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Direct Binding of Reelin to VLDL Receptor and ApoE Receptor 2 Induces Tyrosine Phosphorylation of Disabled-1 and Modulates Tau Phosphorylation

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Summary

The large extracellular matrix protein Reelin is produced by Cajal-Retzius neurons in specific regions of the developing brain, where it controls neuronal migration and positioning. Genetic evidence suggests that interpretation of the Reelin signal by migrating neurons involves two neuronal cell surface proteins, the very low density lipoprotein receptor (VLDLR) and the apoE receptor 2 (ApoER2) as well as a cytosolic adaptor protein, Disabled-1 (Dab1). We show that Reelin binds directly and specifically to the ectodomains of VLDLR and ApoER2 in vitro and that blockade of VLDLR and ApoER2 correlates with loss of Reelin-induced tyrosine phosphorylation of Disabled-1 in cultured primary embryonic neurons. Furthermore, mice that lack either Reelin or both VLDLR and ApoER2 exhibit hyperphosphorylation of the microtubule-stabilizing protein tau. Taken together, these findings suggest that Reelin acts via VLDLR and ApoER2 to regulate Disabled-1 tyrosine phosphorylation and microtubule function in neurons.

Introduction

Layered structures in the mammalian brain arise from the coordinated movements of cohorts of neurons during brain development. One pathway that is critical for correct layering in the neocortex, cerebellum, and hippocampus involves the extracellular matrix-associated protein Reelin. In the cerebral cortex, Reelin controls the final positions of neurons that migrate from the ventricular zone along the radial glial network to their final position in the appropriate cortical layer. Reelin is secreted by a special class of neurons, known as Cajal-Retzius cells, in the outermost layer of the cortex (termed layer 1 or marginal zone). In the absence of Reelin, more neurons than usual are found in the marginal zone, and neurons in lower layers (layers 2 through 6) are found in an inverted stratification. Defects are also found in the layered structures of the cerebellum and hippocampus. The cellular basis of these effects of Reelin is unknown, but it appears that Reelin is a signaling molecule that controls the behavior of several types of migrating neurons (Curran and D'Arcangelo, 1998; Lambert de Rouvroit and Goffinet, 1998).

Inside the cell, the Reelin signal is received by the cytosolic adaptor protein Disabled-1 (Dab1). Dab1 contains a protein interaction domain that binds to nonphosphorylated NPxY motifs in the cytoplasmic tails of several integral cell surface proteins (Pawson and Scott, 1997; Trommsdorff et al., 1998; Homayouni et al., 1999; Howell et al., 1999b). Reelin signaling stimulates tyrosine phosphorylation of Dab1 (Howell et al., 1999a), and subsequently Dab1 protein levels are downregulated by an unknown mechanism (Rice et al., 1998; Howell et al., 1999a). Phosphorylated Dab1 has the potential to recruit nonreceptor tyrosine kinases and their substrates to the cell membrane (Howell et al., 1997a). The downstream targets of this hypothetical kinase cascade initiated by Dab1 are currently unknown but may include cytoplasmic components of the cell migration or adhesion machinery. Indeed, two cytoskeletal proteins, doublecortin (Gleeson et al., 1998) and filamin (Fox et al., 1998), and two integrins (Georges-Labouesse et al., 1998; Anton et al., 1999) are known to be needed for normal migration of cortical neurons.

Several mutations in the Reelin and Dab1 genes have independently arisen or have been generated in mice (Falconer, 1951; Sweet et al., 1996; Howell et al., 1997b; Yoneshima et al., 1997; Sheldon, et al., 1997; Ware et al., 1997). The phenotypes of these animals are indistinguishable, and animals lacking both Reelin and Dab1 have no additional phenotypes suggesting a linear pathway (Howell et al., 1999a). Recently, an identical phenotype was detected in a double knockout strain of mice that lacks two members of the low-density lipoprotein (LDL) receptor gene family, the cell surface proteins VLDLR and ApoER2. These animals are phenotypically indistinguishable from the Reelin-deficient (*reeler*) and Dab1-deficient (*scrambler*, *yotari*, or *Dab1–1*) mice (Trommsdorff et al., 1999). Both receptors can bind Dab1 on their cytoplasmic tails, and, as in *reeler* mice, Dab1 protein levels are dramatically increased in the double mutant animals. These findings suggest that VLDLR and ApoER2 function as obligate components of the Reelin signaling pathway, but their position relative to Dab1 is unclear. VLDLR and ApoER2 could serve as receptors or coreceptors for Reelin and be required for Dab1 tyrosine phosphorylation. Alternatively, VLDLR and ApoER2 could convey a signal from Dab1 to regulate cell adhesion or migration.

In this study we show that both VLDLR and ApoER2 can bind Reelin on their extracellular domains. Like the binding of all known ligands to members of the LDL receptor gene family, this binding required Ca^{2+} (Fass et al., 1997). RAP, a specialized chaperone that prevents the binding of coexpressed ligands to the ectodomains

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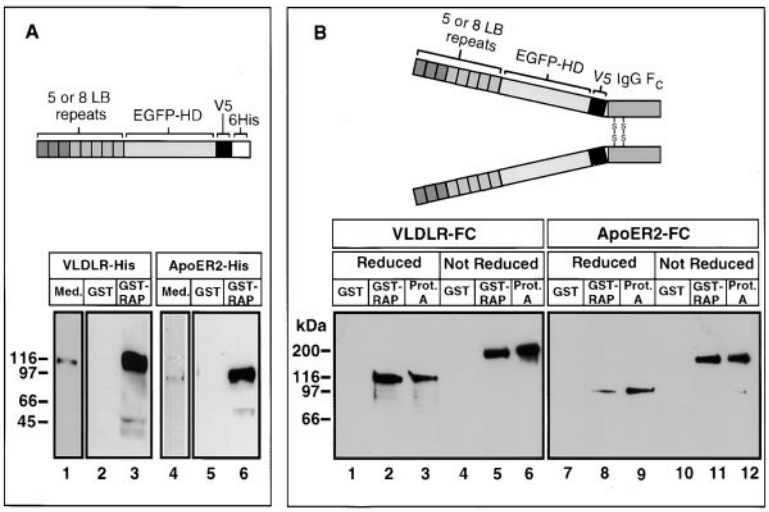

Figure 1. Generation and Properties of Recombinant Receptor Ectodomains

cDNAs encoding the ectodomains of the human VLDLR (containing eight ligand binding repeats followed by its epidermal growth factor precursor homology domain) and the murine ApoER2 (containing five ligand binding repeats followed by its epidermal growth factor precursor homology domain) were fused at their 3′ ends to a V5 antibody epitope and a 6His tag (A) or to a V5 epitope followed by the Fc portion of human IgG (B). The respective expression plasmids were transiently expressed in 293 cells, and culture supernatant containing the soluble fusion proteins was harvested.

