The Extremely Conserved C-terminal Region of Reelin Is Not Necessary for Secretion but Is Required for Efficient Activation of Downstream Signaling

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Reelin is a very large secreted glycoprotein essential for correct development of the mammalian brain. It is also implicated in higher functions and diseases of human brain. However, whether or not secretion of Reelin is regulated and how Reelin transmits signals remain largely unknown. Reelin protein is composed of an N-terminal F-spondin-like domain, Reelin repeats, and a short and highly basic C-terminal region (CTR). The primary sequence of CTR is almost completely conserved among vertebrates except fishes, indicating its importance. A prevailing idea regarding the function of CTR is that it is required for the secretion of Reelin, although this remains unproven. Here we aimed to clarify the function of Reelin CTR. Neither deleting most of CTR nor replacing CTR with unrelated amino acids affected secretion efficiency, indicating that CTR is not absolutely required for the secretion of Reelin. We also found that Reelin mutants without CTR were less potent in activating the downstream signaling in cortical neurons. Although these mutants were able to bind to the Reelin receptor ectodomain as efficiently as wild-type Reelin, quite interestingly, their ability to bind to the isolated cell membrane bearing Reelin receptors or receptor-expressing cells (including cortical neurons) was much weaker than that of wild-type Reelin. Therefore, it is concluded that the CTR of Reelin is not essential for its secretion but is required for efficient activation of downstream signaling events, presumably via binding to an unidentified "co-receptor" molecule(s) on the cell membrane.

Reelin is a very large (>3,400 amino acids) secreted glycoprotein (1) that plays essential roles in brain development (2). Reelin is also involved in adult brain functions (3) and has been implicated in human diseases including schizophrenia (4, 5), autism (6), and Alzheimer disease (7). Most, if not all (8), of Reelin function is mediated by phosphorylation of an intracellular protein, Dab1 (9). In the developing forebrain, Reelin is abundantly expressed in a special type of neuron called Cajal-Retzius cells in the marginal zone (10, 11). It is widely believed that this particular localization is essential for proper function of Reelin, although this remains unproven and has been challenged by some recent studies (12–14). Whether Reelin is a positional cue or a permissive factor (or something else) remains controversial (15, 16), partly because the molecular mechanism regulating the secretion and diffusion of Reelin is unknown.

Reelin protein is divided into three subdomains: the N-terminal F-spondin-like domain that is necessary for oligomerization (17), the eight tandems of Reelin repeats (RR) (2), and the short and highly basic C-terminal region (CTR) (1). The region between the third and sixth RR (RR3 and RR6, respectively) binds to Reelin receptors, very low-density lipoprotein receptor (VLDLR) and apolipoprotein receptor 2 (ApoER2) (18). CTR is 32 amino acids long, comprising less than 1% of the entire Reelin protein. Interestingly, in one of the murine Reelin mutants called Orleans, aberrant Reelin protein (ReelinOrl) lacking a part of the eighth RR (RR8) and CTR is produced, but it is not secreted and accumulates in the endoplasmic reticulum (ER) (19, 20). In addition, an artificial Reelin mutant that terminates in the middle of RR8 was not secreted from transfected COS cells (21). These observations led to the widely accepted idea that CTR is required for Reelin secretion (22, 23). On the contrary, an alternative polyadenylation can give rise to a truncated Reelin protein without the last 33 amino acids (24), and this short form Reelin is secreted when overexpressed in COS cells, although its secretion efficacy is apparently much lower than that of the wild-type (full-length) Reelin (ReelinWT) (24). The mRNA encoding the short form is expressed in similar (i.e. not exactly the same) cell types in developing mouse brain, suggesting it has a role distinct from that of ReelinWT (24). However,
there is no evidence that the short form (CTR-less) Reelin is actually produced and secreted in vivo, and its specific function remains unexplored.

Thus, three questions regarding Reelin CTR remain. First, is it really required for secretion? Second, is it required for any other physiological function? Third, if CTR plays any functional role, what is the molecular mechanism? Herein, we show that the CTR is not necessary for secretion but is required for efficient activation of downstream events.

EXPERIMENTAL PROCEDURES

Animal Care—All of the experimental protocols used here were approved by the Animal Care and Use Committee of Nagoya City University and were performed according to the guidelines of the National Institutes of Health of Japan.

