal processes 10 min after Reelin stimulation (arrowheads). The Nckβ-enriched regions had patterns of actin filaments that varied from actin-poor (C and D) to intense peripheral actin features such as actin rings (star; E and F). Bar, 20 μm.

FIG. 8. Coexpression of tyrosine-phosphorylated Dab1RFP and Nckβ in Rat-2 cells leads to the disruption of the actin cytoskeleton. The actin cytoskeleton, detected with fluorescently labeled phalloidin (white; B, D, F, H, J, and L) observed on the lower plane of Rat-2 fibroblasts transfected with HA-Nckβ (green; A and B), or Dab1RFP, a tyrosine-phosphorylated form of Dab1 (red; C and D), shows the typical mesh pattern. Combined expression of HA-Nckβ and Dab1RFP (E and F) leads to disruption of the actin mesh and clumping of actin filaments. Expression of unphosphorylated Dab1-wt with HA-Nckβ (G and H), or Dab1RFP-5F with HA-Nckβ (I and J) did not disrupt the actin cytoskeleton, nor did expression of Dab1RFP with the SH2 domain mutant of Nckβ (K and L). All images show the plane of the cell closest to the cover glass. Bar, 20 μm.
level of the cells and to the appearance of clumps of actin filaments (Fig. 8E and F). In addition, the majority of the cells expressing Nck\(^{H9252}\) and Dab1RFP had a rounded or unusual morphology. Expression of Dab1RFP-5F, Dab1-wt, or Dab1GFP in conjunction with Nck\(^{H9252}\) overexpression did not disturb the actin scaffolding on the lower surface of the cells (Fig. 8G through J, and data not shown), suggesting that Dab1 phosphorylation is required for the Nck\(^{H9252}\)-dependent disruption of the cytoskeleton observed in these cells. Consistent with this finding, overexpression of Dab1RFP in combination with the Nck\(^{H9252}\)-R312K SH2 domain mutant did not promote reorganization of the actin cytoskeleton in these cells (Fig. 8K and L).

To determine if Reelin might affect the migratory properties of neurons through effects on the actin cytoskeleton, we investigated the appearance of filamentous actin in cells treated with Reelin or control-conditioned medium. The global appearance of actin was similar between treated and untreated samples. However, we did notice a consistent difference in the actin filaments in Nck\(^{H9252}\)-enriched regions of Reelin-treated cells compared to process termini of control-treated cells (Fig. 9). The actin filaments in the Nck\(^{H9252}\)-enriched areas were often localized around the periphery or in tight rings (Fig. 9D and F). Actin filaments were scarce in the center of the Nck\(^{H9252}\)-enriched regions, and we never observed stress fibers.

The Nck family of adaptors is conserved in organisms such as Drosophila melanogaster and Caenorhabditis elegans. These proteins are structurally similar throughout the SH3 and SH2 domains and have conserved downstream effectors such as Pak (21, 33). We made use of the conserved nature of these signaling proteins to test for a genetic interaction between mouse Dab1 and Drosophila Dock in an eye expression model. Both Dab1 and Dock were expressed, separately and in combination, in Drosophila cells with the upstream activator sequence (UAS)-GAL4 system (4). For this study, we used a glass multimer repeat promoter (GMR)-GAL4 line to activate expression of the UAS-Dab1 or UAS-Dock transgenic allele (18). We found that when expressed in the developing Drosophila, Dab1RFP is tyrosine phosphorylated (Fig. 6B). The Dab1RFP-5F protein was expressed at slightly lower levels, but no tyrosine phosphorylation was detectable. The expression of Dab1RFP leads to the roughening of the external eye morphology, which is accompanied by loss of linearity of the ommatidial facets and ommatidial fusions (Fig. 10B, insets). In addition, many of the ommatidia have lost the typical hexagonal shape.

