Reelin-mediated Signaling Locally Regulates Protein Kinase B/Akt and Glycogen Synthase Kinase 3β*

Received for publication, September 9, 2002, and in revised form, September 27, 2002
Published, JBC Papers in Press, October 9, 2002, DOI 10.1074/jbc.M209205200

Uwe Beffert‡§, Gerardo Morfini§%, Hans H. Bock‡§, Huichuan Reyna%, Scott T. Brady%, and Joachim Herz‡¶

From the ‡Department of Molecular Genetics, ¶Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9046

Reelin is a large secreted protein that controls cortical layering by signaling through the very low density lipoprotein receptor and apolipoprotein E receptor 2, thereby inducing tyrosine phosphorylation of the adaptor protein Disabled-1 (Dab1) and suppressing tau phosphorylation in vivo. Here we show that binding of Reelin to these receptors stimulates phosphatidylinositol 3-kinase, resulting in activation of protein kinase B and inhibition of glycogen synthase kinase 3β. We present genetic evidence that this cascade is dependent on apolipoprotein E receptor 2, very low density lipoprotein receptor, and Dab1. Reelin-signaling components are enriched in axonal growth cones, where tyrosine phosphorylation of Dab1 is increased in response to Reelin. These findings suggest that Reelin-mediated phosphatidylinositol 3-kinase signaling in neuronal growth cones contributes to final neuron positioning in the mammalian brain by local modulation of protein kinase B and glycogen synthase kinase 3β kinase activities.

Reelin is a large secreted protein of ~400 kDa that is defective in the ataxic reeler strain (1). In Reelin-deficient mice (2) and humans (3), neurons fail to migrate to their proper positions, resulting in abnormal lamination of the neocortex and the hippocampus. Reelin is also needed for the cortical positioning of Purkinje cells, a requirement for granule cell proliferation and foliation in the cerebellum.

Reelin signaling requires binding to two members of the very low density lipoprotein (LDL) receptor gene family, the very low density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (apoER2), on the surface of the migrating neurons (4, 5). The phenotype of knockout mice in which both of these Reelin receptors have been inactivated by gene targeting is indistinguishable from that of reeler mice, suggesting that both receptors are obligate components of the Reelin-signaling pathway (6).

Further transmission of the signal is dependent upon the cytoplasmic adaptor protein Disabled-1 (Dab1). Dab1-deficient mice are indistinguishable from reeler and vldlr/apoer2 mutant mice (7–9). Dab1 interacts with NPXY motifs in the cytoplasmic domains of several LDL receptor family members (10), including VLDLR and apoER2 (6). Reelin binding to VLDLR and apoER2 induces tyrosine phosphorylation of Dab1 (5, 11). Replacement of tyrosine residues in Dab1 that are phosphorylated in response to Reelin by phenylalanines in knockin mice abolished Dab1 function (12). Furthermore, mice that lack both Reelin and Dab1 are no more affected than animals that lack only Reelin or Dab1 (11), suggesting that they are components of the same pathway.

Tyrosine phosphorylation of Dab1 allows it to interact with nonreceptor tyrosine kinases including Abl and Src family members, suggesting that Dab1 itself might function as a regulator of tyrosine kinase signaling in the cell (13). The phosphotyrosine binding (PTB) domain of Dab1, which mediates the interaction of the adaptor protein with the NPXY motifs in the cytoplasmic domains of the Reelin receptors, also has an independent affinity for phosphatidylinositol 4-phosphate- or PI 4,5-bisphosphate-rich microdomains in the plasma membrane (14). Both of these phospholipids are substrates for phosphatidylinositol 3-kinase (PI3K), a lipid kinase that is activated by tyrosine kinases at the cytoplasmic leaflet of the plasma membrane (15–17).

Here we show that Reelin activates a PI3K-dependent signaling cascade in cultured embryonic neurons, resulting in the activation of protein kinase B (PKB; also called Akt) and the inhibition of glycogen synthase kinase 3β (GSK-3β) by phosphorylation on specific regulatory residues. Genetic evidence indicates that activation of Reelin-induced PI3K signaling is dependent on the presence of VLDLR, apoER2, and Dab1. Reelin signaling results in a reduction of cellular levels of specific phosphorylated forms of the microtubule-associated protein tau. Either inhibition of Reelin binding to its receptors or inhibition of PI3K prevents Reelin-induced activation of this pathway. Furthermore, all components of the Reelin pathway are present or enriched in the axonal growth cones, suggesting that leading processes of migrating neurons are sites where positional cues conferred by Reelin are first received.

* This work was supported by National Institutes of Health Grants HL20948, HL63762, and NS43408 (to J. H.) and NS23368, NS23320, and NS41170 (to S. B.); grants from the Alzheimer Association and the Perot Family Foundation (to J. H.); and grants from the Juvenile Diabetes Foundation and the Welch Foundation (to S. B.). This work was further supported by fellowships from the Canadian Institute of Health Research (to U. B.), the Human Frontier Science Program (to U. B.), the Pew Latin American Fellows Program (to G. M.), and the Deutsche Forschungsgemeinschaft (to H. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

¶ Established Investigator of the American Heart Association and Parke-Davis and the recipient of a Wolfgang-Paul Award by the Humboldt Foundation. To whom correspondence should be addressed: Dept. of Molecular Genetics, UT Southwestern Medical Center, Dallas, Texas 75390-9046. Tel.: 214-648-5633; Fax: 214-648-8804; E-mail: Joachim.Herz@UTSouthwestern.edu.

