Proteins of the CNR Family Are Multiple Receptors for Reelin

Kouji Senzaki,* Masaharu Ogawa,† and Takeshi Yagi*‡
*Laboratory of Neurobiology and Behavioral Genetics
National Institute for Physiological Sciences
Myodaiji, Okazaki 444-8585
†Laboratory for Cell Culture Development
Brain Science Institute
RIKEN
2-1 Hirosawa, Wako-shi, Saitama 351-0198
Japan

Summary

Layering and positioning of neurons require Reelin- and Src family–associated mammalian Disabled (mDab1). Cadherin-related neuronal receptor (*CNR*) genes are expressed in neurons of the cortical layer, but not in Cajal-Retzius cells expressing Reelin. This leads us to hypothesize that CNRs bound to Fyn of the Src family are receptors for Reelin. Herein we confirm the association and colocalization of CNR proteins with Reelin. This binding is blocked by CR-50 antibody against Reelin, as well as by monoclonal antibodies produced against CNRs. Both disturb the signaling pathway from Reelin to mDab1 and the positioning of cortical neurons in vitro. These results strongly suggest that the CNR family proteins are multiple Reelin receptors. In addition, differential conservation of the Reelin-binding domain among terrestrial vertebrates may be pertinent to the diversity or complexity of brains.

Introduction

The central nervous system (CNS) differs in structural organization across species. In particular, the cortex differs considerably in neuronal specialization, laminar differentiation, number and size of cortical areas, the representational patterns of sensory and motor areas, the modular subdivision of areas, and the connection of modules and areas. Nevertheless, the mammalian neocortex in each species is a highly ordered structure. The different classes of neurons localize in an organized radial array of six cellular layers ranging from the pial surface to the white matter. Positioning of a particular neuron at a specific cortical layer is an essential step in forming a well-organized neuronal network in the cortex. During spatial positioning, a series of neuronal migrations occur during which postmitotic neurons leave their birthplace in the ventricular zone and move along glial guidance fibers to their proper places (Walsh and Cepko, 1992; Komuro and Rakic, 1998). The molecular events that regulate the interaction of the migrating cells with the environmental cues which dictate their spatial position are not yet completely understood.

A molecular pathway that regulates the cortical layering and positioning of neurons involves the large extracellular protein Reelin and the cytoplasmic mDab1 protein (reviewed in Curran and D'Arcangelo, 1998). In the cortex, Reelin is generated from Cajal-Retzius neurons and is either associated with the extracellular matrix or with its surface (Ogawa et al, 1995). In *reeler* mice (Falconer, 1951), the gene encoding Reelin is defective (D'Arcangelo et al., 1995; Ogawa et al., 1995). As a result, in *reeler* mice migratory neurons apparently do not receive a critical cue that informs them of their position, leading to an inversion of the cortical layers. These layers normally form from the inside out, with later-born neurons migrating past older ones to form progressively superficial, and thus younger, layers of the neocortex. A specific monoclonal antibody, CR-50, which targets the N-terminal region of the Reelin protein can disturb the cell arrangement of cerebral cortex cells in vitro (Ogawa et al., 1995). This suggests that the N-terminal domain of Reelin plays a significant role in communicating cell positioning information.

The cytoplasmic adaptor protein mDab1 is related to the *Drosophila disabled* gene product. It is predominantly expressed in neurons and has been shown to function downstream of Reelin. mDab1-deficient mice develop a phenotype indistinguishable from *reeler* (Howell et al., 1997b; Sheldon et al., 1997). mDab1 contains a protein interaction domain that binds to NPxY motifs in the cytoplasmic tails of receptors (Pawson and Scott, 1997). Recently, it has been demonstrated that double knockout mice for two cell surface receptors, very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), which possess the NPXY motifs on their cytoplasmic tails, precisely mimic the phenotypes of mice lacking Reelin or mDab1 (Trommsdorff et al., 1999).

mDab1 is tyrosine phosphorylated during mouse embryogenesis, and tyrosine-phosphorylated mDab1 associates with the SH2 domains of Src, Fyn, and Abl (Howell et al., 1997a). A nonreceptor tyrosine kinase signaling pathway would therefore be a potential candidate for mediating transmission of the Reelin signal across the neuronal plasma membrane. It has recently been demonstrated that exogenous Reelin stimulates tyrosine phosphorylation of mDab1 in primary cortical neurons (Howell et al., 1999). This strongly suggests that the Reelin receptor is coupled with tyrosine kinases. Fyn-tyrosine kinase, in particular, has a crucial molecular function in building brain networks and determining patterns of behavior (Yagi, 1999). In loss-of-Fyn-function mice, structural abnormalities were observed in the CA3 region of the pyramidal cell layer in the posterior hippocampus and dentate gyrus regions (Grant et al., 1992; Yagi et al., 1993). The malformation of pyramidal cell layers of the hippocampus is also observed in *reeler* mice (Walsh and Cepko, 1992). Interestingly, we note here that Fyn is also localized in the cortical plate neurons. These data suggest that Fyn also functions in signal transduction from exogenous Reelin.

‡To whom correspondence should be addressed (e-mail: yagi@nips.ac.jp).
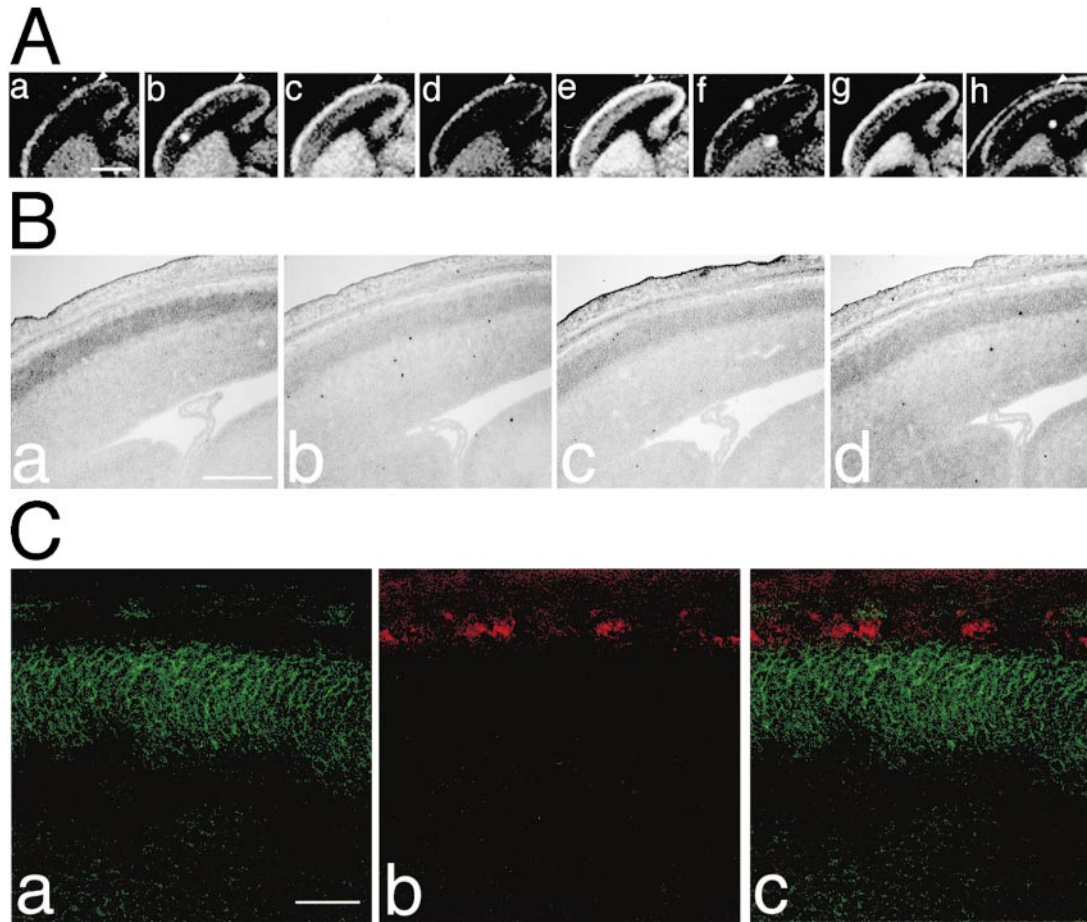
Figure 1. In Situ Hybridization of Embryonic Mouse Cortex at E15

(A) Each *CNR* gene (*CNR1* [a], *CNR2* [b], *CNR3* [c], *CNR4* [d], *CNR5* [e], *CNR6* [f], *CNR7* [g], and *CNR8* [h]) is commonly expressed in the apical cortical region. Arrowheads show the apical cortex. Bar, 200 μm. (a)–(h) are identical magnifications.

(B) Probes used were as follows: *CNR1* (a), *CNR5* (b), *CNR*-CP (c), and *fyn* (d) digoxigenin-labeled antisense. The *CNR*-CP probe covered the identical cytoplasmic regions among *CNR1* to *CNR8*. Bar, 100 μm. (a)–(d) are identical magnifications.

(C) Double staining analysis of the sagittal section of the E15 mouse cortex using digoxigenin-labeled antisense probe from *CNR1* ([a] and [c], green) and CR-50 ([b] and [c], red). CR-50 immunoreactivity (red) shows Reelin expression by Cajal-Retzius cells in the marginal zone and *CNR1* mRNA highly expressed by cells in the cortical plate (green). Bar, 50 μm.