Expression Vectors—The definition and alignment of RR and CTR are determined according to the computerized matching reported by Ichihara et al. (25). The Reelin cDNA construct, pCrl (21), was used to express ReelinWT and as a template for PCR amplification. Expression vectors for ReelinOrl, ReelinΔC-mcs, ReelinFLAG+C, ReelinΔC-FLAG, ReelinΔC-Arg8, ReelinΔC-Glu8, ReelinΔC-Ala8, ReelinΔC-His8, and ReelinΔC-Venus were constructed in pcDNA3.1/Zeo(+) (Invitrogen). ReelinΔC, ReelinRR4, ReelinRR5, ReelinRR6, ReelinRR7, ReelinRR8A, ReelinRR8E, and Reelin3441 were constructed on pcDNA3 (Invitrogen). GR-pCrl (21) was used to express ReelinWT and as a template for PCR amplification. Expression vectors for ReelinOrl, ReelinΔC-mcs, ReelinFLAG+C, ReelinΔC-FLAG, ReelinΔC-Arg8, ReelinΔC-Glu8, ReelinΔC-Ala8, ReelinΔC-His8, and ReelinΔC-Venus were constructed in pcDNA3.1/Zeo(+) (Invitrogen). ER-red fluorescent protein (DsRed2-KDEL) was described previously (26). Addition of green fluorescent protein (GFP) to the C-terminal region of ApoER2 (with the ligand-binding repeats 1–3 and 7–9) was performed using pEGFP-N1 (BD Biosciences). The expression vector for Golgi-targeted GFP was purchased from BD Biosciences. Detailed methods and maps of the expression vectors will be supplied on request.

Antibodies—The mouse monoclonal anti-Reelin antibody G10 and rabbit polyclonal antibody anti-Dab1 were purchased from Chemicon (Temecula, CA). The mouse monoclonal anti-human growth hormone (GH) was a generous gift from Dr. J. Takagi (Institute of Protein Research, Osaka University). Biotinylated cholera toxin B was purchased from Sigma. Alexa 488- or Alexa 594-conjugated secondary antibodies were purchased from Invitrogen. The mouse monoclonal anti-Reelin antibody CR-50 was described previously (10). Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY).

Cell Culture and Transfection—Human embryonic kidney (HEK) 293T, COS-7, and NIH-3T3 cells were plated onto 35-mm dishes in the presence of 1.5 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. To collect Reelin-containing conditioned media, the culture medium was replaced with Opti-MEM (Invitrogen) supplemented with antibiotics after 5 h of transfection and cultured for another 40 h. For immunocytochemistry, the culture medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum after 5 h of transfection and cultured for another 24–40 h.

Primary Culture of Mouse Cortical Neurons—Primary cortical neurons were prepared from embryonic day 15 mice as described previously (27). In brief, cortical lobes were trypsinized for 5 min at 37 °C and dissociated using 0.1% DNase I (Roche). Neurons were plated onto poly-L-lysine-coated 35-mm dishes or 12-well plates and maintained in Neurobasal medium (Invitrogen) containing B27 supplement (Invitrogen), penicillin/streptomycin, and 2 mM L-glutamine.

Dab1 Phosphorylation Assay—Induced phosphorylation of Dab1 in mouse cortical neurons was measured essentially as previously described (27). Briefly, cortical neuronal cells were incubated with Reelin-containing media for 20 min at 37 °C, lysed, and immunoprecipitation was performed with anti-Dab1 antibody. Samples were analyzed by Western blotting using 4G10 first, and then reprobed with anti-Dab1 antibody.

Western Blotting—Samples were separated by SDS–PAGE (5 and 7.5% acrylamide gel for Reelin and Dab1, respectively) or native PAGE without SDS (17), and transferred to a polyvinylidene difluoride membrane. Blotted filters were incubated with the first antibody, washed with Tris-buffered saline with 0.05% Tween 20, incubated with the peroxidase-conjugated secondary antibody, washed, and then visualized with an ECL system (GE Healthcare). Images were captured using a LAS3000 system (Fuji, Tokyo, Japan). Images were analyzed with ImageJ software (version 1.34s, National Institutes of Health). The area of each blot was enclosed, and the total intensity of chemical luminosity was calculated using the following formula: area × (mean intensity − background near the band). The intensity from results using 4G10 was divided by that of anti-Dab1, and normalized with control intensity. The significance of differences between means was determined by one-way analysis of variance and Tukey’s test for multiple pairwise comparisons. Differences were considered significant at p < 0.05. Unless otherwise specified, all data are given as mean ± S.E. from six independent experiments.