(A) Media (1 ml) was incubated with 40 μl GST-Sepharose (lanes 2 and 5) or with 40 μl GST-RAP Sepharose (lanes 3 and 6). Proteins bound to the beads or 40 μl unconcentrated culture supernatant (lanes 1 and 4) were analyzed by Western blotting. VLDLR-V5-His (lanes 1–3) and ApoER2-V5-His (lanes 4–6) were visualized by immunoblotting using an anti-V5 antibody.

(B) Media (1 ml) was incubated with 40 μl GST-Sepharose (lanes 1, 4, 7, and 10), 40 μl GST-RAP Sepharose (lanes 2, 5, 8, and 11), or with 40 μl protein A Sepharose slurry (lanes 3, 6, 9, and 12). Bound proteins were separated by SDS-PAGE under reducing (lanes 1–3 and 7–9) or nonreducing conditions (lanes 4–6 and 10–12). VLDLR-V5-Fc (lanes 1–6) and ApoER2-V5-Fc (lanes 7–12) were visualized by immunoblotting using an anti-V5 antibody. EGDP-HD, epidermal growth factor homology domain.

of LDL receptor gene family members in the endoplasmic reticulum (Herz et al., 1991; Bu et al., 1995; Willnow et al., 1996) blocked Reelin binding to both receptors. RAP also reduced Reelin-induced tyrosine phosphorylation of Dab1 in cultured primary embryonic neurons, implying that transmission of the Reelin signal requires direct binding of Reelin to the VLDLR and ApoER2. Added VLDLR ectodomain also inhibited Reelin-induced Dab1 tyrosine phosphorylation, implying that the VLDLR-interacting region of Reelin is essential for signaling. Furthermore, in *reeler* as well as *vldlr/apoER2* double mutant mice, the phosphorylation level of the microtubule-stabilizing protein tau was dramatically increased, suggesting that loss of Reelin signal input affects the assembly and stability of the neuronal cytoskeleton.

Results

To investigate whether Reelin directly interacts with the extracellular (ecto) domains of VLDLR and ApoER2, we prepared secreted, soluble, and epitope-tagged ectodomains in mammalian cells (Figure 1A). VLDLR and ApoER2 ectodomains containing the authentic signal sequence, ligand binding (LB) domain, and epidermal growth factor precursor homology domain (EGFP-HD) fused to a V5 epitope followed by a hexahistidine (6His) tag were produced in transiently transfected 293 cells (Figure 1A, lanes 1 and 4). Proper folding of the secreted protein was assessed by its ability to bind to a GST-RAP affinity matrix. Binding of RAP to the ligand binding domains of the LDL receptor family members requires the correct folding of their complex disulfide-linked structure (Herz et al., 1991). The secreted ectodomains bound to GST-RAP (Figure 1A, lanes 3 and 6) but not to GST alone (Figure 1A, lanes 2 and 5). Another set of expression vectors in which the ectodomain of either receptor was fused to the Fc portion of human IgG (Figure 1B) allowed the rapid isolation of the recombinant protein from tissue culture supernatants. Both VLDLR-Fc and ApoER2-Fc bound to GST-RAP (Figure 1B, lanes 2, 5, 8, and 11) and protein A (Figure 1B, lanes 3, 6, 9, and 12) with similar efficiency, indicating proper folding of the recombinant protein. In both cases, reduction of the recombinant protein yielded a smaller band, indicating that the unreduced protein is covalently dimerized due to the Fc fusion. LDL receptor ectodomains were produced in an analogous manner (data not shown).

To test direct biochemical interaction of Reelin with either ectodomain, we overexpressed Reelin in 293 cells (Figure 2A, lane 1) using a vector kindly provided by T. Curran and G. D'Arcangelo (D'Arcangelo et al., 1997). Reelin-containing culture supernatant was incubated with protein A Sepharose beads to which LDLR, VLDLR, and ApoER2 ectodomain Fc fusion protein had been bound (Figure 2B, lanes 1–3). Reelin bound to the beads was detected by immunoblotting. VLDLR (Figure 2B, lane 2) and ApoER2 (Figure 2B, lane 3) bound Reelin avidly, whereas the LDL receptor did not (Figure 2B, lane 1). To show that similar amounts of ectodomains had been bound to the beads, we stripped the same filter used in Figure 2B and reprobed it with an anti-V5 antibody that equally recognizes all three Fc fusion proteins (Figure 2C).

On the basis of experiments in which we determined the saturation kinetics of a fixed amount of Fc ectodomains in the presence of variable concentrations of Reelin, we estimate a K_d for this interaction of approximately 500 pM for both VLDLR and ApoER2. This suggests a high-affinity interaction, consistent with the low concentration of Reelin needed to induce detectable tyrosine phoshorylation of Dab1 in cultured neurons (Howell et al., 1999a). To further ascertain that these receptors do not indiscriminately interact with "sticky"

