Phosphatidylinositol 3-Kinase Interacts with the Adaptor Protein Dab1 in Response to Reelin Signaling and Is Required for Normal Cortical Lamination*

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Reelin is a large secreted signaling protein that binds to two members of the low density lipoprotein receptor family, the apolipoprotein E receptor 2 and the very low density lipoprotein receptor, and regulates neuronal positioning during brain development. Reelin signaling requires activation of Src family kinases as well as tyrosine phosphorylation of the intracellular adaptor protein Disabled-1 (Dab1). This results in activation of phosphatidylinositol 3-kinase (PI3K), the serine/threonine kinase Akt, and the inhibition of glycogen synthase kinase 3β, a protein that is implicated in the regulation of axonal transport. Here we demonstrate that PI3K activation by Reelin requires Src family kinase activity and depends on the Reelin-triggered interaction of Dab1 with the PI3K regulatory subunit p85α. Because the Dab1 phosphotyrosine binding domain can interact simultaneously with membrane lipids and with the intracellular domains of apolipoprotein E receptor 2 and very low density lipoprotein receptor, Dab1 is preferentially recruited to the neuronal plasma membrane, where it is phosphorylated. Efficient Dab1 phosphorylation and activation of the Reelin signaling cascade is impaired by cholesterol depletion of the plasma membrane. Using a neuronal migration assay, we also show that PI3K signaling is required for the formation of a normal cortical plate, a step that is dependent upon Reelin signaling.

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3 The abbreviations used are: VLDL, very low density lipoprotein; receptor and apoE receptor 2 (apoER2) function as Reelin receptors and interact with the cytoplasmic adaptor protein Disabled-1 (Dab1) through an Asn-Pro-X-Tyr (NPXV) tetra-amino acid motif in their cytoplasmic domains (for review, see Ref. 4). Binding of Reelin to its receptors induces tyrosine phosphorylation of Dab1 in neurons (5). This event is essential for its function (6) and requires the activity of Src family tyrosine kinases (SFKs) (7, 8). Mice that lack either reelin, dab1, or apoer2 and cldn1 display the typical reeler phenotype, which is characterized by inversion of cortical layers, cerebellar dysplasia, and ataxia.

We have recently reported that Reelin activates phosphatidylinositol-3-kinase (PI3K) in neurons, leading to activation of the serine/threonine kinase Akt (also known as protein kinase B) and inhibition of glycogen synthase kinase 3β, a major kinase for the microtubule-associated protein tau. Reelin-dependent activation of this signaling pathway is apoE receptor-dependent and requires Dab1 (9).

Three classes of PI3K catalyze the formation of phosphoinositides (PIs). These membrane lipids function as second messengers and also mediate the recruitment of adaptor and scaffolding proteins to specific membrane compartments. PI3K signaling, thus, participates in diverse cellular and physiological processes including cell proliferation, inhibition of apoptosis, differentiation, cell motility, membrane trafficking, endocytosis, metabolic regulation, and neoplastic transformation (for reviews, see Refs. 10–12). Only class 1 PI3Ks are sensitive to the widely used inhibitors wortmannin and LY294402, which inhibit the formation of phosphatidylinositol 3,4,5-trisphosphate and, thus, prevent the PI3K-dependent activation of Akt. Class 1 PI3Ks are heterodimers of the p110 catalytic subunit and a regulatory subunit. The latter are multidomain proteins that exist in several isoforms, of which p85α is the longest one. Binding of the regulatory subunit to activating membrane-associated proteins, such as insulin-regulated substrate 1 (IRS1), frequently occurs at phosphorylated tyrosine residues (for review, see Ref. 13). This relieves inhibition on p110 and also brings the complex in proximity to its substrate in the membrane (14–18).

Dab1, like IRS1, is a PTB domain protein that is tyrosine-
phosphorylated in response to an extracellular signal and binds to NXY motifs in the cytoplasmic domains of several transmembrane proteins (for review, see Ref. 4). These similarities prompted us to examine whether Dab1 is directly involved in the Reelin-mediated PI3K activation. We also investigated the role of PI3 in Dab1 membrane targeting and Reelin signaling. Phosphatidylinositol 4,5-bisphosphate has been reported to regulate cytoskeletal reorganization through accumulation in cholesterol-rich plasma membrane compartments called rafts or caveolae (for reviews, see Refs. 19 and 20). These membrane microdomains are enriched in tyrosine kinases and scaffolding proteins and play an important role as cellular signal transduction platforms (for reviews, see Refs. 21–23). Additionally, apoER2 localizes to caveolae in stably transfected Chinese hamster ovary cells (24), and caveolar localization is crucial for the platelet-derived growth factor-induced tyrosine phosphorylation of low density lipoprotein receptor-related protein 1, another member of the low density lipoprotein receptor gene family (25).