Immunocytochemistry—Primary cortical neurons or COS-7 cells were fixed with 4% formaldehyde for 10 min at room temperature and washed with phosphate-buffered saline. Cells were incubated with the indicated primary antibody for 2 h at room temperature, washed four times with phosphate-buffered saline, incubated with the fluorescent secondary antibody for 1 h, and washed with phosphate-buffered saline four times and with water twice. Samples were examined with an LSM510 confocal laser scanning microscope (Carl Zeiss Japan, Tokyo, Japan).

Binding of Reelin to the Ligand Binding Domain of ApoER2—Reelin-containing supernatants were incubated with GH-ApoER2LBD (GH fusion of ligand binding repeats 1–3 and 7 of ApoER2, a generous gift from Prof. J. Takagi, Osaka University), anti-GH antibody and protein G-Sepharose (GE Healthcare) for 1 h at 4 °C, washed with HBAH buffer (Hanks’ balanced salt solution containing 2 mM CaCl2, 0.5 mg/ml bovine serum albumin, 0.1% (w/v) NaN3, and 20 mM Hepes) four times, and suspended in SDS–PAGE sampling buffer, followed by Western blotting analysis.

Binding of Reelin to Cell Membrane—ApoER2-GFP-expressing HEK293T cells were collected with 10 mM Tris, 1 mM EDTA and triturated with a 27-gauge needle. The lysates were centri-
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**FIGURE 1.** Reelin CTR is highly basic, evolutionarily conserved, and not absolutely necessary for secretion. A, alignment of nucleotide sequence encoding Reelin CTR. The amino acid sequence (A.A.) is indicated in the lowest line. Positively charged amino acids are circled. The arrowheads indicate nucleotides that are not conserved among all of the species listed. B, secretion efficiencies of ReelinWT, ReelinOrl, and ReelinΔC in HEK293T cells. The expression vector for ReelinWT (lane 1), ReelinOrl (lane 2), or ReelinΔC (lane 3) was transfected into HEK293T cells, and the culture supernatant (left panel) and cell fraction (right panel) were collected 2 days later. Each fraction was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with anti-Reelin antibody G10. C, secretion efficiency of ReelinWT, ReelinOrl, and ReelinΔC in COS-7 cells. ReelinWT (lane 1), ReelinOrl (lane 2), or ReelinΔC (lane 3) was expressed in COS-7 cells and analyzed as described in B. D, Reelin truncation mutants that terminate in the middle of RR8 are not secreted. ReelinRR7 (lane 1), ReelinRR8A (lane 2), ReelinRR8B (lane 3), or ReelinΔC (lane 4) were expressed in HEK293T cells, and analyzed as described in B. E, Reelin truncation mutants with complete Reelin repeats are secreted, even in the absence of CTR. ReelinWT (lane 1), ReelinRR4 (lane 2), ReelinRR5 (lane 3), ReelinRR6 (lane 4), ReelinRR7 (lane 5), or ReelinΔC (lane 6) were expressed in HEK293T cells and analyzed as in B. F, secretion efficiency of Reelin mutants in NIH-3T3 cells. ReelinWT (lane 1), ReelinΔC (lane 2), ReelinOrl (lane 3), ReelinRR8A (lane 4), or ReelinRR8B (lane 5) were expressed in NIH-3T3 cells and analyzed as described in B. In panels B–F, the arrow and arrowheads indicate full-length and degraded products of Reelin or its mutants, respectively. Positions of molecular weight mass (kDa) are shown on the right side of the panels.

Fuged at 700 × g for 3 min to remove debris, and the supernatant was then collected, and centrifuged at 14,000 × g for 10 min to obtain the membrane fraction. The membrane fraction was then incubated with Reelin-containing conditioned media for 1 h at 4 °C, washed with HBAL buffer 4 times, dissolved with SDS-PAGE sampling buffer, and analyzed by Western blotting. To block nonspecific binding, the assay tubes and the membrane fraction were incubated with 10% bovine serum albumin prior to addition of Reelin-containing media.