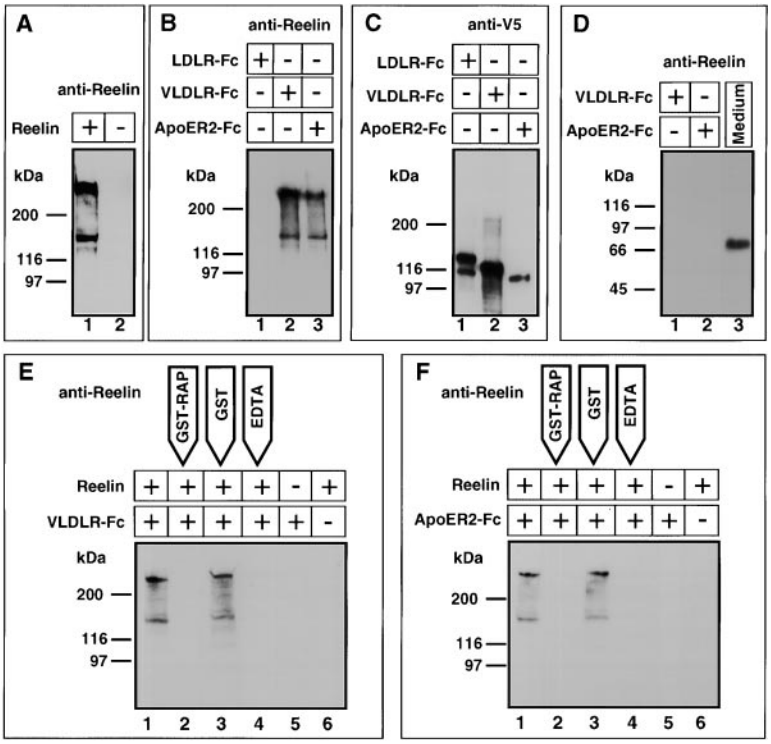

Figure 2. Reelin Binding to Fc Ectodomains and Precipitation by Protein A Sepharose

(A) Immunoblot of 40 μl culture supernatant from 293 cells transfected (lane 1) or not transfected (lane 2) with Reelin expression construct.

(B) Recombinant Fc fusion proteins from 1 ml 293 culture supernatant transfected with either the LDLR-Fc (lane 1), VLDL-Fc (lane 2), or ApoER2-Fc (lane 3) expression plasmid were bound to 40 μl protein A Sepharose slurry, followed by incubation with 500 μl Reelin containing culture supernatant. Reelin protein bound to the beads was detected by immunoblotting with an anti-Reelin antibody (G10).

(C) The same filter shown in (B) was stripped and reprobed with an anti-V5 antibody, showing that comparable amounts of recombinant ectodomains had been bound to the beads.

(D) Sepharose beads containing VLDLR (lane 1) and ApoER2 (lane 2) Fc fusion proteins as described in (B) were incubated with culture supernatant containing recombinant N-Reelin. Proteins bound to the beads and 40 μl of N-Reelin transfected 293 cell culture supernatant (lane 3) were analyzed by immunoblotting using monoclonal G10 anti-Reelin antibody.

(E) Recombinant Fc fusion proteins from 1 ml 293 culture supernatant transfected (lanes 1–5) or not transfected (lane 6) with the VLDL-Fc expression plasmid were bound to 40 μl protein A Sepharose slurry, followed by incubation with 500 ml Reelin containing (lanes 1–4 and 6) or not containing (lane 5) 293 cell culture supernatant without additions (lane 1) or in the presence of 30 μg/ml GST-RAP (lane 2), 30 μg/ml GST (lane 3), or 30 mM EDTA (lane 4). Reelin bound to the beads was detected by immunoblotting using monoclonal G10 anti-Reelin antibody.

(F) Recombinant Fc fusion proteins from 1 ml 293 culture supernatant transfected (lanes 1–5) or not transfected (lane 6) with the ApoER2-Fc expression plasmid were bound to 40 μl protein A Sepharose slurry, followed by incubation with 500 μl Reelin containing (lanes 1–4 and 6) or not containing (lane 5) 293 cell culture supernatant without additions (lane 1) or in the presence of 30 μg/ml GST-RAP (lane 2), 30 μg/ml GST (lane 3), or 30 mM EDTA (lane 4). Reelin bound to the beads was detected by immunoblotting using monoclonal G10 anti-Reelin antibody.

extracellular matrix molecules, we performed analogous in vitro binding experiments using fibronectin. Neither receptor showed any detectable interaction with this control ligand (data not shown).

The monoclonal CR50 antibody has previously been reported to block the biological function of Reelin (Nakajima et al., 1997). This antibody requires an epitope within the N-terminal portion of Reelin for binding (de Bergeyck et al., 1998). These findings raised the possibility that a receptor binding domain of Reelin is located within its N terminus. To investigate whether VLDLR and ApoER2 interact with Reelin through its N-terminal domain, we incubated the receptor Fc fusion proteins with recombinant N-Reelin, which was abundantly secreted by transfected 293 cells (Figure 2D, lane 3). Although both VLDLR and ApoER2 strongly bound to full-length Reelin (Figure 2B, lanes 2 and 3), neither receptor bound to the isolated N-terminal domain. This suggests that either Reelin interaction with the receptors requires other parts of the molecule, or that CR50 binding to its N-terminal epitope on Reelin induces steric hindrance that blocks its biological effect.

To determine whether agents that block ligand binding to VLDLR and ApoER2 would also interfere with the binding of Reelin to these receptors, we incubated Reelin-containing culture supernatant (Figures 2E and 2F, lanes 1–4 and 6) with VLDLR-Fc (Figure 2E, lanes 1–5) and ApoER2-Fc (Figure 2F, lanes 1–5) containing beads in the absence (Figures 2E and 2F, lanes 1, 5, and 6) or presence of GST-RAP (Figures 2E and 2F, lane 2), GST (Figures 2E and 2F, lane 3), or EDTA (Figures 2E and 2F, lane 4). GST-RAP effectively blocks the binding of all known ligands to members of the LDL receptor family (Herz et al., 1991), and Ca^{2+} is required for the stabilization of their ligand binding domains (Fass et al., 1997). RAP was used as an inhibitor because function-blocking antibodies directed against the VLDLR and ApoER2 ectodomains are not available. Both GST-RAP (Figures 2E and 2F, lane 2) and EDTA (Figures 2E and 2F, lane 4) completely blocked the binding of Reelin to the receptor ectodomains, while GST (Figures 2E and 2F, lane 3) had no effect.

