Disabled1 Regulates the Intracellular Trafficking of Reelin Receptors*

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Reelin is a huge secreted protein that controls proper laminar formation in the developing brain. It is generally believed that tyrosine phosphorylation of Disabled1 (Dab1) by Src family tyrosine kinases is the most critical downstream event in Reelin signaling. The receptors for Reelin belong to the low density lipoprotein receptor family, most of whose members undergo regulated intracellular trafficking. In this study, we propose novel roles for Dab1 in Reelin signaling. We first demonstrated that cell surface expression of Reelin receptors was decreased in Dab1-deficient neurons. In heterologous cells, Dab1 enhanced cell surface expression of Reelin receptors, and this effect was mediated by direct interaction with the receptors. Moreover, Dab1 did not stably associate with the receptors at the plasma membrane in the resting state. When Reelin was added to primary cortical neurons, Dab1 was recruited to the receptors, and its tyrosine residues were phosphorylated. Although Reelin and Dab1 colocalized well shortly after the addition of Reelin, Dab1 was no longer associated with internalized Reelin. When Src family tyrosine kinases were inhibited, internalization of Reelin was severely abrogated, and Reelin colocalized with Dab1 near the plasma membrane for a prolonged period. Taken together, these results indicate that Dab1 regulates both cell surface expression and internalization of Reelin receptors, and these regulations may play a role in correct laminar formation in the developing brain.

In the developing forebrain, significant populations of neurons are generated from neuronal progenitors in the neuroepithelium surrounding the lateral ventricle. These neurons then migrate radially toward the pial surface and are deposited at the cortical plate in an “inside-out” order, creating the orchestrated laminar structure of the cerebral cortex (reviewed in Ref. 1). In both humans and mice, the reeler mutation causes abnormal laminar formation in the cerebral cortex, cerebellum, and hippocampus (reviewed in Ref. 2). The causative gene, reelin, encodes a large secreted protein containing 3,461 amino acids (3). Reelin is produced by Cajal-Retzius cells, which are located at the marginal zone of the developing cerebral cortex (4), and it regulates the proper positioning of radially migrating neurons.

Mammalian Disabled1 (Dab1), the homologue of Drosophila Disabled, was first reported to be a binding partner with the Src homology 2 domain of Src kinase (5). Mice carrying an engineered mutation of Dab1 or spontaneous Dab1 mutant mice exhibit phenotypes indistinguishable from reeler mice (6–8). Reelin activates Src family tyrosine kinases (STKs), which then phosphorylate Dab1 at three tyrosine residues (Tyr-198, Tyr-220, and Tyr-233) (9–12). The tyrosine phosphorylation of Dab1 is thought to be critical for Reelin signaling, because the Dab1 mutant phenotype can be rescued by introduction of cDNA encoding the wild type, but not by a mutant Dab1 whose five tyrosine residues are substituted by phenylalanine residues (13). Two members of the low density lipoprotein receptor (LDLR) family, apolipoprotein E receptor 2 (apoER2) and very low density lipoprotein receptor (VLDLR), have been identified as Reelin receptors, and double mutation of these two lipoprotein receptors results in the reeler phenotype (14). These observations led to the widely accepted model that Reelin signal is transduced to Dab1 through apoER2 and VLDLR in migrating neurons.

Members of the LDLR family internalize lipoproteins into cells by clathrin-dependent endocytosis (15). Reelin is also internalized into cells in a receptor-dependent manner (16). The phoshotyrosine binding (PTB) domain of Dab1 interacts with the NPXY motif (N, asparagine; P, proline; X, any amino acid; Y, tyrosine) of the cytoplasmic tail of Reelin receptors in a phosphorylation-independent manner (17). Several cytoplasmic adaptor proteins containing a PTB domain regulate intracellular trafficking of receptors containing the cytoplasmic NPXY motif (18, 19). This evidence led us to hypothesize that Dab1 regulates the intracellular trafficking of Reelin and/or its downstream event in Reelin signaling. The receptors for Reelin belong to the low density lipoprotein receptor family, most of whose members undergo regulated intracellular trafficking. In this study, we propose novel roles for Dab1 in Reelin signaling. We first demonstrated that cell surface expression of Reelin receptors was decreased in Dab1-deficient neurons. In heterologous cells, Dab1 enhanced cell surface expression of Reelin receptors, and this effect was mediated by direct interaction with the receptors. Moreover, Dab1 did not stably associate with the receptors at the plasma membrane in the resting state. When Reelin was added to primary cortical neurons, Dab1 was recruited to the receptors, and its tyrosine residues were phosphorylated. Although Reelin and Dab1 colocalized well shortly after the addition of Reelin, Dab1 was no longer associated with internalized Reelin. When Src family tyrosine kinases were inhibited, internalization of Reelin was severely abrogated, and Reelin colocalized with Dab1 near the plasma membrane for a prolonged period. Taken together, these results indicate that Dab1 regulates both cell surface expression and internalization of Reelin receptors, and these regulations may play a role in correct laminar formation in the developing brain.