**RESULTS**

**CTR of Reelin Is Highly Conserved at Both the Nucleotide and Amino Acid Levels**—Among 32 amino acid residues of CTR, 12 (38%) are basic (circled in Fig. 1A), whereas none are acidic (Fig. 1A). The primary sequence of CTR is extraordinarily conserved: it is completely (100%) conserved among all mammals whose Reelin sequence can be found in the available databases: namely, human, chimpanzee, macaque, dog, mouse, rat, cow, and gray short-tailed opossum. It is also perfectly conserved in chickens and turtles (Fig. 1A). In the crocodile, all but the first one is conserved (Fig. 1A). This prominent conservation is also observed in the DNA sequence encoding CTR: 91 of 99 (92%) nucleotide residues are conserved among all of the species listed in Fig. 1A, for example. From the evolutionary point of view, this observation strongly suggests that CTR has an essential physiological function(s) in the land vertebrates.

As it is a prevailing idea that CTR is required for Reelin secretion, we first compared the secretion efficiency of ReelinWT, ReelinOrl, and mutant Reelin lacking only CTR (ReelinΔC) in HEK293T cells. As shown in Fig. 1B, ReelinWT was efficiently secreted, whereas no ReelinOrl was secreted in HEK293T cells (Fig. 1B, left panel, lanes 1 and 2, respectively). These results were consistent with the previous report (19). Interestingly, ReelinΔC was secreted (Fig. 1B, left panel, lane 3), although its amount was less abundant than that of ReelinWT. It was also observed that ReelinΔC accumulates to a greater extent in the cell fraction than ReelinWT or ReelinOrl (Fig. 1B, right panel). These results were also observed in COS-7 cells (Fig. 1C), suggesting that it is not a cell type-specific event. We also expressed a truncated Reelin mutant that terminated just after RR7 (ReelinRR7) and found that it was secreted (Fig. 1D, lane 1). In fact, other Reelin-truncated mutants with complete Reelin repeats (ReelinRR4, ReelinRR5, and ReelinRR6) were also
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secreted (Fig. 1E, lanes 2–4, respectively). On the other hand, truncated mutants that terminated after subrepeats RR8A or RR8E (ReelinRR8A and ReelinRR8E, respectively (25)) were not secreted (Fig. 1D, lanes 2 and 3, respectively). The same phenomenon was observed when NIH-3T3 cells were used (Fig. 1F), indicating its universality. These results suggested that truncation in the middle of RR leads to total failure of secretion and that CTR is not absolutely required for secretion. However, as ReelinΔC is less efficiently secreted than ReelinWT, it remains possible that CTR plays some role in facilitating secretion.

We next compared intracellular localization of ReelinWT, ReelinOrl, and ReelinΔC. For this analysis, COS-7 cells were chosen as its flat and spreading shape makes it easier for us to observe intracellular organelles, especially ER. ReelinWT was found mainly in the ER and presumably in secretory vesicles (Fig. 2, A–C). ReelinOrl was also localized in the ER but, in comparison to ReelinWT, it tended to accumulate more around the nucleus (Fig. 2, D–F). This was consistent with a previous report that ReelinOrl accumulates in the rough ER in Cajal-Retzius cells (20). Therefore, it was strongly suggested that ReelinOrl (and possibly other truncated mutants that terminate in the middle of RR) is unable to go beyond the ER in its secretory pathway.

Unexpectedly, ReelinΔC showed quite different localization from ReelinWT or ReelinOrl. It accumulated in cells as characteristic tubular structures (Fig. 2G). These structures were observed only when ReelinΔC was expressed, and were positive for ER markers (Fig. 2H). Their appearance and amount was unaffected by brefeldin A treatment (Fig. 2, J–M), indicating that they are dilated ER (like structures) induced by ReelinΔC accumulation. These results were also obtained in 293T and NIH-3T3 cells (supplementary Fig. S1). It was thus indicated that ReelinOrl and ReelinΔC have distinct intracellular fates in the secretory pathway, and thus support the idea that lack of CTR is not directly responsible for the secretion failure of ReelinOrl.