In 1998, we identified a novel cadherin-related neuronal receptor (CNR) family using Fyn binding activity from the mouse brain (Kohmura et al., 1998). Each of the CNR proteins 1–8 contains six extracellular cadherin ectodomains (EC) similar to the cadherins but has a distinct cytoplasmic domain that interacts specifically with the tyrosine kinase Fyn. The cytoplasmic domain bears no similarity to that of the cadherins or any other protein in the database. The CNRs are expressed in the brain and localized to synaptic junctions. While in situ hybridization studies revealed that *CNR* mRNAs are expressed in various regions of the adult brain, differential expression of each *CNR* is observed at a single neuron level at the adult stage (Kohmura et al., 1998). It has recently become apparent that the genomic organization of the *CNR* family (*Pcdhα* in human) contains similarities to that of immunoglobulin and T cell receptor gene clusters (Wu and Maniatis, 1999; Sugino et al., 2000). In addition, a striking feature is the well-conserved EC1 domain among the eight mouse CNR proteins (Kohmura et al., 1998), albeit the EC1 domain of classical cadherins

is required for specific homophilic interactions (Takeichi, 1990). We propose that the EC1 domain of the CNRs may play a similar role by functioning in heterophilic binding among CNR proteins or in binding of all CNR proteins to the same ligand proteins.

Here, we suggest that the CNR family proteins function as multiple receptors for receiving and processing the Reelin signals in forming a well-organized neuronal network in the neocortex. In addition, we propose that differences in the numbers of *CNR* genes containing Reelin-binding domains across terrestrial vertebrates may be attributable for cortical variation of neuronal layers and neural networks of brains.

Results

Expression Patterns of *CNR* Genes and *fyn* in the Cortical Plate Adjacent to Reelin

While each *CNR* is widely expressed in adult mouse brains (Kohmura et al., 1998), it is not known where in the embryonic brain each member of the *CNR* family
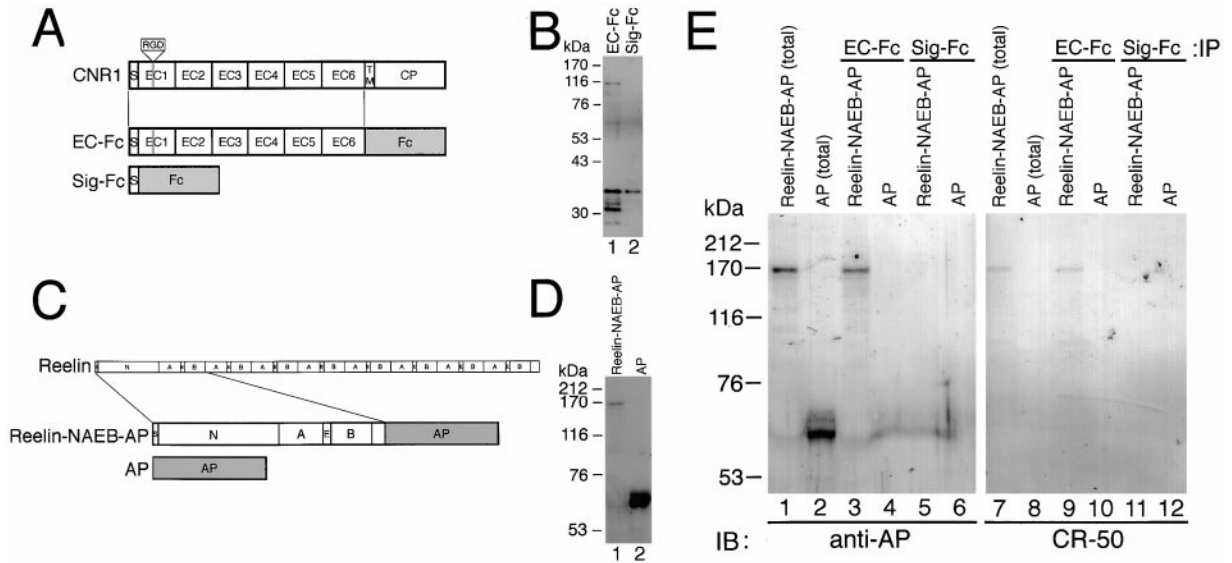
Figure 2. Extracellular Region of CNR1 Binds to Reelin

(A) Diagram of protein structures of native CNR1, artificial EC-Fc, and Sig-Fc proteins.

(B) Immunoblot analysis of recombinant Fc fusion proteins with another anti-human IgG Fc antibody. In conditioned medium from transfected cells, ~110 kDa of EC-Fc (lane 1) or ~38 kDa of Sig-Fc (lane 2) was detected.

(C) Diagram of protein structures of native Reelin, artificial Reelin-NAEB-AP, and AP proteins.

(D) Recombinant Reelin-NAEB-AP (~170 kDa, lane 1) and AP (~65 kDa, lane 2) fusion proteins were detected by immunoblot analysis with an anti-human AP antibody.

(E) Reelin-NAEB-AP could be coimmunoprecipitated with CNR1 EC-Fc protein. By immunoblot analysis with an anti-AP antibody, the 170 kDa band of Reelin-NAEB-AP was detected in total extracts of Reelin-NAEB-AP transfectants (lane 1) and in immunoprecipitants with EC-Fc beads (lane 3), but not by Sig-Fc (lane 5). The 65 kDa AP protein was detected in total extracts of AP transfectants (lane 2), but not in immunoprecipitants with EC-Fc (lane 4) and Sig-Fc (lane 6). By immunoblot analysis with CR-50, Reelin-NAEB-AP was also detected in lanes 7 and 9.

RGD, RGD motif; S, signal peptide; EC, extracellular cadherin ectodomain; TM, transmembrane domain; CP, cytoplasmic domain; Fc, the human immunoglobulin Fc fragment; N, Reelin amino-terminal domain; A, Reelin repeat A domain; E, Reelin EGF-motif; B, Reelin repeat B domain; AP, human placental alkaline phosphatase.

was expressed. To analyze the expression of *CNR*s in the mouse embryonic cortex, we carried out in situ hybridization experiments using mouse E15 brains. In the embryonic forebrain, each of the *CNR1–CNR8* genes was commonly expressed in the apical cortical region and the striatum (Figure 1A, a–h). To further examine the expression pattern in the cortex, we performed in situ hybridization using digoxygenin-labeled RNA probes derived from (a) various N-terminal EC2 and EC3 regions of *CNR1–CNR8*, (b) the identical cytoplasmic regions of *CNR1–CNR8*, and (c) *fyn*. Each of these probes revealed extensive expression of the respective mRNA in the cortical plates (Figure 1B, a–d), while *fyn* expression was also detected in the subventricular region (Figure 1B, d). The identical cytoplasmic regions derived from the "constant region" of the *CNR* gene clusters were also highly transcribed in the cortical layer after the neuronal mitotic stage (Figure 1B, c).

In the cortical plate at embryonic stage day 15, the migration of postmitotic neurons forms cortical layers. The molecular cues (i.e., extracellular protein, Reelin, and the cytoplasmic signal-transducing protein, mDab1) function in normal layering formation. The cortical localization of CNR family proteins bound to Fyn, which is associated with mDab1 (Howell et al., 1997a), is consistent with a model in which members of the CNR family function as obligate components of a pathway that transmits the Reelin signal to mDab1. To further confirm

the expression of *CNR* genes in the cortical plate but not in the marginal zone, we performed double staining analysis with a *CNR1* RNA probe and CR-50, an anti-Reelin antibody. Figure 1C, a–c reveals double staining of *CNR1* mRNA and Reelin protein. Cajal-Retzius cells in the marginal zone were stained by CR-50, but *CNR1* was not detected (Figure 1C, a and c). Thus, cells expressing *CNR1* were proximal and distinct from Cajal-Retzius cells expressing Reelin. Similar patterns were also detected for the other *CNR* genes and their corresponding constant regions (data not shown).

