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Abstract
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The Central Fragment of Reelin, Generated by Proteolytic Processing In Vivo, Is Critical to Its Function during Cortical Plate Development

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Key words: cortical development; CNR1; Dab1; Reelin; ApoER2; VLDLR

Development/Plasticity/Repair

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Key words: cortical development; CNR1; Dab1; Reelin; ApoER2; VLDLR

Introduction

Cortical development proceeds by precursor proliferation in ventricular zones (VZ), followed by neuronal migration to the preplate and cortical plate (CP), and is critically dependent on the function of the Reelin pathway (Lambert de Rouvroit and Goffinet, 1998; Rice and Curran, 2001; Jossin et al., 2003b; Tissir and Goffinet, 2003). Reelin is a glycoprotein of 420–450 kDa that is secreted by several neurons, such as cortical Cajal-Retzius cells. Defective Reelin is the cause of the reeler malformation in mice (D’Arcangelo et al., 1995; Hong et al., 2000) and the Norman-Roberts type lissencephaly in man (Hong et al., 2000) (Online Mendelian Inheritance in Man 257320). In reeler mice, neurons are generated in the VZ like in wild-type animals. Although their initial migration is correct, they form abnormal architectonic patterns at the end of migration. When normal neurons form a dense, laminar CP in which maturation proceeds from inside to outside, reeler mutant neurons form a loose CP in which the gradient of maturation is inverted.

Reelin is thought to deliver a signal to migrating neurons, instructing them to assume their correct position. Their response requires binding of Reelin to at least one of two lipoprotein receptors, very-low-density lipoprotein receptor (VLDLR) and apolipoprotein-E receptor type 2 (ApoER2) (Hiesberger et al., 1999; Trommsdorff et al., 1999), but Reelin does not bind to the closely related low-density lipoprotein receptor (LDLR). The signal is relayed by the Dab1 adaptor that interacts with the cytoplasmic tail of receptors (Howell et al., 1997, 1999, 2000; Sheldon et al., 1997; Ware et al., 1997; Bar et al., 2003; Jossin et al., 2003b). Tyrosine phosphorylation of Dab1 after Reelin binding (Howell et al., 2000; Keshvara et al., 2001) is essential: the reeler-like Dab1 phenotype is rescued by a Dab1 cDNA encoding key tyrosine residues but not when they are mutated to phenylalanine (Herrick and Cooper, 2002). The Fyn and Src tyrosine kinases are implicated in Dab1 phosphorylation (Arnaud et al., 2003; Bock et al., 2004). However, Fyn or Src deficiency do not generate a reeler-like phenotype, suggesting redundancy and/or additional complexity in the pathway. That a reeler-like malformation is induced in brain slices incubated with the Src family kinase inhibitor PP2 further demonstrates the implication of these kinases in Reelin signaling (Jossin et al., 2003a). Fyn docks to the cytoplasmic tail of the cadherin-related neuronal receptor-1 (CNR1),
and CNR1 was reported to bind Reelin, thereby recruiting Fyn into the complex (Senzaki et al., 1999). The N-terminal region of Reelin is considered important, because it contains the epitope of the function-blocking CR50 antibody (Ogawa et al., 1995; Miyata et al., 1997; Nakajima et al., 1997). CR50 interferes with Reelin homopolymerization and with Dab1 phosphorylation (Utsunomiya-Tate et al., 2000; Kubo et al., 2002). On the other hand, the N-terminal moiety of Reelin does not bind to receptors (Hiesberger et al., 1999).

Previous work showed that Reelin is cleaved in vivo at two sites located after domains 2 and 6, resulting in the production of three fragments (Lambert de Rouvroit et al., 1999). To understand further the relationship between the different parts of Reelin and its function during development, we studied the binding in vitro of partial Reelin proteins to ectodomains of the VLDLR and ApoER2 receptors and reassessed the binding of Reelin to CNR1; we tested the ability of partial Reelin proteins to elicit Dab1 phosphorylation in neuronal cultures and their capacity to correct the reeler phenotype in embryonic brain slices; and we generated constructs, tagged with the V5 epitope, were described previously (Hiesberger et al., 1999). Vectors coding for the CNR1 ectodomain, as well as anti-Myc for the others. SR,S V,