The Highly Conserved Structure of CTR Is Not Required for Reelin Secretion—To further investigate the role of CTR in Reelin secretion, we expressed a series of mutant Reelin proteins having either a truncated or an unrelated sequence in place of CTR (Fig. 3A). The secretion efficiency was measured by Western blotting using the culture supernatant and cell fraction from HEK293T cells. Reelin3441 lacking the last 20 amino acids (i.e. without 10 of the 12 basic residues) of CTR was secreted as efficiently as ReelinWT (Fig. 3, B and C, lanes 1 and 3), indicating that the first 12 amino acids is sufficient for secretion facilitation, whereas the region of highly positive charges is not necessary. We next added FLAG epitope (DYKDDDDK), which is rather acidic, in place of CTR (ReelinΔC-FLAG). Quite unexpectedly, ReelinΔC-FLAG was secreted as efficiently as ReelinWT (Fig. 3, B and C, lane 4). We also measured the secretion efficiency of Reelin mutants that have a tandem of the same amino acids (ReelinΔC-Glu8, ReelinΔC-Arg8, ReelinΔC-Ala8, and ReelinΔC-His8). Among them, ReelinΔC-Glu8 and ReelinΔC-Arg8 were efficiently secreted (Fig. 3, B and C, lanes 5 and 6, respectively).
A

| Lane | Name | Sequence |
|------|------|----------|
| 1    | WT   | STRKONYGDSQFGRQHGLRHFYRRRLRYP* |
| 2    | ΔC   | *        |
| 3    | 3441 | STRKONYGDSQSFGHRLRHFYRRRLRYP* |
| 4    | ΔC-FLAG | SSDYKDDDS* |
| 5    | ΔC-Glu8 | SSDYKDDDS* |
| 6    | ΔC-Arg8 | SSDYKDDDS* |
| 7    | ΔC-Venus | SSDYKDDDS* |
| 8    | ΔC-Ala8 | SSDYKDDDS* |
| 9    | ΔC-His8 | SSDYKDDDS* |
| 10   | ΔC-mcs | SSDYKDDDS* |
| 11   | FLAG-C | SSDYKDDDS* |

FIGURE 3. Addition of unrelated sequence in place of CTR can fully rescue Reelin secretion. A, sequence surrounding CTR and its mutants. The FLAG epitope is shown in italics. B and C, expression vectors for ReelinWT or mutant (the lane number is designated in A) were transfected into HEK293T cells, and the culture supernatant (Secreted) and cell fraction (Cell) were collected 2 days later. All samples were subjected to Western blotting analysis using anti-Reelin antibody G10. D, NIH3T3 cells were transfected with the expression vector for ReelinWT (lane 1), ReelinΔC (lane 2), ReelinΔC-FLAG (lane 3), ReelinΔC-Glu8 (lane 4), and ReelinΔC-Ala8 (lane 5). The culture supernatant (Secreted) and cell fraction (Cell) were collected 2 days later and analyzed as described above. The arrows and arrowheads indicate full-length and degraded products of Reelin, respectively. Positions of molecular mass markers (kDa) are shown on the right side of the panels.

respectively) but the other two were not (Fig. 3, B and C, lanes 8 and 9). Interestingly, the secretion efficiency of ReelinΔC-Venus (Venus is a variant of yellow fluorescent protein with 239 amino acids (28)) was no less than that of ReelinWT (Fig. 3, B and C, lane 7). ReelinΔC-mcs, which had five amino acids derived from multicloning sites, was not efficiently secreted (Fig. 3, B and C, lane 10). Insertion of FLAG tag between RR8 and CTR (ReelinFLAG+C) had little effect on secretion efficiency (Fig. 3, B and C, lane 11). Similarly, in NIH3T3 cells, ReelinΔC and ReelinΔC-Ala8 were barely secreted (Fig. 3D, lanes 2 and 5, respectively), whereas ReelinΔC-FLAG and ReelinΔC-Glu8 were quite effectively secreted (Fig. 3D, lanes 3 and 4, respectively). These results clearly indicate that the highly conserved CTR primary sequence is not attributable to it playing a role in Reelin secretion. Most notably, the highly basic nature of CTR was not required for secretion.

