

Reelin Is a Secreted Glycoprotein Recognized by the CR-50 Monoclonal Antibody

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The neurological mouse mutant strain *reeler* displays abnormal laminar organization of several brain structures as a consequence of a defect in cell migration during neurodevelopment. This phenotype is a result of the disruption of *reelin*, a gene encoding a protein that has several structural characteristics of extracellular matrix proteins. To understand the molecular basis of the action of Reelin on neuronal migration, we constructed a full-length *reelin* clone and used it to direct Reelin expression. Here, we demonstrate that Reelin is a secreted glycoprotein and that a highly charged C-terminal region is essential for secretion. In addition, we demonstrate that an amino acid sequence present in the N-terminal region of Reelin contains an

epitope that is recognized by the CR-50 monoclonal antibody. CR-50 was raised against an antigen expressed in normal mouse brain that is absent in *reeler* mice. The interaction of CR-50 with its epitope leads to the disruption of neural cell aggregation *in vitro*. Here, we used CR-50 to precipitate Reelin from reticulocyte extracts programmed with *reelin* mRNA, from cells transfected with *reelin* clones, and from cerebellar explants. The *reelin* gene product seems to function as an instructive signal in the regulation of neuronal migration.

Key words: *reeler*; cerebral cortex; cerebellum; extracellular matrix; glycosylation; mutant mice; neuronal migration

reeler (*rl*) is a mouse autosomal recessive mutation that affects the organization of the developing brain (Caviness and Rakic, 1978; Goffinet, 1984; Rakic and Caviness, 1995). The most striking aspects of the *reeler* phenotype are the abnormal positioning of neurons throughout the cerebral cortex, the cerebellum, and the hippocampus and the aberrant orientation of cell bodies and fibers. In the cerebral cortex of *reeler* mice, neurons generated early in development, normally destined to form the subplate, occupy ectopic positions in superficial cortical layers. Neurons generated at later stages, destined to form the cortical plate, migrate radially but fail to bypass previously generated neurons. Thus, the pattern of neocortical histogenesis in *reeler* mice seems to be inverted. The *reeler* cerebellum is considerably smaller than normal, and it lacks foliation and a Purkinje cell layer (Mariani et al., 1977; Mikoshiba et al., 1980; Goffinet et al., 1984). Neurons positioned abnormally also are encountered in the hippocampus (Caviness, 1973; Stanfield and Cowan, 1979). Although neurogenesis and neuronal connectivity primarily are preserved, brain function is impaired in *reeler* mice, and they exhibit severe motor

defects, including tremors, ataxia, and difficulty in balance and locomotion (Falconer, 1951).

Neuroanatomical analysis suggested that the gene affected by the *reeler* mutation is critical for the appropriate migration of postmitotic neurons (Goffinet, 1984; Caviness et al., 1988). The recent cloning of *reelin*, the gene mutated in *reeler* mice (D'Arcangelo et al., 1995), has provided new insights into the molecular mechanisms underlying the *reeler* defects (Goffinet, 1995; Rakic and Caviness, 1995; Rugarli and Ballabio, 1995). *reelin* encodes a protein of 3461 amino acids with several features of extracellular molecules, including a cleavable signal peptide, several potential glycosylation sites, and EGF-like repeats.

In two strains of *reeler*, *rl* (Falconer, 1951) and *rl^{ts}* (Miao et al., 1994), no *reelin* mRNA was detected (D'Arcangelo et al., 1995). The defect in *rl* originates from a 150 kb deletion in the *reelin* gene (Bar et al., 1995; D'Arcangelo et al., 1995), whereas in *rl^{ts}* it arises from an intragenic deletion of *reelin* sequences associated with the insertion of a transgene (Miao et al., 1994; D'Arcangelo et al., 1995). In a third strain, *rl^{ori}* (Guenet, 1981), a small deletion in *reelin* was described that affects the 3' end of the coding sequence (Curran et al., 1995; Hirotsune et al., 1995). This deletion results from exon skipping as a consequence of the transposition of an active L1 sequence into the gene (Takahara et al., 1996).

In situ hybridization studies revealed that *reelin* is expressed in a complex pattern during neurodevelopment (D'Arcangelo et al., 1995). In the embryonic cerebral cortex, *reelin* mRNA is expressed specifically by a subset of neurons in the most superficial layer, the Cajal–Retzius cells, which are among the first neurons to differentiate in the brain (Marin-Padilla and Marin-Padilla, 1982; Derer and Derer, 1990; D'Arcangelo et al., 1995; Hirotsune et al.,

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1995). In the cerebellum, *reelin* mRNA is expressed very highly in the granule cell layers (D'Arcangelo et al., 1995).

The distribution of *reelin* mRNA is very similar to that recently reported for an epitope recognized by the CR-50 monoclonal antibody. Generated by immunizing *reeler* mice with normal brain homogenate, this antibody interferes with the pattern of aggregation of neocortical cells *in vitro* (Ogawa et al., 1995). In the present study, we generated full-length and truncated *reelin* cDNA clones to investigate the biochemical properties of Reelin and its relationship to the CR-50 epitope.

MATERIALS AND METHODS

Construction of *reelin* clones. The entire *reelin* open reading frame was assembled by fusing five overlapping cDNA clones isolated previously (D'Arcangelo et al., 1995) and subcloning them into the vector pcDNA3 (Invitrogen, San Diego, CA). This vector allows mammalian expression from the human cytomegalovirus (CMV) promoter or *in vitro* transcription from the T7 promoter. The following *reelin* fragments were used: 1.2 kb *NaeI*–*SalI* from p5'BS1, 1.3 kb *SalI*–*NdeI* from pBS2, 3.1 kb *NdeI*–*BspEI* from pBS6, 770 bp *BspEI*–*ApaLI* from p3Rea3, and 4.2 kb *ApaLI*–*EcoRV* from pBS53. The final clone (pCrI) contains the entire *reelin* open reading frame (10,383 bp) plus 95 bp of sequence 5' to the initiator methionine codon and 82 bp of 3' untranslated sequence (*reelin* cDNA nucleotides 188–10,748). To produce an epitope-tagged version of Reelin (pCrIM), we annealed oligonucleotides encoding the human c-Myc epitope 9E10 (OKLISEEDLN) flanked by *BspEI* restriction site sequences and inserted them, in frame, into the unique *BspEI* site present in pCrI. To generate a truncated pCrI3328 clone lacking the C terminus, we eliminated a 500 bp *XhoI* fragment from pCrI. Other expression constructs contain *reelin* cDNA nucleotides 188–865 (pCrI194), 188–1029 (pCrI250), 188–1505 (pCrI407), and 188–1664 (pCrI462) cloned into pcDNA3. pCrI1837 is similar to pCrIM except for the orientation of the c-Myc epitope in the *BspEI* site, which is inverted, thus creating a stop codon immediately after nucleotide 5789. The nucleotide sequence of *reelin* has been deposited in GenBank; the accession number is U24703.