1 The abbreviations used are: LDL, low density lipoprotein; VLDLR, very low density lipoprotein receptor; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; GSK-3β, glycogen synthase kinase-3β; GST, glutathione S-transferase; RAP, receptor-associated protein; Pipes, 1,4-piperazinediethanesulfonic acid; E15, E16, E17, and E18, embryonic day 15, 16, 17, and 18, respectively.
**EXPERIMENTAL PROCEDURES**

**Materials**—PI3K inhibitors wortmannin and LY294002 were purchased from Sigma and Cell Signaling Technology (Beverly, MA), respectively. All other kinase and phosphatase inhibitors were purchased from Calbiochem. All other reagents were from Sigma. Animals deficient for apoER2 (Ibp8) and VLDLR (vldlr) were maintained on a mixed Blalb/c6 background (7). All animals were maintained in accordance with National Institutes of Health and University of Texas Southwestern Animal Resources Center animal care guidelines.

**Activity Assays**—For PKB and CDK5 activities, 300 μg of total protein from cultured neurons were brought to 1 ml in freshly made kinase lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Heps, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 25 mM sodium pyrophosphate, 80 mM glycerol phosphate, 2 mM sodium vanadate, 0.1 mM okadaic acid, and mammalian protease inhibitor mixture (10 μM/ml protease inhibitors)). PKB was immunoprecipitated with 2 μg of mouse monoclonal anti-PKB antibody (SKB1 clone; Upstate Biotechnology, Inc., Lake Placid, NY) and CDK5 with 2 μg of anti-CDK5 antibody (C-8; Santa Cruz Biotechnology). Control immunoprecipitates were done with 2 μg of either normal mouse or rabbit IgG. After overnight rotation at 4 °C, 20 μl of protein G-Sepharose were added, and the tubes were incubated for 2 h. Immunoprecipitates were washed twice with protein lysis buffer and twice with kinase reaction buffer (KB buffer; 10 mM Heps, pH 7.4, 10 mM MgCl2, 0.1 mM EGTA, 1 mM dithiothreitol). Immunoprecipitated PKB and CDK5 were assayed in a total of 4 μl of KB buffer supplemented with 50 μM sodium orthovanadate, 1 μM PKI peptide (New England Biolabs, Beverly, MA), 50 μM microcystin, 50 μM okadaic acid, 100 μM cold ATP/10 μCi of [γ-32P]ATP. Tubes containing immunoprecipitated PKB were incubated with 33 μM crociste-paramyosin fusion protein as substrate (New England Biolabs) and CDK5 immunoprecipitates with 100 μM histone H1 (Calbiochem). After 20 min of incubation at 30 °C, reaction mixtures were stopped by adding 20 μl of 2× Laemmli buffer. Samples were run on SDS-PAGE. Gels were dried after staining with Coomassie Blue. Kinase activity values were obtained using a Typhoon phosphor imager after overnight exposure. Background kinase activity values from immunoprecipitates with nonimmune control antibodies were subtracted.

**Recombinant Reelin**—Stably transfected HEK-293 cells expressing recombinant full-length mouse Reelin (18) were kindly provided by Michael Protzer and Eckard Forster (Radboud University of Freiburg). For a typical experiment, 5 million HEK-293 cells were seeded on 10 8-cm² dishes with 4 × 10^6 cells/dish in 20 ml of Dulbecco’s modified Eagle’s medium (low glucose with 0.2% bovine serum albumin). Cells were grown for 2 days, and Reelin- or mock-conditioned media were collected, centrifuged at 3,000 × g for 15 min, and sterile-filtered. The supernatant was concentrated 20–40-fold using 100-KDa cut-off centrifugal filters (Millipore Corp., Bedford, MA). To ascertain that activation of the PI3K pathway was dependent on Reelin, concentrated Reelin samples were size-fractionated by gel filtration on Superose 6. Fractions containing full-length Reelin (~50% of total protein in the fractions) were pooled and tested for their ability to stimulate PI3K-dependent phosphorylation of PKB and GSK-3β. Results from these control experiments were indisputable from Reelin-conditioned culture supernatant experiments. Control media from untransfected 293 cells were collected, concentrated, and applied to cultured cells under the exact same conditions and in exactly the same relative concentrations as the Reelin-conditioning samples.

