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Reelin Is a Ligand for Lipoprotein Receptors

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Summary

A signaling pathway involving the extracellular protein Reelin and the intracellular adaptor protein Disabled-1 (Dab1) controls cell positioning during mammalian brain development. Here, we demonstrate that Reelin binds directly to lipoprotein receptors, preferably the very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2). Binding requires calcium, and it is inhibited in the presence of apoE. Furthermore, the CR-50 monoclonal antibody, which inhibits Reelin function, blocks the association of Reelin with VLDLR. After binding to VLDLR on the cell surface, Reelin is internalized into vesicles. In dissociated neurons, apoE reduces the level of Reelin-induced tyrosine phosphorylation of Dab1. These data suggest that Reelin directs neuronal migration by binding to VLDLR and ApoER2.

Introduction

The investigation of spontaneous and targeted mutations in mice has led to the identification of a number of genes that are required for appropriate neuronal positioning in developing brain structures. In particular, disruption of the *reelin* gene (D'Arcangelo et al., 1995) causes the classic neurological mutation reeler, which is characterized by ataxia, tremors, imbalance, and a reeling gait that becomes apparent 2 weeks after birth (D'Arcangelo and Curran, 1998; Lambert de Rouvroit and Goffinet, 1998). These behavioral defects are associated with severe hypoplasia of the cerebellum and neuronal ectopia in laminated brain regions, including the cerebral and cerebellar cortices and the hippocampus. *reelin* mRNA encodes a large extracellular glycoprotein containing eight repeated sequences that include EGF-like cysteine motifs (D'Arcangelo et al., 1995, 1997). During embryonic development, *reelin* is expressed by neuronal populations that occupy the most superficial layers of the brain (D'Arcangelo et al., 1995; Ogawa et al., 1995; Rice et al., 1998). These include Cajal Retzius cells in the neocortex and hippocampus and cells in the external germinal layer of the cerebellum. Reelin is thought to direct layer formation by eliciting responses in target neurons, such as cortical plate neurons in the cerebral cortex and Purkinje cells in the cerebellum, that lead to the formation of cell layers and the growth of neuronal processes (D'Arcangelo and Curran, 1998).

A mutant phenotype identical to that observed in *reeler* has also been described in mice carrying mutations in the *disabled-1* (*dab1*) gene (Howell et al., 1997b; Sheldon et al., 1997). Dab1 is an intracellular adapter protein that is expressed in Reelin target cells (Howell et al., 1997a; Rice et al., 1998). Dab1 accumulates in the absence of Reelin, suggesting that Reelin may promote degradation of Dab1 (Sheldon et al., 1997; Rice et al., 1998). The addition of Reelin to primary neuronal cultures results in increased tyrosine phosphorylation of Dab1 (Howell et al., 1999a). Thus, Dab1 is thought to function downstream of Reelin in a signal transduction cascade that controls appropriate cell positioning in the developing brain.

Recently, *reeler*-like defects were described in mice lacking two members of the low-density lipoprotein receptor (LDLR) family, the very low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2) (Trommsdorff et al., 1999). These mice are ataxic, and they exhibit cerebellar hypoplasia and abnormal cortical lamination. Mutation of VLDLR and ApoER2 individually results in subtle defects in cell positioning and reduced dendritic arborization (Trommsdorff et al., 1999). In mammals, the LDLR family comprises five members, LDLR, LDLR-related protein (LRP), Megalin, VLDLR, and ApoER2 (Gliemann, 1998). LDLR functions in the systemic clearance of lipoproteins and in the regulation of lipid transport and metabolism (Brown and Goldstein, 1986). In egg-laying species, related receptors also mediate the uptake of yolk constituents by oocytes (Schneider, 1995). The functions of the mammalian genes related to LDLR are less well defined. All members of this family bind apolipoprotein E (apoE)-containing lipoproteins. In addition, VLDLR, LRP, and Megalin bind and internalize a variety of extracellular ligands including proteases, protease inhibitors, peptide hormones, and vitamin carrier proteins (Gliemann, 1998). VLDLR (Takahashi et al., 1992) has also been referred to as LR8 (LDLR-related protein with eight ligand-binding repeats) to emphasize the fact that its affinity for VLDL is low and that other higher affinity ligands for this receptor may exist (Nimpf and Schneider, 1998). Similarly, ApoER2 has also been referred to as LR7/8B (LDLR-related protein with seven or eight ligand repeats that is expressed in the brain) (Kim et al., 1996; Nimpf and Schneider, 1998). VLDLR and ApoER2 are the most closely related genes among the family members, suggesting that they may have overlapping functions. They are expressed at relatively high levels throughout the brain, including the developing regions affected by Reelin (Kim et al., 1996; Trommsdorff et al., 1999). VLDLR and ApoER2 are present in the cortical plate and in the developing hippocampus during embryogenesis (Trommsdorff et al., 1999). These cell populations express Dab1, and they respond to Reelin that is secreted by Cajal Retzius cells in the marginal zone (Rice et al.,

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1998; Trommsdorff et al., 1999). In the embryonic cerebellum, VLDLR and ApoER2 are present in a differentiating field that contains Purkinje cell precursors (Trommsdorff et al., 1999). Early in cerebellar development, Purkinje cells expressing Dab1 align in a distinct layer beneath the external germinal layer that secretes Reelin (Rice et al., 1998; Trommsdorff et al., 1999).