### Materials and Methods

**Expression of parts of Reelin and other proteins in expression vectors.** The Reelin cDNA construct pCrl, kindly provided by Dr. T. Curran (St. Jude’s Children’s Research Hospital, Memphis, TN) (D’Arcangelo et al., 1997), was used to express Reelin and as a template for PCR amplification. For Reelin constructs, “R” is used for repeat, “N” for N terminus, and “Del” for deletion of a given region. The amplicons for constructs R3–8, R3–6, R3–5, R4–6, R3–4, R4–5, R5–6, R7–8, R4, and R6 (Table 1, Fig. 1) were cloned in the pSecTag2B vector (Invitrogen, San Diego, CA), in-frame with a signal peptide and a C-terminal Myc epitope. Constructs N-R6, Del3–4-5A, N-R5A, and N-R2 were obtained from pCrl by nuclease restriction, followed by ligation. Constructs were verified by sequencing and tested for secretion of the protein by transfection of HEK293T cells. Plasmid pSRFL, kindly provided by K. Nakajima (Keio University, Tokyo, Japan), encodes amino acids 368–3461 of Reelin. This protein (abbreviated ΔN-Reln) does not contain the GI0 and CR50 epitopes (Kubo et al., 2002). The human VLDLR-Fc, human LDLR-Fc, and mouse ApoER2-Fc constructs, tagged with the V5 epitope, were described previously (Hiesberger et al., 1999). Vectors coding for the CNR1 ectodomain, as well as its first ectodomain (EC1) domain, were generated in the same manner as lipoprotein receptor constructs, using specific primers and respective full-length cDNAs as template. The CNR1 ectodomain was amplified using the following primers: 5′-ACCATGGAAATTCTCGGGGGAAGTG-3′ and 5′-ATCCACCAGTGACGCCTACAGTGTTG-3′. The EC1 domain was amplified using the following primers: 5′-CAGGTGACGTGGGAATTTCTCGGGGGAAGTG-3′ and 5′-AAAGCTTGGGAGCGGAGGTTGTCGTG-3′.

**Expression of recombinant proteins and pull-down assays.** For expression of recombinant Reelin and receptor proteins, HEK293T cells were seeded at 1 × 10⁵ cells per 35 mm well and transfected 16–20 hr later with 2 μg of plasmid DNA using calcium phosphate-DNA precipitation in HEPES, as described previously (Kingston, 2001). They were cultured in DMEM with 10% fetal bovine serum (BioWhittaker, Walkersville, MD). One day after transfection, the medium was replaced by 1 ml of serum-free medium, which was collected after 24 hr, replaced, and collected again after 24 hr. Pooled supernatants were used as the source of recombinant protein. The binding of Reelin proteins to lipoprotein receptors was assayed as described previously (Hiesberger et al., 1999) with minor modifications. Briefly, HEK293T cell culture supernatants containing equivalent amount of recombinant LDLR-Fc (in 1 ml), VLDLR-Fc (in 1 ml), or ApoER2-Fc (in 2.5 ml) proteins was mixed with 40 μl of protein A-agarose beads (Roche Products, Hertfordshire, UK) and incubated overnight at 4°C on a rotating wheel. The supernatant was removed, and the beads were incubated with 1 ml of supernatant from HEK293T cells transfected with pCrl or partial Reelin constructs and incubated overnight at 4°C. Beads were washed successively with radioimmunoprecipitation assay (RIPA) buffer (0.15 M NaCl, 1% Triton X-100, 1% SDS, 0.1% sodium deoxycholate, 10 mM sodium phosphate, 2 mM EDTA, and 14 mM 2-mercaptoethanol), 0.5 M NaCl, and RIPA. Inclusion of a 0.5 M NaCl wash was shown to decrease nonspecific binding as evidenced by the absence of binding of Reelin to LDLR-Fc. Proteins