Here we show that activation of PI3K in neurons is regulated by a Reelin-dependent interaction of phosphorylated Dab1 with the PI3K regulatory subunit p85α. We show that the Dab1-containing Reelin signaling complex is associated with the plasma membrane and that the signaling can be modulated by cholesterol depletion of the plasma membrane. Finally, we demonstrate that PI3K activity is required for formation of a normal cortical plate in an in vitro cortical slice migration assay.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies—**PP2 (4-amino-5-(4-chlorophenyl)-7-(4-butyrylpyrazolo)[3,4-d]pyrimidine) was from Biomol Research Laboratories (Plymouth, PA). PP3 (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine) was from Calbiochem. Wortmannin and filipin (F-9765) were from Sigma-Aldrich, STI571 (Gleevec) was from Novartis (Basel, SUI), and LY294002 was purchased from Cell Signaling Technology (Beverly, MA). 1000× stock solutions of the inhibitors were prepared in MeSO. Methyl β-cyclodextrin (MβCD), poly-l-lysine (P-7280), and phosphatase inhibitor cocktails (P-2850 and P-5726) were from Sigma (C-4555); the protease and phosphatase inhibitor cocktails (1% Triton X-100, incubated at room temperature, and homogenized by repeatedly forcing them through a 27-gauge needle. The homogenate was clarified by centrifugation at 20,000 × g for 15 min, and the protein contents of solubilized membrane and soluble fractions were determined using the Bio-Rad detergent-compatible assay. Denatured and reduced samples were separated by SDS-gel electrophoresis (15 μg of protein/lane) and analyzed by immunoblotting.

**Immunoprecipitation Experiments—**Control or Reelin-treated neurons were washed with ice-cold PBS containing 10 mM NaF, 25 mM β-glycerophosphate, and 2 mM sodium orthovanadate (Sigma), centrifuged at 900 × g for 5 min at 4 °C, and resuspended in 1 ml of immunoprecipitation buffer (50 mM Tris, pH 7.5, 0.15 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, supplemented with protease and phosphatase inhibitor cocktails). The neurons were homogenized by forcing through a 27-gauge needle and incubated on ice for 30–60 min. Lysates were cleared by centrifugation at 20,000 × g and 4 °C for 30 min. 900–1000 μl of the supernatant was incubated with 5 μg of the indicated antibody in a total volume of 1 ml and incubated on ice for 1 h. Immune complexes were precipitated with 50 μl of protein A-agarose (for rabbit polyclonal antibodies) or protein G-Sepharose (for mouse monoclonal antibodies) slurry. Pelleted beads were washed once with immunoprecipitation buffer, then three times with washing buffer (immunoprecipitation buffer containing 0.1% Triton X-100), resuspended in 50 μl of SDS loading buffer, reduced with β-mercaptoethanol (Sigma), and boiled. Approximately 5% of the total immunoprecipitate was used to detect the respective immunoprecipitated protein; the rest was used to detect co-precipitated proteins by immunoblotting.

**Immunoblotting—**Neuronal lysates were harvested by adding μg/ml of surfactant to ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM EGTA, 1% (v/v) SDS, 5% (v/v) SDS, 0.25% (v/v) sodium deoxycholate with protease and phosphatase inhibitor cocktails). Lysates were cleared by centrifugation at 4 °C and 20,000 × g for 20 min, adjusted for protein content, mixed with 4× concentrated gel-loading buffer (125 mM Tris, pH 6.8, 50% glycerol, 4% SDS, 5% β-mercaptoethanol), boiled, separated by SDS-gel electrophoresis (10 μg of protein/ lane), and transferred to Hybond-C extra nitrocellulose membranes (Amersham Biosciences). Membranes were blocked in PBS, pH 7.4, with 0.5% Tween 20 and 4% nonfat dry milk and incubated overnight at 4 °C with antibodies diluted in blocking buffer according to the manufacturer’s instructions. Secondary anti-mouse or anti-rabbit anti-IGG conjugated to horsedradish peroxidase (Amersham Biosciences) were used at a 1:5000 dilution for 1 h at room temperature. Bound IGG was detected by ECL (Pierce).

**Embryonic Slice Culture Assay—**Entire fetal brains at E13.5 were embedded in 4% low melting agarose (prepared in Dulbecco’s modified Eagle’s medium/Hanks’ F-12 medium with glutamine, glucose, and antibiotics) and 24 h after culturing, the slices were cut on a vibrotome at 100 μm thick coronal sections were cut, and a slice was processed for histology immediately after sectioning to verify the developmental status before culture. Sections were cultured on collagen-coated polytetrafluoroethylene membranes (Transwell-COL, Costar cat. 3494) in 12-well plates, and 1.5 ml of medium was added to a level just covering the tissue. Cultures were incubated with 1% FCS, 0.1% penicillin and 100 μg/ml streptomycin sulfate) at 37 °C in a 5% CO₂ atmosphere. Between culture days 4 and 6 primary neurons were treated with partially purified Reelin or control-conditioned serum-free medium for the indicated times as described (8).

**Treatment of Cultured Neurons with Inhibitors, Cholesterol Depletion, and Filipin Stain—**Neurons were incubated with various inhibitors compound for 60 min at a final concentration of ME of 0.1% (v/v) without any detectable adverse effect. For cholesterol depletion experiments, neurons were treated with 5 mM MJCD or with 5 mM MJCD preloaded with cholesterol (final cholesterol concentration, 100 μg/ml). For detection of free cholesterol, neurons were fixed in 2% paraformaldehyde in PBS supplemented with 50 μg/ml filipin for 30 min, washed 3 times with PBS, mounted using the Pro-Long antifade kit from Molecular Probes (Eugene, OR), and analyzed by fluorescence microscopy (excitation at 360 nm).

