Reelin is a 3461-residue secreted glycoprotein that plays a critical role in brain development through its action on target neurons. Although it is known that functional reelin protein exists as multimer formed by interchain disulfide bond(s) as well as through non-covalent interactions, the chemical nature of the multimer assembly has been elusive. In the present study, we identified, among 122 cysteines present in full-length reelin, the single critical cysteine residue (Cys2101) responsible for the covalent multimerization. C2101A mutant reelin failed to assemble into disulfide-bonded multimers, whereas it still exhibited non-covalently associated high molecular weight oligomeric states in solution. Detailed analysis of tryptic fragments produced from the purified reelin proteins revealed that the minimum unit of the multimer is a homodimeric reelin linked via Cys2101 present in the central region and that this cysteine does not connect to the N-terminal region of reelin, which had been postulated as the primary oligomerization domain. A surface plasmon resonance binding assay confirmed that C2101A mutant reelin retained binding capability toward two neuronal receptors apolipoprotein E receptor 2 and very low density lipoprotein receptor (VLDLR) on the target neurons [3–5]. Binding of reelin to these receptors induces tyrosine phosphorylation of Dab1, an adaptor protein that is associated with the intracellular region of receptors (6, 7), which is thought to be the critical step in the reelin signaling pathway (for a review, see Ref. 2). 

Reelin protein consists of 3461 amino acid residues including a signal sequence, a “reeler” domain that is also found in F-spondin, a characteristic “reelin repeat” that appears roughly eight times, and a highly basic C-terminal region (1) (see Fig. 1A). Each reelin repeat is 350–390 amino acids in size and can be subdivided into a central EGF module flanked by two subrepeats, A and B (1). Structure determination of a reelin repeat domain revealed that it shows a compact horseshoe-like structure in which subrepeats A and B make direct contact with each other despite the intervention by a central EGF module (8). Furthermore, tandem arrangement of consecutive reelin repeats results in a rodlike structure as evidenced by the crystal structure of a two-domain fragment as well as the electron micrographs of a four-domain fragment (8, 9). The crystal structure of the reeler domain of mouse F-spondin has been determined (10, 11), although the structure of the same domain of reelin has not been solved. Despite the rapid accumulation of three-dimensional structure information for the building blocks of reelin, the molecular architecture of the full-length reelin protein remains poorly defined.

In the reelin primary structure, two regions have been implicated in its biological activity. It was first reported that a functionally important segment resides in an N-terminal region immediately following the F-spondin-like domain because the epitope for a function-blocking anti-reelin antibody CR-50 has been mapped to this region (residues 230–346) (12–15). How-
Covalent Homodimerization of Reelin at Central Region

ever, this region does not seem to bind directly to the receptors, leading to an assumption that CR-50 blocks reelin activity in an indirect manner (5, 16). As the N-terminal region of the reelin has the potential to form a non-covalent homopolymer, which can be inhibited by CR-50, it was postulated that the self-association of reelin protomers through the N-terminal region plays an important role in maintaining its active conformation (17). The second region of functional importance is the central portion of the molecule encompassing the third to the sixth reelin repeat (R3—6). A recombinant fragment corresponding to this segment binds directly to the receptors and can induce biological signaling in the cultured neurons (8, 18, 19). In fact, recent mutagenesis and crystallographic studies revealed that the receptor binding is mediated by a single lysine residue (Lys2467) located in the R6 (9, 20). Furthermore, a recent finding that the cleavage between R2 and R3 by a specific protease greatly diminishes the signaling activity of reelin (21) suggests that the functional integrity is maintained only when both regions (i.e. CR-50 epitope and R6) are present in one molecule.

Reelin is known to form a homo-oligomer via intermolecular disulfide bond(s). Disulfide-linked oligomers have been detected in cell culture supernatant from 293T cells transiently transfected with full-length reelin, in the embryonic brain homogenates prepared from the cerebral cortex, and in human plasma (16, 22). However, neither the number of reelin protomers contained in the functional oligomer nor the Cys residue(s) responsible for the cross-linking has been determined. There are multiple reasons for the difficulty in the biochemical dissection of reelin protein. First, the unusually large molecular mass of a monomer (~400 kDa) precludes the simple size estimation of multimers by SDS-PAGE where they usually do not enter the acrylamide gel under the non-reducing condition. Second, reelin protein produced recombinantly in cells is proteolytically processed at multiple sites and exists as a mixture of various fragments, further complicating the molecular identification. Finally, it has been difficult to obtain pure reelin protein in the large quantity required for a chemical characterisation. As a result, the molecular characterization of reelin protein in nearly all experiments relies on the immunological detection using a limited number of anti-reelin antibodies performed on crude samples such as cell culture supernatants. Importantly, antibody recognizing the central portion of reelin has not been available until recently.

To circumvent these difficulties, we developed a purification protocol for recombinant reelin using an affinity tag system developed in our laboratory (23). Moreover, we utilized monoclonal antibodies recognizing the central portion of reelin established by Goffinet and co-workers (24). By combining these molecular tools with the three-dimensional structural information, we succeeded in identifying the minimum structural unit of reelin as a disulfide-bonded homodimer. Contrary to the traditional view, reelin dimerization was mediated by the cysteine residue present in the central region of reelin near its receptor-binding site. Although the mutant reelin deficient in the critical cysteine residue still formed non-covalently assembled multimer and bound to receptors, it exhibited greatly diminished signaling activity toward the cultured neurons.