**Neuronal Cultures**—Embryonic cortical neurons from mice (E16-E18) or rats (E17-E18) were obtained using standard protocols (19). For wild type mice or rats, embryos were collected at E15. Cortical lobes were isolated, and after removing the meninges, the lobes were chopped into small pieces, pooled, and trypsinized for 15 min at 37 °C. Trypsinization was stopped by the addition of fetal calf serum (one-twentieth volume). After centrifugation, cells were washed twice in Hank’s balanced salt solution (Invitrogen). Neurons were dissociated by triturating 40 times in Hanks’ balanced salt solution with 12.5 mM MgCl2, 0.025% DNase, 0.4 μg/ml trypsin inhibitor, and 2 mg/ml bovine serum albumin with a polished glass Pasteur pipette and transferred to Neurobasal medium containing B27 supplement (Invitrogen), penicillin/streptomycin, and 1 mM glutamine. Cells were plated at ~1,000 cells/mm² on poly-l-lysine (Sigma)-coated plates. Wild type embryonic neurons were obtained from mouse or from rat embryos and yielded identical results in all experiments. For Dab1 or apoER2/VLDLR-deficient cultures, mouse embryos were treated individually until plating onto cell culture dishes, with a hind portion of the embryo used for genotyping.

**Reelin Stimulation**—After 2 days in culture, one-half of the neuronal culture medium was exchanged for fresh medium. On day 3 of plating, cells were stimulated either with mock-conditioned medium or with Reelin (see above). The estimated final concentration of Reelin added to the culture medium was 5 nM. Cells were washed once in PBS and then lyzed with lysis buffer (PBS with 2 mM EDTA, 1% Triton X-100, 0.25% deoxycholic acid, 0.5% SDS, protease inhibitors (mixture of PenStrep-free Roche Complete, Basic Rat Protease Inhibitor, phosphatase inhibitor mixture 1 and 2 (Sigma)). Lysate was centrifuged for 20 min at 4 °C, and supernatants were assayed for protein content.

**Specific Inhibitors**—The glutathione S-transferase (GST) receptor-associated protein (RAP) fusion protein and GST protein were prepared as described (20). GST-RAP and GST were added at 30 μg/ml for 1 h prior to and during Reelin stimulation. PKB inhibitors LY294002 (catalog no. 9901; Cell Signaling Technology) and Wortmannin (W1628; Sigma) were applied at the indicated concentrations 1 h prior to Reelin stimulation and for duration of the assay.

**Immunoblotting**—Equal amounts of protein (10 μg for neuronal cultures and 25 μg for brain fractions) were separated by 4–15% gradient SDS-PAGE, electroblotted onto nitrocellulose membranes, and blocked in Blotto (5% milk in phosphate-buffered saline with 0.05% Tween 20, pH 7.4; Sigma) for 1 h. Membranes were incubated overnight at 4 °C with polyclonal or monoclonal antibodies directed against Dab1, apoER2, CDK5 (sc-173; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-GSK-3β (Ser²¹) (catalog no. 9336; Cell Signaling Technology), GSK-3β (G22320; Transduction Laboratories, Lexington, KY), phospho-PKB (Ser⁴⁷³) (44-622, BioSource, Camarillo, CA), PKB (catalog no. 9272; Cell Signaling Technology), PI3K (66-497; Upstate Biotechnology), phospho-Pten (Ser⁴⁰⁵) (catalog no. 9551; Cell Signaling Technology), Reelin (G10; kindly provided by Andre Goffinet), phosphotyrosine (4G10; Upstate Biotechnology), or phospho-CDK5 (Thr²⁸⁷) (catalog no. 9258; Cell Signaling Technology) or antisera to DAB1 (innogenetics, Ghent, Belgium). After washing, secondary horseradish peroxidase-linked antibodies (Amersham Biosciences) were applied at 1:2000 in Blotto for 1 h, washed, and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to Kodak X-omat Blue XD-1 film (Eastman Kodak Co.).

**Immunoprecipitations**—Reelin-stimulated neurons were harvested in radioimmuno precipitation buffer (PBS with 5% EDTA, 2% EGTA, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholic acid, phosphatase, and protease inhibitors). Samples were normalized for protein and then incubated with Dab1 antibody or preimmune antibodies that were bound to protein A-Sepharose (Sigma) for 4 °C for 2 h, followed by three washes with radiimmunoprecipitation buffer. Samples were then incubated with twice-concentrated gel-loading buffer (4% SDS, 0.4% glycerol, 0.2 M Tris-HCl, pH 6.8, 5.6 mM 2-mercaptoethanol, 5% EDTA, and 0.02% bromphenol blue) at 100 °C for 5 min, followed by 4–15% SDS-PAGE and immunoblotting.

**Isolation of Growth Cone Particles by Subcellular Fractionation**—Fetal rat brain (gestational stage E18) was fractionated as described by Pfenniger et al. (21) to obtain growth cone particles. Briefly, the low speed supernatant of fetal brain homogenate was loaded on a discontinuous sucrose gradient in which the 0.75 and 1 m sucrose layers were replaced with a single 0.83 m sucrose step. This facilitated collection of the interface and increased growth cone particle yield without decreasing purity (22). The 0.32 m/0.83 m interface was washed with 0.32 m sucrose, and centrifuged to yield the GCP fraction, which was resuspended in 0.32 m sucrose for use in experiments.