**Cellular Fractionation of Primary Embryonic Neurons—**After stimulation, neurons were harvested in ice-cold hypotonic buffer A (20 μM NaF, 0.5 mM EDTA, 1% Triton X-100, 0.5 mM protease and phosphatase inhibitor cocktails) subjected to a pressure of 500 p.s.i. for 15 min, and forced through a small hole by slowly releasing the pressure. Nuclei were removed by centrifugation at 900 × g for 10 min, and the postnuclear supernatants were separated into membrane and soluble fractions by ultracentrifugation at 200,000 × g for 60 min. Membrane fractions were resuspended in Tris buffer, pH 7.5 (50 mM, 0.15 mM NaCl, with protease and phosphatase inhibitor cocktails) containing 1% Triton X-100, incubated at room temperature, and homogenized by repeatedly forcing them through a 27-gauge needle. The homogenate was clarified by centrifugation at 20,000 × g for 15 min, and the protein contents of solubilized membrane and soluble fractions were determined using the Bio-Rad detergent-compatible assay. Denatured and reduced samples were separated by SDS-gel electrophoresis (15 μg of protein/lane) and analyzed by immunoblotting.

**Co-immunoprecipitation Experiments—**Control or Reelin-treated neurons were washed with ice-cold PBS containing 10 mM NaF, 25 mM β-glycerophosphate, and 2 mM sodium orthovanadate (Sigma), centrifuged at 900 × g for 5 min at 4 °C, and resuspended in 1 ml of immunoprecipitation buffer (50 mM Tris, pH 7.5, 0.15 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, supplemented with protease and phosphatase inhibitor cocktails). The neurons were homogenized by forcing through a 27-gauge needle and incubated on ice for 30–60 min. Lysates were cleared by centrifugation at 20,000 × g and 4 °C for 30 min. 900–1000 μl of the supernatant was incubated with 5 μg of the indicated antibody in a total volume of 1 ml and incubated on ice for 1 h. Immune complexes were precipitated with 50 μl of protein A-agarose (for rabbit polyclonal antibodies) or protein G-Sepharose (for mouse monoclonal antibodies) slurry. Pelleted beads were washed once with immunoprecipitation buffer, then three times with washing buffer (immunoprecipitation buffer containing 0.1% Triton X-100), resuspended in 50 μl of SDS loading buffer, reduced with β-mercaptoethanol (Sigma), and boiled. Approximately 5% of the total immunoprecipitate was used to detect the respective immunoprecipitated protein; the rest was used to detect co-precipitated proteins by immunoblotting.
RESULTS

Activation of PI3 Kinase by Reelin Requires SFK Activity—
Activation of SFKs and PI3K in neurons in response to Reelin requires Dab1 and the apoE receptors VLDLR and apoER2. To determine the order in which SFKs and PI3K are activated by Reelin we used kinase inhibitors on cultured primary embryonic neurons (Fig. 1). The SFK inhibitor PP2 blocked not only the Reelin-induced Dab1 phosphorylation and SFK activation but also the activation of PI3K by Reelin, as determined with an antibody directed against activated Akt (Fig. 1A, lane 2). Because PP2 can also inhibit c-Abl and c-Kit, we used the inhibitor STI571 to block these kinases without affecting SFK activity (for review, see Ref. 31). This had no effect on PI3K activation by Reelin (Fig. 1A, lane 6). By contrast, the PI3K inhibitors LY294002 and wortmannin had no effect on the activation of SFKs by Reelin, as detected with an antibody directed against the phosphorylated tyrosine in the activation loop (p-Y418[SFK]), whereas Akt phosphorylation at serine 473 was completely blocked (Fig. 1B, lanes 4 and 8). Thus, SFK activation precedes PI3K activation by Reelin.

Potential Mechanisms Leading to Reelin-induced PI3K Activation—Ligand-induced PI3K activation often involves binding of the SH2 domains of the regulatory subunit p85α (Fig. 2A) to phosphotyrosine motifs in growth factor receptor tails or adaptor proteins. Using web-based algorithms (scansite.mit.edu and www.cbs.dtu.dk/services/NetPhos) for detecting sequence motifs that mediate binding to protein interaction domains, we identified conserved putative SH2 domain binding motifs in Dab1 (Fig. 2B) that include Tyr-198 and Tyr-220. These amino acids are specifically phosphorylated in response to Reelin stimulation. The NetPhos algorithm (29) correctly predicts the phosphorylated and nonphosphorylated residues (6, 32) in the cluster of five tyrosine residues located immediately C-terminal of the Dab1 PTB domain (Fig. 2B). Phosphorylation of the potential site involving Tyr-300 has so far not been detected experimentally (6). An alternative mode of interaction between Dab1 and p85α could involve an SH3 domain interaction with proline-rich sequences within Dab1. Such a predicted site surrounds Pro-424. Conceivably, tyrosine phosphorylation of Tyr-198 and Tyr-220 might induce a conformational change exposing this site.