EXPERIMENTAL PROCEDURES

Construction of Multiple Sequence Alignment and Homology Model Building—Based on the sequence alignment for the reelin repeat segments made by Ichihara et al. (25), we have reported a modified version of alignment (26). The alignment was further refined by incorporating the structural information of reelin repeats to maximize the alignment of the predicted β-strands. Homology model building was performed with the program MODELLER (27) using the R5 structure (Protein Data Bank code 2E26) as a template.

Protein Expression, Immunoprecipitation, and Western Blotting—The expression vector for the N-terminally tagged full-length reelin was prepared as described previously (23). The substitutions of cysteine to alanine were introduced into the expression vectors by using the QuikChange strategy (Stratagene). All constructs were verified by DNA sequencing. Transient expression of the full-length reelin (wild type and mutants) in 293T cells was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. Cell culture supernatant was mixed with P20.1 IgG-immobilized Sepharose 4 Fast Flow (GE Healthcare) (23) and rotated at 4°C for 1 h. After washing with 20 mM Tris, pH 7.5, 150 mM NaCl (Tris-buffered saline (TBS)), proteins retained on beads were eluted with 2× SDS-PAGE sample buffer without reducing reagents followed by heat treatment at 95°C for 3 min. Eluates were divided into two equal parts, further incubated with or without 100 mM DTT, and subjected to SDS-PAGE (5% acrylamide gel) followed by electrophoretic transfer onto PVDF membranes. The PVDF membranes were blocked with 5% bovine serum albumin (BSA) in TBS containing 0.05% Tween 20 (TBS-T) and probed with G10 (1 μg/ml) (Chemicon) (28). Blots were visualized using an ECL procedure (GE Healthcare) and recorded using a LAS-1000 minisystem (Fuji, Tokyo, Japan).

Purification of Full-length Reelin—For stable expression, CHO Lec 3.2.8.1 cells (29) were transfected with plasmids encoding N-terminally tagged full-length reelin (wild type or C2101A mutant) by electroporation using a Gene Pulser (BioRad). Cells were plated on 96-well plates and selected for resistance against 1.5 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). The clones with the highest secreted levels of full-length reelin were cultured in roller bottles (Corning). The cell culture supernatant was concentrated by using Acquacide II (Calbiochem) prior to the following purification procedure. Proteins were purified using the P20.1-immobilized Sepharose as described previously (23). Eluted proteins were dialyzed against TBS containing 2 mM CaCl2, and concentrated by ultrafiltration (100-kDa molecular mass cutoff). Protein concentration was determined by BCA assay (Pierce) using BSA as a standard.

Limited Proteolysis of Full-length Reelin by Trypsin and Characterization of Proteolytic Fragments by Western Blotting—Purified wild-type or C2101A mutant reelins were digested with 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Roche Applied Science) in TBS at protease to protein ratios of 1:10, 1:30, 1:100, and 1:300 (w/w). The reaction was allowed to proceed at 20°C overnight. The reaction was stopped by an addition of SDS-PAGE sample buffer followed by
heating at 95 °C for 3 min. Protein samples were separated by 5–20% SDS-PAGE under either a reducing or non-reducing condition. The proteins were transferred from gels to a PVDF membrane and incubated with anti-reelin monoclonal antibody R4B, R5A (both 1:500 diluted ascites fluid; gifts from Dr. André M. Goffinet) (24), or G10 (Chemicon) followed by the ECL visualization described above.

**Size Exclusion Chromatographic Analysis**—Full-length reelin proteins purified with P20.1-immobilized Sepharose were subjected to size exclusion chromatography on a Superose 6 10/300 GL column equilibrated with 150 mM NaCl, 2 mM CaCl$_2$, 20 mM Tris, pH 7.5 at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected and analyzed by Western blotting with G10 as described above.

**Solid-phase Binding Assay**—A solid-phase receptor binding assay was carried out as described previously (8). Briefly, a monoclonal antibody against human growth hormone (hGH) (clone HGH-B, ATCC) was coupled to CNBr-activated Sepharose4B (GE Healthcare). Culture supernatants of 293T cells transfected with the expression vector encoding ApoER2 ectodomain fused C-terminally to hGH (hGH-ApoER2 EC4–6) (9) were mixed with HGH-B-immobilized Sepharose. Beads were washed with TBS and further incubated with cell culture supernatants containing full-length reelin at 4 °C for 2 h. After washing with TBS, proteins retained on beads were eluted with 2x SDS-PAGE sample buffer and subjected to immunoblotting with G10.