**Immunocytochemistry**—Cortical neurons were cultured in Duboceco’s modified Eagle’s medium plus B27 supplement (Invitrogen) for 3 days on dishes coated with 0.3 mg/ml poly-n-lysine. After stimulation with conditioned media or with Reelin, cells were processed for immunochemistry. Briefly, cells were first washed with warm PBS buffer (60 mM Pipes, 25 mM Hepes, 1 mM MgCl₂, pH 7.4) and fixed in 2% paraformaldehyde, 0.01% glutaraldehyde in PHEM at 37 °C for 10 min. After fixation, cells were washed three times with PBS, extracted with 0.2% Triton X-100 for 10 min, and blocked in 10% goat serum for 1 h. Primary antibodies were diluted in block solution and incubated overnight at 4 °C. Immunohistochemistry-specific polyclonal rabbit anti-phospho-PKB (Cell Signaling Technology) and 4G10 antibody were used at a 1:100 dilution. Monoclonal anti-tubulin (DM1A clone) was used at a 1:400 dilution.
RESULTS

To identify the biochemical signaling pathways that are activated by Reelin signaling in the embryonic brain, we developed a sensitive in vitro assay in which we could follow the regulation of cellular kinase activities in whole cell lysates in response to Reelin exposure. To achieve this, it was necessary to reduce Reelin-independent cellular signal input that would otherwise differentially affect the various pools of these kinases and thereby obscure any Reelin-specific effect. This was accomplished by culturing primary embryonic neurons for several days in a minimal medium before stimulation with recombinant Reelin. Reelin-independent kinase activation, for instance by cell adhesion molecules such as integrins, was minimized by plating the cells on polylysine instead of other commonly used mixtures of extracellular matrix components.

Reelin was partially purified from the supernatant of stably transfected 293 cells by column chromatography and size exclusion filtration. Conditioned medium from nontransfected 293 cells served as a control in all experiments. Exposure of cultured neurons to Reelin (Fig. 1A, lane 2) greatly increased the levels of phosphorylated Dab1 in immunoprecipitates (upper panel) as well as in whole cell lysates (middle panel) compared with mock medium (lane 1). Since tau is hyperphosphorylated in animals genetically deficient in Reelin signaling (5), we investigated whether Reelin can directly reduce tau phosphorylation in primary neuronal cultures. Immunoblot analysis of extracts with an antibody directed against the tau phospho-Ser202/Thr205 epitope revealed a marked reduction of phospho-tau in response to Reelin. This suggested that one or more kinases that phosphorylate tau at this site are inhibited as a result of Reelin signaling.

Two multifunctional neuronal kinases implicated in tau phosphorylation are CDK5 and GSK-3β. The activity of the latter is inhibited by phosphorylation of a serine at position 9 by PKB. Enzymatic activity of the total cellular pool of CDK5 was unaffected by Reelin (93.2 ± 4.5% of control). However, the activity of PKB, the primary cellular kinase inhibiting GSK-3β, was increased 2-fold in the same cells (205.6 ± 5.0% of control). This suggested that PKB and GSK-3β might be part of a Reelin signaling cascade. A time course experiment (Fig. 1B) showed that Dab1 phosphorylation reached maximal levels after 20 min of Reelin stimulation (lane 4). Phosphorylation of both GSK-3β and PKB showed a similar pattern of activation, with maximal phosphorylation by 20–30 min and a clear reduction by 60 min. Total levels of GSK-3β and PKB remained unchanged under the same conditions. Enzymatic activity of PKB, but not CDK5, was stimulated by Reelin and correlated with phosphorylation of PKB on Ser473. All subsequent experiments were thus carried out for 20 min.

Maximal activation of PKB requires phosphorylation at two sites: Ser473 and Thr308. To determine whether Reelin differentially affected the phosphorylation of PKB at these sites, we performed the experiment shown in Fig. 1C. Primary embryonic neurons were unstimulated (lanes 1 and 2), exposed to mock medium (lanes 3 and 4), or exposed to Reelin (lanes 5 and 6). Whereas Thr308 phosphorylation was unaffected by any of these conditions, Ser473 phosphorylation was induced only when Reelin was present (lanes 5 and 6) and correlated with Dab1 phosphorylation in both rat and mouse primary cultured neurons (Fig. 1, B and C, respectively). Total levels of Dab1, PKB, and GSK-3β remained unchanged with Reelin treatment.