The CTR Is Necessary for Efficient Induction of Dab1 Phosphorylation in Cortical Neurons—The fact that most of the CTR is necessary for secretion prompted us to investigate the physiological function of the CTR. The phosphorylation of intracellular adaptor protein Dab1 is a widely accepted indication of Reelin signaling activation (2, 3). The primary cortical neurons from embryonic mice were stimulated either with conditioned media containing ReelinWT or mutant Reelin protein, and the Dab1 phosphorylation level was measured by Western blotting. ReelinWT induced robust Dab1 phosphorylation (Fig. 4A, lane 2), whereas ReelinΔC-FLAG, ReelinΔC-Glu8, and ReelinΔC-Venus were much less potent (Fig. 4A, lanes 4–6, respectively). Interestingly, ReelinFLAG+C induced Dab1 phosphorylation only weakly, whereas ReelinΔC-Arg8 was…
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As oligomerization of Reelin is prerequisite for Dab1 phosphorylation (17, 29), we investigated whether CTR affects Reelin binding to these receptors. First, we employed the recombinant ligand-binding domain of ApoER2 fused to human GH (GH-ApoER2LBD) to pull down Reelin (Fig. 5A). Conditioned media containing either ReelinWT or ReelinΔC-FLAG were mixed with GH-ApoER2LBD, anti-GH, and protein G-Sepharose, and the precipitates were analyzed by Western blotting. Control experiments were performed without GH-ApoER2LBD. As shown in Fig. 5C, no difference was observed between ReelinWT and ReelinΔC-FLAG, indicating that CTR is not directly involved in the interaction between Reelin and its receptors. This was consistent with previous reports (18) that the region from RR3 to RR6 was sufficient for the ectodomain of ApoER2 or VLDLR. We next performed similar experiments using membrane fractions isolated from HEK293T cells expressing full-length ApoER2-GFP (Fig. 5B). The results were quite surprising: ReelinWT bound much more strongly (or more stably) to the receptor-bearing cell membrane than ReelinΔC-FLAG (Fig. 5D, lanes 9 and 10, respectively). ReelinFLAG+C and ReelinΔC-Arg8 bound to the receptor-bearing cell membrane more weakly than ReelinWT but slightly more strongly than ReelinΔC-FLAG (Fig. 5D, lanes 11 and 12, respectively). Thus, CTR apparently augments or stabilizes the interaction between Reelin and Reelin receptors on the cell membrane, and this effect is partly, but not solely, mediated by the positive charge of CTR.

We next investigated binding of Reelin to COS-7 cells expressing ApoER2-GFP. Cells were incubated either with ReelinWT or ReelinΔC-FLAG, fixed, and immunostained with anti-Reelin antibody without permeabilization (to detect cell surface but not internalized Reelin). ReelinWT bound to the surface of ApoER2-GFP-expressing COS-7 cells in a rather punctate fashion (Fig. 6, A–C). This binding was dependent on ApoER2-GFP because virtually no staining was observed in control GFP-expressing COS-7 cells or in COS-7 cells expressing no Reelin receptors (27). On the other hand, binding of ReelinΔC-FLAG to ApoER2-GFP-expressing COS-7 cells was barely detectable (Fig. 6, D–F). Consistent with the pull-down experiments using isolated cell membrane (Fig. 5D), ReelinΔC-Arg8 bound to ApoER2-expressing cells rather weakly and ReelinFLAG+C bound only slightly (supplementary Fig. S2). These results demonstrated that CTR is necessary moderately active (Fig. 4A, lanes 3 and 7, respectively). These results highlight three important points. First, CTR is necessary for efficient activation of downstream signaling. Second, CTR must be located just after RR8 to have full activity. Third, the highly basic nature of CTR contributes to its facilitation effect on downstream activation.

As oligomerization of Reelin is prerequisite for Dab1 phosphorylation (17, 29), we investigated whether CTR affects dimer formation and oligomerization of Reelin. SDS-PAGE without a reducing reagent, which is able to evaluate dimer formation of Reelin (29), revealed that ReelinWT, ReelinFLAG, and ReelinΔC-FLAG form dimers to the same extent (Fig. 4C). We also performed native PAGE analysis (17) to evaluate the oligomerization state and found no clear difference between ReelinWT and ReelinΔC-FLAG (Fig. 4D). These results suggested that CTR is not involved in oligomerization of Reelin.