Several members of the LDLR family, including VLDLR and ApoER2, have been shown to bind in vitro to the protein interaction/phosphotyrosine binding (PI/PTB) domain of Dab1 (Trommsdorff et al., 1998, 1999). The cytoplasmic domain of the receptors that binds to Dab1 contains an NPxY motif, an amino acid region that is required for receptor internalization (Chen et al., 1990). Interestingly, Dab1 also binds to an NPxY motif present in amyloid precursor protein (APP) family members, including the amyloid precursor-like protein 1 (APLP1), a transmembrane protein that is prevalent in the brain (Trommsdorff et al., 1998; Homayouni et al., 1999; Howell et al., 1999b). However, mice in which APP family genes have been disrupted do not display ataxia or cortical lamination defects similar to those in *reelin*-, *dab1*-, and VLDLR/APOER2-deficient mice. The phenotype of *VLDLR*$^{-/-}$*ApoER2*$^{-/-}$ mice, together with the association of Dab1 with these receptors, implies that these lipoprotein receptors function in a Reelin-dependent signaling pathway. Several mechanisms of action can be envisaged. For example, the lipoprotein receptors may bind Dab1 and bring it into the proximity of cell surface receptors for Reelin. Alternatively, Reelin may bind VLDLR or ApoER2 directly to exert its function on migrating neurons. To distinguish between these possibilities, we investigated the interaction of Reelin with lipoprotein receptors.

Results

Reelin Binds to VLDLR and ApoER2

Reelin is a large protein of approximately 400 kDa that is relatively nonabundant. It has proven difficult to purify Reelin from brain extracts or to produce it in heterologous systems. To obtain a source of Reelin for our studies, we transfected 293T cells transiently with a full-length *reelin* cDNA clone, pCrl (D'Arcangelo et al., 1997). Reelin secreted from 293T cells was allowed to accumulate in the culture medium for a period of 2 days. Under the conditions used, we routinely obtain approximately 1 μg/ml Reelin as determined by Coomassie blue staining in the conditioned medium (2.5 nM). Consistent with previous findings (Lambert de Rouvroit et al., 1999), we identified three isoforms of approximately 400, 250, and 180 kDa in conditioned medium from transfected cells using antibodies directed against the N-terminal region of Reelin (de Bergeyck et al., 1998) (Figure 1A). Although the ratio of full-length to truncated proteins varied somewhat, all three isoforms were present in brain extracts (Figure 1A). It is likely that the truncated Reelin fragments are generated by the activity of a protease present in the post-Golgi secretory pathway or by an extracellular protease since they are not present in extracts of cells expressing mutated Reelin that cannot be secreted or in brefeldin-treated cells (Lambert de Rouvroit et al., 1999). To determine if Reelin binds to lipoprotein receptors, 293T cells were transfected with expression constructs encoding full-length VLDLR, ApoER2, and LDLR cDNA. As controls, we used mock transfected cells and cells transfected with an expression construct encoding APLP1, a Dab1-binding transmembrane protein. Cells were incubated in the presence of Reelin for 1 hr at 4°C, washed, and analyzed for their ability to retain Reelin by Western blotting. All three Reelin isoforms were found to associate with 293T cells expressing VLDLR and ApoER2, and to a lower extent with cells expressing LDLR. In contrast, no binding was detected using mock transfected cells or cells expressing APLP1 (Figure 1B). Under the conditions employed in this assay, in which an excess of receptor was used, cells expressing either VLDLR or ApoER2 bound approximately 26% of the total applied Reelin, whereas cells expressing LDLR bound approximately 8% of Reelin present in the medium. Although all of the Reelin isoforms were capable of associating with lipoprotein receptors, full-length Reelin bound more efficiently than the truncated proteins. Quantitation of the amount of bound Reelin revealed that 32% of full-length, 17% of the 250 kDa, and 9% of the 180 kDa form of Reelin associated with cells expressing VLDLR and ApoER2 during the course of the experiment (Figure 1B). Similar values were obtained when the experiments were performed at room temperature (Figure 2).

Analysis of the time course of the Reelin-VLDLR interaction indicated that Reelin binding was extremely rapid, as approximately 16% of Reelin present in the medium associated with the receptor within 5 min of incubation

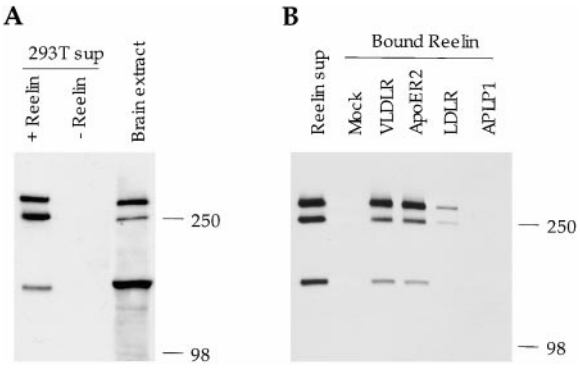

Figure 1. Reelin Binds to Lipoprotein Receptors

(A) Reelin expression in transfected 293T cells and brain extracts. 293T cells were transfected with or without the Reelin expression construct pCrl. Ten microliters of conditioned medium from transfected cells and 10 μg of a protein extract obtained from postnatal mouse brain were analyzed by SDS-PAGE on a 4%–12% gel. Reelin was detected by Western blotting using the N-terminal monoclonal antibodies G10 and E4. Three isoforms of Reelin were detected, a full-length protein of approximately 400 kDa and two truncated proteins of approximately 250 and 180 kDa.

(B) Reelin binds to cells expressing lipoprotein receptors. 293T cells were transfected with expression constructs containing VLDLR, ApoER2, LDLR, or APLP1 or with no DNA (mock). The binding assay was conducted at 4°C using approximately 10^6 transfected cells in the presence of 1 μg/ml Reelin. Bound Reelin was detected by Western blot analysis of cell extracts. Twenty-five percent of the cell lysate containing bound Reelin and 10% of the input Reelin supernatant were analyzed.