p85α would be unlikely to interact directly with the cytoplasmic domains of apoER2 and VLDLR. Although NetPhos identifies the NPXY motif as a likely interaction site for PTB domains, the other tyrosine residues in the cytoplasmic tails of apoER2 and VLDLR are not predicted to be in the proper sequence context where they could mediate SH2 domain interactions with p85α (Fig. 2C). Consistent with this prediction, we were unable to find any indication of tyrosine phosphorylation of apoER2 in immunoprecipitates from Reelin-treated neuronal lysates (Fig. 3A). However, a small amount of a phosphoprotein that comigrates with Dab1 was co-precipitated with apoER2 from Reelin-treated neuronal lysates and likely corresponds to tyrosine-phosphorylated Dab1 (Fig. 3A, lane 6). Reelin Does Not Cause Tyrosine Phosphorylation of the PI3K Subunits—SFK-dependent phosphorylation of the PI3K subunit p85α can activate PI3 kinase by relieving inhibition of the p110 catalytic subunit. We, therefore, tested if Reelin, which activates SFKs in neurons, induces tyrosine phosphorylation of p85α. Neuronal lysates were immunoprecipitated with a p85α antibody, and the precipitates were immunoblotted with a phosphotyrosine-specific antibody (Fig. 3B). The tyrosine-phosphorylated 85-kDa protein that is detected in the Reelin-induced lysate (lane 2) corresponds to tyrosine-phosphorylated Dab1 (p-Dab1; see also Fig. 3C). Although p85α is no longer detectable in the supernatant after immunoprecipitation with an α-p85α antibody, the 85-kDa phosphoprotein was not depleted by the p85α immunoprecipitation (Fig. 3B, lane 4), and no significant tyrosine phosphorylation of immunoprecipitated p85α was detected (lanes 5 and 6). The weakly tyrosine-phosphorylated precipitated protein band at ~85 kDa (lane 6) likely is phosphorylated Dab1 (see next paragraph). The strongly tyrosine-phosphorylated band at ~110 kDa (top panel) corresponds to the catalytic PI3K subunit, which co-precipitates constitutively with p85α. Tyrosine phosphorylation levels of p110 were also not affected by Reelin treatment. These results...
suggest that modulation of tyrosine phosphorylation of PI3K itself is not a major regulatory mechanism for Reelin-induced PI3K activation in neurons.

Reelin Induces Interaction of Dab1 and p85α in Neurons—A common mechanism of ligand-induced PI3K activation involves recruitment of p85α to autophosphorylated receptor kinases or their substrates, resulting in disintegration of the p85α-bound catalytic subunit p110. Because SFK inhibition blocks the tyrosine phosphorylation of Dab1 and PI3K activation by Reelin (Fig. 1A), we hypothesized that Reelin might trigger the recruitment of p85α to Dab1. To test this, we immunoprecipitated Dab1 protein from lysates of control and Reelin-treated primary neuronal cultures and tested for the presence of p85α in the precipitated immune complexes. As shown in Fig. 3C, total Dab1 protein and p-Dab1 were immunodepleted from the immunoprecipitation supernatants (lanes 3 and 4), and p85α indeed co-immunoprecipitated with Dab1 in a manner that correlated with the degree of tyrosine phosphorylation of Dab1 (lanes 6 and 7). No p85α was immunoprecipitated from a Reelin-treated sample with a control antiserum (lane 8) or from lysates in which Dab1 phosphorylation by Reelin had been blocked by the SFK inhibitor PP2 (lane 11).

These data suggest that Reelin induces the recruitment of p85α into a complex with Dab1 in a tyrosine phosphorylation-dependent manner. The converse experiment, in which Dab1 was co-precipitated with an α-p85α antibody in a Reelin-dependent manner supports this conclusion (Fig. 3D). Dab1 was detected only in p85α immunoprecipitates from Reelin-treated neuronal lysates (lane 4) but not from control-treated lysates (lane 3), lysates immunoprecipitated with an irrelevant immunoglobulin (lane 5), or lysates from Reelin-treated cells in which SFKs, and thus, Dab1 phosphorylation had been inhibited with PP2 (lane 11). Neither Fyn (Fig. 3C, lane 6 and 7) nor Src (not shown) could be detected in Dab1 immunoprecipitates from control (lane 6) or Reelin-treated (lane 7) neuronal lysates. This suggests that the interaction between SFKs and p-Dab1 that has been observed in vitro and in transfected cell lines (33) and which involves the SFK-SH2 domain is either transient under these conditions or mutually exclusive and weaker than the p85α-Dab1 interaction. We found the latter to be stable even in the presence of 0.5 M NaCl or 60 mM octylglucopyranoside, a detergent that disrupts cholesterol-rich microdomains of the plasma membrane (data not shown).

Activation of the Reelin Signaling Cascade Occurs at the Plasma Membrane—The Dab1 PTB domain can mediate simultaneous interaction of the protein with the NPXY motif of apoE receptor cytoplasmic domains and with PIs at the inner leaflet of the plasma membrane (34). The affinity of the Dab1 PTB domain for PIs might be important for increasing the apparent affinity for the NPXY motifs in the Reelin receptor tails, which on their own have a relatively low affinity for the Dab1 PTB domain in the micromolar range. The PI interaction might also serve to direct Dab1 into PI-rich specialized membrane compartments such as caveolae and rafts.

