RS1, a Discoidin Domain-containing Retinal Cell Adhesion Protein Associated with X-linked Retinoschisis, Exists as a Novel Disulfide-linked Octamer*

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RS1, also known as retinoschin, is an extracellular protein that plays a crucial role in the cellular organization of the retina. Mutations in RS1 are responsible for X-linked retinoschisis, a common, early-onset macular degeneration in males that results in a splitting of the inner layers of the retina and severe loss in vision. RS1 is assembled and secreted from photoreceptors and bipolar cells as a homo-oligomeric protein complex. Each subunit consists of a 157-amino acid discoidin domain flanked by two small segments of 39 and 5 amino acids. To begin to understand how the structure of RS1 relates to its role in retinal cell adhesion and X-linked retinoschisis, we have determined the subunit organization and disulfide bonding pattern of RS1 by SDS gel electrophoresis, velocity sedimentation, and mass spectrometry. Our results indicate that RS1 exists as a novel octamer in which the eight subunits are joined together by Cys60-Cys219 and Cys110-Cys142 intermolecular disulfide bonds. Subunits within the octamer are further organized into dimers mediated by Cys60-Cys110 bonds. These cysteines lie just outside the discoidin domain indicating that these flanking segments primarily function in the octamerization of RS1. Within the discoidin domain, two cysteine pairs (Cys60-Cys219 and Cys110-Cys142) form intramolecular disulfide bonds that are important in protein folding, and one cysteine (Cys83) exists in its reduced state. Because mutations that disrupt subunit assembly cause X-linked retinoschisis, the assembly of RS1 into a disulfide-linked homo-octamer appears to be critical for its function as a retinal cell adhesion protein.

X-linked retinoschisis (XLRS)† is a common, inherited macular degeneration that affects males early in life (1–3). Affected individuals show a significant loss in central and in some cases peripheral vision, a splitting of the inner layers of the retina, and a loss in the b-wave of the electroretinogram. The gene responsible for XLRS was identified by positional cloning and shown to encode a retinal-specific 224-amino acid protein, known as RS1 or retinoschin, containing a discoidin domain (4).

RS1 is expressed and secreted from photoreceptor cells of the outer retina and bipolar cells of the inner retina as a multisubunit protein (5–8). The secreted protein associates with the surface of rod and cone photoreceptors at the level of the inner segment, outer nuclear, and outer plexiform layers and the surface of bipolar cells within the inner nuclear and inner plexiform layers of the retina. Biochemical studies further show that RS1 is tightly associated with the membrane fraction of retinal cell homogenates (6). RS1 is generally believed to function as a retinal cell adhesion protein, because mice deficient in RS1 have a highly disorganized retina with displacement of bipolar cells into the outer retinal layer, gaps between bipolar cells within the inner retina, disruption of the photoreceptor-bipolar synapse, and progressive degeneration of rod and cone photoreceptors (9).

The dominant structural feature of the RS1 polypeptide is the 157-amino acid discoidin domain, also known as an F5/8 type C domain, which comprises over 75% of the processed polypeptide chain (4). Discoidin domains are present in a wide range of membrane and extracellular proteins where they mediate a variety of cell adhesion and cell signaling processes (10, 11). Some proteins that contain discoidin domains are Factors V and VIII involved in blood coagulation, neuropilins 1 and 2, which mediate nervous system regeneration and degeneration, discoidin domain receptors implicated in cancer metastasis, and discoidin I involved in cellular adhesion during slime mold differentiation and development (10–13). Still other members of the discoidin family play a role in key physiological processes ranging from heart development, milk lactation, and sperm-egg adhesion in vertebrates, to immunity and metamorphosis in the silkworm, and to post-fertilization events in the sea urchin zygote (14–17). Bioinformatic studies have also identified a number of discoidin domain-containing proteins of unknown function (11).

The high resolution structures of the C2 discoidin domain of Factors V and VIII and the b1 discoidin domain of neuropilin 1 saline; ER, endoplasmic reticulum; NEM, N-ethylmaleimide; DTT, dithiothreitol; IAM, iodoacetamide; MS, mass spectrometry; LC, liq uid chromatography; MALDI-TOF, matrix-assisted laser desorption/ ionization time-of-flight; MOPS, 4-morpholinepropanesulfonic acid; bicine, N,N-bis(2-hydroxyethyl)glycine; Bis-Tria, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propene-1,3-diol.
have been determined (13, 18, 19). These domains consist of eight antiparallel $\beta$-strands arranged in a barrel-like structure with two or three loops, or “spikes,” projecting from one end of the core structure. In the case of Factors V and VIII, the spikes are involved in the attachment of these proteins to the phosphatidylserine-rich surface of platelets as a key step in the blood coagulation process (12). The discoidin domain of RS1 has been modeled from the C2 discoidin domain of Factors V and VIII (20, 21), and, like these domains, the structure consists of eight core beta strands and three spike regions.