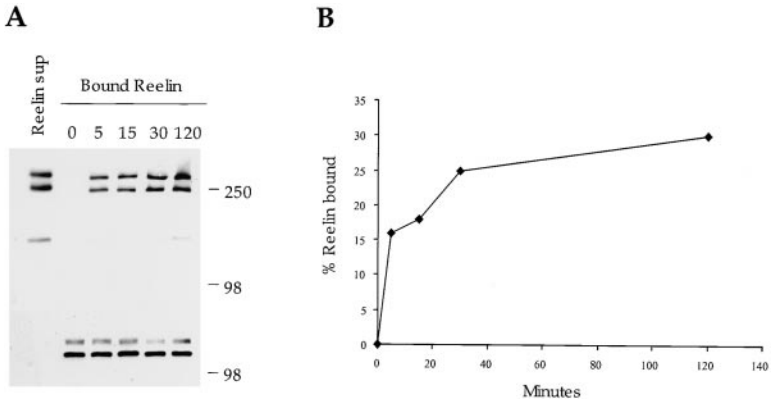

Figure 2. Time Course of Reelin Binding to VLDLR

Binding assays were conducted at room temperature using approximately 10^6 VLDLR-transfected 293T cells and 1 μg/ml Reelin for the indicated times. (A) Cell extracts were examined by Western blotting analysis using antibodies against Reelin (top panel). Twenty-five percent of the cell lysate containing bound Reelin and 10% of the input Reelin supernatant were analyzed. The same filter was reprobed with a polyclonal antiserum directed against VLDLR (bottom panel). (B) The percentage of bound Reelin was plotted against the time of incubation. Numerical values were obtained from the blot shown in (A). Binding approached saturation within 1 hr.

under the experimental conditions used (Figure 2). Similar levels of binding were detected when the binding assay was performed at 4°C (data not shown). Reelin continued to accumulate on VLDLR-expressing cells over a 2 hr incubation period at room temperature, although the binding approached equilibrium within 1 hr (Figure 2). To confirm that similar amounts of receptors were used at each time point, the filter was incubated with an anti-VLDLR antibody (Figure 2A). Similar levels of two proteins of approximately 130 and 150 kDa were detected in all VLDLR-transfected cells (Figure 2A). These bands were absent in mock transfected cells or in cells transfected with other lipoprotein receptors indicating that the antibody is specific for VLDLR (data not shown). The two isoforms of VLDLR may arise from maturation of a precursor protein or by alternative splicing (Iijima et al., 1998).

The association of Reelin with VLDLR was significantly reduced by the anti-Reelin neutralizing monoclonal antibody CR-50, which recognizes an epitope located within Reelin amino acids 250–407 (Ogawa et al., 1995; D'Arcangelo et al., 1997). In contrast, no inhibition was observed in the presence of the G10 monoclonal antibody that recognizes a region located within Reelin amino acids 164–245 (de Bergeyck et al., 1998), or in the presence of normal mouse IgG (Figure 3). The level of inhibition of Reelin binding was dependent on the concentration of CR-50 antibody used in the experiment. Addition of 115 μg/ml of CR-50 antibody led to a 64% inhibition in Reelin binding, whereas inhibition increased to 84% when a concentration of 300 μg/ml of CR-50 was used (Figure 3). Although the CR-50 and the G10 epitopes lie close to each other in the N terminus of Reelin, only CR-50 has been shown to block Reelin function in vitro and in vivo (Ogawa et al., 1995; Del Rio et al., 1997; Miyata et al., 1997; Nakajima et al., 1997). This implies that the interaction between Reelin and VLDLR is functionally significant.

Because a purified preparation of Reelin of known concentration and activity is not currently available, it is not possible to measure the affinity of Reelin for lipoprotein receptors accurately. However, we were able to estimate its apparent affinity by conducting quantitative binding assays using a limiting amount of VLDLR-transfected cells. Under these conditions, the addition of conditioned medium containing approximately 1 μg/ml Reelin (2.5 nM) was sufficient to saturate the receptors (Figure 4). Reelin binding to the VLDLR was modest at a concentration range of 0.08 to 0.31 nM, but it increased dramatically at concentrations above 0.31 nM. Under the conditions used, half-maximum binding occurred at 0.7 nM in two independent experiments (Figure 4B). This indicates that VLDLR is a high-affinity receptor for Reelin. This apparent affinity is comparable to that of the 39 kDa receptor-associated protein (RAP), which

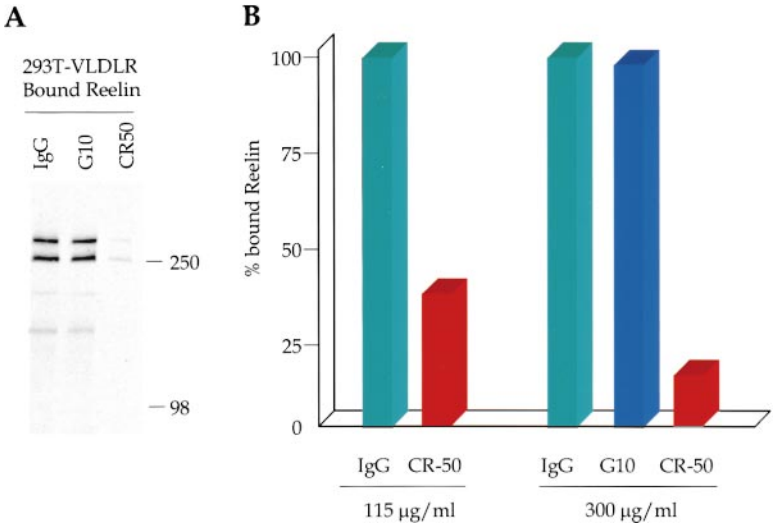

Figure 3. The Neutralizing Antibody CR-50 Inhibits Binding of Reelin to VLDLR

293T cells were transfected with VLDLR, and Reelin binding assays were carried out for 1 hr at room temperature using approximately 10^6 transfected cells. (A) Transfected cells were incubated with 0.4 μg/ml Reelin and 300 μg/ml of the anti-Reelin monoclonal antibodies CR-50 and G10 or mouse IgG as a control. Bound Reelin was detected by Western blot analysis of cell extracts.

(B) Histogram of Reelin binding to VLDLR in two independent experiments. Transfected cells were incubated with 0.8 μg/ml Reelin and 115 μg/ml of CR-50 or mouse IgG or with 0.4 μg/ml Reelin and 300 μg/ml antibodies or mouse IgG as described in (A).