**In Vivo Biotinylation of Reelin Receptors and Surface Plasmon Resonance Analysis**—In vivo biotinylation of soluble ectodomain fragments of reelin receptors within the secretory pathway of mammalian cells was carried out using the experimental design described by Mize et al. (30). An expression plasmid for the Escherichia coli biotin ligase BirA (a gift from Dr. John Strouboulis, Erasmus Medical Center, Rotterdam, The Netherlands) (31) was modified to include a signal sequence of the bovine prolactin at the N terminus and a KDEL endoplasmic reticulum retention signal at the C terminus to yield sBirA-KDEL. To generate expression constructs for soluble and enzymatically biotinylated reelin receptors, a 22-residue biotin acceptor sequence (BAS) (SSLRQLDSQKMEWRSNAGGSV) was appended to the C terminus of GH-ApoER2 EC4–6 or GH-VLDLR (containing residues 28–750 of human VLDLR) was appended to the C terminus of GH-ApoER2 EC4–6) (19, 34), the three bands are likely to represent the full-length reelin receptor (N-t site) and R6 and R7 (C-t site) (19, 34), the three bands are likely to represent the full-length reelin receptor (N-t site) and R6 and R7 (C-t site). Considering the proteolytic processing of reelin that is known to occur at the boundaries between R2 and R3 (N-t site) and R6 and R7 (C-t site) (19, 34), the three bands are likely to represent the full-length reelin (3461 residues), N-R6 fragment (~2670 residues), and N-R2 fragment (~1220 residues), respectively. Under a non-reducing condition, the major G10-reactive band was described previously (20). Kinetic constants were calculated from sensorgram curves using the BIAevaluation software (GE Healthcare) by fitting with a 1:1 Langmuir model.

**Dab1 Phosphorylation Assay**—Induction of tyrosine phosphorylation of Dab1 in mouse cortical neurons was measured as described previously (32). Briefly, cortical neuronal cells were incubated with a series of dilute media containing full-length reelin for 20 min at 37 °C and harvested. Total cell lysates were analyzed by Western blotting using anti-phosphotyrosine antibody (4G10, Millipore) and anti-Dab1 antibody (33).

**Immunocytochemistry**—Primary neurons cultured for 4 days were incubated with cell culture supernatants containing either wild-type or the C2101A mutant version of full-length reelin for 30 min and fixed with 4% formaldehyde. Cells were incubated with 2 μg/ml CR-50 and FITC-conjugated cholera toxin B subunit (Invitrogen) without permeabilization treatment, washed with phosphate-buffered saline, and incubated with anti-mouse IgG-Alexa Fluor 594 (Invitrogen). Samples were examined with an LSM501 confocal laser-scanning microscope (Carl Zeiss, Tokyo, Japan).

**RESULTS**

**Determination of Cysteine Residue(s) Responsible for Intermolecular Disulfide Bond Formation**—As reelin exists as a covalently linked oligomer *in vivo*, at least one cysteine residue should form a specific intermolecular disulfide bridge. Among a total of 122 cysteine residues present in mouse full-length reelin sequence, 100 are predicted to form intramolecular disulfide bridges because of their conservation with the experimentally determined disulfide bonds in reelin repeats (Fig. 1B, denoted by red lines). Four cysteines present in the F-spondin-like domain are also predicted to form intradomain disulfide bonds found in the corresponding domain in mouse F-spondin (10, 11) and are unlikely to participate in the intermolecular disulfide bridge. Moreover, we could further eliminate 10 cysteine residues from the list when we inspected the predicted location of those residues in the homology models of each domain. Five pairs of cysteine residues, namely Cys$_{223}$-Cys$_{226}$ and Cys$_{244}$-Cys$_{343}$ in RX, Cys$_{462}$-Cys$_{540}$ in RZ, Cys$_{2718}$-Cys$_{2733}$ in R7, and Cys$_{3134}$-Cys$_{3215}$ in R8, are all closely positioned in the homology models (α-distances of 5.8, 4.5, 6.7, 4.7, and 3.6 Å, respectively), suggesting intramolecular disulfide bond formation (Fig. 1B, blue lines). As a result, only eight cysteines (Cys$_{256}$, Cys$_{902}$, Cys$_{1199}$, Cys$_{1839}$, Cys$_{1857}$, Cys$_{2101}$, Cys$_{2916}$, and Cys$_{2971}$) remain to be potentially available for the intermolecular disulfide bridging and thus were subjected to further analysis.

When wild-type full-length reelin was transiently expressed in 293T cells and analyzed by immunoblotting using an anti-reelin antibody G10 directed against the N-terminal portion (RX; residues 199–244) (28), three reactive bands with apparent molecular masses of ~400, ~300, and ~150 kDa were visible in the reducing gel (Fig. 2A, lane 10). Considering the proteolytic processing of reelin that is known to occur at the boundaries between R2 and R3 (N-t site) and R6 and R7 (C-t site) (19, 34), the three bands are likely to represent the full-length reelin (3461 residues), N-R6 fragment (~2670 residues), and N-R2 fragment (~1220 residues), respectively.
found near the top of the gel along with a band with an apparent molecular mass of 350 kDa at varying degrees of intensity (Fig. 2A, lane 1). The mobility of the bands indicates that they represent disulfide-bonded reelin multimer and a dissociated monomer, respectively. Next, we mutated each of the eight potential candidate cysteine residues described above to alanine and assessed their oligomer formation. All mutants were secreted into the medium at a level comparable with the wild-type reelin (Fig. 2A, lanes 11–18). However, analysis under a non-reducing condition revealed that one mutant (C2101A) had lost its ability to assemble into the high molecular weight oligomer and migrated solely as a monomer (Fig. 2A, lane 7). This result clearly shows that Cys2101 is involved in the intermolecular disulfide bridge formation and that other cysteines are dispensable for the assembly of covalently linked reelin oligomers.