RAP universally blocks the binding of ligands to LDL receptor family members (20, 23). RAP also blocks the Reelin-inhibited phosphorylation of Dab1 by PKB and GSK-3β requires Reelin binding to both receptors and subsequent Dab1 phosphorylation, we incubated primary mouse neurons in the presence (Fig. 2a, lanes 1, 4, and 6) or absence (lanes 2, 3, 5, and 7) of Reelin, GST-RAP fusion protein (lanes 3–5), or the GST control protein (lanes 6 and 7). A similar experiment was performed in primary rat neurons (Fig. 2b). GST-RAP effectively blocked Reelin-induced Dab1 phosphorylation (Fig. 2a, lane 4), whereas GST-RAP alone had no effect (Fig. 2a, lanes 3 and 5). Reelin-stimulated phosphorylation of PKB at Ser473 was marked...
Dab1 is one of several cytoplasmic adaptor proteins that bind to the intracellular domains of apoER2 and VLDLR (24, 25). To determine whether Dab1 or some other adaptor or scaffolding protein were required for Reelin-mediated activation of the PKB pathway, we prepared neurons from embryos that were wild type, heterozygous, or homozygous for the dab1 knockout allele. As expected, no phosphorylated or total Dab1 protein was detected in dab1−/− neurons (Fig. 3b, lanes 1 and 2), and Reelin-induced phosphorylation of PKB on Ser473 or of GSK-3β on Ser9 occurred only in cells that contained at least one functional dab1 allele (lanes 3–6). Total levels of PKB and GSK-3β were independent of dab1 genotype or Reelin treatment.

To determine whether Reelin stimulation of PKB and inhibition of GSK-3β required activation of PI3K, we incubated neurons with Reelin in the presence or absence of PI3K inhibitors: LY294002 (Fig. 4A) or wortmannin (Fig. 4B). Dab1 phosphorylation by Reelin was unaltered whether or not LY294002 was present (Fig. 4A). However, Reelin-induced phosphorylation of both PKB and GSK-3β were completely blocked by LY294002 (lanes 5 and 6), although total levels of both proteins remained unchanged. Wortmannin also markedly inhibited phosphorylation of PKB at Ser473 (Fig. 4B). Activation of PI3K and subsequent phosphorylation of PKB can also be achieved by inactivation of a negative regulator of PI3K, the lipid phosphatase PTEN. However, no changes in the phosphorylation state of PTEN at its regulatory Ser380 residue were observed under any conditions (data not shown), suggesting that PTEN protein was not involved in Reelin signaling.

Migrating neurons in the mammalian brain probably receive extracellular signals through their leading processes or growth cones. We found that the Reelin receptor apoER2 and the adaptor protein Dab1 as well as PI3K were relatively enriched in purified axonal growth cones from fetal rat brains (Fig. 5A). PKB was also present in growth cones but appeared similarly distributed between growth cones and total cell lysate. The enrichment of nonphosphorylated tau and GAP-43 and the relative depletion of the dendritic marker MAP2 confirm the high degree of enrichment of axonal growth cones that was achieved in this preparation (22). To determine whether Reelin treatment preferentially affected signaling components in growth cones, we used immunocytochemistry to detect activated components of the Reelin signaling pathway in growth cones of cortical neurons after 3 days in culture. Compared with mock-treated neurons, anti-phosphotyrosine immunoreactive components of the Reelin signaling pathway in growth cones were preferentially increased in growth cones with Reelin treatment (Fig. 1A). Furthermore, the levels of all other tyrosine-phosphorylated proteins were unchanged in total extracts from primary embryonic neurons (Fig. 1A), making it likely that the increase in anti-phosphotyrosine immunoreactivity in the growth cones is primarily due to Dab1 phosphorylation. Similarly, phospho-PKB immunoreactivity at Ser473 was preferentially increased in growth cones with Reelin treatment (Fig. 5, H and J) and enriched in filopodia as compared with microtubules (Fig. 5, I and J). These data indicate that Reelin can specifically activate the PI3K/PKB pathway in growth cones of embryonic cortical neurons.

**DISCUSSION**

Genetic and biochemical evidence places the signaling protein Reelin, the neuronal cell surface receptors VLDLR and apoER2, and the cytoplasmic adaptor protein Dab1 in a linear
observed in neurons derived from double receptor knockout cells (lanes 5/H9252 reduced Reelin-induced phosphorylation of PKB and GSK-3 lanes 3 induced with Reelin (embryo. A but not Dab1, is PI3K-dependent. A

Genetic deficiency of apoER2, VLDLR, and Dab1 prevents Reelin-induced PKB phosphorylation. a, mouse embryonic neurons were prepared from E16 embryos from a single mating, generating the three genotypes indicated at the bottom. In wild type (lanes 5 and 6) or heterozygous (lanes 3 and 4) neurons, Reelin induced phosphorylation of Dab1, PKB, and GSK-3β. No induction of PKB or GSK-3β phosphorylation was observed in cells lacking Dab1 protein (lanes 1 and 2).

B

Fig. 4. Reelin-induced phosphorylation of PKB and GSK-3β, but not Dab1, is PI3K-dependent. A, rat embryonic neurons (E16) were preincubated in the absence (lanes 1–3) or presence (lanes 4–6) of the PI3K inhibitor LY294002 (10 μM) for 20 min and then either not induced (lanes 1 and 4), exposed to mock medium (lanes 2 and 5), or induced with Reelin (lanes 3 and 6) for 20 min. LY294002 markedly reduced Reelin-induced phosphorylation of PKB and GSK-3β, whereas Dab1 phosphorylation remained unaffected. Total protein levels for Dab1, PKB, and GSK-3β remained unchanged by LY294002 treatment, although background phosphorylation levels of PKB and GSK-3β were reduced. B, mouse embryonic neurons (E15) were either preincubated (lanes 3 and 4) with the PI3K inhibitor wortmannin (100 nM) or not (lanes 1 and 2) and exposed to Reelin (lanes 2 and 4) or mock-conditioned medium (lanes 1 and 3). Reelin-induced PKB phosphorylation was markedly reduced by Wortmannin treatment, but phosphorylation of Dab1 was unaffected.