To investigate whether other known components of the Reelin signaling pathway are recruited into this membrane-associated complex, we separated postnuclear supernatants of control (Fig. 4, lanes 1 and 3) and Reelin-treated neurons (lanes 2 and 4) into the membrane (lanes 1 and 2) and cytosolic (lanes 3 and 4) fractions. The distribution of several components of the Reelin signaling cascade between these fractions was then examined by immunoblotting. Dab1, its major physiological kinase Fyn, and the Reelin receptor apoER2 were found predominantly in the membrane fraction. By contrast, Akt was restricted almost completely to the cytosolic fraction, whereas p85α and Cdk5, a neuronal serine/threonine kinase that can also phosphorylate Dab1, were present in both fractions. The phosphorylation state of the proteins (p-Dab1, SFK-(Tyr(P)-418) and Ser(P)-473-Akt) did not affect their subcellular distribution. In particular, no significant redistribution of p85α to the membrane compartment in response to Reelin treatment was observed. It cannot be ruled out, however, that changes in the subcellular localization of a small but nevertheless important subfraction of a protein might escape detection at this level of sensitivity. Indeed, only ~5% of the total p85α pool associate with the platelet-derived growth factor receptor upon ligand stimulation (35).

Cholesterol Depletion Affects Reelin Signaling—The relative enrichment of phosphatidylinositol 4,5-bisphosphate in raft-like domains, phospholipid- and cholesterol-rich plasma membrane patches, prompted us to examine the effects of raft disintegration on Reelin signaling in neurons. Phosphatidylinositol 4,5-bisphosphate serves not only as a PI3K substrate and precursor of phosphatidylinositol 3,4,5-trisphosphate, but it also mediates the targeting of proteins containing PI binding domains to the plasma membrane, where it participates in the regulation of endocytosis and cytoskeletal assembly.
Depletion of membrane cholesterol with MβCD was used to disrupt cholesterol- and PI-enriched membrane rafts in cultured neurons. The activation of downstream signaling components by Reelin was significantly reduced in cholesterol-depleted neurons (Fig. 5A, lanes 9 and 10), whereas total protein levels were not significantly affected by the treatment. The reduced intensity of the bands at ~130 and 180 kDa in the phosphotyrosine immunoblot, which are not regulated by Reelin, indicates a general reduction of kinase-mediated signaling in cholesterol-depleted neurons, consistent with an important role of raft-like membrane structures in neuronal signaling.

Pretreatment of neurons with cholesterol-loaded Mβ CD, which prevents cholesterol depletion from the cells, completely prevented the negative effect of Mβ CD-induced raft disruption on neuronal Reelin signaling (lanes 5 and 6). Efficient depletion of free cholesterol from cultured Mβ CD-treated neurons (Fig. 5B) was verified by monitoring the fluorescence emissions from filipin-stained cells.

Inhibition of PI3K Signaling Prevents Formation of a Lamini- cated Cortical Plate—We next sought to test whether inhibition of PI3K activity in an in vitro neuronal migration model would prevent the normal Reelin-dependent formation of a
cortical plate in embryonic mouse brains. Wild type embryonic mouse brain slices, which express Reelin endogenously, were incubated in the absence or presence of the PI3K inhibitor LY294002. After 2 days in which the slices were allowed to proceed through development in vitro, the slices were fixed and stained for histological examination (Fig. 6A). At E13.5, when the embryonic brains had been harvested, the preplate had split, but the cortical plate was only just beginning to form (top panel). After 2 days in culture, a normal, well formed cortical plate had developed (middle panel). Formation of an ordered cortical plate was completely prevented by treatment with the PI3K inhibitor (bottom panel), although cell proliferation was not significantly affected, as judged by the comparable number of cells in the treated and untreated slices.

To demonstrate that we had successfully inhibited PI3K activity in the slices, we prepared protein lysates of identically treated slices and immunoblotted them with an antibody against total Akt and with the anti-p-Akt antibody (Fig. 6B). The effect of wortmannin, another PI3K inhibitor, could not be evaluated because this substance was not functionally stable over the 2 day incubation period. These data suggest that Reelin-dependent PI3K activation may be functionally important in the developing neocortex.

**DISCUSSION**

The large signaling molecule Reelin regulates the lamination of the neocortex and the cerebellum during embryonic development (for reviews, see Refs. 1–4). It is involved in the specification of the radial glia (36, 37) and also modulates synaptic plasticity in the mature brain (38, 39). Transmission of the signal to the neurons requires the Reelin receptors VLDLR and apoER2 and the cytosolic PTB domain adaptor protein Dab1, which interacts with NPXY motifs in the intracellular domains of the receptors (26, 27, 34). Translation of the signal into a cellular response requires tyrosine phosphorylation of Dab1 and subsequent activation of SFKs and PI3K (7, 8). PI3K signaling is also activated in response to Reelin, resulting in activation of Akt, inhibition of glycogen synthase kinase 3β, and modulation of the phospho-

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**Fig. 4.** Reelin-dependent Src family kinase activation and Dab1 tyrosine phosphorylation in neurons occur at the plasma membrane. Subcellular fractionation of control (lanes 1 and 3) and Reelin-treated (lanes 2 and 4) cultured neurons. Postnuclear supernatants were prepared as described under “Experimental Procedures” and separated into membrane (lanes 1 and 2) and cytosolic soluble (lanes 3 and 4) fractions by ultracentrifugation. The fractions were separated by SDS-gel electrophoresis and analyzed by immunoblotting (20 μg of protein/lane). Phosphorylated and non-phosphorylated Dab1 and Fyn, the main Dab1 kinase, were found predominantly in the membrane fraction whether the cells had been treated with Reelin or not. By contrast, the PI3K substrate Akt and its activated form, p-Akt (Ser-473), were mainly found in the soluble fraction. p85α and the serine/threonine kinase Cdk5 were present in both fractions. ApoER2 serves as a marker protein for the membrane compartment.