Analysis of Purified Full-length Reelin—The critical involvement of Cys2101 in the interchain disulfide bond formation provided a way to investigate the molecular organization of reelin maintained by disulfide bridges. Thus, we expressed and purified both wild-type and C2101A mutant versions of full-length reelin protein and analyzed their cross-linking states by comparing the electrophoretic mobilities under reducing and non-reducing conditions (Fig. 2B). Because the level of full-length reelin secreted from transfected cells was generally low (<50 μg/liter), a recently developed affinity tag sequence was attached at the N terminus to facilitate one-step affinity purification from the culture medium using P20.1 antibody (23, 35). Reelin samples thus purified were analyzed by SDS-PAGE under a reducing condition and subjected to silver staining to visualize total proteins. As shown in Fig. 2B, both wild-type (lane 1) and C2101A mutant (lane 3) reelins migrated primarily

---

**Covalent Homodimerization of Reelin at Central Region**

**FIGURE 1. Primary structure of reelin.** A, domain organization of reelin protomer. Subdomains are labeled within or on top of the rectangles that are drawn roughly proportional to their lengths. Positions for the known proteolytic processing sites (N-t and C-t) and eight candidate Cys residues are also indicated. B, multiple sequence alignment of reelin repeats. Subrepeat sequences are arranged in the order of their appearance in the primary structure of full-length reelin. Positions for β-strand segments are denoted by horizontal black bars at the bottom of the alignment and labeled A–K. Cysteines with the experimentally confirmed or structurally predicted disulfide linkages are connected by red or light blue lines, respectively. Predicted lone cysteines are highlighted in yellow. Regions containing epitopes for G10, R4B, and R5A are boxed in blue, magenta, and green, respectively. Note that the sequence for RY and R1A are imperfect and are complementary to each other, suggesting that they form a single folding unit despite the intervention by the RZ segment. res, residue.
Covalent Homodimerization of Reelin at Central Region

A. Non-reducing

Reducing

WT C256A C982A C1195A C1857A C2101A C2191A C2971A kDa 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 m Full length N-R6 N-R2 multimer

B. Silver stain

G10 blot

WT C2101A WT C2101A R NR R NR R NR R NR kDa 1 2 3 4 5 6 7 8

FIGURE 2. Cys\(^{2101}\) of reelin participates in intermolecular disulfide bond. A, effect of alanine substitution of candidate cysteine residues on multimer formation. HEK 293T cells were transiently transfected with full-length reelin expression constructs with the indicated mutation. The secreted reelin proteins were immunoprecipitated (IP) by anti-tag monoclonal antibody P20.1, subjected to SDS-PAGE under either a non-reducing (lanes 1–9) or reducing (lanes 10–18) condition, and analyzed by Western blotting (WB) using anti-reelin G10. B, C2101A mutant reelin does not form a high molecular weight multimer. Wild-type (WT) and C2101A mutant reelins were purified from the culture supernatants of CHO Lec 3.2.8.1 cells stably expressing each protein and analyzed by SDS-PAGE either under a reducing or non-reducing condition using a 5–20% gradient gel followed by silver staining (lanes 1–4) or G10 immunoblotting (lanes 5–8). Asterisks denote contaminating serum albumin, m, multimer.

as doublet bands of >250 kDa (Fig. 2B, lanes 1 and 3). No other major proteins were present in the preparation except for the contaminating serum-derived albumin (asterisk). Western blotting revealed that both of the doublet bands were recognized by the antibody G10 directed against an N-terminal region of reelin (lanes 5 and 7), suggesting that they correspond to full-length reelin (upper band) and N-R6 fragment (lower band), respectively. Because we used as an expression host CHO Lec 3.2.8.1 cells, which produce glycoproteins with structurally homogeneous glycan chains (29), the purified reelin protein exhibited sharp bands on SDS-polyacrylamide gels compared with more diffuse bands observed when expressed in 293T cells (Fig. 2A). Although the electrophoretic behavior of wild-type and C2101A mutant reelins was nearly identical under the reducing condition, they differed markedly under the non-reducing condition; wild-type reelin formed high molecular weight multimers that could not enter the gel (denoted by “m”), whereas C2101A mutant primarily migrated as a monomer (Fig. 2B, lanes 2, 4, 6, and 8). We therefore concluded that the C2101A mutant is indeed devoid of interchain disulfide bonds.

Next, the purified proteins were digested with trypsin at various enzyme to substrate ratios and analyzed by SDS-PAGE under a reducing condition followed by silver staining (Fig. 3A, “Reducing”). Trypsin digestion of the wild-type reelin produced in addition to the original doublet (bands 1 and 2) a total of eight new fragments (bands 3–10) at varying degrees depending on the concentration of the enzyme (lanes 2–5). Similarly, trypsin digestion of the C2101A mutant reelin yielded an identical set of eight fragments with the same trypsin concentration dependence as the wild type (lanes 7–10). The similarity in the protease susceptibility indicates that the mutation does not cause gross structural disturbance of the reelin protomer.