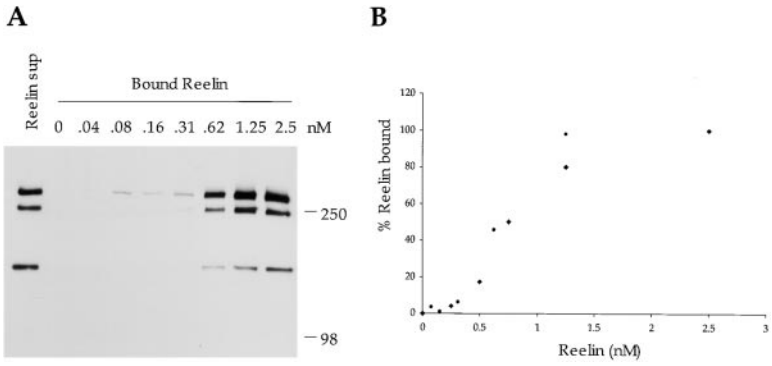

Figure 4. Reelin Binds to VLDLR with High Affinity

(A) Binding assays were conducted at 4°C for 1 hr using a limiting amount of VLDLR-transfected 293T cells (approximately 10^5 cells) and conditioned medium containing the indicated concentrations of Reelin. For concentrations below 2.5 nM, the Reelin-containing medium was diluted consecutively 2-fold with medium conditioned by mock transfected cells. Bound Reelin was detected by Western blot analysis of cell extracts.

(B) The relative amount of Reelin bound to VLDLR-expressing cells from two independent experiments was plotted against the concentration of Reelin (nM). The amount of Reelin bound at the 2.5 nM concentration represents 100% binding. In one experiment (circles), numerical values were obtained from the blot in (A). In another experiment (diamonds), binding was conducted at 4°C for 3 hr using a limiting amount of VLDLR-transfected 293T cells (approximately 10^5 cells). We determined that Reelin was not depleted from the conditioned medium during the course of the experiments by dot blot analysis.

has been shown to bind VLDLR with high affinity (Battey et al., 1994).

Reelin Is Internalized by Cells Expressing VLDLR

Lipoprotein receptors internalize many extracellular ligands through a mechanism involving their cytoplasmic NPxY motifs (Chen et al., 1990). We used an immunocytochemical approach to determine whether Reelin is internalized following association with VLDLR. COS-7 cells expressing VLDLR were incubated with Reelin for 15 min at 37°C, and Reelin and VLDLR were visualized by immunocytochemistry. Consistent with the binding assays described above using 293T cells, Reelin associated only with COS-7 cells that expressed VLDLR (Figures 5A–5C). In nonpermeabilized cells, Reelin was distributed across the surface of cells expressing VLDLR (Figures 5D and 5E). An additional population of Reelin molecules was revealed after permeabilization of the same cells by treatment with detergent (Figure 5F). This population of Reelin was relatively minor, and it appeared to be associated with large vesicular structures (Figures 5F and 5I). No internalized Reelin was detected when VLDLR-transfected cells were incubated with Reelin at 4°C (Figures 5J–5L). These results indicate that the association of Reelin with VLDLR results in internalization of Reelin.

Reelin Binding to Lipoprotein Receptors Is Calcium Dependent

The ligand binding domains of LDLR family members contain disulphide bridges as well as calcium-binding residues (Fass et al., 1997). Binding of lipoprotein to LDLR is absolutely dependent on the presence of extracellular calcium (van Driel et al., 1987). To investigate whether the association of Reelin with VLDLR and ApoER2 is also calcium dependent, we treated VLDLR- and ApoER2-transfected cells with Reelin in the presence and absence of 1 mM EGTA. The addition of this chelating agent completely abolished binding of Reelin to VLDLR and ApoER2 (Figure 6A). This effect was abrogated by addition of 3 mM calcium, and similar results were obtained using 1 mM EDTA (data not shown).

apoE Inhibits the Interaction of Reelin with VLDLR and ApoER2

apoE is a major constituent of very low-density lipoproteins, and it binds to all members of the LDLR family (Mahley, 1988). Three major isoforms, apoE2, apoE3, and apoE4, are encoded by different alleles of human APOE. Therefore, we investigated the effect of apoE-containing lipoproteins and recombinant apoE isoforms on the interaction of Reelin with VLDLR and ApoER2. Recombinant apoE3, the most common isoform of apoE, significantly inhibited the binding of Reelin to both VLDLR and ApoER2 (Figure 6A). apoE3 reduced the binding of 1 μg/ml Reelin to ApoER2 by approximately 50%. Comparison of the effects of purified apoE-VLDL and recombinant apoE isoforms indicated that apoE3 and apoE4 consistently inhibited Reelin binding to VLDLR more efficiently than apoE2 or apoE-VLDL in several experiments. In a typical experiment (Figure 6A), apoE3 and apoE4 inhibited Reelin binding to VLDLR by approximately 60%, whereas the level of inhibition by apoE2 was approximately 10%. This is consistent with previous studies indicating that apoE2 binds LDLR less efficiently than apoE3 and apoE4 (Weisgraber et al., 1982). Interestingly, only a modest inhibition of Reelin binding was observed in the presence of apoE-VLDL, raising the possibility that VLDLR may interact preferentially with apoE3 and apoE4 proteins rather than lipoprotein particles or that apoE proteins may bind to Reelin directly.