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Regulated Trafficking of Reelin Receptors

Receptors. Previously, it was reported that Dab1 enhances cell surface expression of LDLR, another Dab1-binging receptor, in a heterologous cell line by inhibiting endocytosis of the receptor (20). In the present study, we demonstrated that Dab1 up-regulates cell surface Reelin receptors by a distinct mechanism, in which it enhances translocation of the receptors to the plasma membrane. We also showed that tyrosine phosphorylation of Dab1 initiates intracellular trafficking of Reelin and its receptors.

EXPERIMENTAL PROCEDURES

Expression Vectors—The expression vectors encoding mouse Reelin (pCrl, Venus (pCS-Venus), and human VLDLR and mouse apoER2 (pCDNA3.1-VLDLR and pRcCMV-apoER2) were kindly provided by Dr. T. Yamamoto. The mouse Dab1 gene was amplified by reverse transcription-PCR from cDNA of mouse embryonic brain with the following primers: 5'-GGGAATTCTAGTCAACTGAGAACATACT-3' and 5'-CCCCCTCGACTGCTACCTGG-3'. The PCR product was cloned into EcoRI and XhoI sites in pME-FLAG, a derivative vector from pME-18S (21), which carries cDNA corresponding to FLAG polypeptides and multiple cloning sites. The cDNA encoding the FLAG promoter, to make pME-FLAG-Dab1. The SI14T mutant of Dab1 was generated by using two sets of primers: 5'-GGGAATTCTAGTCAACTGAGAACATACT-3' and 5'-GGGTAGTACCTTACCCTGACATTGAG-3'. The PCR products were digested with EcoRI and XhoI and with SnalI and SalI, respectively, and ligated into pME-FLAG to make pME-FLAG-Dab1. The PCR product for constituting the C-terminal of Dab1 was used and cloned into pEGFP vector (GFP), the human VLDLR gene was amplified by PCR with the following primers: 5'-GGGAAGCTTACCGCCATGGGCCACCTGCGCCGACT-3' and 5'-GGGATCCAGGACCATGAGCAGTGC-3'. These products were then digested with XhoI and EcoRI and with XhoI and Xbal, respectively, and subcloned into XhoI and Xbal sites which in pCDNA3.1-Zeo+ (Invitrogen) to make pCrl-C. The cDNA encoding the VLDLR variant, Venus (22), was amplified by PCR with primers containing an EcoRI site at the 5' end, and the product was subcloned into pCrl-C to make pCrl-C-YFP. Finally, the other parts of Reelin cDNA were obtained from pCrl and subcloned into pCrl-C-YFP to make pCrl-YFP.

Cell Lines and Transfection—293T and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum and antibiotics. COS-7 cells were washed four times with serum-free DMEM to remove lipoprotein and transfected with the indicated vectors in DMEM containing 5% lipoprotein-free fetal bovine serum (Thermo Trace, Melbourne, Australia) using TransIt LT1 (Mirus, Madison, WI). Twenty four hours later, the cells were subjected to immunoprecipitation, Western blotting, and immunocytochemistry as described below. To obtain Reelin-containing supernatant, 293T cells (4 x 10^6) were plated onto 10-cm dishes and transfected with pCrl, pCrl-YFP, or empty vector by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. On the following day, the supernatants were then collected, centrifuged, and filtered through a 0.2-μm filter.

Primary Culture of Cortical Neurons—Cortical neurons were obtained from Reelin-deficient reeler or Dab1-deficient yotari embryos (embryonic day 15 (E15)). In brief, cortical lobes were trypsinized for 5 min at 37 °C and then washed with a plated-decidual growth factor balanced salt solution with 12 mM MgCl2 and 0.025% DNase I. Neurons were plated onto poly-L-lysine-coated 35-mm dishes (2 x 10^4 cells/dish) or 18 x 18-mm cover glasses (1 x 10^4 cells/glass), and maintained in Neurobasal medium (Invitrogen) containing B27 supplement (Invitrogen), 2 mM of l-glutamine, 1% ITS, and antibiotics for 4 days. The neurons were then placed at 4 °C to prevent endocytosis and treated with control or Reelin-containing supernatant at 4 °C for 20 min. Subsequently, excess Reelin was removed, and the neurons were incubated in the culture medium for the indicated periods.