pathway that is critical for neuronal migration and proper organization of the mammalian brain (2, 4–9). Reelin-induced Dab1 phosphorylation in primary cortical neurons (11) can be inhibited by blocking Reelin binding to the lipoprotein recep-
tors apoER2 and VLDLR (5). Here we provide genetic and biochemical evidence that the signaling cascade induced by Reelin downstream of Dab1 inhibits GSK-3β and suppresses phosphorylation of the microtubule-associated protein tau. This cascade involves PI3K and PKB kinase activities acting downstream from and dependent on the presence of apoER2, VLDLR, and Dab1.

Our results suggest a model, shown in Fig. 6, whereby Reelin would initially bind to a protein complex on the surface of migrating neurons that contains as obligate components one or both of the lipoprotein receptors apoER2 and VLDLR. A likely place where this might occur is the axonal growth cone, the leading edge of the migrating neuron. Growth cones are the first to make contact with the Reelin-expressing Cajal-Retzius neurons at the border between the cortical plate and the marginal zone (26), and several components of the Reelin signaling complex are enriched in growth cones (Fig. 5). An as yet unidentified tyrosine kinase would then be recruited to the receptor-signaling complex, leading to phosphorylation of Dab1 bound to the cytoplasmic tails of VLDLR and apoER2. Tyrosine phosphorylation of Dab1 is required to activate PI3K, which in turn leads to stimulation of PKB by phosphorylation on Ser473 and inactivation of GSK-3β by phosphorylation on Serβ. Activation of PI3K and PKB-mediated signaling pathways can impact on a broad range of cellular functions (15–17), including gene transcription, protein translation, metabolism, axonal transport, cell survival, growth, and migration. Specificity is probably maintained by temporal as well as spatial restriction of the signal (i.e., primarily to the growth cone). In our model, Reelin-induced suppression of GSK-3β activity may block phosphorylation of GSK-3β substrates (e.g., the microtubule-associated protein tau or the microtubule motor kinesin) (27). Conversely, failure to suppress GSK-3β activity of a specific GSK-3β pool (for instance by defects in Reelin, VLDLR, apoER2, or Dab1) could explain the observed increases in tau phosphorylation in these different mutant mouse strains (5) as well as changes in other GSK-3β-sensitive processes.

This model is based on several lines of evidence. First, Reelin induces phosphorylation of Dab1, PKB, and GSK-3β, an event that requires the presence of VLDLR and apoER2 and binding of Reelin to these receptors. Second, cells deficient in Dab1 are unable to induce phosphorylation of PKB or GSK-3β in response to Reelin. Third, inhibitors of PI3K such as wortmannin and LY294002 inhibit Reelin-induced phosphorylation of PKB and GSK-3β but not of Dab1. Finally, several mediators of
Reelin-mediated Signaling Regulates PKB/Akt and GSK-3β

Fig. 5. Components of the Reelin signaling pathway are present in growth cones. A, total cell lysate and axonal growth cones were prepared from E18 rat brain as described above. 10 μg of protein were loaded in each lane and immunoblotted for the indicated proteins. ApoER2, DAB1, and PI3K are all enriched in growth cone particles relative to total lysate, and PKB is readily detectable, indicating that components of the Reelin pathway are present in growth cones. GAP43 and tau are also enriched in growth cones to different extents, whereas actin is present but not enriched in growth cones relative to lysate. Growth cone particles are primarily derived from growing axons, since MAP2, a dendritic marker, is depleted in this fraction relative to total lysate. B–G, Primary rat cortical neurons were cultured and immunostained for phosphotyrosine after exposure to mock medium (B–D) or to Reelin (E–G) for 20 min. Neuronal axons and growth cones show increased levels of tyrosine-phosphorylated proteins in response to Reelin. Scale bar, 10 μm. H–J, immunostaining of rat cortical neurons reveals enrichment of Ser^473-phosphorylated PKB (H; red in merged panel J) in axonal growth cones relative to tubulin (H; green in merged panel J). Scale bar, 15 μm. Insets show higher magnification of selected growth cones. Scale bar, 5 μm.

Reelin signaling, including apoER2 and Dab1, are highly enriched in axonal growth cones.

Reelin-induced Phosphorylation of PKB and GSK-3β Requires ApoER2, VLDLR, and Dab1—The present results provide genetic (Fig. 3) and biochemical (Fig. 2) evidence that apoER2 and VLDLR are required for Reelin-induced phosphorylation of Dab1, PKB, and GSK-3β. They do not exclude a role for co-receptors in the process. Reelin-induced phosphorylation of Dab1, PKB, and GSK-3β was greatly reduced by RAP, an inhibitor of Reelin binding to both receptors (Fig. 2). Furthermore, PKB phosphorylation was abolished in neurons from mice in which both apoER2 and VLDLR had been genetically disrupted (Fig. 3a). In contrast, cells that contained at least one remaining receptor allele (either VLDLR or apoER2) were still able to activate PKB to some extent. This result correlates with our finding that only those mice that lack all four receptor alleles show the same severe neuronal migration defects as the reeler mutants (6, 28), whereas those missing only three alleles develop milder phenotypes similar to the respective single receptor knockouts.