Reelin CTR Augments Binding Affinity between Reelin and Its Receptor on the Cell Membrane—Binding of Reelin to either ApoER2 or VLDLR is essential for induction of Dab1 phosphorylation (30, 31). We thus investigated whether deletion of CTR affects Reelin binding to these receptors. First, we employed the recombinant ligand-binding domain of ApoER2 fused to human GH (GH-ApoER2LBD) to pull down Reelin (Fig. 5A). Conditioned media containing either ReelinWT or ReelinΔC-FLAG were mixed with GH-ApoER2LBD, anti-GH, and protein G-Sepharose, and the precipitates were analyzed by Western blotting. Control experiments were performed without GH-ApoER2LBD. As shown in Fig. 5C, no difference was observed between ReelinWT and ReelinΔC-FLAG, indicating that CTR is not directly involved in the interaction between Reelin and its receptors. This was consistent with previous reports (18) that the region from RR3 to RR6 was sufficient for the ectodomain of ApoER2 or VLDLR. We next performed similar experiments using membrane fractions isolated from HEK293T cells expressing full-length ApoER2-GFP (Fig. 5B). The results were quite surprising: ReelinWT bound much more strongly (or more stably) to the receptor-bearing cell membrane than ReelinΔC-FLAG (Fig. 5D, lanes 9 and 10, respectively). ReelinFLAG+C and ReelinΔC-Arg8 bound to the receptor-bearing cell membrane more weakly than ReelinWT but slightly more strongly than ReelinΔC-FLAG (Fig. 5D, lanes 11 and 12, respectively). Thus, CTR apparently augments or stabilizes the interaction between Reelin and Reelin receptors on the cell membrane, and this effect is partly, but not solely, mediated by the positive charge of CTR.

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for a stable association between Reelin and ApoER2 on the plasma membranes of live cells.

Finally, and most importantly, we investigated whether the same phenomenon was observed in cortical neurons that are the physiological target of Reelin in vivo. Primary cortical neurons were incubated either with ReelinWT or ReelinΔC-FLAG and immunostained with anti-Reelin antibody without permeabilization. Although some of the neurons express and secrete Reelin, the staining signals were very low unless exogenous Reelin was added, under the conditions we employed (Fig. 6, G and H). I–P, cortical neuronal cells were incubated with either ReelinWT (I–K) or ReelinΔC-FLAG (L–N) for 45 min, fixed, and stained with CR-50 without permeabilization. The cell membrane was visualized using biotinylated cholera toxin B (CTB) followed by Alexa 594-streptavidin (J, M, and O). Under the conditions used in this experiment, endogenous Reelin expressed in neuronal cells was barely detected unless Reelin was added exogenously (O and P). Scale bars, 10 μm.

FIGURE 6. ReelinWT, but not its ΔC mutant, stably binds to live cells and neurons that express Reelin receptors. A–H, COS-7 cells expressing ApoER2-GFP were incubated with either ReelinWT (A–C) or ReelinΔC-FLAG (D–F) for 30 min, fixed, and stained with CR-50 without permeabilization. COS-7 cells expressing control GFP do not bind Reelin (G and H). I–P, cortical neuronal cells were incubated with either ReelinWT (I–K) or ReelinΔC-FLAG (L–N) for 45 min, fixed, and stained with CR-50 without permeabilization. The cell membrane was visualized using biotinylated cholera toxin B (CTB) followed by Alexa 594-streptavidin (J, M, and O). Under the conditions used in this experiment, endogenous Reelin expressed in neuronal cells was barely detected unless Reelin was added exogenously (O and P). Scale bars, 10 μm.

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Finally, and most importantly, we investigated whether the same phenomenon was observed in cortical neurons that are the physiological target of Reelin in vivo. Primary cortical neurons were incubated either with ReelinWT or ReelinΔC-FLAG and immunostained with anti-Reelin antibody without permeabilization. Although some of the neurons express and secrete Reelin, the staining signals were very low unless exogenous Reelin was added, under the conditions we employed (Fig. 6, G and H). I–P, cortical neuronal cells were incubated with either ReelinWT (I–K) or ReelinΔC-FLAG (L–N) for 45 min, fixed, and stained with CR-50 without permeabilization. The cell membrane was visualized using biotinylated cholera toxin B (CTB) followed by Alexa 594-streptavidin (J, M, and O). Under the conditions used in this experiment, endogenous Reelin expressed in neuronal cells was barely detected unless Reelin was added exogenously (O and P). Scale bars, 10 μm.

DISCUSSION

The main findings of this study are: 1) the CTR of Reelin is extremely conserved among vertebrates, 2) most of the CTR is not required for secretion of Reelin as is widely believed, 3) secretion efficiency is indeed affected when CTR is totally removed, but an unrelated sequence can completely substitute for it, 4) CTR is necessary for efficient activation of downstream signaling, and 5) this effect is most likely exerted via binding of CTR to a certain molecule(s) on the cell membrane.