The eye morphology, and ommatidial organization or shape are largely unaffected by Dab1RFP-5F expression (Fig. 10C). To account for the lower expression of the UAS-Dab1RFP-5F...
alleles, we generated flies that carried two copies of the UAS-Dab1RFP-5F transgene and one copy of the GMR-GAL4 transgene. These flies expressed Dab1RFP-5F at levels that were comparable to the Dab1RFP expression levels and did not show defects in external eye morphology (Fig. 6B). The RFP expression did not alter eye morphology from that of the wild type (Fig. 10A). Other controls such as the UAS-Dab1RFP and UAS-Dab1RFP-5F transgenic lines in the absence of GMR-GAL4 were indistinguishable from the normal flies (data not shown).

We observed that in flies that coexpressed Dab1RFP and Dock, the severity of the eye roughness was enhanced (Fig. 10E). In these compound eyes, there were many more fusions, and the size of the ommatidia was variable. In contrast, the eyes of flies expressing the Dab1RFP-5F and Dock proteins were not more disorganized than those of flies expressing the Dock or Dab1RFP-5F protein alone, which showed only minor aberrations (Figure 10C, D, and F). This suggests that the Dab1 phosphorylation sites are required for the genetic interaction observed with Dock in this exogenous expression model.

**DISCUSSION**

The tyrosine phosphorylation of Dab1 was implicated as a requisite step in Reelin signal transduction. Here we show that a consequence of Dab1 tyrosine phosphorylation is the formation of a complex with the Nckβ SH2-SH3 domain adaptor molecule. Members of this family of adaptors appear in species from *Drosophila melanogaster* and *Caenorhabditis elegans* to *Homo sapiens* (14, 34). We show that coexpression of tyrosine-phosphorylated mouse Dab1 and the *Drosophila* Nck family member Dock leads to enhancement of the Dab1 expression phenotype in the eye (Fig. 10). This suggests that Nck family members are conserved signaling partners for Disabled family proteins.

The Nck proteins function in tyrosine kinase-based signal transduction cascades and in many instances connect extracellular signals to alterations in the actin cytoskeleton. Of the known SH3 domain binding partners of the Nck proteins, which number over 20, approximately half have direct or indirect roles in the regulation of actin dynamics (33). We provide evidence that one consequence of a Dab1-Nckβ interaction is alterations in the actin cytoskeleton. Through the analysis of Dab1-signaling partners such as Nckβ, we hope to gain a better understanding of cell biological changes that are induced by Reelin signaling.

Nckβ bound tyrosine-phosphorylated Dab1 to a much greater degree than Nckα (Fig. 1). Since the closely related SH2 domains of these two molecules bind different phosphorylation sites on the platelet-derived growth factor receptor, this specificity difference is not unprecedented (7). In addition, the SH2 domain of Nckβ but not of Nckα was able to bind to the tyrosine-phosphorylated cytoplasmic domain of Ephrin-B1, suggesting that while they are similar, the SH2 domains of Nckα and Nckβ have different specificities (8). In our in vitro assay, Nckβ was capable of binding to Dab1 phosphorylated on either Y220 or Y232, which have similar residues C-terminal to the tyrosine, Q-V-P and D-V-P, respectively. This may suggest that valine and proline in the +2 and +3 position relative to the phosphotyrosine may foster the Nckβ SH2 domain interaction. Both Y220 and Y232 are phosphorylated in embryonic mouse brain (B.Howell, unpublished results); but only Y220 phosphorylation is known to be Reelin inducible (32).

We provide several lines of evidence that support an interaction between Nckβ and Dab1 in vivo. The protein complex is robust enough to support coimmunoprecipitation from cells overexpressing HA-Nckβ and tyrosine-phosphorylated Dab1 in the presence of 1% NP-40 (Fig. 2). We observed Nckβ distribution at the cell periphery of cells expressing tyrosine-phosphorylated Dab1RFP, whereas in cells expressing unphosphorylated versions of Dab1, Nckβ distribution was exclusively cytoplasmic (Fig. 7). Colocalization between Dab1 and Nckβ was also apparent at distal sites in processes of neurons treated with Reelin. This was not the case in neurons treated with control-conditioned medium, where Nckβ protein was predominantly localized in the cell soma (Fig. 4). Taken together, these data suggest that these proteins interact in vivo and that this interaction is dependent upon Dab1 tyrosine phosphorylation.