In vitro transcription/translation. Expression plasmids were transcribed, and the resulting RNA was translated *in vitro* via a reticulocyte lysate transcription/translation system according to the manufacturer's instructions (TnT, Promega, Madison, WI). DNA (2 µg) was transcribed in a 50 µl reaction mix with T7 RNA polymerase. Translation was performed in the presence of 40 µCi of [³⁵S]methionine or a mix of [³⁵S]methionine and [³⁵S]cysteine in the absence of unlabeled methionine. One microliter of the reaction mix was diluted in 100 µl of SDS sample buffer and subjected to SDS-PAGE. The remainder of the reaction mix was diluted in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P40, 0.5% Na deoxycholate, and 0.1% SDS) for immunoprecipitation analysis.

COS cell transfection. COS-7 cells (4×10^5) were plated in 60 mm dishes in the presence of 5 ml of DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and incubated overnight. On the next day, the medium was replaced with Opti-MEM (Life Technologies), and the cells were transfected with the LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. After 5–7 hr of transfection in 2 ml of Opti-MEM, 2 ml of DMEM containing 20% fetal bovine serum was added, and the cultures were incubated overnight. On the next day, the medium was replaced with fresh DMEM containing 10% serum. After one additional day, cells were examined by immunofluorescence analysis, or they were prepared for immunoprecipitation analysis as described below.

Cerebellar primary cultures. The cerebellum was removed by dissection from postnatal day 5–8 (P5–P8) normal or *reeler* B6C3Fe mice, which were generated as the progeny of a *rl* heterozygous cross (Jackson Laboratory, Bar Harbor, ME). Then the tissue was chopped finely with a razor blade and triturated in a plastic pipette. Cells were resuspended in basal Eagle's medium supplemented as described (Fischer, 1982), with 5% horse serum (HyClone, Logan, UT). Explants or dispersed cells (2×10^5 cells/cm²) were plated in tissue culture dishes that had been precoated with 20 µg/ml poly-L-lysine. The cultures were switched to serum-free medium 1 d after plating and incubated for 1–4 d. Extensive neurite outgrowth during this period was indicative of healthy cultures.

Immunoprecipitation. Reelin was produced *in vitro* using rabbit reticulocyte lysates in the presence of [³⁵S]methionine or a mix of [³⁵S]methionine and [³⁵S]cysteine (Amersham, Arlington Heights, IL, or New

England Nuclear, Boston, MA) in the absence of unlabeled methionine. The transcription/translation reaction was diluted in 1 ml of RIPA buffer supplemented with the protease inhibitors leupeptin (20 µg/ml), aprotinin (0.1 mg/ml), and phenylmethylsulfonyl fluoride (PMSF; 2 mM). Aliquots of the diluted mixture were used for immunoprecipitation. To prepare radiolabeled Reelin expressed in COS cells, we incubated cultures overnight in DMEM lacking methionine and cysteine, supplemented with 1% fetal bovine serum and 300 µCi/ml of a mix of [³⁵S]methionine and [³⁵S]cysteine. Reelin expressed in cerebellar cultures was labeled overnight with the [³⁵S] labeling mix in basal Eagle's medium depleted of methionine (Cellgro) and supplemented as previously described (Fischer, 1982). For immunoprecipitation analysis, culture medium was collected from COS or cerebellar cells and diluted 1:1 with 2× RIPA buffer. Cell extracts were prepared in RIPA buffer supplemented with protease inhibitors as described above. Both supernatants and lysates were precleared by centrifugation at 1500 rpm in a refrigerated Eppendorf microfuge for 10 min.

Anti-Myc monoclonal antibody 9E10 (a gift of G. I. Evan, Imperial Cancer Research Fund, London, UK, and M. J. Bishop, University of California, San Francisco, CA, or obtained commercially, Babco, Richmond, CA), CR-50 ascites (Ogawa et al., 1995), rabbit antisera anti-Rlp3 raised against amino acids 1144–1163 of Reelin, or affinity-purified rabbit antibody anti-rp5 raised against amino acids 3443–3461 of Reelin were added to the samples and incubated at 4°C for 2 hr to overnight. Immobilized G-protein agarose beads (Immunopure plus, Pierce, Rockford, IL) were added to precipitate the antibodies, and the samples were incubated for an additional 30 min. Immunoprecipitates were collected by centrifugation and washed three times with RIPA buffer. Samples were resuspended in 20 µl of SDS sample buffer, boiled, and loaded onto SDS polyacrylamide gels (4–12% gradient gels, Novex, San Diego, CA, or 4% Duracryl, Oxford Glycosystems, Abingdon, UK).

Glycosylation analysis. Immunoprecipitates were resuspended in the appropriate glycosidase buffer and digested as follows. For peptide-N-glycosidase F (PNGaseF) digestion, samples were resuspended in 2× PNGaseF buffer (100 mM potassium phosphate, pH 7, 0.4% SDS, and 2% β-mercaptoethanol) and boiled for 5 min. The concentration of Nonidet P-40 was adjusted so that it exceeded SDS by sevenfold in a 20 µl reaction mix. The sample was divided into two aliquots, and PNGaseF (0.2 U, Boehringer Mannheim, Indianapolis, IN) was added to one tube. For neuraminidase digestion, samples were resuspended in 2× neuraminidase buffer (200 mM sodium acetate, pH 5.5, 0.5% SDS, and 0.7% β-mercaptoethanol) and boiled for 5 min. The concentration of Nonidet P-40 was adjusted so that it exceeded SDS by sevenfold in a 20 µl reaction mix. The sample was divided and neuraminidase from *Clostridium perfringens* (1 µl of NeuA, Worthington, Freehold, NJ) was added to one tube. For O-glycosidase, samples were resuspended in 50 mM sodium phosphate, pH 5, and O-glycosidase DS (1 mU, Glyko, Novato, CA) was added to one sample. For chondroitinase ABC digestion, samples were resuspended in 20 mM Tris HCl and 40 mM sodium acetate, pH 8; chondroitinase ABC (10 mU, Boehringer Mannheim) was added to one sample. All samples were incubated for 1 hr at 37°C. After incubation, an equal volume of 2× SDS sample buffer was added to each, and the samples were boiled and loaded onto 4% SDS-polyacrylamide.