We confirmed Reelin binding to the ectodomains of VLDLR and ApoER2 by the converse experiment (Figure 3). In this case, 293 culture supernatant that either did or did not contain Reelin was incubated with soluble, epitope-tagged ectodomains. Reelin was then precipitated using G10 monoclonal antibody (de Bergeyck et al., 1998) and adsorption to protein G. Bound ectodomains were detected by Western blotting using a monoclonal antibody directed against the V5 epitope. Both VLDLR (Figure 3A) and ApoER2 (Figure 3B) ectodomains were coprecipitated with Reelin (Figures 3A and 3B, lane 1). GST-RAP (Figures 3A and 3B, lane 2) and EDTA

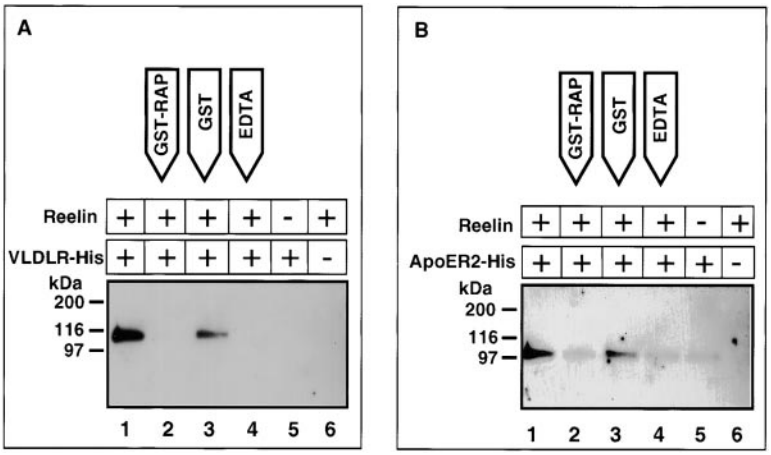

Figure 3. Coprecipitation of Receptor Ectodomains and Detection with Anti-V5 Antibody

Culture supernatant (1 ml) from Reelin transfected (lanes 1–4 and 6) or mock transfected 293 cells (lane 5) was incubated with 10 μl anti-reelin antibody (G10) and precipitated with protein G Sepharose.

(A) Reelin-containing beads were incubated with 1 ml culture supernatant from 293 cells transfected with the VLDLR-His expression plasmid (lanes 1–5) or mock transfected 293 cells (lane 6). GST-RAP (30 μg/ml; lane 2), 30 μg/ml GST (lane 3), or 30 mM EDTA (lane 4) were added where indicated.

(B) Reelin-containing beads were incubated with 1 ml culture supernatant from 293 cells transfected with the ApoER2-His expression plasmid (lanes 1–5) or mock transfected 293 cells (lane 6). GST-RAP (30 μg/ml; lane 2), 30 μg/ml GST (lane 3), or 30 mM EDTA (lane 4) were added where indicated.

(Figures 3A and 3B, lane 4), virtually completely blocked the association of the ectodomains with Reelin, while GST (Figures 3A and 3B, lane 3) had no effect. Ectodomains were not precipitated in the absence of Reelin (Figures 3A and 3B, lane 5) and no V5 immunoreactive protein was precipitated in the absence of soluble ectodomains (Figures 3A and 3B, lane 6). Equal efficiency of immunoprecipitation of Reelin (Figures 3A and 3B, lanes 1–5) was ascertained by immunoblotting of the precipitate (data not shown).

We next tested whether inhibition of Reelin binding to LDL receptor family members on primary embryonic neurons would affect tyrosine phosphorylation of Dab1 (Figure 4A). Adherent cultures of primary neurons from embryonic *reeler* mouse brains were prepared and incubated for 1 hr with 1 (Figure 4A, lane 4) or 5 (Figure 4A, lane 6) μg/ml of GST-RAP, or with 5 μg/ml GST (Figure 4A, lane 5). The cultures were then stimulated for 15 min with 293 cell culture supernatant containing (Figure 4A, lanes 1 and 3–6) or not containing (Figure 4A, lane 2) Reelin. Following the incubation, cells were lysed and Dab1 was precipitated with an antibody prepared against a carboxy-terminal peptide (Trommsdorff et al., 1999). Immunoprecipitated proteins were separated by SDS gel electrophoresis and immunoblotted with an anti-phosphotyrosine antibody (Figure 4A, upper panel) and the anti-Dab1 antibody (Figure 4A, lower panel). In immunoprecipitates prepared with nonimmune serum, no phosphotyrosine containing protein was precipitated (Figure 4A, lane 1). When the neurons were incubated in the absence of Reelin (Figure 4A, lane 2), weakly phosphorylated Dab1 protein was precipitated from the lysates. When Reelin, in the absence (Figure 4A, lane 3) or presence (Figure 4A, lane 5) of GST was included during incubation of the neuronal cell culture, tyrosine phosphorylation levels of Dab1 were increased. When GST-RAP, at 1 (Figure 4A, lane 4) or 5 μg/ml (Figure 4A, lane 6) was present in the culture supernatant, the increase in Dab1 tyrosine phosphorylation was prevented, suggesting that Reelin stimulation of Dab1 phosphorylation requires an interaction with the ligand binding domains of LDL receptor family members.

We also performed a competition experiment (Figure 4B) in which we included the recombinant VLDLR ecto-

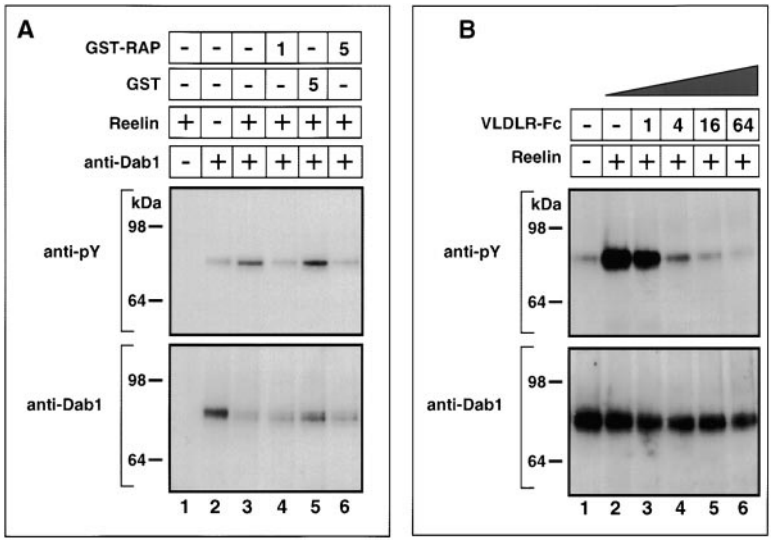

Figure 4. RAP and VLDLR Fc Proteins Interfere with Reelin-Induced Dab1 Tyrosine Phosphorylation

Neuronal cultures from reeler brains were treated with Reelin conditioned (+) or control (−) culture supernatant and immunoprecipitates obtained with an anti-Dab1 (+) or nonimmune (−) antibody were assayed for Dab1 tyrosine phosphorylation by immunoblotting with an anti-phosphotyrosine antibody.