Immunocytochemistry—The following antibodies were used for immunocytochemistry: mouse anti-Reelin (CR-50, 1:1000; Ref. 4), rabbit anti-Dab1 (1:2000; Chemicon, Temecula, CA), rabbit anti-βIII-tubulin (1:2000; Covance, Princeton, NJ), rabbit anti-calreticulin (1:400; Chemicon), and mouse anti-FLAG (0.5 μg/ml; Sigma). Primary neurons or COS-7 cells on the cover glasses were fixed with 4% paraformaldehyde for 10 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS, and treated with 3% normal goat serum to block non-specific binding. Cells were then and dissociated by triturating with the indicated antibodies for 1 h at room temperature. After being washed with PBS, cells were further incubated with secondary antibodies conjugated with Alexa-488 or Alexa-594 (Molecular Probes, Eugene, OR). Samples were examined with a fluorescence microscope (BX50, Olympus, Tokyo, Japan) or a confocal microscope (FV300, Olympus).

Quantitation of Fluorescence—To quantitate internalization of Reelin, photographs of neurons were acquired under the same conditions using a fluorescence microscope at low magnification (x20 objective). All images were analyzed with an image analyzer without any mathematical processing. The area of each soma was enclosed, and the total intensity of fluorescence within each area was calculated using the following formula: pixel number x (mean fluorescence of enclosed region – background within the cell).

The fluorescence images of Reelin were merged with those of Dab1, and overlapping signals were selected (D-Reelin). Finally, the percentage of Reelin colocalizing with Dab1 was calculated using the following formula: 2 the fluorescence of D-Reelin/2 the fluorescence of A-Reelin x 100.

Proportion of the Amount of Cell-bound Reelin—Primary cortical neurons and transfected COS-7 cells were treated with supernatant containing YFP-Reelin and untagged Reelin, respectively, for 20 min at 4 °C. Cells were washed five times with culture medium without any supplement and lysed with SDS-PAGE loading buffer. The protein samples were separated by 5–7.5% SDS-PAGE and subjected to Western blotting using the following antibodies: mouse anti-Reelin (G10, 0.5 μg/ml, Chemicon), rabbit anti-Dab1 (1:500; Covance, Princeton, NJ), rabbit anti-VLDLR and anti-apoER2 (1:2000, kindly provided by Dr. Kobayashi), rabbit anti-Dab1 (1:2000; Chemicon), mouse anti-FLAG (0.2 μg/ml; Sigma), and mouse anti-ph-bain (0.2 μg/ml; Sigma). Blotted filters were visualized with an ECL system (Amersham Biosciences).

Immunoprecipitation—The surface proteins of transfected cells were labeled with sulfo-succinimidyl-6-(biotin-amido)hexanoate (sulfo-NHS-LC-biotin, Pierce) according to the manufacturer's instructions. In brief, cells were washed with ice-cold PBS four times and incubated with 0.125 mg/ml of sulfo-NHS-LC-biotin for 15 min at 4 °C. Cells were then lysed with RIPA buffer (10 mM phosphate buffer (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) containing 1 mM sodium orthovanadate and protease inhibitors. The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and biotinylated proteins were precipitated with streptavidin-conjugated agarose (Pierce). Precipitated proteins were washed with RIPA buffer six times and analyzed by Western blotting.

Detection of Dab1 Phosphorylation—Primary cortical neurons were lysed with RIPA buffer containing 1 mM sodium orthovanadate and protease inhibitors. The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatants were subjected to immunoprecipitation using anti-Dab1 antibody (Santa Cruz Biotechnology; 5 μg/ml) and protein-G-Sepharose (Amersham Biosciences). The immunoprecipitated samples were washed with RIPA buffer four times, eluted with the loading buffer in boiling water, separated by 7.5% SDS-PAGE, and transferred to nitrocellulose. The membrane was probed with anti-phosphotyrosine (anti-Tyr(P)) mouse monoclonal antibody (1:2000; PY20, Pharmingen, San Diego) conjugated with horseradish peroxidase. The membrane was reprobed with anti-Dab1 (1:2000; Chemicon) to normalize the loading amount of precipitated proteins.

Immunoprecipitation—Transfected cells were lysed with TNE lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton
X-100) containing 1 mM sodium orthovanadate and protease inhibitors at 4 °C for 15 min. The lysates were centrifuged, and the supernatants were precipitated with 5 μg/ml of anti-FLAG antibody. The precipitated samples were washed with TNE buffer four times, separated by 7.5% SDS-PAGE followed by Western blotting using anti-FLAG, anti-VLDLR, and anti-α3P2 antibodies as described above.