Previously, it was demonstrated that addition of Reelin to primary neurons causes phosphorylation of Dab1 on tyrosine residues (Howell et al., 1999a). To determine whether apoE inhibits Reelin signaling in neurons, we conducted several experiments by treating dissociated embryonic cortical neurons with Reelin in the presence or absence of apoE3. In a typical experiment (Figure 6B), addition of Reelin alone to dissociated neurons caused a 3-fold increase in the levels of tyrosine phosphorylation of Dab1. Addition of 50 μg/ml apoE3 reduced the Reelin-induced increase in tyrosine phosphorylation by approximately 50%. apoE3 alone also caused a slight decrease in the basal level of tyrosine phosphorylation of Dab1 (10%). These data suggest that

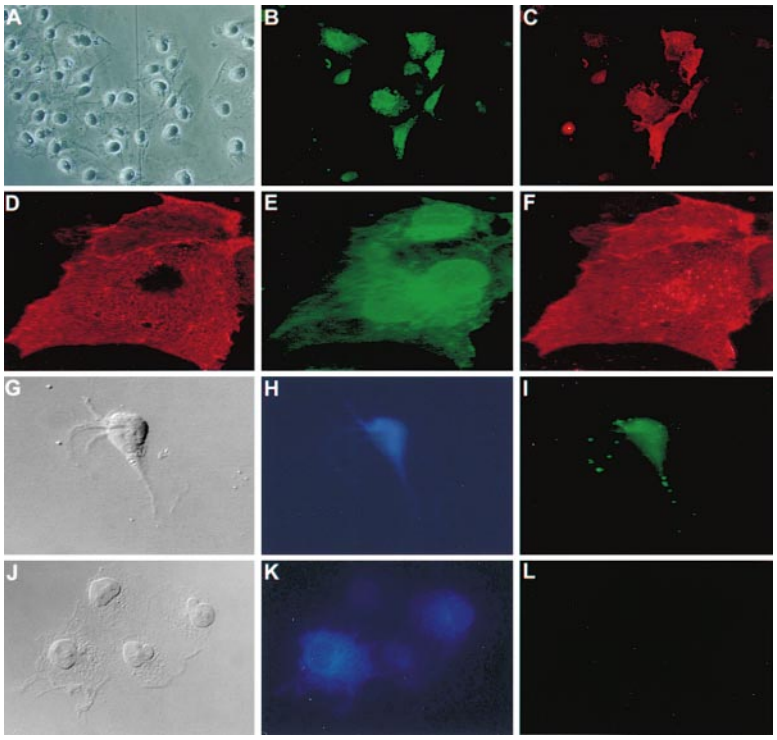

Figure 5. Cells Expressing VLDLR Internalize Reelin

COS-7 cells were transfected with a VLDLR expression construct and treated with Reelin-containing conditioned medium for 15 min at 37°C. Cells were then fixed and permeabilized, except in (D). VLDLR was detected using a rabbit polyclonal antibody directed against the intracellular domain. Reelin was detected using the mouse monoclonal antibody E4.

(A-C) Comparison of Reelin binding and VLDLR expression in transfected cells. (A) Transfected cells visualized by phase contrast. (B) VLDLR-expressing cells were identified using FITC-conjugated anti-rabbit antibodies (green). (C) Reelin bound to the cells was visualized using Texas Red-conjugated anti-mouse antibodies (red). Only cells that expressed VLDLR were positive for Reelin.

(D–F) Sequential staining was performed to demonstrate internalization of Reelin. (D) Nonpermeabilized cell expressing Reelin on the cell surface visualized as described in (C). Following permeabilization by treatment with detergent, the same cell was stained for VLDLR (E) and once more for Reelin to detect the intracellular protein (F). Reelin appears to be localized to large intracellular vesicles.

(G-L) Transfected cells were incubated with Reelin in parallel at room temperature (G-I) or at 4°C (J-L). Cells were fixed and permeabilized before incubating with antibodies against VDLDR and Reelin. VLDLR was visualized with an AMCA-conjugated anti-rabbit antibody (blue, [H] and [K]). Reelin was visualized with a FITC-conjugated anti-mouse antibody (green, [I] and [L]).

apoE3 interferes with the ability of Reelin to activate a Dab1-dependent signaling pathway in neurons.

Discussion

A Model for Reelin Function in Brain Development

The data presented here indicate that Reelin functions by binding to the lipoprotein receptors, VLDLR and ApoER2, to direct cell positioning in the developing brain (Figure 7). The apparent affinity of Reelin for VLDLR is similar to that of RAP, a well-known ligand for lipoprotein receptors. However, RAP is an intracellular chaperone and not a physiological extracellular ligand (Willnow et al., 1996). The high affinity of Reelin for these receptors suggests that Reelin may be a natural ligand for VLDLR and ApoER2 in the brain. The interaction of Reelin with VLDLR results in internalization of Reelin, a phenomenon that is well documented for all the extracellular ligands of LDLR family members (Brown and Goldstein, 1986;

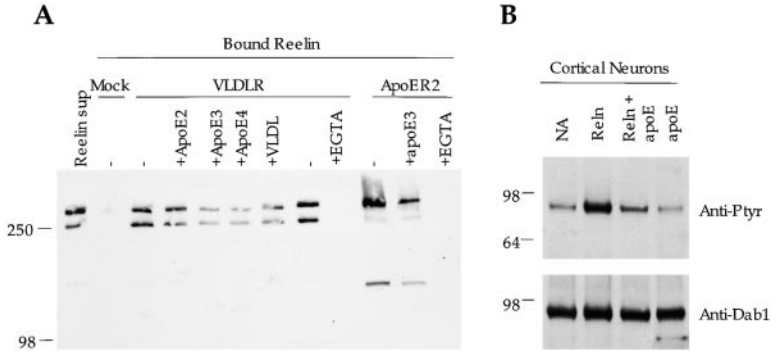

Figure 6. Inhibition of Reelin Binding to VLDLR- and ApoER2-Expressing 293T Cells by EGTA and apoE Isoforms

(A) 293T cells were transfected with VLDLR, ApoER2, or with no DNA (mock). Approximately 10^6 transfected cells were incubated at 4°C with 1 μg/ml Reelin alone (-) or in the presence of 1 mM EGTA, 25 μg/ml of the indicated recombinant apoE isoforms, apoE2, apoE3, and apoE4, or purified apoE-VLDL (VLDL). Bound Reelin was detected by Western blot analysis of cell extracts.