Reelin CTR has been extremely well conserved during the evolution of land animals (Fig. 1A). Among 31 completely conserved residues, two are methionine (one codon) and 13 (Lys, Asn, Gln, Tyr, His, and Phe) are encoded by two codons. This means that the probability of change in the primary sequence of CTR would be quite high, and thus indicates that Reelin CTR has been under tremendous constraint during the course of evolution. Loss-of-function experiments in lower vertebrates will be of great help in understanding how CTR contributes to the formation of a wide variety of brain structures in various vertebrates.

We found that a mutant Reelin protein lacking only the CTR is secreted but that none of the mutants that terminate in the middle of RR are secreted (Fig. 1, B–F). Therefore, it is suggested that an abrupt termination in the middle of RR, not the absence of CTR, is the main cause of secretion failure of ReelinOrl (19, 20). Deletion of the entire CTR did reduce the secretion efficacy (Fig. 1, B, C, E, and F), but deletion of the last 20 amino acids, including 10 of the 12 basic residues (Reelin3441) had no effect on secretion (Fig. 3, B and C, lane 3). Thus the highly basic nature of CTR is not necessary to facilitate secretion. Furthermore, Reelin mutants in which CTR has been replaced with unrelated sequence such as the FLAG epitope (ReelinΔC-FLAG), eight arginines (ReelinΔC-Arg8) or glutamates (ReelinΔC-Glu8) are secreted as efficiently as ReelinWT (Fig. 3), suggesting that the highly conserved structure of CTR is not involved in the facilitation of secretion. It remains unknown whether or not Reelin secretion is in any way regulated. It is noteworthy that the above results were obtained using cultured cell lines and it is thus important to confirm them using neuronal cells that endogenously express Reelin such as Cajal-Retzius cells (10, 11) or GABAergic neurons (32). However, it is quite difficult with currently available techniques to induce expression only in these cells. We are currently making a knock-in mouse in which ReelinΔC-FLAG is expressed instead of ReelinWT, and our investigation using this mouse is anticipated to address this issue.

We found that Reelin mutants lacking CTR or that have an insertion between RR8 and CTR are less active in inducing Dab1 phosphorylation. How can this be explained? Unfortunately,
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For an axon guidance molecule, Semaphorin 5A is Plexin-B3, but its physiological outcome is influenced by whether Semaphorin 5A binds to chondroitin sulfate or to heparan sulfate proteoglycans (38). Similar observations were reported for Wnt, sonic hedgehog, netrin, transforming growth factor β, and so on (39, 40). We are now attempting to identify the molecule with which Reelin interacts, and thus far we found that Reelin does not bind to heparin (supplementary Fig. S3). It should also be noted that layer formation in the forebrain was normal in mice with no heparan sulfate (37), suggesting that heparan sulfate is not a required component of Reelin signaling.

Our findings and the proposed model on the function of Reelin CTR are schematically shown in Fig. 7. Alternative splicing (24) gives rise to ReelinΔC in addition to ReelinWT (containing positively charged CTR). The highly conserved structure of Reelin CTR is not required for its secretion as is widely believed, and thus both ReelinWT and ReelinΔC are secreted. ReelinWT binds to ApoER2/VLDLR and the negatively charged molecules on the neurons, inducing Dab1 phosphorylation effectively. Alternatively, CTR may directly interact with the membrane lipid bilayer. In any case, it would be reasonable to think that this reaction occurs only in the vicinity of the Reelin-producing cells, because interaction between ReelinWT and the receptor-bearing cell membrane is quite strong and stable, and Reelin is internalized into these cells (27, 30). It is intriguing to assume that the same molecule that binds to Reelin CTR binds to Src family tyrosine kinase family that is responsible for Dab1 phosphorylation (35, 41) in its cytoplasmic region, although this is at present highly speculative. On the other hand, ReelinΔC may diffuse more easily because its binding to ApoER2/VLDLR is rather transient. Therefore, ReelinΔC may function in the distal and weak signal transduction. This dual mode of Reelin action may be advantageous for the precise regulation of neuronal migration and may imply that Reelin plays both positional and permissive roles. The next challenges will be to identify the CTR-binding molecule, to clarify its role in signal transduction, and to understand the physiological significance of CTR in vivo. We are currently working on these questions. In particular, we are close to obtaining a ReelinΔC-FLAG knock-in mouse, which is certainly anticipated to reveal how important CTR is in vivo.

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