**Pharmacological Experiments**—For inhibition of the activity for STKs, primary neurons were treated with either 5 μM of PP2 or its inactive analogue PP3 (EMB Biosciences, San Diego) for 1 h at 37 °C. After Reelin treatment, the neurons were further incubated for 20 min at 37 °C in the presence of either PP2 or PP3. For prevention of endocytosis, neurons were treated with 0.5 M sucrose for 5 min at 4 °C after Reelin treatment. Subsequently, the supernatant was removed, and the neurons were further incubated for 20 min at 37 °C in the presence of hypertonic sucrose. To examine the subcellular localization of VLDLR-GFP, transfected cells were treated with 100 μg/ml of cycloheximide (Nacalai Tesque, Kyoto, Japan) just prior to fixation.

**RESULTS**

**Internalization of Reelin in Dab1-deficient Neurons**—Because Dab1 is a member of the PTB domain-containing protein family, whose functions include regulation of intracellular trafficking of receptors, we examined the role of Dab1 in trafficking of Reelin receptors. Primary cortical neurons (E15 + 4 days in vitro) from Dab1-deficient embryos and their wild-type or heterozygous littermates were first incubated with exogenous Reelin at 4 °C to prevent endocytosis. After excess Reelin was washed out, the cells were incubated at 37 °C for 10 min followed by immunocytochemistry with antibodies against Reelin (Fig. IA, green) and βIII-tubulin (red). Because the anti-Reelin antibody never detected signals in culture (except for Cajal-Retzius cells) in the presence of the control culture supernatant under the present conditions (data not shown), all the Reelin-positive signals were considered to be derived from the exogenously added Reelin. Whereas confocal images at high magnification revealed that Reelin was detected in Dab1-deficient neurons (Fig. IA, bottom, arrow), the total signal in Dab1-deficient neurons was weaker than those in wild-type or heterozygous neurons (Fig. IA, top). We measured the relative fluorescence of Reelin in the soma of βIII-tubulin-positive neurons with an image analyzer (Fig. 1D). The fluorescence intensity in Dab1-deficient neurons (1.00 ± 0.30) was lower than those in wild-type (1.60 ± 0.56) or heterozygous neurons (1.40 ± 0.39).

We next biochemically compared the amount of Reelin binding to the neurons. To distinguish the exogenously applied Reelin from the endogenous one, yellow fluorescent protein (YFP)-tagged Reelin was utilized. After confirming that our anti-GFP antibody specifically reacts with YFP-Reelin but not untagged Reelin (Fig. IC), YFP-Reelin was incubated with primary cortical neurons at 4 °C for 20 min, and whole cell lysates were analyzed by Western blotting using the anti-GFP antibody. Dab1-deficient neurons contained a 44% lower level of YFP-Reelin than wild-type neurons and a 29% lower level of YFP-Reelin than heterozygous neurons (Fig. 1D). Taken together, these results indicate that efficient binding of Reelin to neurons requires Dab1.

**Effect of Dab1 on Cell Surface Expression of Reelin Receptors**—We next measured the binding of Reelin to COS-7 cells overexpressing Reelin receptors, because COS-7 cells do not express Reelin, its receptors, or Dab1 endogenously. The experiments were carried out under lipoprotein-free conditions to exclude the effects of lipoprotein. As shown in Fig. 2A, cotransfection of wild-type Dab1 (Dab1WT) in COS-7 cells expressing VLDLR (left panels) and apoER2 (right panels) enhanced the binding of Reelin by 2.6- and 1.3-fold, respectively. By contrast, the amount of Reelin binding to the cells was not clearly enhanced by cotransfection of Dab1ST, a mutant in which serine 114 was substituted by threonine, and which does not bind to Reelin receptors (data not shown; see Ref. 24).

We next measured the cell surface expression of Reelin receptors in COS-7 cells. Cell-surface proteins were labeled with sulfo-NHS-LC-biotin, and then the biotylated proteins were precipitated with avidin beads, followed by Western blotting using antibodies against Reelin receptors. As shown in Fig. 2B, cell surface expression of both receptors (especially the slowly migrating form, SMF) was significantly increased in the presence of Dab1WT, but not Dab1ST, when compared with the total amount of the receptors (Fig. 2B, bottom panel). The differences in apparent molecular weight between the SMF and the rapidly migrating form (RMF) are probably because of the differences in glycosylation states (25, 26). In other words, the SMF and the RMF correspond to mature and immature proteins, respectively. Therefore, it was suggested that Dab1 selectively enhances cell surface expression of mature Reelin receptors through direct association with them.