Immunofluorescence. COS cells were transfected and plated on glass slide chambers (Nunc, Naperville, IL). Two days after transfection, cells were rinsed with PBS, fixed with 2% paraformaldehyde, and permeabilized with 1% Triton X-100. Primary mouse monoclonal antibodies 9E10 (anti-Myc) or CR-50 ascites were diluted 1:200 in PBS containing 2.5% normal horse serum (Vector Laboratories, Burlingame, CA). Cells were incubated with the primary antibody for 1–2 hr at room temperature and then washed and incubated for 30 min with the secondary antibody, FITC-labeled rabbit anti-mouse (Dako, Carpinteria, CA), diluted 1:80 as described above. Cells were rinsed twice with PBS before mounting on coverslips with fluorescence mounting medium (Vectashield, Vector Laboratories). The staining was analyzed under a microscope with phase-contrast and fluorescence settings (BX60, Olympus Optical, Tokyo, Japan).

RESULTS

Generation of *reelin* clones

To facilitate the investigation of Reelin function, we assembled a full-length *reelin* construct by the consecutive cloning of five contiguous cDNAs, as described in Materials and Methods. The full-length construct contains 95 bp of sequence 5' to the initiator

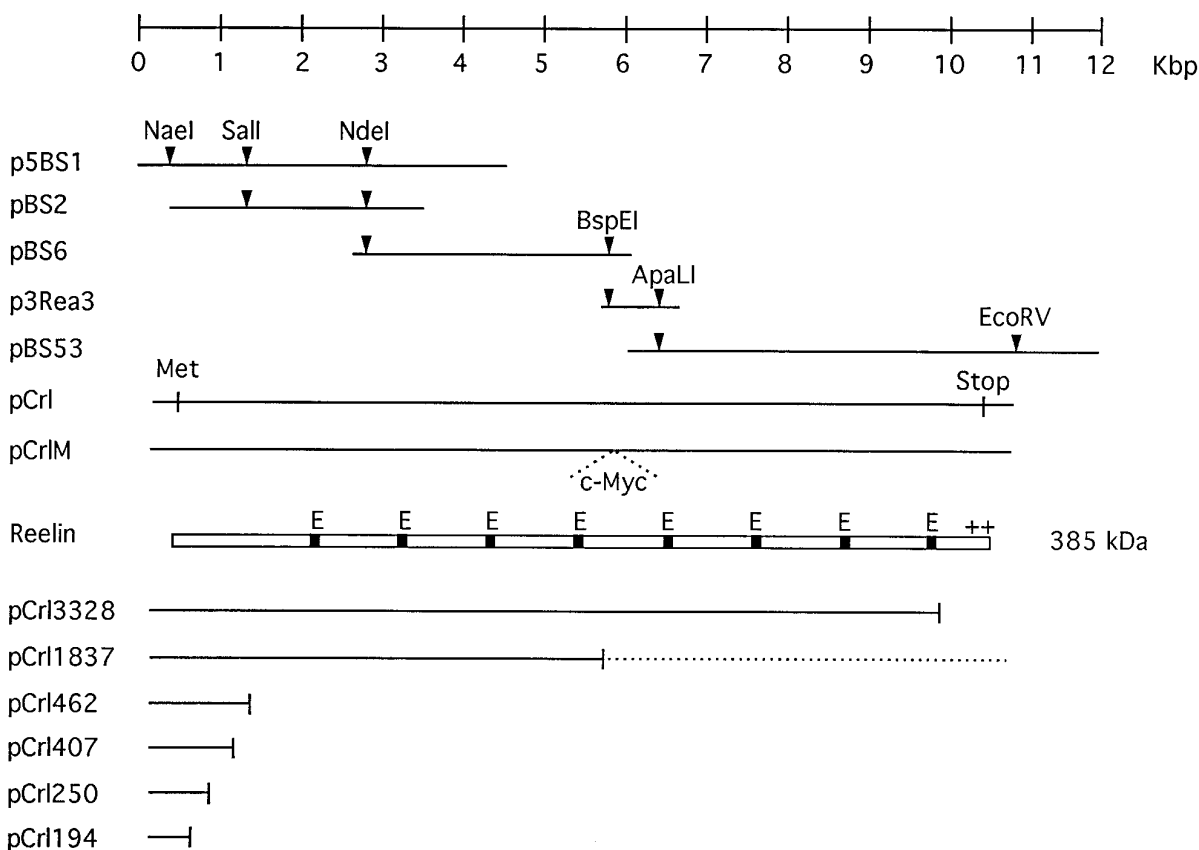


Figure 1. Assembly of full-length and truncated *reelin* cDNAs. Five overlapping plasmids (*lines*) encoding portions of the *reelin* cDNA (p5BS1, pBS2, pBS6, p3Rea3, and pBS53) were digested with the indicated restriction enzymes and cloned consecutively into pcDNA3 to generate a full-length clone that contains the entire open reading frame under the control of the mammalian CMV and the bacteriophage T7 promoters (*pCrI*). The initiator methionine codon (*Met*) and the stop codon (*Stop*) are indicated. After signal peptide cleavage, the full-length protein (*box*) is predicted to be 385 kDa in size. EGF-like repeats (*E*, *black boxes*) and a highly positively charged region in the C terminus (++) are indicated. A double-stranded oligonucleotide encoding a c-Myc epitope, 9E10 (*c-Myc*), was cloned in frame into the unique *BspEI* restriction site of pCrI to generate the pCrIM construct. Other expression constructs (*lines*) encoding truncated Reelin proteins in pcDNA3 are shown below the protein diagram. The numbers contained in the expression construct names refer to the last encoded amino acid of Reelin. The pCrI1837 contains an inverted c-Myc oligonucleotide at the *BspEI* restriction site, which puts the C-terminal half of the protein out of frame (*dotted line*).

methionine codon and 82 bp of 3' untranslated sequence. The open reading frame of 10,383 bp is sufficient to encode a protein of 3461 amino acids. After cleavage of the signal peptide, the molecular mass of Reelin was predicted to be ~385 kDa. The full-length open reading frame was placed under the control of the CMV promoter for expression in mammalian cells (pCrI, Fig. 1). This vector also contains a promoter sequence for the T7 RNA polymerase, which allows transcription *in vitro*. To facilitate detection of the recombinant protein, we inserted a short DNA sequence encoding a human c-Myc epitope in frame in the fourth Reelin repeat, as illustrated (pCrIM, Fig. 1).

Several truncated clones expressing portions of the Reelin sequence also were generated. These contain truncations at amino acid 194 (pCrI194), 250 (pCrI250), 407 (pCrI407), 462 (pCrI462), 1837 (pCrI1837), and 3328 (pCrI3328), as illustrated in Figure 1.