(A) Addition of the indicated amounts (in micrograms per milliliter) of GST-RAP (RAP) but not GST to neurons inhibited Reelin-induced tyrosine phosphorylation of Dab1 (upper panel). Similar levels of Dab1 were recovered (lower panel, immunoblotting for Dab1).

(B) Preincubation of Reelin with increasing amounts of VLDLR Fc inhibits Reelin-induced Dab1 tyrosine phosphorylation. VLDLR-Fc protein concentrations are indicated as arbitrary units.

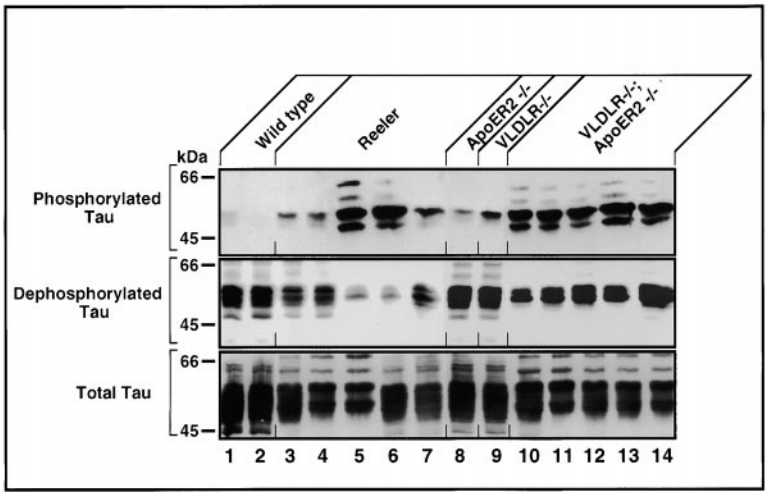

Figure 5. Hyperphosphorylation of Tau in *reeler* and *vldlr/apoER2* Double Knockout Mouse Brain

Brains from 20- to 23-day-old wild-type (lanes 1 and 2), *reeler* (lanes 3–7), *apoER2*$^{-/-}$ (lane 8), *vldlr*$^{-/-}$ (lane 9), and *vldlr*$^{-/-}$;*apoER2*$^{-/-}$ (lanes 10–14) mice were homogenized and extracts were prepared as described in Experimental Procedures. Proteins (10 μg/lane) were separated by reducing SDS gel electrophoresis, transferred to nitrocellulose filters, and incubated with specific monoclonal antibodies. Monoclonal antibody AT-8 specifically recognizes tau phosphorylated on residues 202 and 205 (upper panel). Monoclonal antibody Tau-1 recognizes tau that is not phosphorylated on residues 199 and 202 (middle panel). Monoclonal antibody anti-human tau cross-reacts with the mouse protein and recognizes tau independent of its phosphorylation state (lower panel).

domain Fc fusion protein in the culture supernatant. In this experiment, we sought to determine whether binding of Reelin to the ectodomains in solution prevented its functional interaction with its receptors on the neuronal surface. 293 culture supernatant containing (+) or not containing (−) Reelin was preincubated in the absence or presence of various concentrations of VLDLR-Fc fusion protein before adding to neuronal cultures. Dab1 was precipitated from the cell lysates, proteins were separated by SDS gel electrophoresis, and blots were probed with anti-phosphotyrosine (Figure 4B, upper panel) and anti-Dab1 (Figure 4B, lower panel) antibodies. In the absence of Reelin (Figure 4B, lane 1), Dab1 was only weakly phosphorylated. In the presence of Reelin (Figure 4B, lane 2), Dab1 tyrosine phosphate levels increased dramatically. VLDLR-Fc protein efficiently blocked Dab1 tyrosine phosphorylation in a dose-dependent manner (Figure 4B, lanes 3–6), indicating that signaling by Reelin requires the same epitope(s) with which VLDLR interacts.

vldlr/apoER2 double knockout mice are phenotypically indistinguishable from *reeler* and *Dab1* mutant mice (Trommsdorff et al., 1999). Taken together with the present results, these findings suggest that Reelin signals directly through VLDLR and ApoER2 by inducing Dab1 tyrosine phosphorylation. Ultimately, Reelin regulates neuronal migration and could do so by altering the cytoskeleton. We have previously shown that in mice lacking both ApoER2 and VLDLR, the cellular distribution of the microtubule-associated protein 2 (MAP2) is altered and the structure of dendritic trees is impaired (Trommsdorff et al., 1999). To further define a potential link between the Reelin signaling pathway in *reeler*, *vldr* and *apoER2* mutant mice and alterations in the cytoskeleton, we examined the neuronal microtubule-stabilizing protein tau (Figure 5). Hypophosphorylated tau binds to and stabilizes neuronal microtubules, whereas hyperphosphorylation of tau leads to its dissociation from the microtubules and disruption of the axonal cytoskeleton (Merrick et al., 1997). Hyperphosphorylation of tau is also thought to promote tangle formation in the neurons of patients afflicted with Alzheimer's disease (Matsuo et al., 1994; Billingsley and Kincaid, 1997). Antibodies specific for phosphorylated (Figure 5, upper panel) (Mercken et al., 1992) or dephosphorylated (Binder et al., 1985) (Figure 5, middle panel) tau were used to probe brain extracts from 20-day-old wild-type (Figure 5, lanes 1 and 2), *reeler* (Figure 5, lanes 3–7), *apoER2*$^{-/-}$ (Figure 5, lane 8), *vldlr*$^{-/-}$ (Figure 5, lane 9), and double mutant mice (Figure 5, lanes 10–14). An antibody that detects tau irrespective of the phosphorylation level was used as a control (Figure 5, lower panel). In both *reeler* (Figure 5, lanes 3–7) and double knockout (Figure 5, lanes 10–14) brains, cellular tau was hyperphosphorylated compared to wild-type controls. Cellular levels of unphosphorylated tau were commensurately decreased. Phosphorylated tau was only mildly increased in single knockout brains (Figure 5, lanes 8 and 9). These differences in tau phosphorylation correlate well with the observed histological disorganization of neuronal microtubules in these mutant strains (Rice et al., 1998; Trommsdorff et al., 1999). The difference in the amount of phosphorylated and nonphosphorylated tau between individual *reeler* animals may be explained by their nonuniform strain background. *reeler* animals used in this experiment were the F_2-generation of inbred *reeler* mice obtained from the Jackson Laboratories and 129SvEv animals that had been maintained by isolated inbreeding in our colony since 1991.