### Table 1. Constructs used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>PCR Digestion</th>
<th>Amino acids in Reelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reelin</td>
<td>Eco47, NotI</td>
<td>1 to 3461</td>
</tr>
<tr>
<td>N-R6</td>
<td>Yes</td>
<td>1 to 2712</td>
</tr>
<tr>
<td>R3–8</td>
<td>Yes</td>
<td>1220 to 3428</td>
</tr>
<tr>
<td>R3–6</td>
<td>Yes</td>
<td>1220 to 2664</td>
</tr>
<tr>
<td>Del3–4-5A</td>
<td>A gel, Clot</td>
<td>Deletion from 1287 to 2181</td>
</tr>
<tr>
<td>N-R5A</td>
<td>Clot, NotI</td>
<td>1 to 2181</td>
</tr>
<tr>
<td>N-R2</td>
<td>A gel, NotI</td>
<td>1 to 1286</td>
</tr>
<tr>
<td>R3–5</td>
<td>Yes</td>
<td>2664 to 3428</td>
</tr>
<tr>
<td>R4–6</td>
<td>Yes</td>
<td>2664 to 3428</td>
</tr>
<tr>
<td>R5–6</td>
<td>Yes</td>
<td>2664 to 3428</td>
</tr>
</tbody>
</table>
| R7–8 | Yes | 3461 of Reelin. This protein (abbreviated ΔN-Reln) does not contain the GI0 and CR50 epitopes (Kubo et al., 2002). The human VLDLR-Fc, human LDLR-Fc, and mouse ApoER2-Fc constructs, tagged with the V5 epitope, were described previously (Hiesberger et al., 1999). Vectors coding for the CNR1 ectodomain, as well as its first ectodomain (EC1) domain, were generated in the same manner as

**Figure 1.** Receptor binding. The different Reelin constructs are shown, together with their ability to coprecipitate with LDLR-Fc, ApoER2-Fc, and VLDLR-Fc. No construct binds to LDLR only full-length Reelin and proteins N-R6, R3–6, and R3–8 are able to bind to VLDLR and ApoER2, and they bind similarly to both. The lane labeled Control is a Western blot of supernatant from cells transfected with the different plasmids. Blots were revealed with anti-Reelin G10 for the constructs that contain region H, with anti-Reelin 12 and 14 for the constructs that contain the C-terminal repeat (de Bergeyck et al., 1998), and with anti-Myc for the others. Sa, Sv, Signal peptides of Reelin and encoded by the pSecTag vector; SP, region of Reelin with similarity with F-spondin; H, unique region. Arrows indicate the two Reelin cleavage sites.
were eluted from the beads by boiling for 3–5 min in SDS gel loading buffer, separated by SDS-PAGE (Duracryl, 5%), and transferred to a polyvinylidene difluoride (PVDF) membrane using a transblot (Bio-Rad, Hercules, CA). Blots were revealed with either anti-Reelin G10 (de Bergeyck et al., 1998) or anti-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA) to reveal, respectively, Reelin or partial Reelin polypeptides or with anti-V5 antibody (Invitrogen) to reveal receptor-Fc constructs. Secondary antibodies were goat anti-mouse antibodies coupled with HRP (Cytomation; Dako, High Wycombe, UK). Chemiluminescent revelation was performed using the Super Signal West Pico kit (Pierce, Rockford, IL). Each fusion protein was tested at least three times.

For estimation of interactions between Reelin and CRN1, recombinant ectodomain-Fc fusion proteins were generated as follows. Briefly, 30 dishes (60 mm) were transfected with the expression vector, and, 24 hr later, the medium was replaced with medium lacking serum but containing 0.2% BSA. After another 24 hr, culture supernatant was harvested, and the recombinant secreted fusion proteins were purified using protein A-Sepharose as described for VLDLR-Fc (Hiesberger et al., 1999). Recombinant proteins were concentrated ~300 times, and Western blotting using anti-V5 antibody was performed to evaluate their relative concentration. For pull-down experiments, equal amounts of CRN-Fc proteins were incubated with 30 μl of protein A-Sepharose beads, 500 μl of BSA buffer (30 mM Tris-Cl, pH 7.4, 130 mM NaCl, 2 mM CaCl2, and 0.2% BSA), and 500 μl of supernatant from HEK293 cells transfected with pCrl and incubated for 3 hr at 4°C. Beads were spun down and washed four times with BSA buffer and once with BSA-free buffer. Bound proteins were analyzed by Western blotting using either anti-V5-HRP conjugate or anti-Reelin-HRP conjugate.