The CR-50 monoclonal antibody recognizes Reelin

The staining pattern of CR-50, a monoclonal alloantibody raised in *reeler* mice against normal brain homogenate (Ogawa et al., 1995), is very similar to the distribution of *reelin* mRNA. Furthermore, this antibody interferes with the pattern of aggregation of neocortical cells *in vitro* (Ogawa et al., 1995). To determine

whether CR-50 recognizes Reelin, we expressed the full-length *reelin* construct containing a c-Myc epitope (pCrIM) *in vitro*, using a coupled transcription/translation system. Reelin was detected as a single polypeptide of ~385 kDa on SDS-polyacrylamide gels. It was immunoprecipitated by CR-50, as well as by antibodies specific for the c-Myc epitope, and by antibodies raised against amino acids 1144–1163 of Reelin (Rlp3; Fig. 2, lanes 2–4). In contrast, no Reelin was precipitated in the absence of antibodies or by preimmune sera (Fig. 2, lanes 1, 5). This suggests that the epitope recognized by CR-50 is encoded by the primary amino acid sequence of Reelin, because many post-translational modifications, such as glycosylation, do not occur in the reticulocyte lysate.

Immunocytochemistry was used to confirm the finding that CR-50 recognizes Reelin. CR-50 was used previously to demonstrate the presence of a *reeler*-related antigen in brain sections and in cultured neurons (Ogawa et al., 1995; Miyata et al., 1996). COS cells were transfected with or without pCrIM, fixed, and incubated with CR-50, as described in Materials and Methods. After incubation with FITC-labeled secondary antibody, positive cells were visualized under a fluorescent microscope. Approximately 5% of the cells transfected with pCrI revealed a strong intracellular signal (Fig. 3), whereas no signal was detected in cells transfected

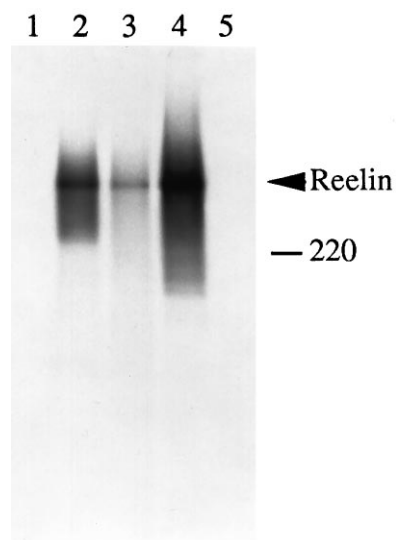


Figure 2. Expression of full-length Reelin *in vitro*. A plasmid encoding full-length Myc-tagged Reelin (pCrIM) was transcribed *in vitro* by the T7 RNA polymerase and translated by using a rabbit reticulocyte lysate. The [³⁵S]-labeled final product was analyzed on a 4–12% SDS-polyacrylamide gel. Aliquots of the reaction were treated with no antibody (lane 1), monoclonal antibody against c-Myc (9E10, lane 2), monoclonal antibody CR-50 (lane 3), polyclonal anti-Reelin peptide Rlp3 (lane 4), or preimmune serum (lane 5). The immunoprecipitated Reelin protein is ~385 kDa.

in the absence of pCrI (data not shown), demonstrating the specificity of CR-50 for Reelin. The CR-50 staining pattern, which was particularly intense near the nucleus, was similar to that obtained with the anti-Myc antibody (data not shown).

CR-50 recognizes the N terminus of Reelin

To map the CR-50 epitope on Reelin, we used a series of truncated *reelin* expression clones (Fig. 1) in an *in vitro* transcription/translation assay. Each construct encoded a truncated Reelin protein of the expected size (Fig. 4A), which was subjected to immunoprecipitation with the CR-50 antibody. As for full-length Reelin (Fig. 2, lane 3), the CR-50 antibody was able, specifically, to immunoprecipitate truncated proteins containing amino acids 1–1837 (pCrI1837), 1–462 (pCrI462), and 1–407 (pCrI407), but not proteins containing only amino acids 1–194 (pCrI194) or 1–250 (pCrI250) (Fig. 4B). No appreciable signal was detected when a control IgG antibody used for immunoprecipitation (data not shown). These data demonstrate that the CR-50 epitope is located between Reelin amino acids 251 and 407.

Reelin is a secreted glycoprotein

Analysis of the phenotype of *reeler* mice led to the proposal that they lack a signaling molecule required for the regulation of cellular interactions during neuronal migration. This was confirmed and extended by studies using chimeric *reeler* and normal mice, which demonstrated that at least some Purkinje cells from *reeler* mice are able to differentiate properly when exposed to a normal cerebellar environment (Terashima et al., 1986). One interpretation of these data is that the *reeler* gene encodes a protein that provides an extrinsic signal for migrating neurons. This is consistent with the predicted amino acid sequence of Reelin, which indicates the presence of a cleavable signal peptide at the N terminus. This feature, together with the sequence similarity to F-spondin and the presence of EGF-like repeats,

suggests that Reelin is a secreted protein. To investigate this possibility formally, we looked for the presence of secreted Reelin in medium from COS cells expressing pCrIM and in medium from cerebellar explants.

COS cell cultures were labeled overnight with a mixture of [³⁵S]methionine and [³⁵S]cysteine, as described in Materials and Methods. Media were collected and processed for immunoprecipitation with antibodies directed against the c-Myc epitope (Fig. 5A) and CR-50 (Fig. 5B). A c-Myc antibody specifically precipitated Reelin (~400 kDa) from the medium obtained from COS cells transfected with pCrIM (Fig. 5A, lanes 3, 4), but not from that obtained from control cultures (Fig. 5A, lanes 1, 2). Similarly, CR-50 specifically precipitated Reelin from the medium of transfected COS cells (Fig. 5B, lanes 1–4). Other protein bands were detected occasionally in immunoprecipitation assays; however, these represent either cross-reacting cellular proteins or degradation products of Reelin. To analyze the expression of endogenous Reelin, we prepared cultures from developing cerebelli dissected at P5–P8. After 1–4 d in culture, cells were labeled biosynthetically with [³⁵S]methionine and [³⁵S]cysteine. Reelin (~400 kDa) was clearly detected both in CR-50 immunoprecipitates from the culture medium and from cell extracts of cultured cerebellum (Fig. 5B, lanes 5, 6). Additional bands may correspond to Reelin proteolytic fragments or may result from antibody cross-reactivity. The size of Reelin produced by cerebellar cells was similar to that of the protein synthesized in COS cells. Immunoprecipitated Reelin produced by COS or cerebellar cells migrated at a higher apparent molecular weight than that expressed *in vitro* in reticulocyte extracts, which is predicted to be ~385 kDa (Fig. 5A,B, lanes 5, 7, respectively). This suggests that Reelin undergoes post-translational modification in intact cells. These data confirm the finding that CR-50 recognizes Reelin specifically, and they demonstrate that Reelin is a secreted protein that is post-translationally modified.