Next, to identify the form of Reelin receptors to which Dab1 binds, communoprecipitation experiments were performed. Most surprisingly, Dab1 immunoprecipitated the SMF, but not the SMF, of Reelin receptors (Fig. 2C). These results strongly suggest that Dab1 binds to immature Reelin receptors and does...
not stably associate with the receptors at the plasma membrane.

**Effect of Dab1 on Subcellular Localization of Reelin Receptors**—Because Dab1 enhanced the cell surface expression of Reelin receptors without increasing their total amount, we hypothesized that Dab1 regulates the subcellular localization of Reelin receptors. To test this hypothesis, we constructed an expression vector encoding VLDLR tagged with green fluorescent protein (VLDLR-GFP, Fig. 3A), when expressed alone, exhibited a mesh-like distribution throughout the cytoplasm but was not stably associated with the receptors at the plasma membrane (Fig. 3E). Dab1ST had no effect on the localization of VLDLR-GFP (Fig. 3E). Based on these findings, it was concluded that Dab1 enhances the cell surface expression of VLDLR through direct interaction with its cytoplasmic NPXY motif.

**Spatial and Temporal Changes in the Subcellular Localization of Dab1 and Internalized Reelin in Primary Cortical Neurons**—Because tyrosine phosphorylation of Dab1 is known to be essential for the function of Reelin (13), numerous studies have been performed to identify the proteins binding to phosphorylated Dab1 (5, 10, 27, 28, 30). The result of the immunoprecipitation experiment (Fig. 2C) strongly suggested that Dab1 does not stably associate with Reelin receptors at the plasma membrane. This result raises the following question: how Dab1 is phosphorylated when the receptors bind to Reelin. To obtain a clue to this question, primary cortical neurons from reeler embryos were treated with either control or Reelin-containing medium, and the subcellular localization of Dab1 was analyzed. As shown in the top panel of Fig. 4A, Dab1 was diffusely distributed throughout the cytoplasm in the absence of Reelin, as reported previously (31). By contrast, Dab1 punctately accumulated at the cell periphery in the presence of Reelin (Fig. 4A, bottom).

We therefore investigated the spatial and temporal changes in the subcellular localization of Dab1 and Reelin in primary cortical neurons after Reelin treatment as described in Fig. 1, and we compared them with the kinetics of tyrosine phosphorylation of Dab1. Dab1-positive puncta were observed with Reelin around the cell periphery at 2 min (Fig. 4B, middle panels), when tyrosine phosphorylation of Dab1 was already detectable (Fig. 4C). This punctate signal of Dab1 was never
observed in Dab1-deficient neurons (data not shown). At 20 min, Reelin was observed within the soma, where Dab1 was rarely observed (Fig. 4B, bottom, arrowheads). By contrast, a small population of Reelin-positive vesicles at the cell periphery contained Dab1 signals (arrows). These results suggested that Dab1 is recruited to Reelin receptors, and its tyrosine residues are phosphorylated only when they bind to Reelin and that Dab1 dissociates from Reelin-containing vesicles after internalization of Reelin has been completed.

Role of STKs Activation in Internalization of Reelin—We next examined the effect of tyrosine phosphorylation of Dab1 on the trafficking of Reelin receptors and internalized Reelin in primary neurons. Primary neurons from reeler embryos were treated with the STKs inhibitor PP2, its inactive analogue PP3, or vehicle followed by Reelin treatment as described in Fig. 1. Subsequently, the neurons were incubated at 37 °C for 20 min and subjected to immunocytochemistry. As shown in Fig. 5A, top and bottom panels, Reelin was internalized into neurons treated with vehicle and PP3, respectively. However, in the PP2-treated neurons, Reelin was distributed near the plasma membrane and was well colocalized with Dab1 at the same time point. Based on fluorescence intensity and pixel number, we calculated the relative proportions of Reelin colocalized with Dab1 near the plasma membrane (Fig. 5B). In the PP2-treated neurons, more than 80% of total Reelin colocalized with Dab1 near the plasma membrane. By contrast, less than 30% of total Reelin was found at the plasma membrane with Dab1 in control or the PP3-treated neurons. These results suggest that inhibition of STKs prevents internalization of Reelin in cortical neurons.