CNR Proteins Bind to Reelin

The observed *CNR* expression patterns prompted us to examine whether CNR family proteins associated with Fyn were receptors for Reelin protein. To produce the extracellular domain of CNR (amino acids 27–692) or the N-terminal region of Reelin (amino acids 28–911), we transfected transgenes pCNR1EC-Fc or pReelin-NAEB-AP into human embryonic kidney 293 (HEK293)-EBNA cells. The CNR1 EC-Fc protein is a fusion of the extracellular domain of CNR1 to human immunoglobulin Fc fragment (Fc) (Figure 2A). This protein is detectable by immunoblotting with anti-Fc antibody in conditioned medium as a ~110 kDa protein, consistent with the combined sizes of the CNR1 extracellular region and Fc (Figure 2B). As a control, we also produced a ~38 kDa Sig-Fc protein, which contains the signal peptide region of
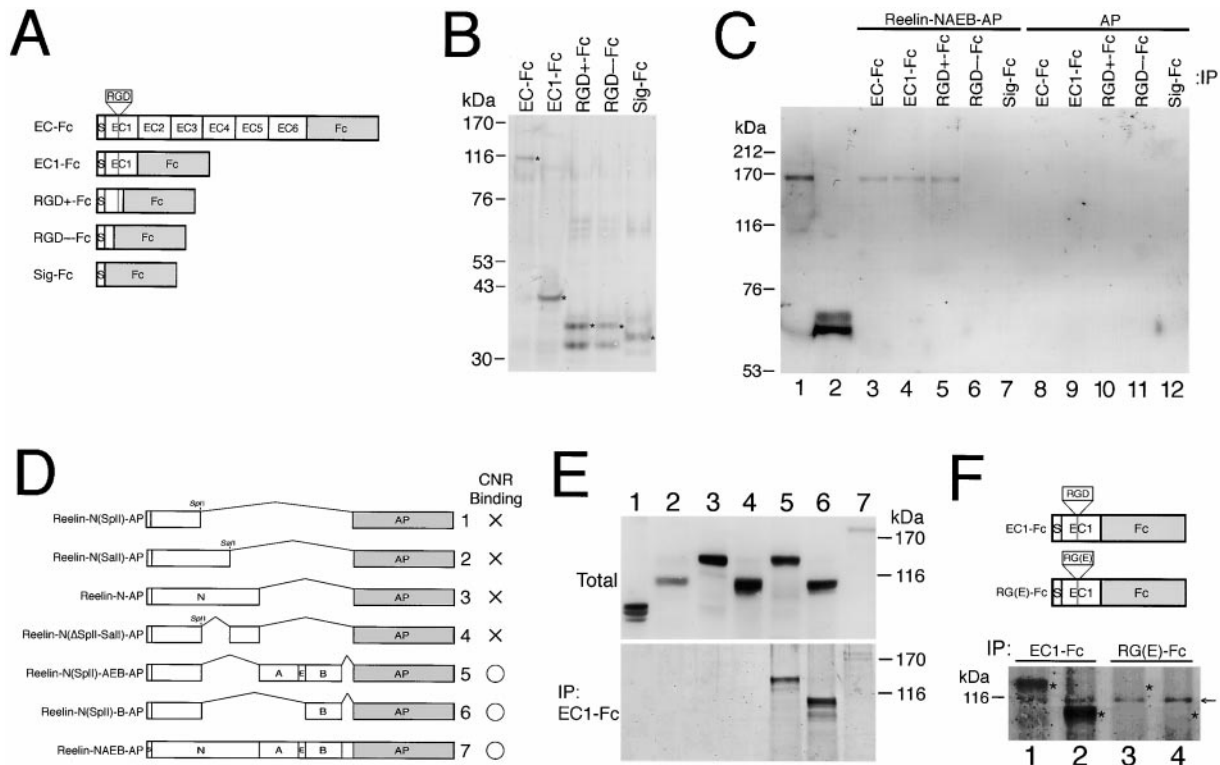
Figure 3. The EC1 Domain of CNR1 Conserved among CNR Family Proteins and the First Reelin Repeat B Domain of Reelin Are Necessary for Their Binding

(A) Schematic structures of Fc fusion proteins containing different portions of the CNR1 extracellular domain.

(B) Expression of the recombinant Fc fusion proteins. Bands of ~110 kDa of the EC-Fc (lane 1) or ~38 kDa of the Sig-Fc (lane 2) were detected. Proteins immunoprecipitated by anti-human Fc antibody were stained by immunoblot analysis with another anti-human IgG Fc antibody. Asterisks show the presumable band sizes.

(C) Confirmation of Reelin-binding domain of CNR1. By immunoblotting with anti-AP antibody, Reelin-NAEB-AP was detected in transfected cell total extracts (lane 1) and in the immunoprecipitants with EC-Fc (lane 3), EC1-Fc (lane 4), and RGD$^+$-Fc (lane 5) beads, but not with RGD$^-$-Fc (lane 6) and Sig-Fc (lane 7). The AP protein was found in the transfected cell extract (lane 2), but not in all immmnoprecipitants (lanes 8–12).

(D) Schematic structures of AP fusion proteins containing different portions of Reelin. Their respective CNR binding activities are shown on the right.

(E) Confirmation of the binding domain of Reelin as the RBD of CNR family proteins. By immunoblotting with anti-AP antibody, Reelin-N(SpII)-AP protein (lane 1), Reelin-N(SalI)-AP protein (lane 2), Reelin-N-AP protein (lane 3), Reelin-N(ΔSpII-SalI)-AP protein (lane 4), Reelin-N(SpII)-AEB-AP protein (lane 5), Reelin-N(SpII)-B-AP protein (lane 6), and Reelin-NAEB-AP protein (lane 7) were detected in total cell lysates. In the immunoprecipitants with the CNR1 EC1-Fc beads, the Reelin-N(SpII)-AEB-AP, Reelin-N(SpII)-B-AP, and Reelin-NAEB-AP proteins were detected (lanes 5–7), but not the other AP fusion proteins (lanes 1–4).

(F) RGD motif of CNR was necessary for binding the B domain of first Reelin repeat. Schematic structures of EC1-Fc and RG(E)-Fc fusion proteins are shown. Reelin-N(SpII)-AEB-AP and Reelin-N(SpII)-B-AP were not immunoprecipitated by RG(E)-Fc (lanes 3 and 4). These were detected by immunoblot analysis with an anti-AP antibody. Asterisks and an arrow show the presumable band sizes and nonspecific bands, respectively.

CNR1 and Fc (Figure 2B). The Reelin-NAEB-AP protein is a fusion of the N-terminal region of Reelin to alkaline phosphatase (AP). The protein is detectable with anti-AP antibody in cell lysates as a ~170 kDa species, consistent with the combined sizes of the N-terminal portion of Reelin and AP (Figure 2D). Using these fusion proteins, we performed coimmunoprecipitation experiments to test the direct binding of CNR1 EC-Fc to Reelin-NAEB-AP. As shown in Figure 2E, the Reelin-NAEB-AP protein was precipitated by beads conjugated with the CNR1 EC-Fc protein, but not with the control CNR1 Sig-Fc protein. The AP protein by itself was not precipitated with CNR1 EC-Fc protein. These results provide evidence for a direct interaction between the extracellular region of CNR1 and the N-terminal segment of Reelin.

Which regions of the CNR1 extracellular domain mediate the interaction between CNR1 and Reelin? We produced various fusion proteins comprised of Fc fused to different portions of the extracellular region of CNR1 (Figure 3A). Expression of these proteins was confirmed by immunoblotting with anti-Fc antibody in conditioned medium from transfected cells (Figure 3B). As shown in Figure 3C, the Reelin-NAEB-AP protein (amino acids 28–911) was precipitated by beads conjugated with CNR1 EC-Fc (positive control), EC1 (amino acids 1–133)-Fc, and RGD$^+$ (amino acids 1–97)-Fc proteins, but not with RGD$^-$ (amino acids 1–67)-Fc and Sig (amino acids 1–39)-Fc proteins. These results indicate that the Reelin-binding domain is localized between Ser-68 and Gly-97 of CNR1 protein. This amino acid sequence is completely conserved among CNR1–8 (Kohmura et al., 1998). This suggests that CNR1–8 all possess binding activity to Reelin protein. The EC1 domains of CNR1–8 are unusually well conserved in comparison to other

members of the cadherin superfamily. These results imply that the conserved EC1 domain of the CNR family uniformly functions in binding to Reelin. Furthermore, therein lies the possibility that homophilic interactions of the EC1 region of the CNR family are of less significance; nevertheless, classical cadherins and protocadherins possess homophilic binding activity. Within the Reelin-binding sequence, there is an RGD motif that is known to function in protein–protein interactions, such as those reported for fibronectin and integrin (Pierschbacher and Ruoslahti, 1984). To further delineate the binding site of CNR1 to Reelin, we performed site-directed mutagenesis of CNR1 EC1-FC, with the RGD sequence changed to RGE. Interestingly, RG(E)-Fc did not bind to Reelin protein (data are shown in Figure 3F as binding assay with Reelin-N(SpII)-AEB-AP and Reelin-N(SpII)-B-AP proteins). This result indicates that the Asp residue of RGD in CNR1 is necessary for the interaction with Reelin protein.

Reelin is a secreted glycoprotein that has several structural characteristics of extracellular matrix proteins. Reelin contains an N-terminal region of similarity with F-spondin and eight internal repeats of 350–390 amino acids. Each Reelin repeat contains an A and B domain. The A and B domains are separated by an EGF-like motif (D'Arcangelo et al., 1995, 1997). An amino acid sequence present in the N-terminal region of Reelin contains an epitope that is recognized by the CR-50 monoclonal antibody, and the interaction of CR-50 with its epitope leads to the disruption of neural cell aggregation in vitro. To determine which domains of Reelin mediate the interaction with CNR family proteins, we constructed various fusion proteins containing different portions of the N-terminal region of Reelin and AP (Figure 3D). Expression of these proteins was confirmed by immunoblotting with anti-AP antibody in cell lysates from cells transfected with the respective transgenes (Figure 3E). As shown in IP: EC1-Fc of Figure 3E, Reelin-NAEB-AP (positive control), Reelin-N(SpII)-AEB-AP, and Reelin-N(SpII)-B-AP proteins were precipitated by beads conjugated with the EC1-Fc protein of CNR1, while Reelin-N(SpII)-AP, Reelin-N(SalI)-AP, Reelin-N-AP, and Reelin-N(ΔSpII-SalI)-AP proteins were not. These results indicate that the B domain of the first Reelin repeat is necessary for binding to the EC1 domain of CNR1.