**Fig. 5.** Cholesterol depletion of cultured neurons with methyl β-cyclodextrin. A, cholesterol depletion of primary embryonic neurons impairs Reelin signaling. Three-day old cultured rat neurons were preincubated with 5 mM MβCD complexed with 100 μM cholesterol (lanes 3–6) or with MβCD without cholesterol (lanes 7–10) or left untreated (lanes 1 and 2) before control (lanes 1, 3–4, 7–8) or Reelin treatment (lanes 2, 5–6, 9–10). Cholesterol depletion with MβCD potently decreased Reelin-induced tyrosine phosphorylation of Dab1 and subsequent activation of SFKs and PI3K (lanes 9 and 10). Note that the total protein levels (Dab1, Cdk5, and the unspecific band seen with the SFK-(Tyr(P)-418 (α-p-Y418)) antibody, marked with an asterisk) remain virtually unaltered in the cholesterol-depleted neurons. Lanes 3 and 4, 5 and 6–8, and 9 and 10 represent independent duplicate experiments. B, MβCD treatment efficiently depletes cholesterol from neurons. Cultured rat neurons (400× magnification) were not treated (a and c) or treated (b and d) with MβCD and stained with filipin. A decrease in fluorescence indicates effective removal of free cholesterol from the treated neurons.
In vitro bryonic cortex at the preplate (histology of three vibratome slices. Sections were prepared from em-
zyon cortical plate (top; the pial surface is at the
ventricular zone; IZ, intermediate zone. Bar = 100 μm. B, immunoblot (WB) of total
Akt and of Ser(P)-473-Akt in extracts of two slices cultivated without (+) and with (−) LY294002. Inhibition of Akt phosphorylation by
LY294002 reflects inhibition of PI3K.

**Fig. 6. PI3K inhibition prevents cortical plate formation.** A. histology of three vibratome slices. Sections were prepared from em-
byronic cortex at the preplate (PP) stage and processed immediately after sectioning (0 days in vitro (0DIV)) or incubated for days in vitro (2DIV) under control conditions or in the presence of 50 μM LY294002. The appearance of a cortical plate (CP) bracketed between the cell poor marginal zone (MZ) and subplate (SP) is evident in the control slice (2DIV) but is abnormal in the presence of LY294002. The ventricle is at the bottom; the pial surface is at the top of each panel. VZ, ventricular zone.

**Table 1.** The insulin signaling pathway in primary embryonic neurons, where insulin-dependent tyrosine phosphorylation of IRS trig-
gers its interaction with the SH2 domains of p85α, which allows the associated p110 subunit to become active. Like Dab1, IRS contains an N-terminal PTB domain, which mediates its binding to an NPXY motif in the insulin receptor tail when it is tyrosine-phosphorylated in response to insulin. A PI
binding pleckstrin homology domain contributes to the mem-
brane association of IRS (40). In contrast to the IRS proteins, the Dab1 PTB domain strongly prefers unphosphorylated NPXY motifs (34).

Thus, in a possible scenario, phosphorylation of the VLDLR or apoER2 NPXY motif might function as a switch, turning off
Dab1-dependent signaling and potentially activating another
phosphotyrosine-dependent pathway. However, we found no
evidence for tyrosine phosphorylation of the apoER2 intracel-
ular domain in the absence or presence of Reelin, making such a mechanism unlikely. Instead, Dab1 phosphorylation, which
appears to be amplified from a low basal level by receptor dimerization (41), SH2 domain interaction (33), and amplifi-
cation of SFK activity (7, 8), serves to recruit PI3K into a complex where it becomes activated. This model is supported by several lines of evidence. First, tyrosine phosphorylation of Dab1 is necessary for Reelin-triggered PI3K activation, and SFKs are
required for this to occur (Fig. 1). Second, the Reelin-dependent interaction of p85α and Dab1 correlates with the levels of Dab1
ubiquitylation state of the microtuble-stabilizing protein tau (9). It was unclear, however, how PI3K is activated and whether this
precedes or is a consequence of prior SFK activation and whether PI3K signaling is necessary for Reelin-controlled neu-
ronal migration. In this study we have now shown that SFKs are
activated independent of PI3K and that SFK function is
required for subsequent PI3K activation. The latter involves the
formation of a complex of the regulatory p85α subunit of PI3K with tyrosine-phosphorylated Dab1. Inhibition of PI3K
activity prevents the formation of a normal cortical plate in an
in vitro slice migration assay. These results establish a conti-
guous biochemical pathway that begins at the plasma mem-
brane and continues to regulate the dynamic architecture of the
microtubular network.