To further confirm that Reelin is not effectively internalized into the PP2-treated neurons, cell surface Reelin (sReelin) and internalized Reelin (iReelin) were stained with anti-Reelin antibody before and after permeabilization, respectively. In brief, cultured neurons were fixed and first stained with anti-Reelin antibody and a secondary antibody conjugated with Alexa-488. Subsequently, they were permeabilized and stained with the same antibody and a secondary antibody conjugated with Alexa-594 (Fig. 5C). The PP2-treated neurons had bright signals containing sReelin near the plasma membrane, and weak iReelin-signals were partially overlapped with them at 20 min, but they were rarely detected within the soma of the neurons (Fig. 5C, top panels). In contrast, iReelin was detected within the presence of the neurons treated with vehicle, and this signal was not detected (data not shown) or PP3 (Fig. 5C, bottom panels) at the same time point. These results reveal that Reelin is not effectively internalized in the presence of PP2.

Finally, to determine whether tyrosine phosphorylation of Dab1 precedes internalization of Reelin, primary neurons treated with Reelin were incubated in the presence of hypertonic sucrose, which severely inhibited internalization of the receptor-ligand complex (32). After confirming that hypertonic sucrose effectively prevented Reelin internalization, resulting in localization of Reelin at the cell periphery with Dab1 (Fig.
Antibodies. The examined with a confocal microscope. The neurons from 0.5 M sucrose for 5 min at 4 °C. Subsequently, the neurons were incubated for 20 min at 37 °C, and subcellular localization of Reelin and Dab1 was indicated that Dab1 also regulates intracellular trafficking of Reelin receptors. Plasma level and that it initiates intracellular trafficking of Reelin and Reelin receptors. Phosphorylated Dab1, but it largely remains unclear (2). In the present study, we propose a novel model of the molecular mechanisms of brain malformation in Dab1-deficient mice. Although Dab1 selectively up-regulated cell surface expression of the mature forms of Reelin receptors, it communoprecipitated immature forms, but not mature forms, of Reelin receptors (Fig. 2C). These results strongly suggest that Dab1 does not stably associate with Reelin receptors at the plasma membrane. An alternative possibility is that the O-linked sugar chains of Reelin receptor interfere with Dab1 binding after cell lysis. However, this is unlikely because Dab1 communoprecipitates mature forms of amyloid precursor protein, which contains O-linked sugar chains, in the cell lysate (17). As reported previously (16), ApoE, a common ligand of all mem-

**FIG. 5. Effect of PP2 on internalization of Reelin.** A, the subcellular localization of Reelin and Dab1 in the presence of PP2. Primary cortical neurons from reeler embryos were cultured for 1 h in the presence of 0.05% DMSO (vehicle, top), PP2 (5 μM, middle), or PP3 (5 μM, bottom) followed by Reelin treatment as described above. After unbound Reelin was removed, the neurons were further incubated for 20 min at 37 °C in the presence of each reagent. Neurons were stained with anti-Reelin (left) and anti-Dab1 (center) antibodies after fixation and examined with a confocal microscope. The right panels are merged images of Reelin (green) and Dab1 (red). Bar, 10 μm. B, quantitative analysis of colocalization of Reelin with Dab1. The proportion of Reelin colocalizing with Dab1 was calculated with an image analyzer as described under “Experimental Procedures.” C, cell surface Reelin (sReelin, left) and internalized Reelin (iReelin, center) were distinguished immunocytochemically in either the PP2- (top) or PP3-treated (bottom) neurons and examined with a confocal microscope. The right panels are merged images of sReelin (green) and iReelin (red). Light blue lines indicate the cell periphery. Bar, 10 μm. D, subcellular localization of Reelin and Dab1 in the presence of hypertonic sucrose. Primary cortical neurons from reeler embryos were treated with exogenous Reelin as described above. The neurons were further incubated with 0.5 M sucrose for 5 min at 4 °C. Subsequently, the neurons were incubated for 20 min at 37 °C, and subcellular localization of Reelin and Dab1 was examined with a confocal microscope. The bottom panel is a merged image of neurons stained with anti-Reelin (green) and anti-Dab1 (red) antibodies. The top and middle panels are Reelin and Dab1 signals, respectively, of the boxed region in the bottom panel. Arrowheads indicate the position of Reelin-containing vesicles. Bar, 5 μm (top) and 10 μm (bottom). E, phosphorylation of Dab1 in the presence and absence of hypertonic sucrose. Neurons treated with exogenous Reelin were incubated for 10 min at 37 °C in the presence or absence of sucrose. Cell lysis, immunoprecipitation (IP), and Western blotting (WB) were performed as described in Fig. 4B.
How Does Dab1 Regulate Internalization of Reelin?—Reelin was internalized into COS-7 cells expressing Reelin receptors in the absence of Dab1 (Fig. 3C) (16), and PP2 did not prevent internalization of Reelin into cells transfected with either VLDR or apoER2 alone (data not shown). By contrast, internalization of Reelin was severely inhibited by PP2 in neurons (Fig. 5), and we also observed that internalization of Reelin was unaffected by PP2 in Dab1-negative cells (data not shown). These observations suggest that activation of STKs is essential for internalization of Reelin that depends on (or is regulated by) Dab1. The activation of STKs by Reelin results in degradation of Dab1 by the ubiquitin-proteosome pathway (37, 38), suggesting that the degradation of Dab1 may permit internalization of Reelin. Ubiquitination of Dab1 is carried out by Cbl, a ubiquitin-protein isopeptide ligase for degradation of receptor tyrosine kinases (38). One such receptor, epidermal growth factor receptor, binds to Cbl at the plasma membrane (41), but it is internalized into Cbl-deficient fibroblasts (42). Taken together, these results strongly suggest that internalization of Reelin precedes the degradation of Dab1, and the degradation is not involved in internalization of Reelin. Alternatively, factors that bind to phosphorylated Dab1, such as Nckβ (27) or CrkL (10, 30), may be involved in the regulation of Reelin internalization.