The same samples were then analyzed under a non-reducing condition to investigate the interchain disulfide connectivities (Fig. 3A, “Non-reducing”). For the C2101A mutant (lanes 16–20), the SDS-PAGE pattern of the digests was essentially the same as that obtained under the reducing condition (lanes 6–10) with bands 1′–10′ apparently corresponding to bands 1–10 in the reducing gel. This clearly shows the absence of disulfide bridges connecting the major proteolytic fragments produced from the C2101A mutant and confirms the predominant role of Cys\(^{2101}\) in the covalent intermolecular cross-linking. In contrast to the mutant, analysis of the wild-type reelin digests under a non-reducing condition (lanes 11–15) resulted in a distinct band pattern. Specifically, bands 1′–4′ and 6′ seen with the C2101A mutant disappeared, whereas four new bands (bands A–D) with larger sizes appeared together with the high molecular weight multimer at the top of the gel. As bands A–D showed up only after the trypsin treatment, they cannot be composed of bands 1′ (full length) and 2′ (N-R6) that were already present in the starting material. Therefore, it is likely that bands A–D represent multichain fragments composed of polypeptides corresponding to missing bands 3′, 4′, and 6′ and that they are disulfide-bonded through Cys\(^{2101}\).

Immunoblot Analyses of Tryptic Fragments from Wild-type Full-length Reelin—To determine the disulfide bond pairing involving Cys\(^{2101}\), we needed to analyze the behavior of the fragments containing this residue. Two monoclonal antibodies established by Goffinet and co-workers (24) were ideal for this purpose because their epitopes lie very close to Cys\(^{2101}\). They were raised against an antigen corresponding to residues 1796–2115, and epitopes for R4B and R5A were mapped to regions 1810–1825 in subrepeat B of R4 and residues 1985–2058 in subrepeat A of R5, respectively (24) (Fig. 1B). The tryptic digests of the wild-type reelin described above were subjected to the immunoblot analyses using these antibodies as well as G10 to estimate the location of each fragment in the reelin primary structure. First, digests were separated under a reducing condition and probed with three antibodies (Fig. 3B, “Reducing”). Among the 10 polypeptides in the digest, antibodies R4B and
R5A commonly recognized bands 1, 2, 3, 4, and 6 (lanes 1–10). Because a small amount of reactive band 4 was already present in the purified reelin prior to the trypsin treatment (lanes 1 and 6), this 150-kDa fragment likely represents R3–6 fragment known to exist in the physiological condition due to the proteolytic cleavage occurring at the R2/3 and R6/7 boundaries (19). Interestingly, bands 8 (76 kDa) and 9 (38 kDa) were exclusively recognized by R4B and R5A, respectively. These two fragments were produced simultaneously with a concomitant disappearance of band 6, indicating that they originated from this 120-kDa fragment. It is also obvious from the molecular weight that band 6 is a subfragment of band 4 (i.e., R3–6). Compared with the blot probed with the antibodies against the central region of reelin, the G10 blot showed a different set of reactive bands including bands 5 and 7 (lanes 11–15). Although some of the G10-reactive bands (e.g., bands 6.1, 8.1, and 9.1) had electrophoretic mobilities similar to those of fragments from the central region (i.e., bands 6, 8, and 9, respectively), they were produced at a different trypsin concentration and thus represent distinct fragments.

The interchain disulfide bonding pattern could be deduced when the same digests were resolved under a non-reducing condition (Fig. 3B, “Non-reducing”). It is clear that all fragments containing the R4B/R5A epitope region are linked by a disulfide bond(s) to constitute larger fragments corresponding to bands A, B, and D (lanes 16–25), whereas many G10-reactive poly-
Covalent Homodimerization of Reelin at Central Region

The identities of bands were deduced as described in the text and are schematically drawn as in Fig. 1A. The theoretical molecular mass for each fragment is shown next to the rectangle with the putative amino acid residue numbers in parentheses. The position of Cys\(^{2101}\) is indicated by a vertical red line at the top. Epitope segments for three antibodies (G10, R4B, and R5A) are denoted as bars in the background. At the bottom, the possible arrangement of the reelin multimer is shown with the partially formed disulfide bonds or non-covalent lateral association represented by dotted lines.

predicted to be trypsin-sensitive, we could assign the location for the major fragments in the reelin primary structure (Fig. 4). As described above, bands 1, 2, 4, and 5 can be confidently assigned as full-length (N-R8), N-R6, R3–6, and N-R2 fragments, respectively. As band 3 is smaller than band 2 by a size roughly corresponding to one reelin repeat, we predict that it represents a fragment corresponding to N-R5. Cleavage of band 4 (R3–6) at the same site would produce R3–5, whose predicted size matches with band 6 (120 kDa). The exclusive presence of R4B and R5A epitopes in bands 8 and 9, respectively, indicates the presence of a trypsin-sensitive site in the region between residues 1826 and 1984. Double lysines at residues 1935 and 1936 are putatively assigned as the cleavage site to split bands 8 and 9 from band 6 (R3–5) because they are located at the tip of the flexible J-K loop (Fig. 1B), and the expected molecular weights agree with the observed sizes (Fig. 4). In contrast to these fragments that originated from the central region, all G10-reactive fragments are expected to be the subfragments of band 5 (N-R2) considering the very N-terminal location of the epitope. Assuming that they all retain an intact N terminus, C-terminal truncation positions were estimated based on the apparent molecular weight of each fragment. Thus, we putatively assign bands 6.1, 7, 8.1, and 9.1 as N-terminal fragments terminating at Lys\(^{1112}\), Arg\(^{834}\), Arg\(^{647}\), and Lys\(^{321}\), respectively (Fig. 4).