Discussion

Double knockout mice that carry mutations in two members of the LDL receptor gene family, *vldlr* and *apoER2*, are phenotypically indistinguishable from Reelin- and Dab1-deficient mice. In addition, Dab1 protein level is increased, reminiscent of the *reeler* mutant (Rice et al., 1998), suggesting that Reelin signaling is perturbed. Thus, it has been proposed that VLDLR and ApoER2 lie on the same signaling pathway as Reelin and Dab1. The role of VLDLR and ApoER2 in transmitting the signal is not clear, however. These cell surface proteins might be needed as coreceptors or receptors for Reelin, or they might be involved downstream of Dab1 in regulating the adhesion or migration of the responding cell.

Here we show that Reelin binds to the extracellular domains of the VLDLR and ApoER2. Binding is inhibited by RAP, a universal inhibitor of ligand binding to LDL receptor family members, or by chelation of Ca^{2+} ions. Competition of Reelin binding to cultured primary embryonic neurons with RAP or with soluble VLDLR ectodomain prevents Reelin-induced tyrosine phosphorylation of Dab1. Reelin-induced tyrosine phosphorylation of Dab1 is known to be inhibited by EDTA (Howell et al., 1999a). These results clearly place the VLDLR and ApoER2 between Reelin and Dab1 in the signaling pathway. In addition, we have discovered an additional phenotypic similarity between *reeler* and *apoER2/vldlr* mutant mice. These mutant brains exhibit hyperphosphorylation of the microtubule-stabilizing protein tau. This finding suggests that tau phosphorylation is regulated directly or indirectly by the Reelin-ApoER2/VLDLR-Dab1 signaling pathway and that the tau phosphorylation state may contribute to the altered distribution of MAP2 protein and microtubules in mutant brains.

RAP and EDTA are not exclusive inhibitors of VLDLR and ApoER2, and thus they might also act on other targets on neurons. Taken together with the genetic evidence, however, the present data indicate that VLDLR and ApoER2 are intrinsic and obligate components of the Reelin signaling pathway that regulate neuronal migration and positioning during brain development. Both VLDLR and ApoER2 are likely to function as receptors for Reelin on the surface of migrating neurons and to be required for transmitting the signal to the adaptor protein Dab1, which binds to their cytoplasmic tails. Tyrosine phosphorylation of Dab1 presumably primes cytoplasmic kinase cascades that control cell shape by remodeling and stabilization of the neuronal cytoskeleton (Trommsdorff et al., 1999). However, it is far from evident how VLDLR and ApoER2 would regulate tyrosine phosphorylation. Neither receptor has been reported to associate with tyrosine kinases or phosphatases. One hypothesis is that there is a coreceptor for Reelin that has associated tyrosine kinase activity. Reelin could stimulate Dab1 tyrosine phosphorylation by bringing together the coreceptor-linked tyrosine kinase and the VLDLR/ApoER2-associated substrate Dab1. Indirect evidence for a coreceptor arises from consideration of the functional domains of Reelin. Reelin contains a unique N-terminal region related to F-spondin followed by eight large repeats (D'Arcangelo et al., 1995). Nakajima et al. (1997) have shown that the N-terminal region is essential for in vivo function by using a monoclonal antibody to this region to disrupt hippocampal development. This antibody also disrupts an in vitro aggregation assay for Reelin function and interferes with Reelin-dependent axonal targeting (Ogawa et al., 1995; Del Rio et al., 1997). However, recombinant Reelin N terminus neither binds to VLDLR or ApoER2 (Figure 2D) nor stimulates tyrosine phosphorylation of Dab1 in primary neurons (B. W. H., unpublished data), suggesting that the N-terminal portion of Reelin may interact with another cell surface protein on neurons, while the repeats in the remainder of the molecule interact with VLDLR and ApoER2.

The molecular defects caused by mutations in the *vldlr* and *apoER2* genes prevent neurons from migrating to their proper cortical position along the radial glial fiber network and from forming normal dendritic spines. A decreased capacity to remodel or form a stable cytoskeleton may be the underlying cause. The phosphorylation state of the microtubule-stabilizing protein tau is a well-established marker for microtubule stability (Billingsley and Kincaid, 1997; Merrick et al., 1997). Hyperphosphorylated tau is incapable of associating with and stabilizing microtubules. In humans, hyperphosphorylated tau is prone to form paired helical filaments (PHF) (Kosik et al., 1988), which are responsible for the tangles seen in neuronal cell bodies in the brains of patients afflicted with Alzheimer's disease. Mutations in tau that increase its propensity to form tangles are the cause for frontotemporal dementia (Hong et al., 1998). Furthermore, the apolipoprotein E-4 (apoE4) isoform is known to decrease the age of onset of Alzheimer's disease in the general population (Schmechel et al., 1993) and in patients bearing tau mutations. Our findings reveal a possible mechanism by which apoE could modulate cellular processes involved in neurodegeneration. Both VLDLR and ApoER2 are known to bind apoE on their extracellular domains. There, it would compete with other extracellular molecules possibly including, but not limited to, Reelin, which continues to be expressed in adult brains. A subtle alteration of cellular signals initiated by members of the LDL receptor gene family might affect the stability of the neuronal cytoskeleton, accelerating neuronal cell death. In support of such a model is the finding that apoE2, an isoform of apoE that binds poorly to the LDL receptor and the other family members, is protective against Alzheimer's disease compared to apoE4 and also to apoE3, the most common allele in the general population.