The redistribution of Dab1 into the processes was rapid. We measured Nckβ in process tips 10 to 15 μm from the cell soma 10 min after Reelin stimulation (Fig. 9). This fast anterograde movement could be mediated by kinesins. The Dab1 PTB domain interacts with two proteins that have been implicated in kinesin-mediated transport. The amyloid precursor protein (APP) binds kinesins directly, and ApoER2 was shown to interact indirectly through association with Jun interacting proteins (30, 49). Dab1 could therefore act to shuttle bound proteins anterograde into distal processes through binding APP or ApoER2.

Nck family proteins are thought to participate in remodeling the actin cytoskeleton upon recruitment to the membrane (35). Since Dab1 is targeted to the membrane through its PTB domain, which binds to cell surface receptors and phospholipids (24, 29, 46), we sought to determine if exogenous expression of tyrosine-phosphorylated Dab1 in the presence of high levels of Nckβ would alter actin profiles. Overexpressing Nckβ with Dab1RFP led to disruption of the cytoskeleton. Instead, clumps of actin filaments were observed on the lower plane of coexpressing cells (Fig. 8). The disruption of the actin cytoskeleton appears to require the interaction of the two proteins, since either alone had little or no effect. Consistent with this, reorganization of the actin cytoskeleton was not induced when either the Dab1RFP-5F or Nckβ SH2 domain mutant R312K was substituted for the wild-type proteins. This suggests that tyrosine-phosphorylated Dab1 is capable of substituting for extracellular signals that recruit Nckβ to effect changes in the actin cytoskeleton.

The demonstration that Nckβ binds Dab1 and redistributes in Reelin stimulated nascent neurons suggests Reelin may regulate actin dynamics. We did not observe a global difference in actin profiles between fixed neurons from cultures stimulated with Reelin or control conditioned medium. The actin cytoskeleton in the Reelin-induced Nckβ-enriched regions, however, differs from patterns typically observed in growth cones or process terminals of control cultures (Fig. 9). While the actin filament distribution was variable, a circle of actin filaments was often seen demarcating the Nckβ-enriched regions.
We consistently observed that the Nckβ-enriched regions were devoid of actin stress fibers, which are routinely observed at process termini in the control cultures.

Other systems have provided clues to Nckβ function downstream of extracellular signals. In *D. melanogaster*, the Nck family member Dock transmits signals that regulate the targeting of axonal growth cones. In its absence, the growth cones of the R1 to R6 photoreceptor cells fail to terminate in the lamina and instead migrate into the medulla (14). In mammals it has recently been shown that Nckβ interacts with the transmembrane Ephrin-B1 ligand, which regulates cell sorting and axonal repulsion (8). In response to stimulation with Eph receptors, expressed on adjacent cells, the transmembrane ligand becomes tyrosine phosphorylated, promoting an interaction with the Nckβ SH2 domain. This interaction leads to the loss of stress fibers as well as the redistribution of paxillin from the cell periphery into the cytoplasm, suggesting a loss of focal contacts (8).

These cellular changes may be promoted by the numerous Nckβ SH3 domain-binding proteins. Among them, a number regulate the activity of the Rho family of small GTPases. These include Dock180, an exchange factor for Rac, hrrnPK, which binds to Vav, also a RacGEF, and Pak1, which regulates both Rac and Rho (8, 33, 48). Interestingly it has recently been demonstrated that Reelin stimulation leads to the activation of phosphoinositide 3-kinase (2), which in some signaling scenarios leads to activation of Rac. The kinase CDK5 activator p35 was suggested to regulate neuronal positioning in part by regulating the activity of Rac and the Pak1 kinase (22, 37). The Reelin and CDK5 pathways have synergistic roles in regulating neuronal placement (39). It will therefore be interesting to determine if Reelin acts through Daβ1 and Nckβ to regulate the activity of the Rho family GTPases.

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