Involvement of the Intracellular Trafficking of Phosphorylated Dab1 in Reelin Signaling—Internalization is essential for the activation of downstream effectors in some signaling systems such as Notch, Sonic Hedgehog, and transforming growth factor β signaling (reviewed in Ref. 43). Recently, it was shown that internalization of receptor tyrosine kinases is critical for the activation of PI3K (29), which is also implicated in Reelin signaling (28, 39). In our preliminary experiments, activation of PI3K was severely inhibited when endocytosis of Reelin receptors was blocked. Thus, we assume that the phosphorylation of Dab1 initiates the Reelin signaling by triggering intracellular trafficking of the vesicles containing Reelin, its receptors, and phosphorylated Dab1.

Model of the Intracellular Trafficking of Reelin Receptors Regulated by Dab1—Based on our results, we propose the following model, in which Dab1 regulates intracellular trafficking of Reelin receptors in three steps (Fig. 6): In the first step, Dab1 binds to Reelin receptors at the Golgi apparatus and prevents their retrieval from the Golgi to the ER. In the second step, Dab1 translocates Reelin receptors to the plasma membrane, but it does not stably bind to the receptors at the plasma membrane before binding of Reelin. In the third step, Dab1 clusters with the receptors at the plasma membrane and is phosphorylated in response to Reelin, initiating the process of endocytosis of Reelin-bound receptors.