Monoclonal antibodies against VLDLR and ApoER2. The cDNA encoding the extracellular part (amino acids 28–355) of human VLDLR fused to myelin basic protein at its N terminus and carrying a 6-His tag at the C terminus, cloned in pMalc2x (New England Biolabs, Beverly, MA), was generously provided by D. Blaas (Institute of Biochemistry, Vienna, Austria). A recombinant protein corresponding to the extracellular part of mouse ApoER2 (amino acids 36–390) carrying a 6-His tag at the N terminus was cloned in pProex-1 (Invitrogen). Fusion proteins were produced in Escherichia coli, purified on an Ni-NTA agarose column (Qiagen, Hilden, Germany), and used to immunize mice deficient in the corresponding protein (Frykman et al., 1995; Trommsdorff et al., 1999).

Monoclonal antibodies were obtained by splenocyte fusion and produced as ascites fluid as described previously (de Bergeyck et al., 1998). Severed in isolation, and clones 7G10 (anti-VLDLR) and 2H8 (anti-ApoER2) were selected because they corresponded to myelin basic protein at its N terminus and carrying a 6-His tag at the C terminus, cloned in pMalc2x (New England Biolabs, Beverly, MA), was generously provided by D. Blaas (Institute of Biochemistry, Vienna, Austria). A recombinant protein corresponding to the extracellular part of the LDL receptor (Frykman et al., 1995; Trommsdorff et al., 1999). The relative concentrations of Reelin and ΔN-Reln were adjusted similarly. Finally, the relative concentrations of R3–6 and R3–8 were adjusted using an anti-Myc antibody. Other antibodies were used as described in Section 3.6.2, R3–8 were used by Howell et al. (1999). Supernatants were concentrated approximately eightfold (Biomax columns; Millipore, Bedford, MA) before use. Because construct R3–8 does not contain the 142 epitope, its relative concentration was adjusted to that of Reelin, using antibodies 12 and 14 directed against the C-terminal part of Reelin (de Bergeyck et al., 1998). The relative concentrations of Reelin and ΔN-Reln were adjusted similarly. Finally, the relative concentrations of R3–6 and R3–8 were adjusted using an anti-Myc antibody. Other antibodies were used as described in Section 3.6.2.

Primary cortical cell cultures or embryonic slices were rinsed with culture medium and stimulated for 20 min at 37°C with ligand provided as 500 μl of concentrated (spin column; Millipore) transfected cell supernatant. Cells were lysed in an NP-40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Nonidet-P-40) supplemented with 50 mM NaF, 2 mM Na3VO4, 1 mM phenylarsine oxide, 2 mM EDTA, and protease inhibitor mixture (Complete; Roche Products) at 4°C for 10 min. Lysates were clarified by centrifugation and incubated overnight at 4°C with a rabbit polyclonal antibody raised against a peptide containing the 12 C-terminal amino acids of Dab1, followed by an incubation of 2 hr with protein A–agarose beads (Roche Products). The beads were washed three times with NP-40 buffer, and proteins were eluted by boiling for 5 min in SDS gel loading buffer and analyzed on an 8% SDS–PAGE. The proteins were transferred to a PVDF membrane and revealed with a mouse monoclonal anti-Dab1 antibody (E1) or an anti-phosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology).

Results

The central part of Reelin mediates receptor binding

Reelin (Fig. 1) starts with a signal peptide of 27 residues that is followed by a region with similarity to F-spondin (amino acids 28–190). A unique segment among amino acids 191 and 500 is followed by eight repeats of ~350 amino acids (repeat 1, residues 501–860; repeat 2, 861–1220; repeat 3, 1221–1596; repeat 4, 1597–1947; repeat 5, 1948–2314; repeat 6, 2315–2661; repeat 7, 2662–3051; and repeat 8, 3052–3428). The protein terminates with a basic stretch of 33 amino acids (3429–3461). In vivo, Reelin is processed by cleavage at two sites located, respectively, between repeats 2 and 3 and repeats 6 and 7 (Lambert de Rouvroit et al., 1999) (Fig. 1).