(B) Reelin-induced tyrosine phosphorylation of Dab1 is inhibited by apoE. Cortical neurons obtained from embryonic day 16.5 mice were incubated with 1 μg/ml Reelin in the presence or absence of 50 μg/ml recombinant apoE3. Dab1 was immunoprecipitated using polyclonal antibodies and subjected to Western blot analysis using the anti-phosphotyrosine antibody 4G10 (top panel) and antibodies specific for Dab1 (bottom panel).

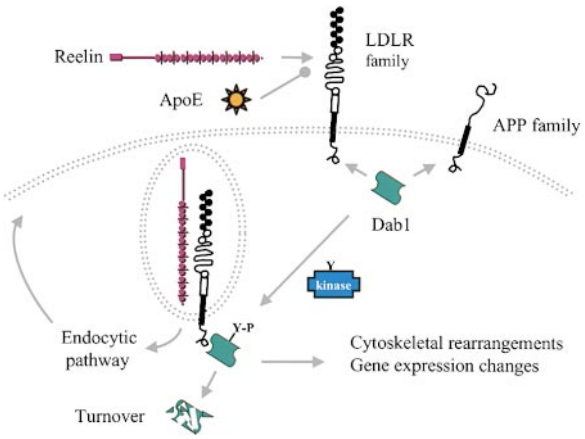

Figure 7. A Model for Reelin Signaling

Extracellular Reelin binds lipoprotein receptors of the LDLR family, and it is internalized. Binding of Reelin to lipoprotein receptors is inhibited by the presence of apoE. Dab1 associates with the cytoplasmic domain of lipoprotein receptors. Internalization of the Reelin-lipoprotein receptor complexes into endocytic vesicles results in increased tyrosine phosphorylation of Dab1 by recruitment of an unidentified kinase(s) and in increased turnover of Dab1. Dab1 also associates with other transmembrane proteins including APP family members. Reelin-induced modification of Dab1 phosphorylation may represent a critical step in the transmission of a Reelin signal from the extracellular environment to intracellular events such as second messenger activation and altered gene expression. Through these events, Reelin directs the formation of cortical layers during brain development. The Reelin signaling pathway may also contribute to events associated with neuronal regeneration or degeneration in the adult brain.

Gliemann, 1998). Since Dab1 binds to an intracellular domain of lipoprotein receptors, internalization of the Reelin-lipoprotein receptor complex may also cause endocytosis and subsequent degradation of Dab1. This could explain previous observations that Dab1 accumulates in ectopic neurons in mice lacking Reelin or VLDLR and ApoER2 (Rice et al., 1998; Trommsdorff et al., 1999).

The induction of tyrosine phosphorylation of Dab1 by Reelin has been shown to depend on the presence of divalent cations (Howell et al., 1999a). Here, we demonstrate that the interaction of Reelin with VLDLR and ApoER2 requires calcium (Figure 6A). Similarly, the ligand binding domains of LDLR require calcium in order to bind lipoproteins (van Driel et al., 1987). Taken together, these observations suggest that Reelin may bind to the ligand binding domains of VLDLR and ApoER2. The interaction of Reelin with VLDLR is also inhibited by the CR-50 antibody, which recognizes an epitope present in the N-terminal region of Reelin. The CR-50 epitope appears to represent a three-dimensional structure as it is destroyed by fixation or denaturation, and it maps to a region of greater than 100 amino acids. The importance of this structure is underscored by the fact that CR-50 inhibits cortical layer formation in rotating cultures (Ogawa et al., 1995), formation of the Purkinje cell layer in cerebellar explants (Miyata et al., 1997), and axonal branching of entorhino-hippocampal fibers (Del Rio et al., 1997), and disrupts formation of the hippocampus in vivo (Nakajima et al., 1997). Based on our findings, it now appears that the inhibitory effects of CR-50 are a consequence of its ability to block binding of Reelin to lipoprotein receptors.

We did not find any evidence for a coreceptor molecule in our binding studies since Reelin did not associate with 293T or COS cells in the absence of transfected lipoprotein receptors. Furthermore, if another transmembrane protein was required for Reelin binding to lipoprotein receptors, it would have to be a highly expressed and relatively ubiquitous protein, as we were able to demonstrate very high levels of Reelin binding to the transfected cells. Although we cannot formally rule out the possibility that other cell surface molecules participate in Reelin signal transduction, the simplest interpretation of our data is that VLDLR and ApoER2 function as receptors for Reelin.

VLDLR and ApoER2 are transmembrane proteins that do not contain intrinsic tyrosine kinase activity. This indicates that Reelin induces Dab1 tyrosine phosphorylation by recruiting an as yet unidentified protein kinase. Mammalian Dab1 can be phosphorylated in vitro by Abl, Src, Fyn, Lyn, and Arg (Howell et al., 1997a; data not shown). Thus, it is possible that members of the nonreceptor tyrosine kinase family phosphorylate Dab1 in response to Reelin. However, tyrosine phosphorylation of Dab1 is undiminished in brain extracts derived from mice lacking Src, Abl, or Fyn (Howell et al., 1997a). In addition, recently we identified a tyrosine kinase activity that associates with Dab1 (our unpublished data). This activity is present in brain extracts from mice lacking Abl, Fyn, Lyn, Lck, Src, or Yes. Thus, at the present time, it is not clear whether none of these tyrosine kinases phosphorylate Dab1 in vivo or whether they have overlapping functions.

Role of the Reelin Pathway in the Adult Brain

Reelin is expressed at high levels in postnatal brain, long after neuronal migration is complete (Ikeda and Terashima, 1997; Alcantara et al., 1998). Sites of high expression include the hippocampus, the entorhinal cortex, the olfactory bulb, and the cerebellum. Thus, there is a correlation between high levels of Reelin expression and the requirement for the maturation of neuronal processes and synaptic remodeling. A role for Reelin in synaptogenesis and axonal branching has already been suggested in the postnatal hippocampus (Del Rio et al., 1997; Borrell et al., 1999). VLDLR, ApoER2, and Dab1 are also expressed in the postnatal brain (Kim et al., 1996; Rice et al., 1998). Thus, it is conceivable that the components of the Reelin pathway that participate in the termination of neuronal migration in the embryonic brain may also be involved in the growth and remodeling of neuronal processes in the adult brain.