Binding Features of CNR Family and Reelin Proteins

We next measured the binding affinity of CNR1 EC1-Fc to Reelin-NAEB-AP in equilibrium binding experiments using surface plasmone resonance. We found that the specific binding curves of CNR1 EC1-Fc to Reelin-NAEB-AP displayed saturation and could be fitted to the Hill equation (Figure 4A). The predicted value for the dissociation constant (K_D) for binding of CNR1 EC1-Fc to Reelin-NAEB-AP was 1.7 ± 0.11 nM. We further confirm the binding activities between the full-length Reelin protein and the extracellular domains of CNR1, CNR2, and CNR3 proteins. These binding curves also displayed saturation and could be fitted to the Hill equation (Figure 4B). The dissociation constants for binding of CNR1, CNR2, and CNR3 to full-length Reelin proteins were approximately 1.8, 1.7, and 1.6 nM, respectively. These values are comparable to those typical of ligand and receptor interactions; for example, 0.85–1.5 nM of Sema
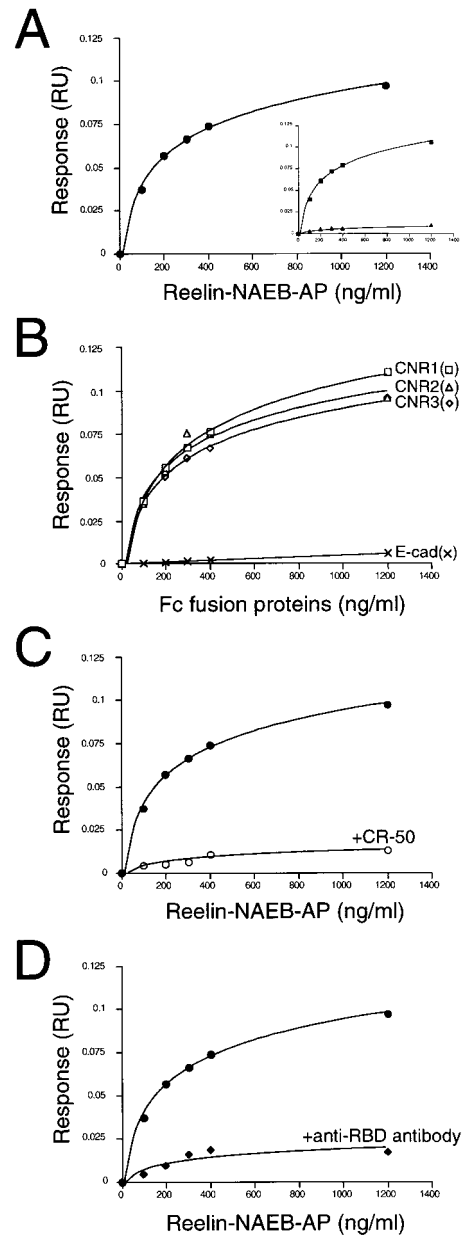

Figure 4. Equilibrium Binding of CNR Family to Reelin Proteins

(A) Surface plasmon resonance dose–response curves for Reelin-NAEB-AP or AP binding to immobilized CNR1 EC1-Fc. Specific binding was determined by subtraction of values obtained for Reelin-NAEB-AP binding to CNR1 EC1-Fc and control AP binding to CNR1 EC1-Fc. Inset shows raw data (filled squares, Reelin-NAEB-AP fusion protein binding to CNR1 EC1-Fc; filled triangles, control AP binding to CNR1 EC1-Fc). The K_D values for interactions of Reelin-NAEB-AP with EC1-Fc were 1.62–1.84 nM.

(B) Surface plasmon resonance dose–response curves for the extracellular domains of CNR1 (squares), CNR2 (triangles), CNR3 (lozenges), and E-cadherin (crosses) to immobilized full-length Reelin.

(C) After incubating 100 μg/ml CR-50 Fab fragment with Reelin-NAEB-AP at 27°C for 1.5 hr, the interaction response between Reelin-NAEB-AP and CNR1 EC1-Fc was detected. CR-50 blocked the binding of Reelin-NAEB-AP to CNR1 EC1-Fc (open circles).

(D) After binding anti-RBD antibody to CNR1 EC1-Fc, the interaction response between Reelin-NAEB-AP and CNR1 EC1-Fc was detected. The anti-RBD antibody blocked the binding of Reelin-NAEB-AP to CNR1 EC1-Fc (filled lozenges). Filled circles in (C) and (D) show the specific binding of Reelin-NAEB-AP fusion proteins to CNR1 EC1 similarly to (A).
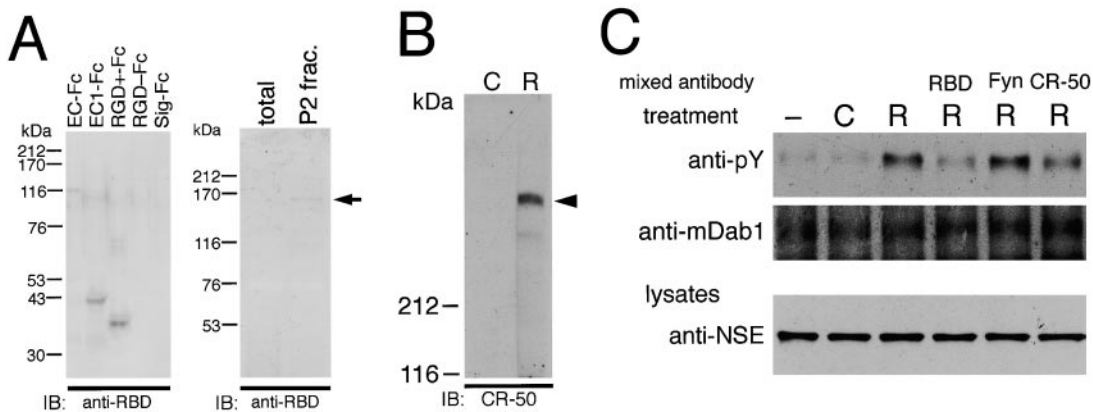
Figure 5. A Specific Monoclonal Antibody against the Reelin-Binding Domain of CNRs Block Reelin-Stimulating Tyrosine Phosphorylation of mDab1 in Embryonic Forebrain Cells

(A) Characterization of anti-RBD antibody by immunoblot analysis with CNR1 Fc fusion proteins and embryonic brain extracts. Anti-RBD antibody recognizes EC-Fc, EC1-Fc, and RGD⁺-Fc, but not RGD⁻-Fc and Sig-Fc. The recombinant proteins were detected by anti-human Ig Fc antibody (Figure 3B). The protein bands of ∼160 kDa (arrow) were detectable by anti-RBD antibody in the P2 fraction. In total fraction, ∼160 kDa bands were very faint.

(B) Detection of full-length Reelin protein (arrowhead) in conditioned medium by immunoblotting with CR-50. C, conditioned medium transformed by control vector, pCDNA3; R, conditioned medium transformed by Reelin expression vector, pCrl.

(C) Reelin-induced tyrosine phosphorylation of mDab1 is blocked by anti-RBD and CR-50 antibodies. Cells isolated from E16 mice forebrains were treated for 0 min (−) or 10 min (C) with control conditioned medium and were treated for 10 min with Reelin conditioned medium (R) with no, anti-RBD (RBD), anti-Fyn (Fyn), and CR-50 antibodies. The tyrosine phosphorylated levels or protein amounts of immunoprecipitated mDab1 proteins were detected by immunoblotting with anti-phosphotyrosine (top) or anti-mDab1 (middle), respectively. Equivalent protein concentration in cell lysates was confirmed by immunoblotting with anti-NSE (neuron specific enolase) antibody (bottom).

III and neuropilin (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). These results confirm that multiple CNR family proteins can bind to Reelin at concentrations comparable to receptor and ligand interaction. In contrast, the extracellular domain of E-cadherin hardly binds to Reelin protein (Figure 4B). The well-conserved EC1 domain among CNR family proteins may specify an extracellular cadherin domain for binding Reelin.

CR-50 antibody disturbs the marked positional sorting of neuron aggregates (Ogawa et al., 1995). This strongly implies that CR-50 inhibits the interaction between Reelin and functional Reelin receptors for layering and positioning cortical neurons. To confirm that CNR proteins were functional Reelin receptors, we needed to determine the effect of CR-50 on binding activity between CNR1 and Reelin proteins. We measured the binding affinities of CNR1 EC1-Fc to Reelin-NAEB-AP in equilibrium binding experiments in the presence of Fab fragment of CR-50. Surprisingly, the Fab fragment efficiently inhibited binding between the CNR1 EC1-Fc to Reelin-NAEB-AP (Figure 4C). While the CR-50 recognizes the SpII-SalI portion of the N-terminal region of Reelin (D'Arcangelo et al., 1997), our results confirmed the CNR-binding domain of Reelin to be within the first Reelin repeat B. Nevertheless, CR-50 efficiently blocked the protein–protein interaction between CNR1 and Reelin. It is possible that the N-terminal portion of Reelin may have a complicated protein structure. This can result in the CR-50 epitope and the first Reelin repeat B to be proximally located in the protein structure to form a binding pocket for CNR1. However, the inhibition of the binding of CNR1 EC1 to Reelin by CR-50 strongly suggests that the CNR family proteins are functional receptors for Reelin.

A Monoclonal Antibody Targeted to the Reelin-Binding Domain of CNR Proteins Blocks the Signaling Cascade from Reelin to mDab1 and Disturbs Reaggregation of Cortical Neurons

Monoclonal antibodies were produced against the CNR peptide sequence containing an RGD motif sequence (see Experimental Procedures). We characterized the monoclonal antibodies by immunoblotting with several fusion proteins produced in HEK293-EBNA cells transfected with the respective transgenes, and protein extracts of membrane fractions of the cerebral cortex from embryonic day 15 mice. The raised antibody (2H12) recognized CNR1 EC-Fc, EC1-Fc, and RGD⁺-Fc fusion proteins, but not RGD⁻-Fc and Sig-Fc (Figure 5A). Bands of ∼160 kDa in size were detected in P2 membrane fraction proteins from the embryonic mouse cerebral cortex (Figure 5A). The band size was coincident with bands stained with 6-1B anti-CNR1 antibody (Kohmura et al., 1998). Bands due to nonspecific staining were not detected in total and P2 fractions. The 2H12 antibody efficiently blocked protein interaction between CNR1 EC1 and Reelin (Figure 4D). This suggests that the 2H12 antibody exhibits special binding activity to the Reelin-binding domain of CNRs, and that the epitope recognized by 2H12 is contained within the binding sequence of CNRs to Reelin. We therefore named 2H12, anti-Reelin binding domain (RBD) antibody.