Recombinant full-length Reelin and partial Reelin polypeptides were assayed for binding to the extracellular region of VLDLR and ApoER2, as well as LDLR (negative control), as shown in Figure 1. Binding to LDLR was undetectable for all secreted proteins. Construct N-R2, composed of the spondin similarity region, the unique segment and repeats 1 and 2, thus corresponding to the N-terminal processing fragment, did not...
bind lipoprotein receptors. Even construct N-R5A, which includes the first four Reelin repeats and part of repeat 5, did not bind significantly, confirming that the N-terminal moiety of Reelin is not involved. Similarly, all secreted constructs that contain one, two, or three Reelin repeats, including construct R7–8 that corresponds to the C-terminal processing fragment, failed to bind detectably to VLDLR or ApoER2. In contrast, construct N-R6, which encodes the N-terminal part up to and including repeat 6, as well as proteins R3–6, corresponding to the central fragment generated by processing, and R3–8, which contains repeats 3–8, bound to VLDLR and ApoER2 similarly to full-length Reelin. Although detectable binding required at least four repeats, suggesting that binding capacity increased with repeat number, this is not the sole factor. For example, construct Del3–4-5A, in which repeats 3, 4, and the N-terminal part of repeat 5 are deleted, as well as protein N-R5A did not bind detectably to either receptor, although they contain more than four Reelin repeats. Altogether, the results of these experiments suggested that the central Reelin repeats 3–6, which correspond to the central fragment generated by processing in vivo, are particularly important for receptor binding.

The cadherin-related neuronal receptor CNR1 does not bind Reelin in vitro

Previous work suggested that the protocadherin CNR1 binds the N-terminal region of Reelin via its EC1 domain (Senzaki et al., 1999). Because Fyn and related kinases are important in Reelin signaling (Arnaud et al., 2003; Bock and Herz, 2003; Jossin et al., 2003a) and CNR1 binds Fyn in its cytoplasmic tail, CNR1 was considered a perfect candidate for a Reelin coreceptor that could bring Fyn in the signaling complex (Senzaki et al., 1999; Yagi, 2001). However, the Reelin-alkaline phosphatase (AP) fusion protein used in this binding study was extracted from cell lysates and not secreted, suggesting that it may not be properly folded. Given the potential importance of that mechanism, we sought to verify the binding in vitro between the whole ectodomain or the EC1 domain of CNR1 and secreted Reelin, as described in Materials and Methods. In contrast to this previous report, we were unable to detect any interaction between the ectodomain or the EC1 region of the CNR1 protein and Reelin, whereas binding of Reelin to lipoprotein receptors was consistently observed in the same conditions (Fig. 2).

The central part of Reelin triggers Dab1 phosphorylation

The ability of the different Reelin constructs to induce tyrosine phosphorylation of the Dab1 adapter was assessed using primary cortical neuronal cultures. As illustrated in Figure 3A, full-length Reelin, N-terminally deleted Reln (ΔN-Reln), and proteins R3–8, N-R6, and R3–6 were all able to induce Dab1 phosphorylation, whereas all other Reelin polypeptide constructs were inactive. The addition to native Reelin of proteins N-R2, R3–4, or R5–6 did not modify its capacity to induce Dab1 phosphorylation (data not shown). Some background phosphorylation was observed in the absence of exogenous Reelin or in the presence of small Reelin proteins (Fig. 3A). This background was variable from experiment to experiment. It reflected presumably Reelin-independent phosphorylation, because it was detected even when reeler mutant neurons were used and as tyrosine phosphorylation was assessed using antibody 4G10, which is not sequence specific. Because the capacity to induce Dab1 phosphorylation probably depends on the concentration of activating ligand, it was important to use similar concentrations to compare the activity of different fusion proteins. Using the normalization described in Materials and Methods, there was a clear correlation between the ability to bind to receptors and the capacity to stimulate Dab1 phosphorylation. This indicated that R3–6 is sufficient and that no other region is absolutely required to initiate the Dab1-dependent branch of Reelin signaling, at least in cells in vitro. In some experiments, full-length Reelin appeared slightly more active in triggering Dab1 phosphorylation than the proteins that lacked the N-terminal part, suggesting that this N-terminal region might have an enhancing effect on Dab1 phosphorylation. Reelin preparations contain some proportion of cleavage products, and this proportion increases significantly in the presence of neurons, even during the short (20 min) incubation time of the assay. To detect a putative interference of the N-terminal sequence, Dab1 phosphorylation assays were performed by using R3–8 alone or in the presence of N-R2. No difference was observed, clearly indicating that the N-terminal fragment did not interfere with the function of the protein.