The identities of the multichain fragments of larger sizes (bands A–D) are more difficult to determine because the size estimation by the electrophoretic mobilities cannot be accurate for such high molecular weight proteins. Nevertheless, the pattern of appearance under each condition combined with the molecular sizes strongly suggest that bands A, B, and D are composed solely of the central fragments linked via Cys\(^{2101}\), representing R3–6 homodimer, R3–6/R3–5 heterodimer, and R3–5 homodimer, respectively (Fig. 4). Conversely, band C seems to contain N-terminal fragments only and may represent the dimerized form of N-R2 fragment (i.e. band 5). In contrast to the Cys\(^{2101}\)-mediated dimerization, however, the efficiency of this dimerization may be low because the majority of band 5 stays as a monomer under the non-reducing condition (as band 5\(^{1/10}\)). As elimination of any single cysteine residue within the N-R2 region did not abolish multimerization (Fig. 2A, lanes 2–4), it is possible that this N-terminal dimerization occurs through several cysteines at limited efficiency.

Biological Activity of Dimerization-deficient Reelin—The disappearance of covalently linked multimer in C2101A mutant suggests that the mutation may affect the overall multimeric assembly. However, size exclusion chromatography analysis of C2101A reelin revealed that it exists as a high molecular mass complex similar to the wild-type reelin; the wild-type reelin eluted as a relatively sharp peak at around the 2,500–600-kDa region on a Superose 6 column, whereas the mutant showed a slightly broader size distribution of 2,500–400 kDa (Fig. 5). This result suggests that full-length reelin can assemble into a non-covalent multimer even in the absence of the Cys\(^{2101}\)-mediated disulfide bond albeit with a slightly reduced average size. To evaluate the functional significance of Cys\(^{2101}\)-mediated homodimerization, we assessed the receptor binding activity of C2101A mutant by pulldown assay. As shown in Fig. 6A,
C2101A mutant reelin bound to the soluble ectodomain fragment of ApoER2 at an efficiency similar to that of the wild-type, indicating that the covalent dimerization via Cys2101 is not essential for the receptor binding activity of reelin. This observation is consistent with our previous finding that the monomeric fragment composed of R5 and R6 (R5–6) is capable of binding to the receptor (9). However, it is possible that the mutant reelin has reduced binding affinity toward the receptor because the pulldown experiment was performed at a fixed concentration of reelin and cannot be used for the quantitative comparison. We therefore performed surface plasmon resonance (SPR) experiments on the interaction between reelin and its receptors, ApoER2 and VLDLR. To this end, receptor ectodomain fragments engineered to contain biotinylation sequence at the C terminus were produced in mammalian cells and immobilized onto a streptavidin-coated sensor chip surface. Fig. 6B shows the binding sensorgrams of full-length reelin to the immobilized receptors. When the data were fitted using a single binding site model, the dissociation constants (K_D) of the wild-type reelin multimer toward ApoER2 and VLDLR were 0.11 and 0.86 nM, respectively. These values are in good agreement with those obtained by Andersen et al. (36) using the same type of assay. When C2101A mutant reelin was subjected to the SPR experiments, we observed binding to both receptors with a concentration dependence similar to that of wild-type reelin (Fig. 6B, lower panels). However, the affinity values derived from the multicurve fitting were 5–6-fold lower than those of the wild type for both receptors (i.e. 0.62 and 4.6 nM for ApoER2 and VLDLR, respectively). This reduction in the receptor binding activity may be due to the lower average size of the multimer because the valency of the analyte (solution-phase binder) in SPR experiments can greatly affect the apparent solution affinity.

Next, we checked whether the mutant reelin retains biological activity in a more physiologically relevant assay system using cultured neurons. Primary cultures of neurons derived
from the cortex of mouse embryonic brain were incubated with the cell culture supernatants containing wild-type or C2101A mutant reelin followed by evaluation of the tyrosine phosphorylation of cellular Dab1. To our surprise, C2101A mutant could not induce Dab1 phosphorylation even when undiluted culture supernatant was used, whereas Dab1 phosphorylation was clearly observed with wild-type reelin upon up to 4-fold dilution (Fig. 7, top panel). Immunoblot analysis of the supernatants with G10 antibody revealed that the there was no difference in the expression level of reelin protein between wild type and C2101A mutant (Fig. 7, bottom panel), indicating that the activity of the mutant was less than ¼ of the wild-type activity. The lack of signaling activity of the mutant prompted us to check whether C2101A reelin successfully interacted with the cultured neurons. As shown in Fig. 8, binding of wild-type reelin to the neuronal membrane was confirmed by the immunostaining with CR-50, whereas almost no CR-50 positive signal was visible in cells treated with C2101A mutant. Again, incubation with up to 4-fold diluted culture supernatant containing wild-type reelin resulted in a detectable level of CR-50 staining, indicating that the effective affinity of the C2101A mutant reelin to the neuronal cell surface is at most ¼ (but can be much lower) of that of the wild-type protein.