Here, we show that binding of Reelin to VLDLR and ApoER2 is inhibited by apoE. The APOE gene is expressed at high levels throughout the brain, where it is thought to function in phospholipid homeostasis as well as growth factor modulation by interacting with LRP (Gutman et al., 1997). apoE also accumulates to very high levels in response to neuronal injury in the central and peripheral nervous systems (Skene and Shooter, 1983; Poirier et al., 1991). Furthermore, epidemiological studies have shown that the apoE4 allele is a susceptibility factor for Alzheimer's disease (Strittmatter and

Roses, 1995). The ability of apoE isoforms to interfere with Reelin binding to VLDLR correlates with their respective affinities for the ligand binding domains of lipoprotein receptors. This observation raises the intriguing possibility that Reelin may contribute to some of the complex processes involved in neurodegeneration.

In addition to the effect of apoE on Reelin signaling described above, several other pieces of circumstantial evidence link the Reelin pathway to neuropathology. The PI/PTB domain of Dab1 has been shown to bind to the NPxY motif present in the cytoplasmic domain APP family members (Trommsdorff et al., 1998; Homayouni et al., 1999; Howell et al., 1999b). A proteolytic product of APP, APPβ, is a major constituent of the amyloid plaques that are characteristic of Alzheimer's disease. Furthermore, mutations in APP have been linked to autosomal dominant familial Alzheimer disease, the most common form of late-onset dementia (Price and Sisodia, 1998). Mice lacking the cyclin-dependent kinase 5 (cdk5) or its activating subunit p35 display an abnormality in cortical lamination that is similar but not identical to that observed in *reeler* mice (Ohshima et al., 1996; Chae et al., 1997). Therefore, it is conceivable that cdk5 activity may be involved in downstream signaling events activated by Reelin. Interestingly, Cdk5 immunoreactivity increases in neurons that exhibit early stage neurofibrillary tangles, a hallmark of Alzheimer's disease, and cdk5 can phosphorylate tau, a microtubule-associated protein that accumulates in a hyperphosphorylated form in neurofibrillary tangles (Imahori and Uchida, 1997). Cdk5 can also phosphorylate Dab1 efficiently in vitro on serine and threonine residues (our unpublished data). These findings raise the possibility that Dab1 may play a role in physiological and pathophysiological processes involving APP. While none of these observations provides compelling evidence that the Reelin pathway is involved in Alzheimer's disease, collectively they suggest that it may be interesting to explore the relationship between the molecular mechanisms that regulate cell migration during brain development and neurodegenerative processes.

Lipoprotein Receptors in Brain Development

The unexpected role of lipoprotein receptors in brain development was revealed recently by the finding that VLDLR and ApoER2 double knockout mice resemble Reelin- and Dab1-deficient mice (Trommsdorff et al., 1999). Mice lacking one of the receptor genes display only a modest phenotype, indicating that these two receptors may have overlapping functions. Our observation that Reelin binds equally well to VLDLR and ApoER2 is consistent with this view. VLDLR and ApoER2 selectively bind apoE-containing lipoproteins, whereas LDLR binds apoE- and apoB-100-containing lipoproteins. It has been previously shown that, in order to bind LDLR with high affinity, apoE must be complexed with lipids (Innerarity et al., 1979). Here, we demonstrate that recombinant apoE proteins are more effective than apoE-containing VLDL at inhibiting Reelin binding to VLDLR and that recombinant apoE3 can interfere with phosphorylation of Dab1 in dissociated neurons. These findings suggest that, at the concentrations used in our study, recombinant apoE may either be able to modulate lipoprotein receptors or bind to Reelin. Further studies will be necessary to elucidate the mechanism whereby apoE inhibits the interaction of Reelin with lipoprotein receptors.

VLDLR and ApoER2 display an overall modular structure similar to that of the LDLR (Gliemann, 1998; Willnow, 1999). These receptors contain ligand binding repeat regions similar to those of complement type A and regions of similarity with the epidermal growth factor (EGF) precursor, including six cysteine repeat motifs. VLDLR and at least one isoform of ApoER2 contain eight ligand binding repeats, one more than LDLR. Despite these structural similarities, several studies suggest that the ligand binding specificities and the physiological roles of VLDLR and ApoER2 may differ from those of the LDLR (Kim et al., 1996; Nimpf and Schneider, 1998). First, VLDLR and ApoER2 are expressed in several tissues, including the brain, but not in liver, whereas LDLR is expressed predominantly in the liver. Second, unlike LDLR, VLDLR and ApoER2 do not bind to low-density lipoprotein particles. Third, very low-density lipoprotein particles bind to LDLR with a higher affinity than to VLDLR. The modest role played by VLDLR in lipoprotein metabolism is also emphasized by the fact that the plasma lipoprotein profile is unaffected in mice lacking VLDLR (Frykman et al., 1995). Taken together, these findings suggested that VLDLR does not function primarily as a receptor for lipoproteins. The results presented here suggest that Reelin may be a physiological ligand for VLDLR and ApoER2 in the developing, and perhaps in the adult, brain.

Experimental Procedures

Plasmid Constructs

The following mammalian expression constructs were used: pCrl (D'Arcangelo et al., 1997), encoding mouse *reelin* cDNA, a construct containing the human VLDLR, a construct containing a brain splice variant of the mouse ApoER2 cDNA (Brandes et al., 1997), pLDLR-17 (Russell et al., 1989), containing the human LDLR cDNA, and a construct encoding mouse APLP1 cDNA (Homayouni et al., 1999).