In primary neuronal culture, mDab1 tyrosine phosphorylation is stimulated by exogenous Reelin (Howell et al., 1999). Here, we measured Reelin-induced tyrosine phosphorylation levels of mDab1 in cortical neurons with or without anti-RBD antibody for CNR family proteins. The exogenous full-length Reelin was prepared by transfecting HEK293T cells with an expression vector,
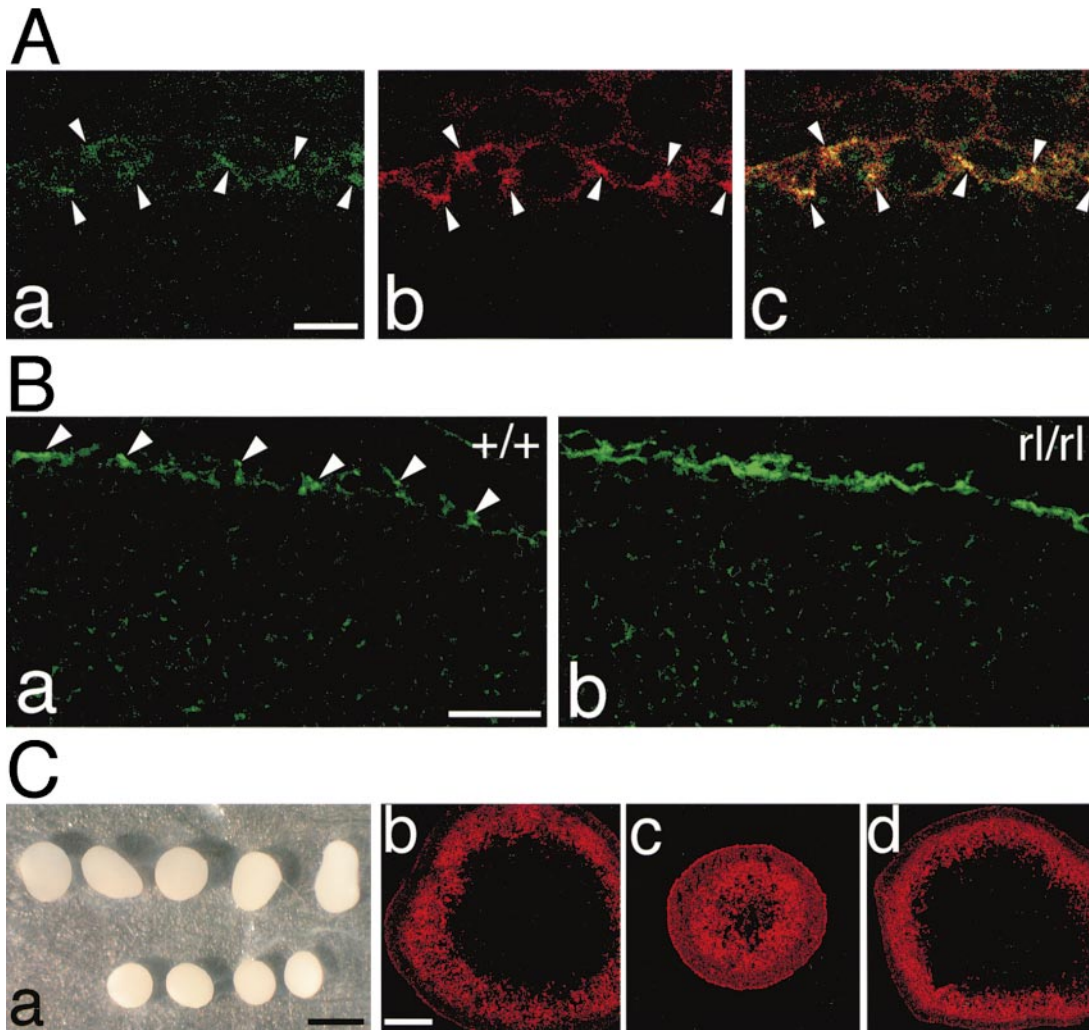
Figure 6. Localization and Function of CNR Proteins Bound to Reelin during the Development of Cortical Neurons

(A) Colocalization of CNR and Reelin proteins in the marginal and cortical regions of E15 mouse. Double staining analysis of E15 using anti-RBD antibody ([a] and [c], green) and CR-50 ([b] and [c], red). CR-50 immunoreactivity (red) shows Reelin expression by Cajal-Retzius cells in the marginal zone, and CNR protein localization (green) also enriched in the marginal zone. Many strong stained dots (arrowheads) by anti-RBD and CR-50 antibodies are double stained and colored to yellow (c). Bar, 10 μm cortex at E15 in (a). (a), (b), and (c) are identical magnifications.

(B) Alteration of CNR protein localization in the *reeler* mutant cortex at E15. Immunohistochemical staining by anti-RBD antibody (2H12) in the wild-type (a). In *reeler* cortex, the staining strength was increased, but the discrete dots in the wild-type were decreased (b). Bar, 25 μm in (a). (a) and (b) are identical magnifications.

(C) Reaggregation culture experiment of cerebral cortical cells in response to anti-RBD CNR antibody. (a) Upper five aggregates were exposed to anti-Fyn rat IgG antibody (200 μg/ml), and lower four aggregates to anti-RBD rat IgG antibody (200 μg/ml). Bar, 1 mm. (b) In the control aggregate (exposed no antibody), MAP2-labeled neurons are uniformly concentrated and aligned symmetrically in the outer zone of the aggregate. In the aggregate-exposed anti-RBD antibody (200 μg/ml), MAP2-labeled neurons appear loose and are concentrated in the middle of the aggregate (c), while another control (with anti-Fyn antibody) shows no effect (d). Bar, 250 μm in (b). (b), (c), and (d) are identical magnifications.

pCrl (Figure 5B). In a control experiment, a 10 min exposure to 250 pM Reelin induced an approximately 4-fold increase in phosphotyrosine content of mDab1. The increased level was not affected by anti-Fyn monoclonal antibody. Nevertheless anti-RBD antibody to the CNR family efficiently inhibited the Reelin-induced tyrosine phosphorylation level of mDab1 (Figure 5C). These results confirmed that CNR family proteins were located in a functional signaling pathway from exogenous Reelin to cytoplasmic mDab1. Similarly, the Fab fragment of

CR-50 for Reelin also disturbed the Reelin-induced tyrosine phosphorylation level of mDab1 (Figure 5C). The Reelin-induced tyrosine phosphorylation of mDab1 is inhibited by inhibitors of Src family kinases (Howell et al., 1999). Therefore, during developmental positioning of cortical neurons, the binding interaction between Reelin and CNR family proteins induces enzymatic activation of Src family tyrosine kinase containing Fyn, resulting in the phosphorylation of the tyrosine residues of mDab1.

In fixed embryonic day 15 mouse cerebral cortex, the immunoreactivity of anti-RBD antibody was enriched in the marginal zone coincidentally with CR-50 immunoreactivity by confocal laser scanning fluorescence microscopy (Figures 6A, a and c; and Figure 6B, a). The double labeling was most intense in the discrete dots along the cell surface and dendritic fibers of Cajal-Retzius neurons (Figure 6A, c). As the mRNAs of the *CNR* family were expressed in cortical neurons but not in the marginal zone (Figure 1C), the CNR proteins may be transported and enriched in the tips of neuronal fibers. The colocalization observations provided evidence that the Reelin-binding domain of CNRs and Reelin directly interact in vivo. Examination of the localization of CNR proteins in the homozygous *reeler* embryonic cortex lacking Reelin revealed an increased signal in the marginal zone of the *reeler* cortex (Figure 6B, b). However, the discrete dots were decreased in number of the *reeler* cortex in contrast to the wild type. The dots are also detected even in a cultured single Cajal-Retzius neuron by staining with CR-50 (Ogawa et al., 1995). Therefore, the discrete dots indicate the localization of Reelin protein along the cell surface of Cajal-Retzius neurons. The decrease in the number of dots of CNR localization in the *reeler* marginal zone strongly suggests the in vivo colocalization of Reelin and CNR proteins. In addition, the staining of CNR in the *reeler* cortex also suggests that CNR expression is regulated independently of the Reelin signal. The colocalization of Reelin and CNR family proteins further confirms that CNR family proteins are in vivo Reelin receptors.