**DISCUSSION**

It is known that reelin forms a covalent dimer via intermolecular disulfide bond(s) (16, 22). In the present study, we determined the critical cysteine residue participating in the formation of the covalent dimer and showed that the dimer formation via this cysteine residue is required for the full biological activity of reelin. Careful inspection of the primary sequence guided by the structural information extracted eight potential candidates for the intermolecular disulfide formation of 122 cysteines, and mutational experiments identified Cys2101 present in the fifth reelin repeat as the sole responsible residue. As Cys256 included in the candidate list was the only cysteine residue present in the epitope segment for the CR-50, an antibody that is known to disrupt oligomerization of reelin (17), we initially expected this residue to be responsible for the interchain disulfide. However, mutation of Cys256 to Ala did not affect the covalent dimer formation of full-length reelin. In fact, this residue is unlikely to be functionally important because it is not conserved in human reelin. In contrast, Cys2101 is conserved across species from sea urchin, Ciona, and lancelets to humans. Furthermore, structural consideration also supports the involvement of Cys2101 in the homodimer formation. In the crystal structure of R5–6 fragment, this residue is located on a surface-exposed loop at the top ridge of R5A domain with its side chain pointing outward (9). Although R5–6 region seems to adopt a stiff rodlike structure inferred by electron microscopy (8), the location of Cys2101 is compatible with the approach of two reelin molecules in either an antiparallel or diagonal fashion to form the disulfide-linked dimer. Moreover, Cys2101 is located at a molecular face opposite from the receptor-binding site (i.e. Lys2467) (9, 20), allowing the resultant dimer to bear well exposed receptor-binding sites.

The fact that C2101A mutant reelin still oligomerizes (Fig. 5) strongly suggests that there is another self-association site(s) present in the full-length reelin, most likely in its N-terminal region. As C2101A mutation led to a great reduction in the signaling activity, however, it is clear that a multimeric state alone is insufficient to keep the reelin fully active. It seems that a special higher order structure maintained by both covalent and non-covalent intermolecular interactions is required for the full activity. Consistent with this view, it has been reported that the complete cleavage of the N-t site in full-length reelin results in greatly reduced activity (21). One can envision such “special higher order structure” by side-by-side joining of disulfide-bonded dimers (Fig. 4, bottom) similar to what has been observed in the high molecular weight multimerization of the plasma protein von Willebrand factor (37).
almost infinite lateral propagation of von Willebrand factor multimers that could become >20,000 kDa in size, however, gel filtration experiments suggest that the reelin multimer contains a much smaller number of molecules, probably 10 monomers at most (Fig. 5). There may be a structural or chemical mechanism that limits the multimer size during the biosynthesis of reelin.

Although the purified mutant reelin exhibited reduced binding affinity toward ApoER2 and VLDLR in SPR experiments (Fig. 6B), the maximal binding was not severely affected as evident from the bead pulldown assay (Fig. 6A). Therefore, it is not clear whether the apparent lack of signaling and binding activity toward neurons in mutant reelin (Figs. 7 and 8) is attributable solely to the reduced solution affinity toward the two known receptors. A possibility remains that reelin utilizes an interaction with an as yet identified co-receptor(s) to achieve productive binding to neurons, which is critically dependent on the fine structure maintained by the intermolecular disulfide bond. The highly basic C-terminal region of reelin may mediate such a secondary interaction because removal of this segment is known to reduce signaling activity of reelin (38, 39).

The physiological importance of the higher order structure of reelin described above may seem to contradict with the recently proposed hypothesis that the R3–6 fragment released by N-t and C-t cleavage functions as a major signaling molecule in tissues (24). However, these two views are not mutually exclusive because these two molecular forms may constitute a mechanism of functional differentiation of reelin. According to the model of Jossin et al. (24), the cleaved R3–6 fragment (which should exist as a dimer as evidenced by the current work) diffuses away from the original environment and reaches the target neurons at the cortical plate where it is internalized and initiates downstream events. It is possible that the intact (i.e. uncut) reelin multimers that remain confined near the site of secretion do not just constitute a dormant pool but may act on a different target by using additional receptor(s). Although a detailed characterization of the molecular architecture of the functional reelin multimer awaits further study, the present biochemical characterization will help to design an experimental strategy to test the new model of reelin signaling.

Acknowledgments—We thank Drs. André M. Goffinet and John Strouboulis for the generous gifts; Keiko Tamura-Kawakami, Emiko Mihara, and Maiko Nampo for excellent technical support; and Mayumi Nakano for preparation of the manuscript.