So that the nature of the post-translational modifications of Reelin that result in an apparent increase in molecular weight might be investigated, CR-50 immunoprecipitates from transfected COS cell medium and from medium removed from cerebellar explants were digested with a series of glycosidases, and the products were analyzed by SDS-PAGE. CR-50 immunoprecipitated Reelin from the medium of COS cells transfected with pCrIM (Fig. 6A, lanes 2–10), but not from the medium obtained from mock-transfected cells (Fig. 6A, lane 1). After digestion with PNGaseF, an enzyme that specifically cleaves asparagine-linked N-glycans, the apparent molecular mass of Reelin was reduced (Fig. 6A, compare lanes 3 and 4). Digestion with O-glycosidase, an enzyme that cleaves O-linked carbohydrate chains attached to serine and threonine residues, resulted in a modest decrease in the apparent mass of Reelin (Fig. 6A, lanes 5, 6). No appreciable effect was observed after digestion with neuraminidase A (Fig. 6A, lanes 7, 8), which cleaves sialic acid groups, or with chondroitinase ABC (Fig. 6A, lanes 9, 10), which cleaves chondroitin sulfate and dermatan sulfate side chains from proteoglycans. CR-50 also precipitated the endogenous Reelin from the medium of cerebellum explants obtained from normal mice (Fig. 6B, lanes 2–5), but not from explants obtained from *reeler* mice (Fig. 6B, lane 1). Treatment with PNGaseF (Fig. 6B, lane 3) and, to a lesser extent, O-glycosidase (Fig. 6B, lane 5) resulted in an apparent size reduction, whereas neuraminidase A (Fig. 6B, lane 4) had no effect. These data indicate that Reelin is subject to N-linked and, to a lesser extent, to O-linked glycosylation.

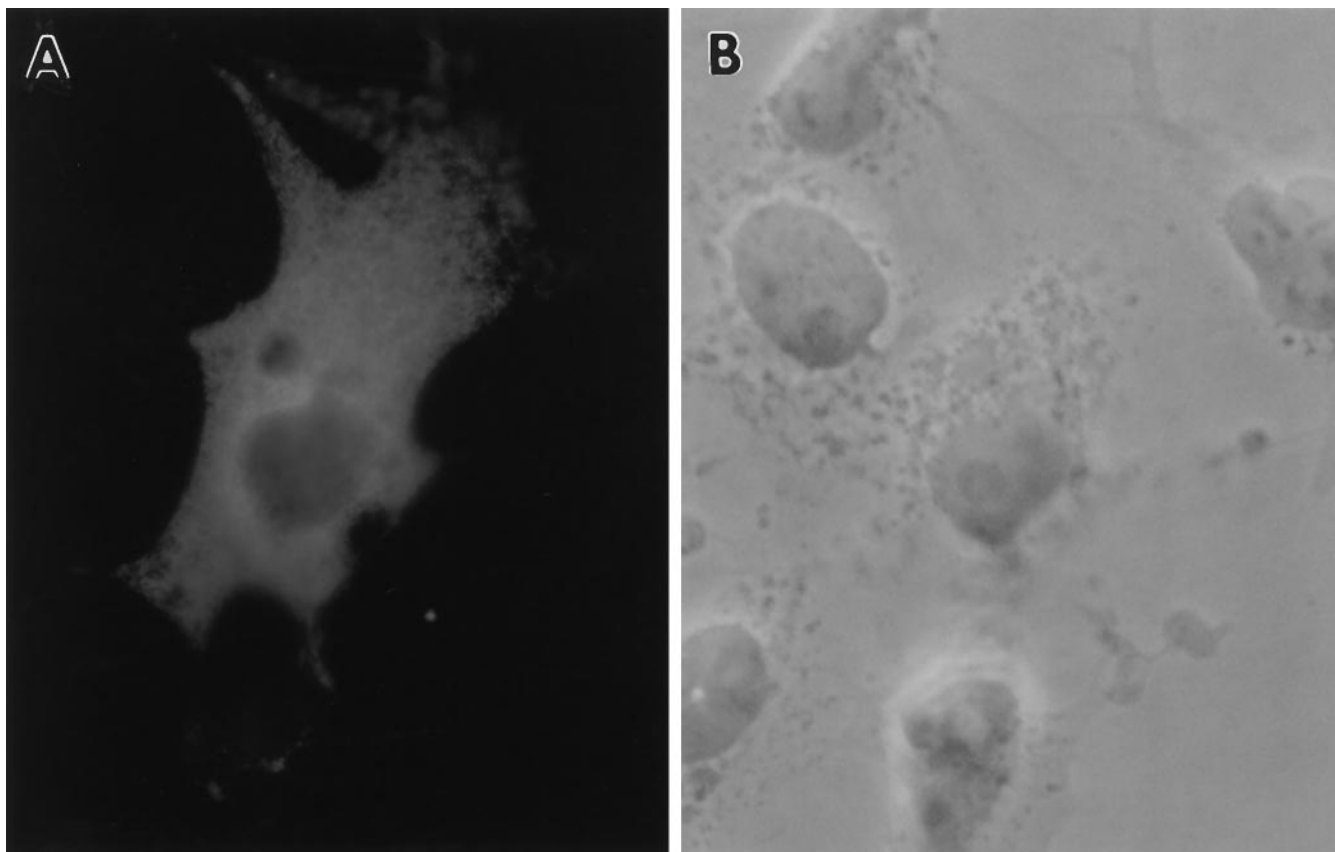


Figure 3. Intracellular localization of Reelin in COS cells. After transfection with pCrIM encoding full-length Reelin under the control of the CMV promoter, COS cells were fixed, permeabilized, and incubated for 1 hr with the primary CR-50 antibody and for an additional 30 min with FITC-tagged anti-mouse secondary antibody. *A*, Fluorescent image of a cell expressing Reelin (80 \times magnification). *B*, Phase-contrast image of the same cell field.