Cultured primary embryonic forebrain neurons are a well
characterized model system in which most of the biochemical
mechanisms that are activated by Reelin binding at the neu-
ronal plasma membrane have been worked out. Analysis of
neuronal lysates and coimmunoprecipitation experiments now
show that the regulatory p85α subunit of PI3K complexes with
tyrosine-phosphorylated Dab1 to activate the enzyme (Fig. 3).
The same principal mechanism is used by the IRS proteins in
the insulin signaling pathway in primary embryonic neurons,
where insulin-dependent tyrosine phosphorylation of IRS trig-
gers its interaction with the SH2 domains of p85α, which allows the associated p110 subunit to become active. Like Dab1, IRS contains an N-terminal PTB domain, which mediates its binding to an NPXY motif in the insulin receptor tail when it is tyrosine-phosphorylated in response to insulin. A PI
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ular domain in the absence or presence of Reelin, making such a mechanism unlikely. Instead, Dab1 phosphorylation, which
appears to be amplified from a low basal level by receptor dimerization (41), SH2 domain interaction (33), and amplifi-
cation of SFK activity (7, 8), serves to recruit PI3K into a complex where it becomes activated. This model is supported by several lines of evidence. First, tyrosine phosphorylation of Dab1 is necessary for Reelin-triggered PI3K activation, and SFKs are
required for this to occur (Fig. 1). Second, the Reelin-dependent interaction of p85α and Dab1 correlates with the levels of Dab1
tyrosine phosphorylation, and the SFK inhibitor PP2 prevents co-immunoprecipitation of p85α and Dab1 in response to Reelin
treatment (Fig. 3). The phosphorylation level of neither PI3K
subunit was altered by Reelin stimulation, indicating that di-
ter tyrosine phosphorylation of this enzyme is not involved.
Furthermore, Fyn and Src, the main SFKs that are involved in
Reelin signaling, were not detected in p85α immunoprecipi-
tates from Reelin-stimulated neurons (Fig. 3), which argues
against a mechanism involving a scaffold that organizes SFKs
and PI3K in an activation complex (42).

IRS-1 activation of PI3K can be suppressed by serine phos-
phorylation (for review, see Ref. 13). Intriguingly, Dab1 is also
serine-phosphorylated by Cdk5 (43), although this is not mod-
ulated by Reelin. Cdk5, together with glycogen synthase kinase
3β, mediates phosphorylation of tau and also regulates micro-
tubule function by other mechanisms. This raises the possibil-
ity of a regulatory mechanism where Cdk5 might control gly-
cogen synthesis kinase 3β activation by Reelin.

How could Dab1 activate PI3K directly? One possible mech-
anism might involve the direct interaction of the p85α SH2
domain with phosphotyrosines in Dab1. The five clustered ty-
rosine residues that follow the PTB domain (Fig. 2) are obvious candidates for
potential Reelin-dependent tyrosine phosphorylation sites had
been mutated to non-phosphorylatable phenylalanines (6).

However, this does not completely rule out a Reelin-induced phosphorylation of this site in wild type neurons, which could
depend either on a conformational change or on another un-
identified tyrosine kinase that requires phosphorylation of the
mutated tyrosines for activation. Alternatively, a conforma-
tional change induced by phosphorylation of Tyr-198 and Tyr-
220 might result in exposure of the proline-rich potential Src
homology 3 domain binding site around residue 424 (Fig. 2B).

**Table 2.**
A functional role for the C-terminal region of Dab1 in neuronal migration is supported by findings in mice that express only one functional hypomorphic allele of the naturally occurring disruption of the catalytic subunits p110 \( \alpha \) and \( \gamma \) is not informative in this respect. Likewise, inhibition of Plk3 activity with the inhibitor LY294002 in a cortical slice migration assay prevented the formation of a normal cortical plate (Fig. 6), suggesting that Plk3 is necessary to mediate this Reelin-dependent step and plays a role in regulating cortical lamination during the development of the mammalian brain.