In addition to its discoidin domain, RS1 contains a 23-amino acid leader or signal sequence, followed by a 39-amino acid segment known as the Rs1 domain. The leader sequence plays an essential role in the insertion of the nascent RS1 polypeptide chain into the ER membrane. It is subsequently cleaved in the lumen of the ER by a signal peptidase as a key step in the formation of RS1 into the ER membrane resulting in mislocalization of the Rs1 3R10 antibody was purified and coupled to CNBr-

were transfected and membranes from bovine retinal membranes for 20 min in transfer buffer (25 mM Tris, 192 mM glycininc containing 2% methanol.

For analysis of the C35S/C40S/C42S/C58S mutant, a 6.5% nonreducing gel was used, and the protein was transferred for 20 min in transfer buffer containing 7% methanol. Blots were blocked with 0.5% skim milk in PBS and labeled with the Rs1 3R10 antibody containing 0.5% skim milk in PBS and 0.1% Tween 20 for 1 h. The blots were then washed, labeled with goat-anti-mouse Ig conjugate for 20 min in 1% milk and 0.1% Tween 20, washed, bound protein was eluted with 4% SDS in column buffer.

**Gel Electrophoresis and Western Blotting**—Proteins were denatured in a reducing solution (10 mM Tris, pH 6.8, 1% SDS, 10% glycerol) in the presence or absence of 4% $\beta$-mercaptoethanol and separated on 6.5% or 10% polyacrylamide SDS gels or gradient polyacrylamide SDS gels as indicated. For 6.5% or 10% polyacrylamide SDS gels, a denaturing mixture of 10 mM Tris, pH 6.8, 1% SDS, and 10% glycerol was used. For gradient gels, the above mixture was used except 1% SDS was replaced with 2% lithium dodecyl sulfate, and 2 mM EDTA was also included. The gels were transferred to Immobilon P membranes for 20 min in transfer buffer (25 mM Tris, 192 mM glycininc containing 2% methanol.

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CHAPS. After elution with column buffer containing 4% SDS, the sample was denatured in SDS solution and analyzed by SDS gel electrophoresis.

Velocity Sedimentation of Native RS1—Bovine retinal RS1, recombinant WT RS1, and RS1 containing the C59S/C223S mutation were sedimented through a 5–20% (w/w) linear sucrose gradient containing PBS at 4 °C for 17 h and 72,449 g in a Beckman TLS-55 rotor. Bovine retinal RS1 was obtained by urea and CHAPS extraction as described above. The secreted fraction of transfected WT and C59S/C223S RS1 was concentrated by ammonium sulfate precipitation as described above before subjecting to velocity sedimentation. Mouse IgG having a molecular mass of 160 kDa was run as a standard. All samples were spun for 20 min at 100,000 g in a Beckman TLA 100.4 rotor prior to loading onto the sucrose gradient to remove any insoluble material. Three-drop fractions were collected, run on reducing gels, and blotted with the Rs1 3R10 antibody. The intensity of the labeled bands was quantified with the Odyssey Infrared Imaging System. Sucrose concentration in each fraction was determined by refractometry to plot the percentage of each protein species versus the percentage of sucrose.

In-gel Proteolytic Digestion of RS1 for Mass Spectrometry—In-gel digestion was performed by punching out the Coomassie Blue-stained RS1 bands with a glass Pasteur pipette. The gel pieces were washed with water several times to remove acetic acid. They were then destained in a 1:1 mixture of 100 mM ammonium bicarbonate and 100% acetonitrile several times. After shrinking and drying the gels with 100% acetonitrile, the gel pieces were incubated with either 50 mM NEM for nonreducing samples, or 10 mM DTT (Sigma) followed by 50 mM IAM (Sigma) for reducing samples, washed with ammonium bicarbonate, and dried from 100% acetonitrile. The gel pieces containing the samples were then incubated with the proteases at 12 ng/μl for 30 min on ice. The protease solution was removed, and the gel pieces were overlaid with 50 mM ammonium bicarbonate and 5 mM calcium chloride. Non-reduced samples also contained 0.6 M urea. The samples were digested for 18 h at 37 °C. The solution was collected in a tube and the gel pieces were re-extracted with 50 mM ammonium bicarbonate/66% acetonitrile (basic extraction) and with 5% formic acid/66% acetonitrile (acidic extraction). The samples were pooled, dried in a SpeedVac, and resuspended in 10 μl of 50 mM ammonium bicarbonate. For MALDI-TOF mass spectrometry, protease-digested RS1 (2 μl) was applied to an H4 chip (Ciphergen, Fremont, CA). The sample was dried and washed with two quick rinses of water before applying 20% α-cyanohydroxycinnamic acid matrix in a solvent with 50% acetonitrile and 0.1% trifluoroacetic acid. Samples were analyzed on a surface-enhanced laser desorption ionization-time of flight (Ciphergen). Masses obtained were average masses. A Qstar XL LC/MS/MS (Applied Biosystems, Foster City, CA) was used for MS/MS sequencing of the RS1 peptides. Trypsinized samples were obtained as above and lyophilized and reconstituted in formic acid. A PepMap C18 column, with a 3-mm particle size and 100-Å pore size column from LC Packings (Amsterdam, Netherlands) was used for peptide separation. Solvents B and A contained 20% acetonitrile in water and 5% acetonitrile in water, respectively. LC conditions started at 2% solvent B, with a gradient to 60% B over 60 min, to 95% B at 93 min, and held for 3 min before returning to 2% B. Masses obtained were monoisotopic masses. The enzymes used were trypsin (Promega, Mad-
RESULTS