To examine the physiological effects of the anti-RBD CNR antibody on the histogenetic assembly of cortical cells, we performed reaggregation experiments using dissociated cerebral cortical cells isolated from E14 embryos. In control experiments, the dissociated cortical cells formed a single spherical aggregate (1000–1300 μm in diameter after 5 days). The aggregate was fixed after 5 days of incubation, and cryostat sections were immunostained with anti-MAP2 antibody to elucidate the neuronal arrangement within the aggregate. MAP2-labeled neurons were uniformly concentrated and aligned symmetrically in the outer 1/3 zone of the aggregate (Figure 6C, b). This result is consistent with previous observations (Ogawa et al., 1995). When cortical cells were exposed to anti-RBD CNR antibody (200 μg/ml) from initial cultivation, they also formed a single spherical aggregate, but their diameters were smaller (700–1000 μm) than those in the control (lower of Figure 6C, a). In addition, a control anti-Fyn rat IgG antibody (200 μg/ml) exhibited no effects (upper part of Figure 6C, a). When the sections of the small aggregate were stained by anti-MAP2 antibody, the staining neurons were loosely arrayed and concentrated in the middle of the aggregate (Figure 6C, c), while anti-Fyn antibody exhibited no effects (Figure 6C, d). The superficial zone (less stained by anti-MAP2 antibody) was formed even in the presence of anti-RBD antibody. Thus, anti-RBD CNR antibody disturbed the positioning of cortical neurons in vitro, but the malformation of the aggregate by the anti-RBD antibody resulted in a different pattern from that of CR-50 antibody targeted to Reelin (Ogawa et al., 1995). Hence, it is possible that CNR family proteins function not only as receptors for Reelin in cortical layer
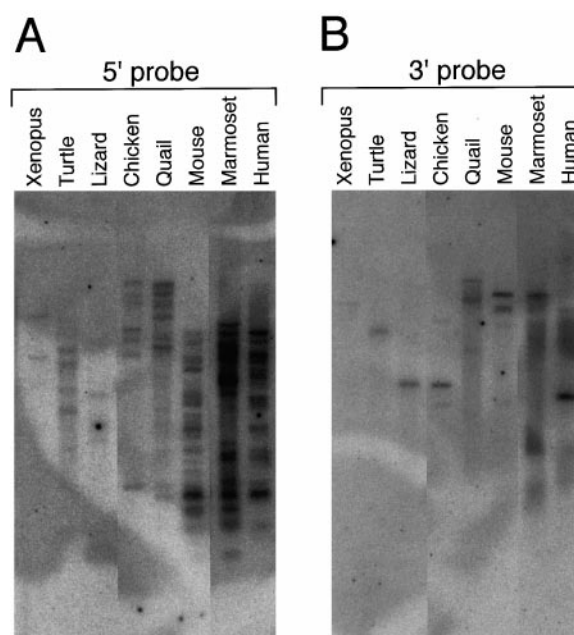

Figure 7. Evolutionary Diversity of the Reelin-Binding Domain of *CNR* Family Genes

Genomic DNA samples collected from human, marmoset, mouse (C57BL/6), quail, chicken, lizard, turtle, and frog (*Xenopus laevis*) were digested with EcoRI and analyzed by Southern blot using probes to the EC1 region (A) and the identical cytoplasmic region among *CNR1–8* (B) of the mouse CNR1.

formation, but also in homophilic CNR interactions or heterophilic interactions in the cortical neurons.

Evolutionary Features of the EC1 Domain of the *CNR* Family

The Reelin-binding EC1 domains are unusually conserved among CNR family proteins in mice (Kohmura et al., 1998). To determine whether the *CNR* family genes are conserved in other organisms, genomic DNA samples from several vertebrate species were analyzed by Southern blot analysis. Using the Reelin-binding EC1 region of *CNR1*, we detected hybridization with human, marmoset, mouse, quail, chicken, lizard, turtle, and frog DNA. Surprisingly, the number of detectable bands was different among terrestrial vertebrates. Approximately 20, 10, 5, and 2 bands were found in mammals, birds, reptiles, and amphibians, respectively (Figure 7A). On the other hand, one to three bands were commonly detected among these species by probing with the cytoplasmic constant region in *CNR* genes (Figure 7B). It is known that the constant region is divided into three short exons in human genomic DNA (Wu and Maniatis, 1999; Sugino et al., 2000). Hence, while the Reelin-binding domain of the *CNR* family is conserved, the gene copy number varies among terrestrial vertebrates. The layering and positioning of neurons are markedly diverse among terrestrial vertebrate brains (Marín-Padilla, 1998). Our data suggest that alterations in the duplication of *CNR* family genes containing the Reelin-binding EC1 domain may be a basis for the evolutionary diverse or complex patterns of layering and positioning of neurons.

Discussion

Reelin is expressed by pioneer Cajal-Retzius neurons that occupy the marginal zone of the neocortex, and it regulates layering and positioning of neurons. The *CNR* family transcripts are extensively expressed in nearly all of the neurons in the developing cortical plate but are not found in the Cajal-Retzius cells. We performed coimmunoprecipitation experiments with fusion proteins of CNR1 and Reelin to demonstrate the affinity of Reelin for CNR1 and to determine the binding site of CNR1 to Reelin. Interestingly, the amino acid sequence comprising the binding site of CNR1 that interacts with Reelin is identically conserved among CNR1 through CNR8. In addition, the extracellular domains of CNR1–3 proteins exhibit highly similar binding affinities to Reelin. Therefore, the CNR1–8 proteins may interact with Reelin through a common mechanism. An anti-RBD CNR monoclonal antibody that recognizes the conserved amino acid sequence can block the signaling pathway from Reelin to mDab1 in the cortical neurons. Furthermore, CNR family proteins colocalize with Reelin in the marginal zone of embryonic day 15 mice as demonstrated by immunostaining with the anti-RBD CNR antibody. This suggests that multiple CNR family proteins possess Reelin binding affinities and functional receptor activities. The candidacy of CNR family proteins as Reelin receptors is strengthened further by our demonstration of the blockage of the interaction between CNR1 and Reelin by the CR-50 antibody. CR-50 may inhibit the functional interaction of Reelin to its receptor, because the antibody disturbs the arrangement of cerebral cortical cells in vitro (Ogawa et al., 1995). Furthermore, intraventricular injection of CR-50 at the embryonic stage disrupts the organized development of the hippocampus in vivo, converting it to a *reeler* pattern (Nakajima et al., 1997). In addition, an anti-RBD CNR antibody, which inhibits the interaction between CNR1 EC1 and Reelin proteins, inhibits Reelin-induced tyrosine phosphorylation of mDab1 in cerebral cortical cells and disrupts the cortical cell arrangement. These results provide evidence that the CNR family proteins are functional Reelin receptors.

Signaling Cascades of the CNR Family
in Cortical Neurons

mDab1-deficient mice have been described that closely resemble *reeler*, yet they express *reelin* mRNA and protein (Howell et al, 1997b). In contrast, mDab1 is expressed at a higher level, but tyrosine is phosphorylated to a lower extent in *reeler* mutant embryo brains. Furthermore, the tyrosine phosphorylation of intracellular mDab1 protein is stimulated by exogenous Reelin (Howell et al, 1999). This suggests that Reelin regulates neuronal positioning by inducing mDab1 tyrosine phosphorylation. Here, the inhibition of the Reelin-induced tyrosine phosphorylation of mDab1 by anti-RBD CNR antibody (Figure 5C) strongly suggests that multiple CNR family proteins function as Reelin receptors. In addition, Fyn, which is associated with CNR proteins, binds to mDab1 (Howell et al, 1997a), and Fyn and mDab1 are also expressed in the cortical layers.

Very recently, cell surface receptors of VLDLR and ApoER2, which can bind to mDab1 via their cytoplasmic tails, bind to Reelin and are concerned with Reelin-induced tyrosine phosphorylation of the mDab1 (D'Arcangelo et al., 1999; Hiesberger et al., 1999). The estimated values for the K_D are, from 0.08 to 0.5 nM, less than 1.6 to 1.7 nM on CNR proteins (Figure 4). It is possible that CNR family proteins, Fyn (Src family), mDab1, and VLDLR or ApoER2 form a Reelin–receptor complex in the plasma membrane of the cortical neurons. Major cytoskeletal changes are required during the active migration of neurons (Rivas and Hatten, 1995; Rakic et al., 1996): the neuronal cell extends a neuronal process toward the target destination, followed by a translocation of the nucleus and the cytoplasmic components (Rakic, 1971; Komuro and Racik, 1998). Pathways invoked downstream of the Reelin–receptor complex, may include Doublecortin, which associates with microtubules (Francis et al., 1999), and Cdk5 and p35, which influence the reorganization of the actin cytoskeleton (Nikolic et al., 1998) (Figure 8A).

The identically conserved C-terminal region among CNR1–8, which is derived from the constant region of the *CNR* gene cluster in mouse genomic DNA (Kohmura et al., 1998), similarly to the *Pcdh*α cluster in human genomic DNA (Wu and Maniatis, 1999; Sugino et al., 2000), is commonly associated with Fyn (Kohmura et al., 1998; unpublished data). In the present report, we have shown that the N-terminal identical amino acid sequence among proteins CNR1–8 of the CNR family comprises the binding activity to Reelin protein. These data suggest that extracellular Reelin signaling is linked to cytoplasmic Fyn tyrosine kinase signaling through multiple CNR family proteins. Nevertheless, the functional roles attributed to the diversity of the CNR family proteins remain to be elucidated.

About the CNR Family

The N-terminal EC1 domain of cadherins is required for specific homophilic interactions. Variations in the sequences of extracellular domains can lead to diversity in cell–cell interactions (Takeichi, 1990). However, the EC1 domains of the CNR family containing the Reelin-binding sequence are unusually well conserved. Our present data indicating that the EC1 domains of CNR family uniformly bind to Reelin protein are concordant with their unusual conservation. The heterophilic binding activity of cadherins, akin to these Reelin–CNR interactions, has been reported between the EC1 domain of E-cadherin and integrin αEβ7 (Karecla et al., 1996). Therefore, the potential for homophilic or other heterophilic binding activities of the EC1 domains of CNR proteins exists. The differences in the malformations that result in the presence of anti-RBD antibody in comparison to malformations resulting from the presence of CR-50 in the reaggregation experiments of cortical cells also suggest these possibilities. Further characterization of the EC1-binding domains will be paramount in understanding the molecular mechanisms of migration, axonal extension, dendrite formation, or synaptogenesis.