The difference in tau phosphorylation we have observed in F_2-hybrid reeler animals correlated well with the phenotypic expression of the mutation in the individual animals. Animals in which tau was hyperphosphorylated generally appeared to be more severely affected, being retarded in growth and exhibiting pronounced whole body tremor, as compared to their less affected homozygous reeler littermates (M. T. and J. H., unpublished data). Severely affected animals died around 20 days after birth, around the same time as has been reported for *vldlr*$^{-/-}$;*apoER2*$^{-/-}$ (Trommsdorff et al., 1999) and Dab1–1 (Howell et al., 1997b) mice. The lethality of the Dab1–1 mutation seen in the 129Sv and 129Sv/C57BL6 mixed genetic background is also suppressed in different strain backgrounds (B. W. H. and J. A. C., unpublished data). These observations suggest that modifier genes can considerably alter the phenotypic expression of mutations in the Reelin signaling pathway. We presently do not know whether disruption of the signaling cascades, in which VLDLR and ApoER2 participate, is the primary cause of the observed changes in tau phosphorylation or whether more indirect mechanisms are involved. We are not aware, however, of other animal models in which wide variations of tau phosphorylation have been generated by a combination of naturally occurring and induced mutations and variations in strain background.

In conclusion, our results show that VLDLR and ApoER2 are Reelin-binding proteins expressed on embryonic neurons. Their interaction with Reelin is needed

for the downstream induction of Dab1 tyrosine phosphorylation. Coupled with the genetic evidence from knockout mice (Trommsdorff et al., 1999), we propose a Reelin-VLDLR/ApoER2-Dab1 pathway that regulates neuronal positioning in cortex, hippocampus, and cerebellum during development. Our findings have further identified microtubule-stabilizing proteins as one of the targets of this pathway and provide a possible rationale for explaining how apoE might modulate the onset of neurodegeneration and dementia in man.

Experimental Procedures

Mouse Strains

reeler, *vldlr*, and *apoER2* knockout mice have been described (Falconer, 1951; Trommsdorff et al., 1999). *vldlr*$^{-/-}$ and *apoER2*$^{-/-}$ animals used in this study were bred for multiple generations on a mixed 129SvEv × C57BL/6J background. *reeler* animals used in the experiment in Figure 5 were the F_2 generation of inbred B6C3Fe-a/a-Reln^{rl} animals obtained from the Jackson Laboratories crossed with descendants of 129SvEv mice that had been maintained through isolated inbreeding in our colony since 1991.

Construction of Ectodomain Expression Vectors

Human LDLR, human VLDLR, and mouse ApoER2 ectodomains were amplified by PCR from plasmids containing the respective full-length cDNAs. VLDLR ectodomain was amplified using the following primers: 5′-GAATTCACCATGGGCACGTCCGCGCTCTGGG-3′ and 5′-AAAGCTTTGACAGTCTCGGCCATTTTCCTC-3′. ApoER2 ectodomain was amplified using the primers 5′-GAATTCACCATGGGCCG CCCAGAACTGGGCGTGCT-3′ and 5′-AAAGCTTGGTGCTCGGTAG CATTTCTTCATGT-3′. LDLR ectodomain was amplified using the primers 5′-ACCATGGGGCCCTGGGGCTGGAAATTG-3′ and 5′-CCTCAC GCTACTGGGCTTCTTCTC-3′. PCR products were cloned into the pcDNA3.1/V5/His-TOPO vector (Invitrogen) containing the V5 monoclonal antibody epitope and a 6His tag that allows purification on NTA-columns. All expression vectors were verified by sequencing and designated VLDLR-His, ApoER2-His, and LDLR-His.

Expression vectors in which the receptor ectodomains had been fused to the constant human IgG domain were constructed by amplifying the Fc domain with primers 5′-ACCGGTGACGTCGAGTC CAAATCTTGTGACAAAACTCAC-3′ and 5′-GTTTAAACTCATTTAC CCGGAGACAGGGAGAG-3′. The resulting PCR product was digested with AgeI and PmeI and ligated into the VLDLR and ApoER2 expression plasmids digested with the same enzymes. This replaces the 6His tag with the Fc region. The resulting vectors were designated LDLR-Fc, VLDLR-Fc, and ApoER2-Fc.

The N-terminal Reelin-6His (N-Reelin) expression plasmid (pN-Reelin) was constructed as follows: a PCR product containing the N-terminal domain of Reelin and corresponding to the first 437 coding amino acids (containing the F-spondin homology region and the CR-50 binding domain) was amplified using the primers 5′-ACCATGGAG CGCGGCTGCTGGGCGCCGCG-3′ and 5′-TGCTCCCAAGATATCC CATCCTGTGGG-3′. The product was cloned into pcDNA3.1/V5/His-TOPO (Invitrogen).

Cell Culture, Transfection, and Expression of Recombinant Proteins

Recombinant proteins were expressed in 293 cells as previously described (Hiesberger et al., 1998). Briefly, 30 dishes (60 mm) of 293 cells were grown to 70% confluency in DMEM (low glucose) containing 10% FCS and cotransfected with 1 μg pVA plasmid and 4 μg of either the pCRL (full-length Reelin expression plasmid) (D'Arcangelo et al., 1997), pN-Reelin, pVLDLR-Fc, or ApoER2-Fc expression plasmid using the Stratagene MBS Kit. Twenty-four hours later, the medium was changed to DMEM (low glucose) containing 0.2% BSA. After another 24 hr, culture supernatant was harvested and directly used in experiments. Alternatively, the recombinant secreted proteins were purified by affinity chromatography on NTA-agarose or protein A Sepharose columns. GST-RAP and GST were expressed in BL21DE3 bacteria and purified as previously described (Herz et al., 1991).

Pull-Down Experiments

293 cell culture supernatant (1 ml) containing recombinant LDLR-Fc, VLDL-Fc, or ApoER2-Fc proteins was mixed with 40 μl protein A Sepharose and rotated for 4 hr at 4°C. The Sepharose was then spun down and incubated with 500 μl 293 cell culture supernatant transfected with pVA plasmid and pCRL or with pVA and pN-Reelin, $CaCl_2$ was added to a final concentration of 1 mM, and the mixture was rotated at 4°C for 4 hr. GST-RAP or GST were added to a final concentration of 30 μg/ml and EDTA was added to a final concentration of 30 mM during incubation, where indicated. Sepharose beads were washed six times with LBB (30 mM Tris-Cl [pH 7.2], 120 mM NaCl, 2 mM $CaCl_2$), resuspended in 50 ml 2× protein sample buffer containing 100 mM DTT and boiled for 5 min. Proteins were transferred to nitrocellulose for 60 min at 12V using a semidry cell (Trans-blot SD, Bio-Rad). Membranes were blocked in PBS containing 0.05% Tween-20 and 5% nonfat dry milk and incubated with anti-Reelin G10 antibody (1:1000) for 1 hr. Bound IgG was visualized with goat-anti-mouse Ab-HRP and ECL (Pierce).