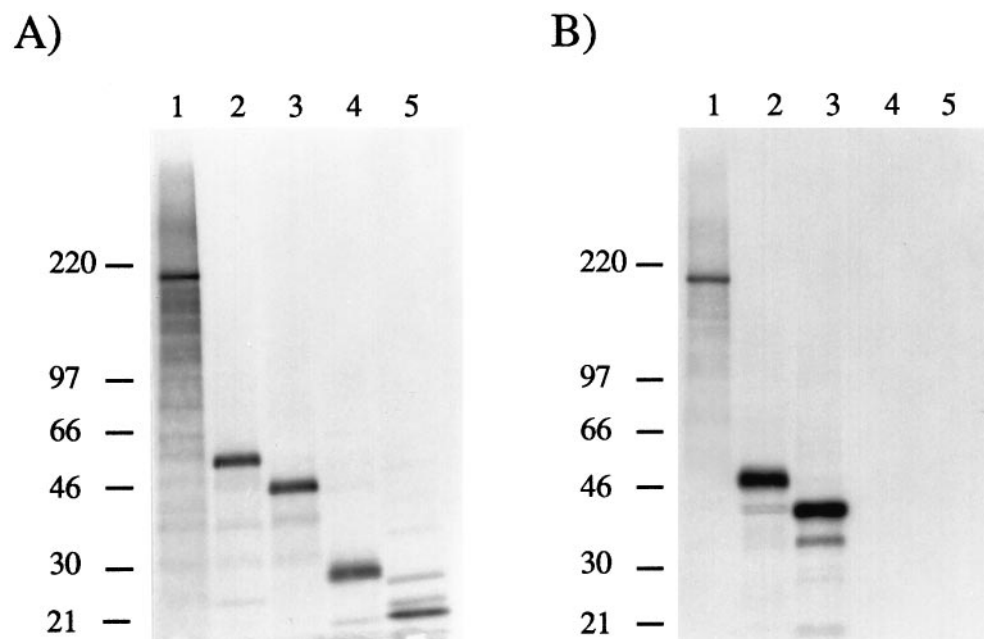


Figure 4. CR-50 epitope mapping. Plasmids encoding truncated Reelin proteins were transcribed *in vitro* by the T7 RNA polymerase and translated by using a rabbit reticulocyte lysate in the presence of [35 S]methionine and [35 S]cysteine. *A*, The products of pCr11837 (206 kDa, lane 1), pCr1462 (51 kDa, lane 2), pCr1407 (46 kDa, lane 3), pCr1250 (27.5 kDa, lane 4), and pCr1194 (22 kDa, lane 5) were analyzed on a 4–12% SDS-polyacrylamide gel. *B*, The same *in vitro* translated proteins as in *A* were subjected to immunoprecipitation with the monoclonal antibody CR-50 and analyzed on a 4–12% SDS-polyacrylamide gel. Because all truncated proteins except those encoded by pCr1194 and pCr1250 are recognized by CR-50, the epitope is located between amino acids 251 and 407 of Reelin.

The C terminus of Reelin is required for secretion

Several independent alleles of the *reeler* mutation have been described. In two strains, *rl* (Falconer, 1951) and *rl^{ts}* (Miao et al., 1994), the mutation is a consequence of a relatively large deletion of *reelin*, which results in a complete loss of expression (Bar et al.,

1995; D'Arcangelo et al., 1995). In contrast, mice of the *rl^{or1}* strain (Guenet, 1981) have a small deletion of a 220 bp exon near the 3' end of *reelin*, which results in a frame shift in the C terminus of Reelin (Curran et al., 1995; Hirotsune et al., 1995; Takahara et al., 1996). The predicted protein product of the *rl^{or1}* gene lacks 205

Figure 5. Reelin is modified and secreted by COS or cerebellar cells. *A*, COS cells (lanes 1–4) were transfected with (lanes 3, 4) or without (lanes 1, 2) pCrIM. The [³⁵S]-labeled supernatant was subjected to immunoprecipitation with no antibody (lanes 1, 3) or with anti-Myc antibody 9E10 (lanes 2, 4). A specific band of ~400 kDa (top arrowhead) was detected only in the sample containing both pCrIM and anti-Myc. The predicted 385 kDa *in vitro* translated [³⁵S]-labeled product of pCrIM was immunoprecipitated with the anti-Myc antibody (lane 5). The protein produced *in vitro* (bottom arrowhead) seems to have a lower molecular mass than that obtained in COS cells. *B*, The supernatant from COS cells transfected with no DNA (lanes 1, 2) or pCrIM (lanes 3, 4), cerebellar cell supernatant (lane 5) or cerebellar cell lysates (lane 6), and *in vitro* translation reaction of pCrIM (lane 7) were labeled with [³⁵S], as described in Materials and Methods, and subjected to immunoprecipitation with the CR-50 antibody. All immunoprecipitates from transfected COS or cerebellar cells migrated at a higher apparent molecular weight (top arrowhead) than the *in vitro* translated product (bottom arrowhead). The position of a 220 kDa molecular weight marker is indicated.

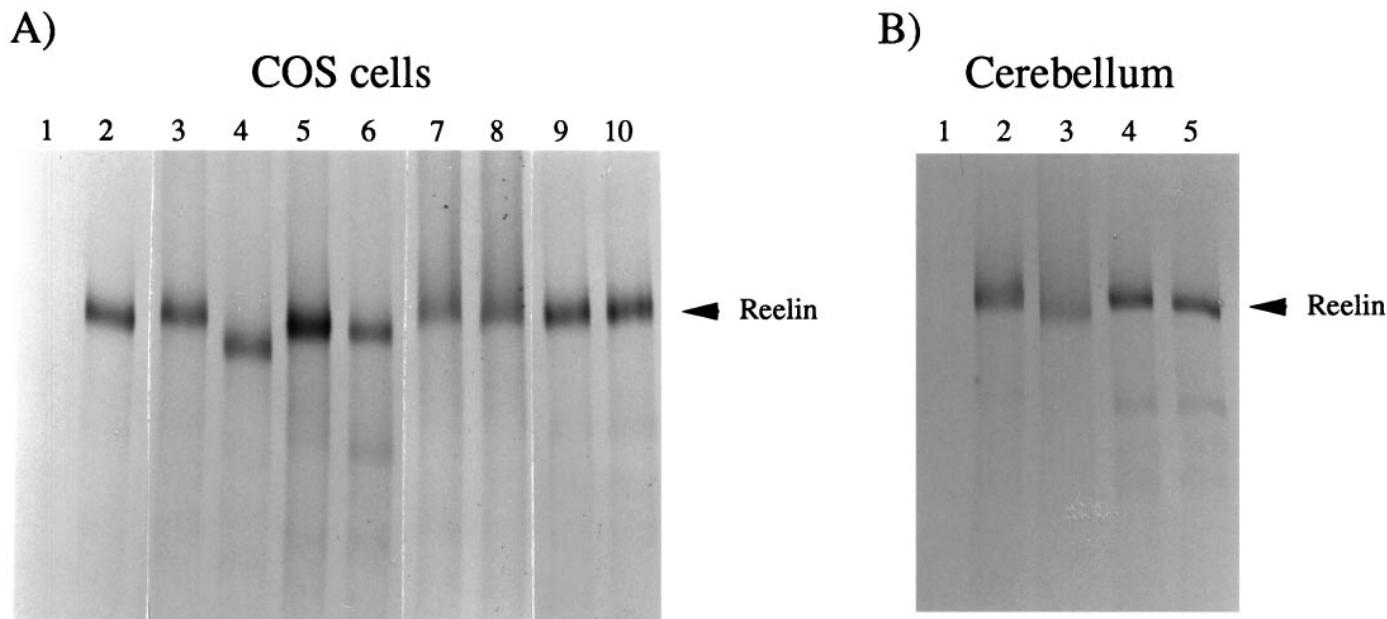
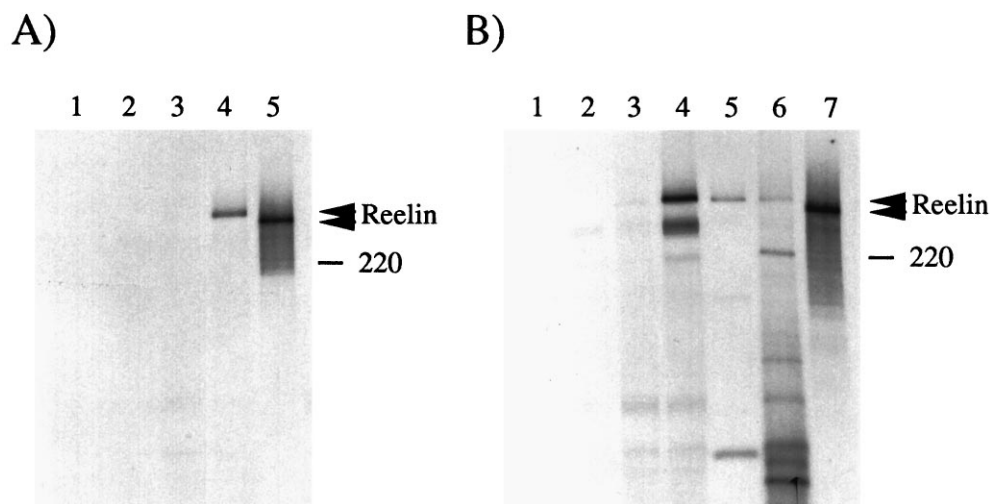