Antibodies

The anti-Reelin mouse monoclonal antibody G10 (de Bergeyck et al., 1998) was obtained as ascites fluids. The anti-Reelin mouse monoclonal antibodies E4 (de Bergeyck et al., 1998) and CR-50 (Ogawa et al., 1995) were Protein A purified from hybridoma cell culture supernatants. The rabbit anti-VLDLR polyclonal antibody, raised against the C-terminal region of human VLDLR, was obtained as whole serum. Purified mouse IgG was purchased from Jackson Laboratories. Secondary antibodies used for immunofluorescence were FITC-conjugated anti-rabbit IgG (Jackson Laboratories), Texas Red-conjugated anti-mouse IgG (Molecular Probes), and AMCA-conjugated anti-rabbit IgG (Molecular Probes). The secondary antibodies used for Western blot analysis were HRP-conjugated anti-mouse or anti-rabbit IgG (Amersham). Polyclonal goat anti-Dab1 antibodies used for immunoprecipitation were affinity purified. HRP-conjugated 4G10 anti-phosphotyrosine antibody (Pierce) was used to detect phosphorylated Dab1.

Cell Culture and Transfection

293T and COS-7 cells were obtained from ATCC. Cells were cultured in Dulbecco's modified Eagle medium (BioWhitaker) supplemented with 10% fetal bovine serum (Life Technologies), 10 U/ml penicillin/streptomycin mixture (Life Technologies), and 2 mM GlutaMAX (Life Technologies) at 37°C under 5% CO_2. Cells were transfected with

plasmid constructs using the Fugene6 transfection reagent (Boehringer Mannheim) according to the manufacturer instructions. Cortical neurons were dissociated from embryonic day 16.5 mice derived from the mating of two *reeler* heterozygotes (obtained from The Jackson Laboratory). Cerebral cortical neurons from ten embryos were dissociated in ice-cold Hank's Balanced Salt Solution, pooled, and triturated in serum free Dulbecco's modified Eagle medium.

Reelin Expression

293T cells were transfected with the full-length mouse Reelin expression construct pCrl. The following day, the culture medium was replaced with a serum-reduced medium (Nephrigen, Celox Laboratories). After 2 more days, the conditioned medium was collected and stored at 4°C. To calculate the amount of Reelin produced, the conditioned medium from mock and Reelin-transfected cells was concentrated 20-fold using Centricon 100 filters (Amicon) and analyzed by SDS-PAGE. Reelin was detected by staining with Coomassie Brilliant Blue. By comparison with protein markers, we estimated the Reelin concentration to be approximately 1 μg/ml in the original conditioned medium.

Reelin Binding Assay

For binding assays, 293T cells were transfected with or without lipoprotein receptors or APLP1 and washed in phosphate buffer saline (PBS) before distributing into Eppendorf tubes. Cells were incubated with conditioned medium containing Reelin or with medium collected from a mock transfection at 4°C or room temperature. Cells were then washed once more with PBS and solubilized in a buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton-X, 1 mM EDTA, 0.1 mg/ml aprotinin, 0.1 mg/ml leupeptin, 20 mM sodium fluoride, and 1 mM sodium orthovanadate. Cell extracts were clarified by centrifugation at 13,000 × g for 10 min at 4°C and subjected to Western blot analysis to reveal the presence of bound Reelin.

Western Blot Analysis

293T cell extracts were analyzed by SDS-PAGE on 4%–12% gels (Novex). Proteins were blotted onto nitrocellulose membranes and incubated with a mixture of anti-Reelin monoclonal antibodies E4 and G10 at a 1:1000 dilution and HRP-conjugated anti-mouse IgG antibodies at a 1:10000 dilution. To detect VLDLR, filters were reprobed with a 1:1000 dilution of rabbit polyclonal antiserum directed against VLDLR and HRP-conjugated anti-rabbit IgG antibodies at a 1:10000 dilution. Immunoreactive Reelin and VLDLR proteins were detected by enhanced chemiluminescence (ECL) using the SuperSignal West Dura Extended Duration Substrate (Pierce). Immunoprecipitated Dab1 was analyzed by SDS-PAGE on 4%–12% gels and blotted onto nitrocellulose membranes. The blots were incubated first with the HRP-conjugated 4G10 anti-phosphotyrosine antibody and subsequently with anti-Dab1 antibodies. The blots were developed by ECL using the ECL-Plus kit (Amersham).

Quantitative Analysis of Reelin Binding

To quantitate the amount of Reelin bound to cells expressing lipoprotein receptors, we first generated a standard curve by spotting increasing amounts of a Reelin-containing medium on a nitrocellulose membrane. The filter was probed with anti-Reelin antibodies as described for Western blot analysis. Reelin was detected by ECL, and numerical values were obtained by spot densitometry using the ChemiImager program and a gel imaging apparatus (Alpha Innotech). For quantitation studies, the intensity of Reelin bands was measured by spot densitometry using X-ray films exposed to the ECL reagents for variable amounts of time. Only numerical values that fell within the linear range of the standard curve were used for quantitation.

Immunofluorescence

COS-7 cells were transfected with full-length human VLDLR. Two days after transfection, the cells were treated with Reelin conditioned medium for 15 min at 37°C or 4°C. After treatment, the cells were fixed with 4% paraformaldehyde and blocked in 2.5% horse serum (Vector), and cell surface staining was performed using the E4 anti-Reelin monoclonal antibody at a 1:500 dilution from a 0.5 mg/ml stock. Subsequently, the cells were permeabilized with 0.1% Triton X-100 in PBS, blocked in 2.5% horse serum, and reincubated with the E4 antibody and with anti-VLDLR antiserum used at a 1:1000 dilution. Secondary antibodies were used at a 1:1000 dilution.

Dab1 Phosphorylation Analysis

Dissociated neuronal cultures were treated in suspension with or without Reelin-containing medium. Cells were lysed in a buffer containing 50 mM Tris, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM Na vanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. After clarifying the extracts, Dab1 was immunoprecipitated and analyzed by Western blotting as described above. Quantitative analysis of Dab1 phosphorylation was performed by densitometry as described above for the Reelin binding studies.

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