Reelin-mediated activation of PKB and phosphorylation of GSK-3β was also dependent on Dab1 (Fig. 3b). Cells that were heterozygous for Dab1 retained the ability to phosphorylate PKB and GSK-3β in response to Reelin. Taken together, these results show that apoER2, VLDLR, and Dab1 are required for Reelin-induced regulation of PKB- and GSK-3β-mediated signaling and that activation of this pathway appears to be necessary for proper neuronal positioning in the developing mammalian brain.

Reelin Signaling Requires PI3K Activity—PKB activity can be regulated by at least two distinct mechanisms: directly through PI3K activation or by inactivation of PTEN, a phosphatase that hydrolyzes the PI3K product phosphatidylinositol 3,4,5-trisphosphate (29). No changes were observed at the Ser^308 phosphorylation site in PKB in response to Reelin. Taken together, these results demonstrate that PI3K activity is required for phosphorylation of PKB at
Ser473, since both kinase inhibitors effectively eliminated the Reelin-induced phosphorylation of PKB (Fig. 6). Consistent with this finding is a recent report that shows that PI3K activity is required for migration of cortical interneurons (30).

Neither PI3K inhibitor affected the ability of Reelin to induce Dab1 phosphorylation, indicating that Dab1 phosphorylation precedes activation of PI3K in this sequence of events. Phosphorylation of PKB at Thr308, a second site that is important for its activation, was not affected by Reelin. These findings identify Ser473 phosphorylation of PKB as a distinct branch of a PI3K-dependent signaling pathway that is controlled by Reelin in embryonic neurons.

The sequence of signaling events initiated by Reelin as proposed here is naturally linear. However, most signaling pathways are multifaceted, branching and converging at various points. For example, CDK5, another kinase that can phosphorylate tau, is also implicated in neuronal migration (31, 32). Mice deficient in CDK5 or its activators p35 and p39 display severe neuronal migration defects similar to but distinct from those seen in reeler mice (32, 33). We observed no changes in CDK5 activity in any of the mutant mouse strains or in response to Reelin stimulation of primary neuronal cultures. These findings suggest that CDK5 is not a direct downstream target of Reelin and its receptors, although it may act on a common target.

The identity of such a target remains to be determined. Tau hyperphosphorylation could be a causal event for neuronal migration defects or a secondary consequence. GSK-3β and CDK5 are major kinases responsible for normal and pathological phosphorylation of tau protein in vivo, and Reelin appears to affect tau phosphorylation through GSK-3β. However, tau knockout animals display normal brain organization with no indication of neuronal migration defects (34). This suggests that changes in tau phosphorylation might occur parallel to alteration of neuronal migration patterns.

Interestingly, both CDK5 and GSK-3β have been implicated in regulation of kinesin-driven motility in neurons. GSK-3β specifically inhibited anterograde, but not retrograde, fast axonal transport in vivo (27). GSK-3β was enriched in neuronal growth cones, where delivery of membrane proteins and Reelin signaling occurs. Similarly, inhibition of CDK5 specifically inhibits anterograde fast axonal transport (35). The essential role of fast axonal transport in neuronal function and the finding that both GSK-3β and CDK5 play a role in regulating fast axonal transport suggest that regulation of these pathways by extracellular cues such as Reelin may be a key step in neuronal migration. Consistent with this model, GSK-3β in growth cones has been implicated in signaling by the axonal growth inhibitor protein semaphorin 3A (36). Regulation of GSK-3β through extracellular cues such as Reelin and semaphorins could modulate delivery of newly synthesized material to a specific subcellular compartment.

In conclusion, our results provide unequivocal genetic evidence that Reelin activates a PI3K-dependent signaling cascade through the lipoprotein receptors apoER2 and VLDLR and the adaptor protein Dab1. This activation occurs in axonal growth cones, suggesting that integration of the Reelin signal at the leading processes of migrating neurons may be crucial for activating the molecular transport machinery that moves the cell body to its final position, thereby determining the lamination of the mammalian cortex.

Acknowledgments—We are indebted to Wen-Ling Niu, Robin Wray, Jenny Hayes, Patrick Pratumrat, and LaMetria Blair for invaluable technical support; Eduardo Churuquina for image processing; Karl Pfenninger and Keith Mikule for the GCP preparation; and Tom Curran and Eckardt Förster for Reelin expression vectors and for sharing the Reelin expressing 293 cell line. We also thank Li-Huei Tsai, Eric Johnson, and Ann Marie Pendergast for critical comments.