Figure 6. Reelin is glycosylated. *A*, COS cells were transfected with no DNA (lane 1) or with pCrIM (lanes 2–10). Cells were labeled with [³⁵S], and Reelin was immunoprecipitated from the supernatant with the CR-50 antibody. Immunocomplexes were resuspended in gel loading buffer (lanes 1, 2) or in the appropriate glycosidase buffer and incubated with (lane 4) or without (lane 3) PNGaseF, with (lane 6) or without (lane 5) *O*-glycosidase, with (lane 8) or without (lane 7) neuraminidase A, and with (lane 10) or without (lane 9) chondroitinase ABC. After digestion, gel loading buffer was added, and the samples were analyzed on a 4% SDS polyacrylamide gel. *B*, Cerebellar cells were obtained from postnatal day 7 *reeler* (lane 1) or normal (lanes 2–5) mice. Cells were labeled with [³⁵S], and Reelin was immunoprecipitated from the supernatant with the CR-50 antibody. Immunocomplexes were resuspended in gel loading buffer (lanes 1, 2) or in the appropriate glycosidase buffer and digested with PNGaseF (lane 3), neuraminidase A (lane 4), or *O*-glycosidase (lane 5). Samples were analyzed on a 4% SDS polyacrylamide gel.

C-terminal amino acids and contains 70 novel amino acids from an out-of-frame region. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that *reelin* mRNA is present in *rl^{ortl}* mice (Hirotsune et al., 1995), suggesting that the protein also might be present. We noted that the region of Reelin mu-

tated in *rl^{ortl}* contains a short C-terminal region with a very high positive charge that might be required for the function of Reelin. Therefore, we created a truncated version of *reelin* (pCrI3328) lacking a region encoding 133 C-terminal amino acids, which includes this highly charged domain. We confirmed the truncation

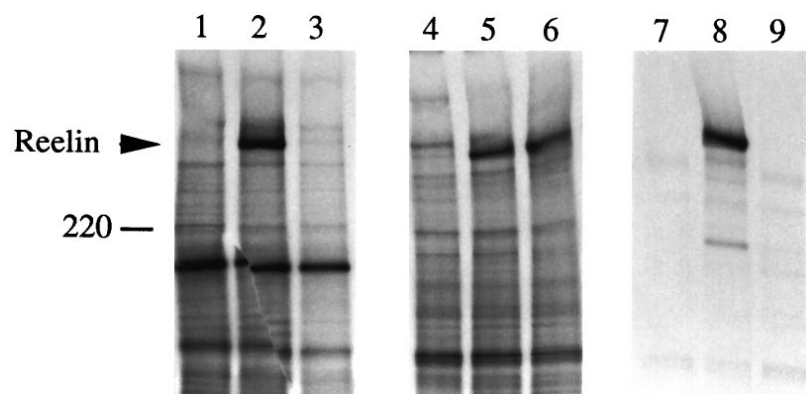


Figure 7. The C terminus of Reelin is required for secretion. COS cells were transfected with no DNA (lanes 1, 4, 7), pCrIM (lanes 2, 5, 8), or pCrI3328 (lanes 3, 6, 9) and labeled with [35 S]. Lysates (lanes 1–6) or supernatants (lanes 7–9) were subjected to immunoprecipitation with anti-rp5 (lanes 1–3) or with CR-50 antibody (lanes 4–9). The anti-rp5 antibody, directed against the C terminus of Reelin, recognized the full-length product of pCrI, but not the truncated product of pCrI3328. CR-50 immunoprecipitated both proteins from the cell lysate, but it only precipitated the full-length protein from the cell supernatant. The migration of a 220 kDa molecular weight marker is indicated.

in the product of this construct with affinity-purified anti-rp5 antibodies directed against C-terminal amino acids 3443–3461 of Reelin. As expected, the anti-rp5 antibodies precipitated Reelin from extracts of COS cells transfected with full-length Reelin (pCrIM), but not from cells transfected with pCrI3328 or from untransfected cells (Fig. 7, lanes 1–3). Bands smaller than 400 kDa are attributable to antibody cross-reactivity. Furthermore, we confirmed that COS cells express high levels of pCrI3328 by immunofluorescence analysis with the CR-50 antibody, which recognizes an N-terminal epitope (data not shown). When the CR-50 antibody was used in the immunoprecipitation assay, both full-length and truncated Reelin were precipitated from extracts of transfected cells (Fig. 7, lanes 4–6). CR-50 also precipitated full-length Reelin from transfected cell supernatants; however, it failed to precipitate the truncated protein (Fig. 7, lanes 7–9). These data demonstrate that the highly charged C terminus of Reelin is required for secretion. Because this region is missing in rl^{ori} , we suggest that the *reeler* phenotype is a consequence of the absence of extracellular Reelin.

DISCUSSION

The data presented here demonstrate that Reelin is a secreted protein that contains the CR-50 epitope. Thus, the neurodevelopmental defect in *reeler* mice is a consequence of the loss of an extracellular signal that is required for the regulation of neuronal migration. This is clearly the case for the *rl* and rl^{tg} mice, because no *reelin* mRNA is expressed in these strains (D'Arcangelo et al., 1995). However, *reelin* mRNA was reported to be present in rl^{ori} mice. The rl^{ori} strain has a deletion in the *reelin* gene that introduces an out-of-frame deletion in the C terminus (Curran et al., 1995; Hirotsune et al., 1995). Interestingly, when we introduced a similar mutation into Reelin, the protein was no longer secreted (Fig. 7), indicating that the C-terminal region is essential for maturation and export. Thus, the rl^{ori} strain also may be missing extracellular Reelin because of a failure in protein transport.