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REFERENCES

1. Rice, D. S., and Curran, T. (2001) *Annu. Rev. Neurosci.* 24, 1005–1039.
2. Tissir, F., and Goffinet, A. M. (2003) *Nat. Rev. Neurosci.* 4, 496–505.
3. Rice, D. S., and Curran, T. (1999) *Genes Dev.* 13, 2758–2773.
4. Herz, J., and Bock, H. H. (2002) *Annu. Rev. Biochem.* 71, 405–434.
5. Howell, B. W., Herrick, T. M., and Cooper, J. A. (1999) *Genes Dev.* 13, 643–648.
6. Howell, B. W., Herrick, T. M., Hildebrand, J. D., Zhang, Y., and Cooper, J. A. (2000) *Curr. Biol.* 10, 877–885.
7. Arnaud, L., Balif, B. A., Forster, E., and Cooper, J. A. (2003) *Curr. Biol.* 13, 9–17.
8. Bock, H. H., and Herz, J. (2003) *Curr. Biol.* 13, 18–26.
9. Beffert, U., Morfini, G., Bock, H. H., Reyna, H., Brady, S. T., and Herz, J. (2002) *J. Biol. Chem.* 277, 49054–49064.
10. Cantley, L. C. (2002) *Science* 296, 1655–1657.
11. Kato, R., Okkenhaug, K., Ahmad, K., White, S., Timms, J., and Waterfield, M. D. (2001) *Annu. Rev. Cell Dev. Biol.* 17, 615–675.
12. Vanhaesebroeck, B., Leevers, S. J., Ahmad, K., Timms, J., Kato, R., Driscoll, P. C., Woosholski, R., Parker, P. J., and Waterfield, M. D. (2001) *Annu. Rev. Biochem.* 70, 535–602.
13. Shepherd, P. R., Whymers, D. J., and Siddle, K. (1998) *Biochem. J.* 333, 471–490.
14. Funaki, M., Katagiri, H., Inukai, K., Kikuchi, M., and Asano, T. (2000) *Cell. Signal.* 12, 135–142.
15. Voloshin, H., Schnizler, D. G., Hadari, Y. R., Taylor, S. I., Accili, D., and Zick, Y. (1995) *J. Biol. Chem.* 270, 18083–18087.
16. Yenush, L., Makati, K. J., Smith-Hall, J., Ishibashi, O., Myers, M. G. Jr., and White, M. F. (1996) *J. Biol. Chem.* 271, 24300–24306.
17. Bazzini, G., Ingrosso, A., Brancaccio, A., Sciacchitano, S., Esposito, D. L., and Falasca, M. (2000) *Mol. Endocrinol.* 14, 823–836.
18. Jacobs, A. R., LeRoith, D., and Taylor, S. I. (2001) *J. Biol. Chem.* 276, 40795–40802.
19. Caroni, P. (2001) *EMBO J.* 20, 4332–4336.
20. Yin, H. L., and Janmey, P. A. (2003) *Annu. Rev. Physiol.* 65, 761–789.
21. Anderson, R. G. (1998) *Annu. Rev. Biochem.* 67, 199–225.
22. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol.* 14, 111–136.
23. Simons, K., and Tomine, D. (2000) *Nat. Rev. Mol. Cell Biol.* 1, 31–39.
24. Riddell, D. R., Sun, X. M., Stannard, A. K., Soutar, A. K., and Owen, J. S. (2001) *J. Lipid Res.* 42, 998–1002.
25. Bouche, P., Liu, P., Gotthardt, M., Hiesberger, T., Andersen, R. G., and Herz, J. (2002) *J. Biol. Chem.* 277, 15507–15513.
26. Trommsdorff, M., Borch, J. P., Margolis, B., and Herz, J. (1998) *J. Biol. Chem.* 273, 33556–33560.
27. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimip, J., Hammer, R. E., Richardson, J. A., and Herz, J. (1999) *Cell* 97, 709–711.
28. de Burgos-Cay, V., Naerhuizen, B., Goffinet, A. M., and Lambert de Rouvroy, C. (1998) *J. Neurosci. Methods* 82, 17–24.
29. Blom, N., Gammeltoft, S., and Brunak, S. (1999) *J. Mol. Biol.* 294, 1351–1362.
30. Yaffe, M. B., Leparc, G. G., Lai, J., Obata, T., Volinia, S., and Cantley, L. C. (2001) *Nat. Biotechnol.* 19, 348–353.
31. Buchdunger, E., O’Reilly, T., and Wood, J. (2002) *Eur. J. Cancer* 38, 528–536.
32. Keshvara, L., Benhayon, D., Magdaleno, S., and Curran, T. (2001) *J. Biol. Chem.* 276, 16008–16014.
33. Howell, B. W., Gertler, F. B., and Cooper, J. A. (1997) *EMBO J.* 16, 121–132.
34. Howell, B. W., Lanier, I. M., Frank, R., Gertler, F. B., and Cooper, J. A. (1999) *Mol. Cell. Biol.* 19, 5179–5188.
35. Domin, J., Dhand, R., and Waterfield, M. D. (1996) *J. Biol. Chem.* 271, 21614–21621.
36. Forster, E., Tielisch, A., Saum, B., Weiss, K. H., Johanssen, C., Grazus-Porta, D., Muller, U., and Froscher, M. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99, 13178–13183.
37. Hartfuss, E., Forster, E., Bock, H. H., Hack, M. A., Leprince, P., Luque, J. M., Herz, J., Frotscher, M., and Gut, M. (2003) *Development* 130, 4597–4609.
38. Weeber, E. J., Beffert, U., Jones, C., Christian, J. M., Forster, E., Sweet, J. D., and Herz, J. (2002) *J. Biol. Chem.* 277, 39944–39952.
39. Dittyauer, A., and Schachner, M. (2003) *Nat. Rev. Neurosci.* 4, 456–468.
40. Vainshtein, I., Kozacina, K. S., and Roth, A. A. (2001) *J. Biol. Chem.* 276, 8073–8078.
41. Kubo, K., Mikoshihi, K., and Nakajima, K. (2002) *Neurosci. Res.* 43, 381–388.
42. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) *Science* 263, 1609–1612.
43. Keshvara, L., Magdaleno, S., Benhayon, D., and Curran, T. (2002) *J. Neurosci.* 22, 4869–4877.
44. Herrick, T. M., and Cooper, J. A. (2002) *Development* 129, 787–796.
45. Stelt, P., C., Jeon, H., Song, H. K., Herz, J., Eck, M. J., and Blacklow, S. C. (2003) *Structure (London)* 11, 569–579.
46. Murata, H., Hreske, R. C., and Mueckler, M. (2003) *J. Biol. Chem.* 278, 21607–21614.
47. Foukas, L. C., and Okkenhaug, K. (2003) *Arch. Biochem. Biophys.* 414, 13–18.