REFERENCES

1. Falconer, D. S. (1951) J. Genet. 50, 192–201
2. D’Arcangelo, G., Miao, G. G., Chen, S. C., Soares, H. D., Morgan, J. I., and Curran, T. (1995) Nature 374, 719–723
3. Hong, S. E., Shugart, Y. Y., Huang, D. T., Shahwan, S. A., Grant, P. E., Hourihan, J. O., Martin, N. D., and Walsh, C. A. (2000) Nat. Genet. 26, 95–99
4. D’Arcangelo, G., Homayouni, R., Keshvarya, L., Rice, D., Sheldon, M., and Curran, T. (1999) Neuron 24, 471–479
5. Hiesberger, T., Trommdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A., and Herz, J. (1999) Neuron 24, 481–499
6. Trommdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimip, J., Hammmer, R. E., Richardson, J. A., and Herz, J. (1999) Cell 97, 689–701
7. Howell, B. W., Hawkes, R., Soriano, P., and Cooper, J. A. (1997) Nature 389, 733–737
8. Sheldon, M., Rice, D. S., D’Arcangelo, G., Yamasaki, H., Nakajima, K., Mikoshiba, K., Howell, B. W., Cooper, J. A., Goldowitz, D., and Curran, T. (1997) Nature 389, 725–730
9. Ware, M. L., Fox, J. W., Gonzalez, J. L., Davis, N. M., Lambert de Rouvroit, C., Russo, C. J., Chu, S. C., Jr., Goffinet, A. M., and Walsh, C. A. (1997) Neuron 19, 239–249
10. Trommdorff, M., Borg, J. P., Margolis, B., and Herz, J. (1998) J. Biol. Chem. 273, 33556–33560
11. Howell, B. W., Herrick, T. M., and Cooper, J. A. (1999) Trends Biochem. Sci. 24, 557–566
12. Cantrell, D. A. (2001) J. Cell Sci. 114, 1439–1445
13. Kato, R., Okkenkahn, K., Ahmad, K., White, S., Timms, J., and Waterfield, M. D. (2001) Annu. Rev. Cell Dev. Biol. 17, 615–675
14. D’Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., and Curran, T. (1997) J. Neurosci. 17, 23–31
15. Banker, G., and Goslin, K. (1988) Nature 336, 185–186
16. Herz, J., Goldstein, L. J., Strickland, D. K., Ho, Y. K., and Brown, M. S. (1991) J. Biol. Chem. 266, 21232–21238
17. Pfenninger, K. H., Ellis, L., Johnson, M. P., Friedman, L. B., and Soslo, S. (1983) Cell 35, 573–584
18. Lobbe, R., Helman, S. M., Wood, M. R., Quirrega, S., de la Houssaye, B. A., Miller, V. E., Negre-Aminou, P., and Pfenninger, K. H. (1996) Brain Res. Dev. Brain Res. 96, 83–96
19. Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M., and Agrawes, W. S. (1996) J. Biol. Chem. 271, 17401–17404
20. Gotthardt, M., Trommdorff, M., Nevitt, M. P., Shelton, J., Richardson, J. A., Stockinger, W., Nimip, J., and Herz, J. (1999) J. Biol. Chem. 275, 25616–25624
21. Stockinger, W., Brandes, C., Faschling, D., Hermann, M., Gotthardt, M., Herz, J., Schneider, W. J., and Nimip, J. (2000) J. Biol. Chem. 275, 25625–25632
22. Nadarajah, B., Brunstrom, J. E., Zentzledinger, J., Wong, R. O., and Pearlman, A. L. (2001) Nat. Neurosci. 4, 143–150
23. Morfini, G., Szefenby, G., Illuru, R., Ratner, N., and Brady, S. T. (2002) EMBO J. 23, 281–293
24. Lambert de Rouvroit, C., and Goffinet, A. M. (1998) Adv. Anat. Embryol. Cell Biol. 150, 1–106
25. Scheid, M. P., and Woodgett, J. R. (2001) Nat. Rev. Mol. Cell Biol. 2, 760–768
26. Pollex, F., Wulff, K. L., Dijkhuizen, P. A., Vitalis, T., and Ghosh, A. (2002) Development 129, 3147–3160
27. Lew, J., Huang, Q. Q., Qi, Z., Winkelten, R. J., Abersold, R., Hunt, T., and Wang, J. H. (1994) Nature 371, 423–426
28. Oshikawa, T., Ward, J. M., Huh, C. G., Langenecker, G., Yevarea, Pant, H. C., Brady, R. O., Martin, L. J., and Kulkarni, A. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11173–11178
29. Ko, J., Humbert, S., Brunson, R. T., Takahashi, S., Kulkarni, A. B., Li, E., and Tsai, L. H. (2001) J. Neurosci. 21, 6758–6772
30. Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato, Yoshitake, R., Takesi, Y., Noda, T., and Hirakawa, N. (1994) Nature 369, 488–491
31. Ratner, N., Bloom, G. S., and Brady, S. T. (1998) J. Neurosci. 18, 7717–7726
32. Eichkoff, B. J., Walsh, F. S., and Doherty, P. (2002) J. Cell Biol. 157, 211–217