The central fragment R3–6 rescues the reeler phenotype in slices

In brain slices cultivated from E13.5 normal and reeler embryos, the formation of a cortical plate with normal and reeler features, respectively, can be observed after 2 d in vitro, as shown in Figure 4, A and B. When slices prepared from reeler embryos were cultivated in the presence of full-length Reelin (Fig. 4C), recombinant Reelin R3–8, composed of the repeats 3–8 (Fig. 4D), or Reelin R3–6, composed of the minimal central region required for receptor binding and Dab1 phosphorylation (Fig. 4E), the cortical plate that developed in vitro showed an organization nearly similar to that in normal slices. First, a thin cell-poor marginal zone was visible at subpial level, whereas the marginal zone in reeler slices was barely detectable. Second, the compaction and...
radial orientation of cortical plate cells was better defined than in control reeler slices. Third, immunostaining with the antibody CS56, a preplate marker directed against a chondroitin sulfate proteoglycan epitope, showed that preplate splitting, typically defective in reeler mutant cortex, was partially restored in slices treated with Reelin, R3–8, or R3–6. This was confirmed in rescue experiments performed after labeling of preplate cells by BrdU injection of the pregnant mother on E10.5 (Fig. 5). As a control, no phenotype rescue was observed in reeler slices incubated with the N-terminal region of Reelin (N-R2), which does not bind to receptors and does not trigger Dab1 phosphorylation. As mentioned above, all preparations of Reelin contain a proportion of cleavage products, and this proportion increases during incubation with slices, making it difficult to assess the relative functional potency of native Reelin versus the R3–8 and R3–6 active fragments.

**Anti-receptors antibodies stimulate Dab1 phosphorylation but do not correct the reeler phenotype in slices**

Different antibodies against the VLDLR and ApoER2 extracellular regions were added to primary neuronal cultures in isolation and in combination, and Dab1 phosphorylation was estimated. Among the combinations tested, two antibodies, 7G10 (against VLDLR) and 2H8 (against ApoER2), were able to increase modestly Dab1 phosphorylation when added separately but triggered a strong phosphorylation when they were added together (Fig. 3B). This result suggested that, in some conditions, the binding to lipoprotein receptors of ligands unrelated to Reelin is sufficient to initiate the Dab1-dependent component of the Reelin cascade. Interestingly, similar or higher concentrations of stimulating antibodies were unable to correct the reeler trait in slices, although Dab1 phosphorylation was increased (Figs. 4F, 6). Dab1 protein levels are increased in reeler mutant brains, and the incubation of reeler slices for 24 hr with active Reelin R3–8 resulted in a downregulation of Dab1, approximately to the level in normal slices (Fig. 6). However, whereas incubation with stimulating antibodies increased Dab1 phosphorylation as mentioned above, it did not induce a downregulation of Dab1 protein concentrations (Fig. 6). This would suggest that Dab1 phosphorylation alone is not sufficient to transmit the entire Reelin signal.