Coimmunoprecipitations of Ectodomains with Reelin

G10 monoclonal anti-Reelin antibody (10 μl) was added to 1 ml culture supernatant from 293 cells transfected with pVA and pCRL and rotated for 3 hr at 4°C. Protein G Sepharose (40 μl) was then added and rotation was continued for another 3 hr at 4°C. The beads were then spun down by brief centrifugation in an Eppendorf centrifuge, and 1 ml culture supernatant from 293 cells transfected either with pVLDLR-His or pApoER2-His and $CaCl_2$ (final concentration 1 mM) were added. GST-RAP (30 μg/ml), GST (30 μg/ml), and EDTA (30 mM final concentration) were added where indicated and incubation at 4°C was continued over night. The next day beads were washed three times in LBB, and bound proteins were separated on 4%–15% polyacrylamide gels (Bio-Rad) and analyzed by Western blotting using an HRP-coupled anti-V5 antibody (Invitrogen).

Purification and Concentration of VLDLR-Fc Protein

VLDLR-FC fusion protein was purified by recycling 300 ml of 293 cell culture supernatant repeatedly over a 400 μl protein A Sepharose column. The column was washed with 5 ml LBB and VLDLR-Fc was eluted with 900 μl 100 mM glycine (pH 3.0). Tris (pH 8.0) and BSA were added to final concentrations of 100 mM and 0.2%, respectively. After dialysis against LBB, concentrate VLDLR-Fc was stored at −20°C.

Primary Neuron Culture and Reelin Treatment

Reelin was collected as described previously (Howell et al., 1999a) in the conditioned serum-free culture supernatant of pCRL-transfected 293 cells. Supernatants from pCDNA3-transfected cells was used as a control.

Embryos from heterozygous *reeler* crosses were collected and genotyped as described previously (D'Arcangelo et al., 1996; Howell et al., 1999a). Neurons were harvested and cultured essentially as described (Howell et al., 1999a) with minor changes. The E16 embryonic forebrains were dissociated separately, and neuron suspensions were divided equally into six dishes (20 mm) precoated with poly-L-lysine and E.C.L. (entactin, collagen IV, laminin; UBI) and grown according to Maeda and Noda (1998). Neurons derived from a single *reeler* homozygous mutant brain were used for each experiment. In experiments to determine the effects of GST or GST-RAP on Reelin signaling, neuronal cultures were pretreated for 1 hr with proteins in culture media and subsequently treated with Reelin conditioned culture supernatant and GST or GST-RAP. The effects of ApoER2-Fc or VLDLR-Fc ectodomains on Reelin activity were determined by incubating the purified ectodomains with Reelin conditioned media for 1 hr on ice before adding the mixture to the cultured neurons. In all cases, neurons were treated with Reelin or control conditioned culture supernatant at 37°C for 15 min and reactions were stopped by aspirating the media and lysing cells in RIPA buffer (0.15 M NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate [SDS], 0.1% sodium deoxycolate, 10 mM sodium phosphate, 2 mM EDTA,

14 mM 2-mercaptoethanol, 50 mM NaF, 2 mM NaV04, 1 mM phenylarsine oxide, 20 μg aprotinin/ml). Samples were immunoprecipitated with anti-Dab1 C-terminal antibody (Trommsdorff et al., 1999). Proteins were eluted in 2× concentrated gel loading buffer (4% SDS, 40% glycerol, 0.2 M Tris-HCl [pH 6.8], 5.6 M 2-mercaptoethanol), resolved by SDS-gel electrophoresis, and immunoblotted with anti-Dab1 (B3) polyclonal antibody or with an anti-phosphotyrosine monoclonal antibody (4G10; UBI).

Preparation of Brain Extracts and Western Blotting for Phosphorylated Tau

Frozen 20- to 23-day-old mouse brains were thawed, cut into pieces, and homogenized in 10 vol (w/v) of homogenization buffer (0.1 M MES [pH 6.8], 0.5 mM MgSO4, 1 mM EGTA, 2 mM dithiothreitol, 0.75 M NaCl, 2 mM PMSF, 20 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1 mM benzamidine, 25 mM β-glycerophosphate, 10 mM *p*-nitrophenylphosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μM okadaic acid) as described (Matsuo et al., 1994). The homogenate was clarified by centrifugation at 14,000 × g at 4°C. The supernatant was placed in a boiling water bath for 5 min, then chilled on ice for 5 min and centrifuged at 14,000 x g at 4°C to remove precipitated proteins. The concentration of protein in the supernatant, which contains tau and other heat-stable MAPs, was determined with the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

Phosphorylation of tau in brains from knockout animals was monitored using phospho-specific tau antibodies. Ten micrograms of the boiled supernatant protein was separated by SDS-PAGE and transferred to nitrocellulose membranes and incubated with blocking buffer (20 mM Tris-HCl [pH 7.4], 137 mM NaCl, 0.2% Tween-20, and containing 5% nonfat dry milk) for 1 hr at room temperature. Membranes were then incubated with one of the following tau antibodies diluted in blocking buffer containing 2% nonfat dry milk for 1 hr at room temperature. Monoclonal antibody AT-8 was purchased from Polymedco and used at a 1:5000 dilution. AT-8 specifically recognizes the phosphorylated forms of an epitope containing Ser-202 and Thr-205, according to the numbering of the longest form of human tau (Mercken et al., 1992). Monoclonal antibody Tau-1 (a gift from Dr. Lester Binder) was used at a dilution of 1:1000. Tau-1 specifically recognizes the dephosphorylated form of an epitope containing Ser-199 and Ser-202 (Binder et al., 1985). Anti-human tau (Upstate Biotechnology) that recognizes tau independently of phosphorylation (Kosik et al., 1988) was used at a final concentration of 1 μg/ml to detect total tau protein. The membranes were washed with blocking buffer and incubated with a 1:5000 dilution of horseradish peroxidase–conjugated sheep anti-mouse IgG secondary antibodies (Amersham). Immunoreactive bands were visualized using the SuperSignal West Pico Peroxide Solution (Pierce).

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