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REFERENCES

1. Gleeson, J. G., and Walsh, C. A. (2000) Trends Neurosci. 23, 352–359
2. Tissir, F., and Goffinet, A. M. (2003) Nat. Rev. Neurosci. 4, 496–505
3. D’Arcangelo, G., Miao, G. G., Chen, S. C., Soares, H. D., Morgan, J. I., and Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H., and Mikoshiba, K. (1995) Neuron 14, 899–912
4. Howell, B. W., Gertler, F. B., and Cooper, J. A. (1997) EMBO J. 16, 121–132
5. Howell, B. W., Hawkes, R., Soriano, P., and Cooper, J. A. (1997) Nature 389, 733–737
6. Howell, B. W., Hawkes, R., Soriano, P., and Cooper, J. A. (1997) Curr. Biol. 13, 9–17
7. Sheldon, M., Rice, D. S., D’Arcangelo, G., Yoneshima, H., Nakajima, K., Mikoshiba, K., Howell, B. W., Cooper, J. A., Goldowitz, D., and Curran, T. (1997) Nature 389, 730–732
8. Yoneshima, H., Nagata, E., Matsumoto, M., Yamada, M., Nakajima, K., Miyata, T., Ogawa, M., and Mikoshiba, K. (1997) Neurosci. Res. 29, 217–223
9. Arnaud, L., Ballif, B., Forster, E., and Cooper, J. A. (2003) Curr. Biol. 13, 2764–2767
10. Ballif, B. A., Arnaud, L., Arthur, W. T., Gurus, D., Imamoto, A., and Cooper, J. A. (2004) Curr. Biol. 14, 606–610
11. Bock, H. H., and Herz, J. (2003) Curr. Biol. 13, 18–26
12. Kesbmvra, L., Boulhoayon, D., Magdaleno, S., and Curran, T. (2001) J. Biol. Chem. 276, 16008–16014
13. Howell, B. W., Herrick, T. M., Hslebrand, J. D., Zhang, Y., and Cooper, J. A. (2000) Curr. Biol. 10, 877–885
14. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R. E., Richardon, J., and Herz, J. (1999) Cell 97, 689–701
15. Herz, J., and Bock, H. H. (2002) Annu. Rev. Biochem. 71, 405–434
16. D’Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D. S., Sheldon, M., and Curran, T. (1999) Neuron 24, 471–479
17. Howell, B. W., Lanier, L. M., Frank, R., Gertler, F. B., and Cooper, J. A. (1999) Mol. Cell. Biol. 19, 5179–5188
18. Mishra, S. K., Kefel, P. A., Hawrylyuk, M. J., Agostinelli, N. R., Watkins, S. C., and Traub, L. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16099–16104
19. Gotthardt, M., Trommsdorff, M., Nevitt, M. F., Shelton, J., Richardson, J. A., Stockinger, W., Nimpf, J., and Herz, J. (2000) J. Biol. Chem. 275, 25616–25624
20. Takebe, Y., Seiki, M., Fujisawa, J., Hey, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1998) Mol. Cell. Biol. 8, 466–472
21. Nagai, T., Ibar, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyaakiwai, A. (2002) Nat. Biotechnol. 1, 87–90
22. De Gerbykes, Y., Nakajima, K., Lambert de Rouvroit, C., Naerhuymen, B., Goffinet, A. M., Miyata, T., Ogawa, M., and Mikoshiba, K. (1997) Brain Res. Mol. Brain Res. 50, 85–90
23. Yun, M., Keshvara, L., Park, C. G., Zhang, Y. M., Dickerson, J. B., Zheng, J., Rock, C. O., Curran, T., and Park, H. W. (2003) J. Biol. Chem. 278, 36572–36581
24. Magrane, J., Casaroli-Marano, R. P., Reina, M., Gafve, M., and Vilaro, S. (1999) FEBS Lett. 451, 36–62
25. May, P., Bock, H. H., Nimpf, J., and Herz, J. (2003) J. Biol. Chem. 278, 37386–37389
26. Prematara, V., Oehlaki, P. G., Chen, K., Gropman, A., Myers, S., Min, K. T., and Howell, B. W. (2000) Mol. Cell. Biol. 23, 7210–7221
27. Bock, H. H., Josin, Y., Liu, P., Forster, E., May, P., Goffinet, A. M., and Herz, J. (2003) J. Biol. Chem. 278, 38772–38779
28. Sato, M., Ueda, Y., Takagi, T., and Umezawa, Y. (2003) Nat. Cell Biol. 5, 1016–1022
29. Huang, Y., Magdaleno, S., Hopkins, R., Slaughter, C., Curran, T., and Keshvara, L. (2004) Biochem. Biophys. Res. Commun. 318, 204–212
30. Howell, B. W., Herrick, T. M., and Cooper, J. A. (1999) Genes Dev. 3, 643–648
31. Shah, B. H., Alberto Olivares-Reyes, J., Yeshilayka, A., and Catt, K. J. (2002) Mol. Endocrinol. 16, 610–620
32. Hoppe, H. C., and Joner, K. A. (2000) Cell. Microbiol. 2, 569–575
33. Malabiabarrena, A., Jimenez, M. A., Rico, M., and Alarcon, B. (1995) EMBO J. 14, 2257–2268
34. Kamikura, D. M., and Cooper, J. A. (2003) Genes Dev. 17, 2788–2811
35. Riddell, D. R., Sun, X. M., Stannard, A. K., Soutar, A. K., and Owen, J. S. (2003) J. Lipid Res. 42, 998–1002
36. Arnaud, L., Ballif, B. A., and Cooper, J. A. (2003) Mol. Cell. Biol. 23, 9293–9302
37. Sato, M., Ueda, Y., Takagi, T., and Umezawa, Y. (2003) Mol. Cell. Biol. 23, 33877–33884
38. Beffert, U., Morfini, G., Bock, H. H., Reyna, H., Brady, S. T., and Herz, J. (2002) J. Biol. Chem. 277, 49958–49964
39. Beglova, N., Jeon, H., Fisher, C., and Blacklow, S. C. (2004) Mol. Cell 16, 281–292
40. De Melker, A. A., van der Horst, G., Calafat, J., Jansen, H., and Borst, J. (2001) J. Biol. Chem. 276, 2187–2194
41. Duan, L., Miura, Y., Dimri, M., Majumder, B., Dodge, I. L., Reddy, A. L., Ghosh, A., Fernandes, N., Zhou, P., Mullan-Blom, B., Rock, C. O., Bowtell, D., Naramura, M., Gu, H., Band, V., and Band, H. (2003) J. Biol. Chem. 278, 28950–28960
42. Gonzalez-Gaitan, M. (2003) Nat. Rev. Mol. Cell. Biol. 3, 213–224