**Discussion**

The present results show that, like full-length Reelin, a partial protein composed of repeats 3–6 is able to bind to VLDLR and ApoER2 receptors in vitro, to trigger Dab1 tyrosine phosphorylation in neuronal cultures, and to rescue the reeler trait in embryonic brain slices, suggesting that this part of Reelin is functionally critical during cortical plate development. In vivo, Reelin is cleaved at two sites, and comparison with partial Reelin constructs indicated that cleavage proceeds between repeats 2 and 3 and between repeats 6 and 7 (Lambert de Rouvroit et al., 1999;
Figure 5. Partial rescue of preplate splitting in reeler slices. Preplate elements were labeled with the anti-chondroitin sulfate antibody CS-56 (A, C, E) or by BrdU injection of the pregnant mother on E10.5 (B, D, F). In normal embryos (A, B), the appearance of the CP results in preplate splitting, with preplate cells located in the marginal zone (MZ) and superplate (SP). In reeler embryos (C, D), no such splitting occurs, and all preplate cells gather in the superplate (SPP). When reeler slices are cultured in the presence of R3–8 (E, F), R3–6, or native Reelin (data not shown), a partial splitting of the preplate population is observed. Scale bar, 90 μm. IZ, Intermediate zone; CSPG, chondroitin sulfate proteoglycan.

Jossin et al., (2003b). Thus, the central fragment produced in vivo corresponds to the functionally active segment defined in this study.

Using our qualitative assay, partial Reelin proteins bind equally to VLDLR and ApoER2, suggesting that homologous regions of receptors are implicated, although native Reelin has a higher affinity for ApoER2 than VLDLR (Andersen et al., 2003). This fits with observations that VLDLR and ApoER2 null mutant mice are subtle, whereas mice deficient in VLDLR have a phenotype that, however, failed to occur in slices treated with antibodies.

Partial rescue of preplate splitting in Figure 5.

Figure 6. Dab1 phosphorylation and protein levels in slices. Reeler slices were cultured in control conditions, in the presence of active R3–8 or stimulating antibodies and compared with normal slices. Dab1 phosphorylation and protein levels were estimated after 4 and 24 hr in vitro. After 4 hr, both the antibodies and R3–8 induced a phosphorylation of Dab1 that was comparable with that in normal slices. After 24 hr, R3–8 resulted in a downregulation of Dab1 protein levels that, however, failed to occur in slices treated with antibodies.

Like the full-length protein, Reelin fragments containing repeats 3–8 and 3–6 are able to stimulate Dab1 phosphorylation and to rescue the reeler cortical plate phenotype in a slice assay, suggesting that the central region containing repeats 3–6 is sufficient to fulfill the functions of Reelin during cortical plate development. The observation that most biological functions of Reelin are recapitulated by its central fragment show that the cleavage that occurs in vivo does not inactivate the protein. Because all preparations of native Reelin contain processing fragments and their proportion increases during incubation with herins of the CNR family (Senzaki et al., 1999). The phenotypes of mice deficient in β1 integrins are distinctively different from reeler (Graus-Porta et al., 2001; Magdaleno and Curran, 2001). Nevertheless, the increase in the amount of the N-terminal fragment of Reelin in α–3 integrin-deficient brains compared with brains from control mice (Dulabon et al., 2000) suggests a functional link between integrins and Reelin that should be studied further. It has been suggested that the protocadherin CNR1 binds extracellularly to the N-terminal region of Reelin. This molecule also binds the Fyn tyrosine kinase at its C-terminal tail and could thereby participate in Dab1 phosphorylation (Senzaki et al., 1999). However, although this mechanism is appealing and generates wide interest, our present observations fail to confirm significant interactions between Reelin and CNR1. A likely explanation for these divergent results lies in the reagents that were used to examine this possible interaction. The secreted CNR1 ectodomain fusion proteins were identical in both studies. However, whereas we used full-length, secreted and biologically active Reelin, Senzaki et al. (1999) used a Reelin–AP fusion construct that failed to be secreted from transfected cells and instead had to be released, presumably from an aggregated state in the endoplasmic reticulum, by detergent solubilization. The finding that misfolded Reelin–AP fusion proteins might bind CNR1–Fc nonspecifically is supported by our unpublished observations that a presumably incompletely folded LDLR–Fc fusion protein obtained from transfected cell lysates interacts with Reelin in vitro, whereas the same protein, harvested from the culture medium, does not. Thus, the present results make a physiological role of CNRs as Reelin coreceptor less likely.