The EC2 and EC3 domains are the most diverse domains among the mouse CNR family. This suggests that distinct CNR functions are generated from the EC2 and EC3 domains. It is possible that the sequestration of the
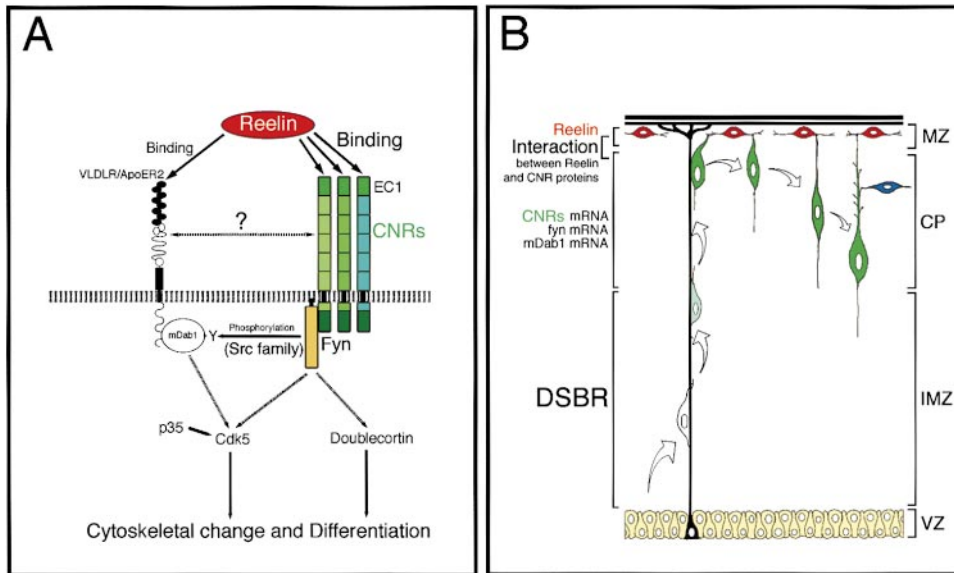
Figure 8. Role of Reelin, CNR Family, Fyn, and mDab1 in Cortical Plate Formation

(A) Hypothetical pathway of Reelin signaling. Reelin directly interacts with multiple CNR proteins on the neuronal surface. Since these CNRs are commonly associated with Fyn-tyrosine kinase, the Reelin signal activates Fyn, and Fyn tyrosine-phosphorylates mDab1 or the other cytoplasmic proteins. The VLDLR- or ApoER2-bound mDab1 is tyrosine phosphorylated and induces activation of the downstream components. After binding to Reelin, multiple CNRs may function in specified cell adhesion or facilitate certain developmental commitments in the cortical neurons.

(B) A schematic of neuronal development in the cortex. Mitotic neurons are localized in the ventricular zone (VZ). Postmitotic neurons migrate along the guidance fiber to the surface. At this postmitotic stage, DNA double strand break repair reactions (DSBR) in the neurons are necessary for survival (Gao et al., 1998). In the cortical surface, migrating neurons contact Cajal-Retzius cells. Here, we show that CNR family proteins expressed in neurons directly bind to Reelin expressed by Cajal-Retzius cells. After this interaction, neurons detach from the fiber, develop a terminal dendritic bouquet, grow the apical dendrites, and initiate the synaptogenesis. mRNAs of *CNR*s, *fyn*, and *mDab1* are expressed in the cortical layer. CNR proteins possessing the Reelin-binding domain are extensively localized in the surface of Cajal-Retzius cells in the marginal zone and colocalized with Reelin.

EC1 domains with Reelin protein exposes the different EC2 and EC3 domains of the CNRs, inducing new homophilic interactions or interactions with other extracellular proteins. The extracellular Reelin may induce the differential cell adhesion activity of the cortical neurons.

We have reported the localization of CNR proteins in maturing synaptic membranes and their differential expression at the individual neuron level in the adult brain (Kohmura et al., 1998). These features are involved in establishing complex networks of neuronal connections in the brain. On the other hand, *reelin* transcripts gradually disappeared from Cajal-Retzius cells at postnatal stages, concomitant with their appearance in subsets of GABAergic neurons distributed throughout the neocortical and hippocampal layers. Prominent expression of *reelin* was detected in the olfactory bulb and cerebral cortex, whereas it is restricted to subsets of GABAergic interneurons in adult brains (Alcantara et al., 1998). These expression patterns suggest that the interactions of CNR family proteins with Reelin have multiple roles in neuronal circuit development and adult brain function. Furthermore, CR-50 antibody, which inhibits binding between Reelin and CNR family proteins, also disturbed the ingrowth of enthorinal afferents into the hippocampus (Del Rio et al., 1997), indicating that the signal transduction pathway from Reelin to the CNRs may not exclusively be used in neuronal layering or positioning.

Positive Selection Model for Neuronal Layering and Positioning

Three models have been proposed to explain the phenotypes of Reelin- and mDab1-deficient mice (Curran and D'Arcangelo, 1998). One model suggests that Reelin acts as a repellent that induces the cortical preplate to split into the marginal zone and subplate. The failure of the preplate to separate in *reeler* causes the cortical plate to develop ectopically underneath the subplate neurons. In another model, a Reelin gradient may selectively induce cortical plate neurons to migrate past the subplate neurons. In a third model, Reelin is thought to act as a stop signal that instructs migrating neurons to detach from their glial guidance fiber, along which they migrate and proceed with their differentiation program.

The proteins of the CNR family are enriched in the marginal zone of the embryonic mouse brain. This indicates that the discovery that the CNR family functions as multiple Reelin receptors is consistent with each of the above models, but the stop signal model is more suitable than the repellent and attractive models. The neuronal expression patterns of the multiple CNR family proteins exhibiting binding to Reelin may be regulated during neuronal differentiation. We therefore propose another "positive selection model," whereby Reelin cues select functional neurons through the expression of diverse CNR family proteins. These cues, via the CNR family, induce neuronal differentiation and positioning

by developing a terminal dendritic bouquet, detaching from their glial guidance fiber, growing the apical dendrites, and initiating synaptogenesis (Figure 8B). The positive selection model is similar to the stop model. This model may be more feasible in light of the heretofore described features of the CNR family (i.e., genetic diversity, binding interactions with Reelin, protein structure for cell adhesion, localization in the synaptic junction, and coupling with Fyn-tyrosine kinase). Furthermore, the coupling of CNR proteins to tyrosine kinase signaling (Kohmura et al., 1998) and the division of their genomic structure into "variable" and "constant" regions is reminiscent of the immunoglobulin and T cell receptor genes (Wu and Maniatis, 1999; Sugino et al., 2000). These immunological receptor molecules function in positive and negative selection during the maturation of immune cells; hence, the positive selection model for Reelin may be conceptually broader than the stop model with regard to the development of brain formation or function. Interestingly, DNA double strand break repair reactions are necessary for differentiation of cortical plate neurons that express CNR genes (Gao et al., 1998) (Figure 8B).

The Role of the CNR Family Genes in the Evolution of Brain Formation and Function

Extant vertebrates are currently comprised of diverse groups, each with diverse and numerous species. The molecular evolution of the Hox gene is a candidate for the emergence of morphological diversity and complexity among animals (Meyer, 1998; Prince et al., 1998). In contrast, the brain differs markedly in structural organization and function across species. Simple changes in the genome may produce profound effects that give rise to the diversity and complexity of brain structure and function within a species. Such changes can result, for example, in the production of a new type of cell, in lengthening of the proliferation time of an area of the germinal epithelium, in the migration of a set of neurons away from the germline area, or in the radial alignment of the glial cells. The CNR family genes encoding our proposed multiple Reelin receptors are arranged in genomic clusters (Wu and Maniatis, 1999; Sugino et al., 2000), which may correspond, respectively, to function in neuronal migration, and differentiation and synaptogenesis during brain development. Interestingly, the Reelin-binding domains of the CNR genes among terrestrial vertebrates are conserved, but their numbers are divergent. The number of stained bands representing the Reelin-binding domains of CNR genes gradually increased from amphibia to mammals in parallel with the transition from brain simplicity to complexity. Nevertheless, the cytoplasmic constant region remained unchanged among terrestrial vertebrates. Thus, the alteration or duplication of the variable region of CNR genes leads us to question the relatively simple, "more genes, more complexity" model of evolutionary brain diversification in terms of phenotypic complexity. Therefore, the evolution of the CNR gene cluster provides a particularly intriguing foundation for the hypothesis that genomic and brain complexity are causally linked. Further sequence analysis of the CNR gene clusters among various vertebrates will be necessary to elucidate our understanding of the evolutionary diversity and complexity of brain formation or function. Nevertheless, the comparison of gene numbers and structures of CNRs among vertebrates will provide new insights into the molecular mechanisms involved in the formation, function, and evolution of vertebrate brains.

In conclusion, the present findings demonstrate a novel role for the CNR gene family in cellular signaling events that extends beyond that of cell adhesion molecules. Several unusual molecular features of the members of the CNR family, including their unusual genomic organization, diverse gene numbers, conserved EC1 domain, and binding to Fyn-tyrosine kinase, support the framework for the Reelin theory for brain structure and function.