The demonstration that the CR-50 epitope is encompassed within the *reelin* coding sequence allows interpretation of the effects of the CR-50 monoclonal antibody in terms of the function of Reelin. CR-50 recognizes an antigen in normal brain that is absent in *reeler* (Ogawa et al., 1995). The antigen distribution mainly parallels *reelin* mRNA expression in the developing cerebral, cerebellar, and hippocampal cortices. In addition, CR-50 applied extracellularly recognizes cell types, such as Purkinje cells in the cerebellum, that do not express *reelin* mRNA (Miyata et al., 1996a). This could reflect the interaction of extracellular Reelin with a receptor located on the surface of Purkinje cells. CR-50

alters the aggregation pattern of cortical cell cultures *in vitro* (Ogawa et al., 1995), the organization of the Purkinje cell plate in cerebellar explants (Miyata et al., 1996b), and the formation of hippocampal layering *in vivo* (Nakajima et al., 1996). These effects closely resemble the phenotype of *reeler* mice in which Reelin is absent. Our demonstration that CR-50 recognizes a Reelin peptide epitope indicates that the antibody interferes with Reelin activity directly.

In cerebral, cerebellar, and hippocampal cortices, neuronal cells located superficially, near the pia, synthesize and secrete Reelin from the earliest stages of development. Other neuronal cell types, located in deeper positions, may become responsive to the Reelin extracellular signal by virtue of the presence of an as yet unidentified cell surface receptor. These target cells migrate from the ventricular layer toward more superficial layers along radial fibers. The termination of their migration, and therefore their ultimate location, is determined by the Reelin signal. However, the exact outcome of the interaction with Reelin with its receptor is likely to vary according to the cell type.

At early stages of mouse cerebral cortex development (embryonic day 10–12), a preplate composed of Cajal–Retzius and subplate cells is present. The Cajal–Retzius cells are neurons destined to reach the most superficial layer (layer I or marginal zone), where they fully differentiate into horizontal cells (Marin-Padilla and Marin-Padilla, 1982; Derer and Derer, 1990). The subplate neurons are destined to reside in the deepest layer of the cortical plate (layer VIB; Shatz et al., 1988). In normal brain, the Cajal–Retzius cells produce and secrete Reelin into the preplate extracellular environment. The preplate then splits into two components, the marginal zone and the subplate layer, and the cortical plate develops between them (embryonic day 13–16) (Marin-Padilla, 1978). Reelin remains closely associated with the surface of Cajal–Retzius cell bodies and processes (Ogawa et al., 1995), some of which may descend into the cortical plate that is developing underneath (Marin-Padilla, 1978). In the *reeler* brain, Cajal–Retzius cells do not produce extracellular Reelin, the preplate does not split, and the cortical plate develops ectopically underneath subplate neurons. Because the failure of the preplate to split is the earliest event associated with the *reeler* phenotype (Goffinet, 1979), it is possible that subplate neurons represent target cells that are repelled by Reelin. This interpretation is consistent with the observation that dissociated cells from the *reeler* cortex are more adhesive than normal, particularly the cells that are born early (embryonic day 10–12; Hoffarth et al.,

1995). The separation of subplate cell bodies from the Cajal–Retzius cell layer may be a consequence of an active repulsion event associated with cell body or dendrite retraction, or it could result from maturation-dependent translocation of subplate cell bodies, a consequence of apical dendrites anchoring to the Reelin-rich marginal zone (Marin-Padilla, 1988). Alternatively, Reelin might preferentially attract cortical plate neurons to migrate past the subplate toward the marginal layer. This hypothesis requires either that Reelin must be able to diffuse into the developing cortical plate or that Cajal–Retzius processes must come in direct contact with cortical plate neurons. Given the solubility of Reelin demonstrated in the present study and the observation that some Cajal–Retzius cell processes descend into the cortical plate (Marin-Padilla, 1978), this latter hypothesis is also viable.

In the developing hippocampus, Cajal–Retzius-like cells in the marginal zone express Reelin (data not shown), which orchestrates the laminar organization of the hippocampus proper and the dentate gyrus. These cells, like the neocortical Cajal–Retzius cells, are generated very early during development and occupy a superficial layer (Soriano et al., 1994). However, a more detailed analysis of the early cell interactions involved in hippocampus formation is required to formulate a hypothesis on the mode of action of Reelin in this as well as other structures affected by the *reeler* mutation.

In the embryonic cerebellum, Reelin is produced by neuronal precursors in the nuclear transitory zone that are destined to give rise to cells of the deep nuclei and by postmitotic neurons in the deepest part of the external granular layer and in the internal granular layer (D'Arcangelo et al., 1995; Ogawa et al., 1995; Miyata et al., 1996a). No expression was detected in Bergmann glia or in Purkinje cells. In the normal cerebellum, Purkinje cells form a plate underneath the external granule layer, and they develop complex dendritic trees in the molecular layer. Reelin-positive postmitotic granule neurons migrate inwardly, crossing the molecular layer and the Purkinje cell body layer to form the internal granule layer, where they lose Reelin extracellular immunoreactivity (Miyata et al., 1996a). In *reeler* mice, Purkinje cells do not form a cortical plate, but they remain ectopically located in the deep cerebellar nuclear mass. Because Purkinje cells accumulate Reelin on the extracellular membrane (Miyata et al., 1996a), it is conceivable that they express a receptor that mediates an increased adhesion with Reelin-producing granule cells. Recent functional studies demonstrate that the CR-50 antibody interferes with the formation of the Purkinje cell layer (Miyata et al., 1996b). Thus, it is conceivable that a common Reelin-dependent mechanism underlies the formation of both the cortical plate in the cerebral cortex and the Purkinje cell layer in the cerebellar cortex.

A general consideration from all the reported studies is that Reelin is expressed by specific neuronal populations to help other neurons determine their correct positioning in developing laminar structures. So far, Reelin expression or binding activity has not been described in radial fibers despite the fact that the orientation and the end feet of radial fibers are abnormal in the *reeler* brain (Derer, 1979). Recent findings suggest that Cajal–Retzius cells produce a factor that affects radial fiber development (E. Soriano, personal communication). Thus, it is possible that Reelin may affect the formation of the radial glia scaffold indirectly. It is to be hoped that some of the questions concerning the development of laminar organization in brain structures will be resolved by the identification and characterization of proteins that interact with Reelin, including potential cell surface receptors and possibly other extracellular signaling molecules.

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