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slices, it is not presently possible to assess the relative activity of full-length and fragments of Reelin. In explant cultures, Reelin processing was best inhibited by zinc chelators such as o-phenanthroline (Lambert de Rouvroit et al., 1999), which proved, however, too toxic to be used in our slice culture assay. Although the correction of the reeler phenotype in slices presumably does not mimic faithfully the in vivo situation, the effect obtained is comparable with the partial rescue observed in transgenic mice that express the reelin cDNA under control of the nestin promoter (Magdaleno et al., 2002). The observation that the addition of exogenous Reelin to reeler slices is able to correct their phenotype suggests that Reelin does not need to be produced locally in the marginal zone to fulfill its role. In the transgenic experiments mentioned above (Magdaleno et al., 2002), ectopic Reelin expression in ventricular zones similarly resulted in significant phenotype rescue. This suggests that Reelin could put neurons in a permissive state to respond to another signal in the marginal zone rather than provide a direct instruction such as a stop (Tissir and Goffinet, 2003).

Unlike Reelin or R3–8, the same anti-receptor antibodies that are able to stimulate Dab1 phosphorylation do not have correcting activity in slices and do not induce the downregulation of Dab1 protein concentration. Thus far, the relative kinetics of Dab1 phosphorylation under Reelin or antibody stimulation could not be compared, and the identity of phosphorylated tyrosine residues in both conditions remains to be defined. With those reservations, our results indicate that integration of the Reelin signal might require more than Dab1 phosphorylation and confirm a previous report that the Reelin-induced decrease of Dab1 protein level is partly independent of Dab1 tyrosine phosphorylation (Howell et al., 2000).

Our results can best be explained by postulating the need for Reelin-dependent recruitment of other cell surface molecules, possibly the integrins (Dulabon et al., 2000; Rodriguez et al., 2000), but probably not CNR1, into the complex. Although these recruited components of the Reelin signaling complex would obviously not be required for induction of Dab1 phosphorylation, these membrane proteins or associated cytoplasmatic factors might be themselves substrates for the intracellular kinases that are stimulated by Reelin-binding to ApoER2 and VLDLR. It is possible that, for instance, phosphorylation of these biological effector molecules by Reelin-activated kinases could regulate neuronal migration and positioning by modulating cell adhesion. Alternatively, a Dab1-independent action of Reelin could be provided by its reported intrinsic serine protease activity that is able to modulate synaptic maturation (Quattrocchi et al., 2003). Thus far, however, the part of Reelin implicated in this activity is not fully defined.

The N-terminal region of Reelin is not absolutely required for activity, at least in the in vitro and ex vivo assays that we used. Other work pointed to the importance of the N-terminal moiety that contains the epitope of the monoclonal antibody CR50 (Ogawa et al., 1995), which was reported to have function blocking activity (Miyata et al., 1997; Nakajima et al., 1997). The N-terminal region of Reelin mediates aggregation, and the formation of homopolymers increases the ability of Reelin to stimulate Dab1 phosphorylation. Both homopolymer formation and Dab1 phosphorylation were decreased by CR50 (Utsunomiya-Tate et al., 2000; Kubo et al., 2002). Our results show that full-length Reelin is indeed slightly more efficient than partial proteins in the Dab1 phosphorylation assay. As a result of the use of transfected cell supernatants and neuronal or slice cultures, the assays that are presently available cannot be quantified using purified components. In vivo, homopolymer formation could increase the effective Reelin concentration and allow the recruitment of more receptor units, thereby increasing signal activation.

In conclusion, our results show that Reelin binds to lipoprotein receptors via its central region that corresponds to the middle fragment generated by processing. This could organize a supramolecular complex at the plasma membrane and/or modulate endocytosis and intracellular traffic of signaling molecules. Other components could be recruited by binding to Reelin directly (“coreceptors”) or by cis interactions with lipoprotein receptors and/or Dab1. By analogy with the role of lipoprotein receptor-related protein (LRP) as a coreceptor of PDGF signaling (Boucher et al., 2002; Loukinova et al., 2002) and the implication of LRP6 as a mediator of Wnt signaling (Pinson et al., 2000), it is tempting to speculate that Reelin, VLDLR, ApoER2, Dab1, and cytoplasmatic kinases such as Fyn may work in parallel with another system that remains to be identified.

References
Howell BW, Herrick TM, Cooper JA (1999) Reelin-induced tyrosine phos-