Experimental Procedures

In Situ Hybridization

In situ hybridization was performed as described previously (Watanabe et al., 1992; Schaeren-Wiemers and Gerfin-Moser, 1993) using fyn, CNR1, CNR5, and CNR-CP as templates to synthesize digoxigenin-labeled cRNA probes. Sequences corresponding to the same cDNA regions as the Southern blot with specific probes were used as described previously (Kohmura et al., 1998). The antisense oligonucleotide probes for CNR1–8 are described in Kohmura et al. (1998). Immunostaining and in situ hybridization double labeling were performed using CNR1 as templates to synthesized digoxigenin-labeled cRNA probes. The hybridized sections were treated with CR-50 (Ogawa et al., 1995) and anti-digoxygenin sheep antibody, and CR-50 antibodies and cRNAs were visualized with Cy3-conjugated anti-mouse IgG and FITC-conjugated anti-sheep IgG, respectively. Mounted sections were examined with a Zeiss laser scan microscope.

Generation of Fusion Proteins

Sequences encoding the extracellular domain (EC: amino acids 1–692), EC1 domain (amino acids 1–133), the region containing the RGD motif (RGD$^+$: amino acids 1–97), the region not containing the RGD motif (RGD$^-$: amino acids 1–67) and the signal sequence (Sig: amino acids 1–39) of mouse CNR1, the extracellular domains of CNR2 (amino acids 1–691), CNR3 (amino acids 1–687), and E-cadherin (amino acids 1–709) were amplified by PCR and cloned into the expression vector pEF-Fc (Mizushima and Nagata, 1990). Sequences encoding the amino-terminal region of mouse Reelin (Reelin-NAEB: amino acids 1–911) and human AP derived from plasmid APTag-2 (Flanagan and Leader, 1990) were amplified by PCR, fused, and cloned into the expression vector pCEP4 (Invitrogen). Reelin-N[SpII], amino acids 1–246; Reelin-N[SalI], amino acids 1–368; Reelin-N[ΔSpII-SalI], amino acids 1–246, 369–498; Reelin-N[SpII]AEB, amino acids 1–246, 499–859; or Reelin-N[SpII]B, amino acids 1–246, 699–859 were amplified by PCR, fused to the sequence encoding AP, and cloned into pCEP4. We performed site-directed mutagenesis of EC1-Fc, changing the RGD sequence into RGE using the TAKARA LA PCR in vitro Mutagenesis Kit (TAKARA). These plasmids were transiently transfected into HEK293-EBNA cells with Lipofectamin PLUS (GIBCO–BRL). The full-length Reelin expression vector (pCrl) or the control vector (pCDNA3) was also used with HEK293T (D'Arcangelo et al., 1997).

Immunoprecipitation

To detect interactions between various CNR1 Fc fusion proteins and various Reelin AP fusion proteins, 500 μl of protein A–agarose beads (CALBIOCHEM) were incubated with 500 μg of anti-Fc antibody (JIRL; Jackson ImmunoResearch Laboratory) in TBS (20 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin) at room temperature for 2 hr. After washing three times with 1 ml of TBS, 30 μl of the beads was incubated with 1 ml of the conditioned medium from HEK293-EBNA transfected with various CNR1 Fc fusion proteins at room temperature for 2 hr. Beads conjugated with recombinant proteins were washed three times with TBS, resuspended in 1 ml of the cell lysate

of HEK293-EBNA cells transfected with various Reelin AP fusion proteins and AP (APTag-4 gift from J. G. Flanagan) with IP buffer (20 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 1% Triton X-100, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin), incubated at 4°C for 12 hr, and washed ten times with 1 ml of IP buffer. Bound proteins were released by boiling beads in 40 μl of SDS sample buffer and analyzed by SDS–PAGE (8% gel) and Western blotting with anti-AP antibody (BioMedTek).

Binding Assays Using Surface Plasmon Resonance-SPR670

Surface plasmon resonance was used to determine quantitatively the strength of the interaction between CNR1 EC1-Fc fusion proteins and Reelin-NAEB-AP fusion proteins in real time, respectively, used with SPR670 (Nippon Laser and Electronics Lab, Nagoya). Anti-Fc antibody (10 μg/ml) was bound to gold membrane chips, and after washing with PBS, was twice blocked with 0.5 M glycine-HCl (pH 5.0). After washing with PBS, binding with CNR1 EC1-Fc fusion proteins was performed in conditioned medium from HEK293-EBNA cells transfected with CNR1 EC1-Fc plasmid. Following another wash with PBS, interactions between CNR1 EC1-Fc fusion proteins and Reelin-NAEB-AP fusion proteins with the concentrated Reelin-NAEB-AP fusion protein were detected using anti-AP antibody coupled to an Affi-Gel 10 Gel (BIO-RAD) column, and conditioned medium from HEK293-EBNA cells transfected with Reelin-NAEB-AP plasmid. When we performed blocking assays, the concentrated Reelin-NAEB-AP fusion protein was incubated with 100 μg Fab fragment of CR-50 at 27°C for 1.5 hr, followed by detection of the interaction between CNR EC1-Fc fusion proteins and Reelin-NAEB-AP fusion proteins, or after binding the anti-RBD antibody to CNR1 EC1-Fc, detection of the interaction between CNR1 EC1-Fc fusion proteins and Reelin-NAEB-AP fusion proteins was performed. For determining the strength of the interactions between the extracellular domains of CNR1, 2, and 3, and E-cadherin to the full-length of Reelin, the Reelin protein was directly bound to gold membrane chips.

Production of Monoclonal Antibodies

For monoclonal antibody production, 200 μg of KLH-conjugated peptide sequence RGDLLEVNLQN was injected into the foot pad of 7-week-old male Fisher rats. Three days after subsequent booster immunization, the dispersed lymphocytes were fused with P3U1 myeloma cells in a 5:1 ratio by polyethylene glycol. Fused cells were selected in HAT medium, and ELISA-positive cells were cloned. Specificity was confirmed by analysis of immunoblot staining patterns of each CNR1 EC-Fc fusion protein and embryonic brain extracts. The total and P2 fraction extracts were obtained as described (Kohmura et al., 1998).

Immunohistochemistry

For immunostaining to detect the expression of CNR family proteins in the E15 mouse cortex, cryostat sections (10 μm) from unfixed frozen embryos were collected and fixed in 2 hr with 4% paraformaldehyde-PBS. Staining was performed with anti-RBD rat monoclonal antibody (2H12, 1:10), and bound antibody was visualized using a FITC-conjugated anti-rat IgG-specific antibody (JIRL). For double immunostaining of the CNR family and Reelin in E15 mouse cortex, staining was performed with anti-RBD rat monoclonal antibody (2H12, 1:10) and anti-Reelin mouse monoclonal antibody (CR-50, 1:50), and bound antibody was visualized using a FITC-conjugated anti-rat IgG specific antibody (JIRL) and a Cy3-conjugated anti-mouse IgG-specific antibody (JIRL). Mounted sections were examined with a Zeiss laser scan microscope.

mDab1 Tyrosine Phosphorylation Experiment

We determined the levels of Reelin-induced tyrosine phosphorylation of mDab1 in embryonic cortical cells as described (Howell et al., 1999). Aliquots (200 μl) of dissociated brain cells were rotated with 100 μl fresh, Reelin- or control-conditioned media, or Reelin-conditioned media with 50 μg/ml of anti-CNR (2H12), anti-Fyn (γC3), or 200 μg/ml Fab fragment of CR-50 at 37°C for 10 min. We estimated that the concentration of Reelin produced is 750 pM. Reactions were stopped by centrifuging the cells at 1000 g and washing with PBS. The cell samples were homogenized in RIPA buffer (Howell et al., 1999). Lysates were clarified by centrifugation at 20,000 g for 30 min at 4°C after a 10 min incubation on ice. Samples were normalized for total protein concentrations and also confirmed by immunoblotting with anti-NSE (Affiniti Research Products Limited), and incubation with anti-mDab1 (B3) (a kind gift from J. A. Cooper) that was cross-linked to protein A–Sepharose with dimethyl pimelimidate at 4°C for 2 hr, followed by three washes with RIPA buffer. Bound proteins were analyzed by immunoblotting with anti-mDab1 and anti-phosphotyrosine antibody (4G10, Upstate Biotechnology).

Reaggregation Experiment

Reaggregation culture of E14 cortical cells was principally performed using the method of DeLong (1970) and Ogawa et al. (1995). Cells were suspended at a density of 3×10^5 cells per milliliter of modified N2/G5 medium supplemented with 5% horse serum in a tube (352057; FALCON). For reaggregation experiments, we used two rat IgG antibodies for CNR EC1 (2H12) or Fyn (γC3) (Kohmura et al., 1998). The tubes were incubated on a rotating holder in an atmosphere of 5% CO_2 with saturated humidity at 37°C and rotated at 5 rpm at an angle of 30°. After 5 days of incubation, cell aggregates were fixed in 4% paraformaldehyde and cryosectioned. Immunostaining of anti-MAP2 antibody (Sigma) was conducted similarly with tissue sections. Mounted sections were examined with a Zeiss laser scan microscope.

Southern Blot Analysis

For the zoo blot, the following tissues were used for each vertebrate: whole blood (human, marmoset) and liver (cat, mouse, chicken, quail, lizard, turtle, frog). Genomic DNA from blood was extracted according to Current Protocols in Human Genetics (Dracopoli et al., 1994). Genomic DNAs were digested with EcoRI. The 5′ and 3′ probes and Southern blotting method are as described (Kohmura et al., 1998).

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