REFERENCES

1. D’Arcangelo, G., Miao, G. G., Chen, S. C., Soares, H. D., Morgan, J. I., and Curran, T. (1995) Nature 374, 719–723
2. Tissier, F., and Goffinet, A. M. (2003) Nat. Rev. Neurosci. 4, 496–505
3. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimip, J., Hammer, R. E., Richardson, J. A., and Herz, J. (1999) Cell 97, 689–701
4. D’Arcangelo, G., Homayouni, R., Keshvare, L., Rice, D. S., Sheldon, M., and Curran, T. (1999) Neuron 24, 471–479
5. Hiesberger, T., Trommsdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A., and Herz, J. (1999) Neuron 24, 481–489
6. Howell, B. W., Hawkes, R., Soriano, P., and Cooper, J. A. (1997) Nature 389, 733–737
7. Sheldon, M., Rice, D. S., D’Arcangelo, G., Yoneshima, H., Nakajima, K., Mikoshiba, K., Howell, B. W., Cooper, J. A., Goldowitz, D., and Curran, T. (1997) Nature 389, 730–733
8. Nogi, T., Yasui, N., Hattori, M., Iwasaki, K., and Takagi, J. (2006) EMBO J. 25, 3675–3683
9. Yasui, N., Nogi, T., Kitao, T., Nakano, Y., Hattori, M., and Takagi, J. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 9988–9993
10. Nagae, M., Nishikawa, K., Yasui, N., Namasaki, M., Nogi, T., and Takagi, J. (2008) Acta Crystallogr. B. Biol. Crystallogr. 64, 1138–1145
11. Tan, K., Duquette, M., Liu, J. H., Lawler, J., and Wang, J. H. (2008) J. Mol. Biol. 381, 1213–1223
12. D’Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., and Curran, T. (1997) J. Neurosci. 17, 23–31
13. Miyata, T., Nakajima, K., Mikoshiba, K., and Ogawa, M. (1997) J. Neurosci. 17, 3599–3609
14. Nakajima, K., Mikoshiba, K., Miyata, T., Kudo, C., and Ogawa, M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 8196–8201
15. Ogawa, M., Miyata, K., Nakajima, K., Tagyu, K., Seike, M., Ikenaka, K., Yamamoto, H., and Mikoshiba, K. (1995) Neuron 14, 899–912
16. Kubo, K., Mikoshiba, K., and Nakajima, K. (2002) Neurosci. Res. 43, 381–388
17. Utsunomiya-Tate, N., Kubo, K., Tate, S., Kainosho, M., Katayama, E., Nakajima, K., and Mikoshiba, K. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 9729–9734
18. Jossin, Y., Bar, I., Ignatova, N., Tissier, F., De Rouvrot, C. L., and Goffinet, A. M. (2003) Cereb. Cortex 13, 627–633
19. Jossin, Y., Ignatova, N., Hiesberger, T., Herz, J., Lambert de Rouvrot, C., and Goffinet, A. M. (2004) J. Neurosci. 24, 514–521
20. Yasui, N., Nogi, T., and Takagi, J. (2010) Structure 18, 320–331
21. Kohn, S., Kohn, T., Nakano, Y., Suzuki, K., Ishii, M., Tagami, H., Baba, A., and Hattori, M. (2009) Biochem. Biophys. Res. Commun. 380, 93–97
22. Lugli, G., Krueger, J. M., Davis, J. M., Persico, A. M., Keller, F., and Smalheiser, N. R. (2003) BMC Biochem. 4, 9
23. Nogi, T., Sangawa, T., Tabata, S., Nagae, M., Tamura-Kawakami, K., Beppu, A., Hattori, M., Yasui, N., and Takagi, J. (2008) Protein Sci. 17, 2120–2126
24. Jossin, Y., Gui, L., and Goffinet, A. M. (2007) J. Neurosci. 27, 4243–4252
25. Ichihara, H., Jingami, H., and Tob, H. (2001) Brain Res. Mol. Brain Res. 97, 190–193
26. Takagi, J. (2008) in Reelin Glycoprotein, Biology, Structure and Roles in Health and Disease (Fetami, S. H., ed) pp. 55–68, Springer, New York
27. Eswar, N., Eramian, D., Webb, B., Shen, M. Y., and Sali, A. (2008) Methods Mol. Biol. 426, 145–159
28. de Bergeyck, V., Naerhuysen, B., Goffinet, A. M., and Lambert de Rouvrot, C. (1998) J. Neurosci. Methods 82, 17–24
29. Stanley, P. (1989) Mol. Cell. Biol. 9, 377–383
30. Mize, G. J., Harris, E. J., Takayama, T. K., and Kulman, J. D. (2008) Protein Expr. Purif. 52, 280–289
31. de Boer, E., Rodríguez, P., Bonte, E., Krijgsfeld, J., Katsantoni, E., Heck, A., Grosfeld, V., and Strouboulis, J. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 7480–7485
32. Morimura, T., Hattori, M., Ogawa, M., and Mikoshiba, K. (2005) J. Biol. Chem. 280, 16901–16908
33. Uchida, T., Baba, A., Pérez-Martinez, F. J., Hibi, T., Miyata, T., Luque, J. M., Nakajima, K., and Hattori, M. (2009) J. Neurosci. 29, 10653–10662
34. Lambert de Rouvrot, C., de Bergeyck, V., Cortvriendt, C., Bar, I., Eckhout, Y., and Goffinet, A. M. (1999) Exp. Neurol. 156, 214–217
35. Tabata, S., Nampo, M., Mihara, E., Tamura-Kawakami, K., Fujii, I., and Takagi, J. (2010) J. Proteomics 73, 1777–1785
36. Andersen, O. M., Benhayon, D., Curran, T., and Willnow, T. E. (2003) Biochemistry 42, 9355–9364
37. Sadler, J. E. (2009) J. Thromb. Haemost. 7, Suppl. 1, 24–27
38. Kohn, T., Nakano, Y., Kitooh, N., Yagi, H., Kato, K., Baba, A., and Hattori, M. (2009) J. Neurosci. Res. 87, 3043–3053
39. Nakano, Y., Kohn, T., Hibi, T., Kohn, S., Baba, A., Mikoshiba, K., Nakajima, K., and Hattori, M. (2007) J. Biol. Chem. 282, 20544–20552