RS1 Assembles as a Disulfide-linked Homo-octamer—In previous studies, RS1 was shown to migrate on SDS gels as a 24-kDa monomer under disulfide-reducing conditions and a high molecular weight oligomer under nonreducing conditions (20). To determine the size of the RS1 oligomer, a C38S/C40S/C42S triple mutant displaying partial disruption of the oligomeric complex was expressed, secreted from HEK 293 cells, and analyzed on nonreducing SDS-polyacrylamide gradient gels. As shown in Fig. 1A, the secreted mutant showed a ladder of eight distinct bands ranging from the 24-kDa monomer to the prominent 185-kDa octameric protein.

The secreted fraction of HEK 293 cells expressing WT RS1 was also analyzed by SDS-gel electrophoresis under nonreducing conditions. As shown in Fig. 1B, a ladder of bands was also evident for WT protein when Western blots were exposed for extended times, with the 185-kDa complex being the most prominent species. Unlike the C38S/C40S/C42S mutant, however, only seven bands were resolved for WT RS1, presumably due to the masking of the heptamer by the dominant octameric complex. This was confirmed by plotting the logarithm of the apparent molecular mass for the six lower bands against the logarithm of the acrylamide percentage at which these species migrated (23). From the linear relation (Fig. 1C), the number of subunits in the prominent top band of the WT complex was

![Image](https://via.placeholder.com/150)

**FIG. 2. Analysis of cysteines responsible for RS1 dimer formation.** Octamer-defective C59S/C223S RS1 mutants with an additional C38S, C40S, C42S, or C83S mutation were expressed in HEK 293 cells, and the cellular and secreted fractions were analyzed by SDS-gel electrophoresis under reducing and nonreducing conditions. Western blots were labeled with the Rs1 3R10 antibody. Although some loss in dimer formation was observed for the C38S and C42S mutants, only the C40S mutant showed complete absence of dimers. The first lane in each gel is the control containing only the background C59S/C223S mutation.

![Image](https://via.placeholder.com/150)

**FIG. 3. RS1 forms an octamer in the absence of dimer formation.** A, two possible models for the assembly of RS1 as dimers within an octameric complex. In the first model (left), the Cys80-Cys82 disulfide bond is responsible for octamer formation; within this complex the Cys10-Cys10 disulfide bond forms dimers between adjacent subunit or opposite subunits (not shown). In the second model (right) a tetramer formed through Cys89-Cys223 disulfide bonds is disulfide-bonded to a second tetramer via Cys80-Cys80 disulfide bonds in a head-to-head arrangement. Cys89-Cys223 disulfide bonds are solid lines; Cys10-Cys10 disulfide bonds are dashed lines. B, WT and a Δ4 Cys mutant containing C38S/C40S/C42S/C83S mutations were expressed in HEK 293 cells. The cellular and secreted fractions were separated by SDS-gel electrophoresis under reducing and nonreducing conditions for detection by Western blotting. The Δ4 Cys mutant forms an octamer in the absence of dimer formation, consistent with the first model shown in A.
calculated to be 7.7. This suggests that WT RS1, like the C38S/C40S/C42S mutant, exists as a disulfide-linked octamer.

Analysis of Cysteine Residues Involved in Disulfide-linked Dimer Assembly—Previously, we showed that the Cys59 and Cys223 residues of RS1 are responsible for disulfide-linked oligomer (octamer) assembly (20). Substitution of these cysteine residues with serine resulted in a loss of the disulfide-linked oligomer and the appearance of a disulfide-linked dimer when analyzed on nonreducing SDS gels. To determine which of the one or more cysteine residues in RS1 are responsible for disulfide-mediated dimer formation, we individually replaced each of four cysteine residues (Cys38, Cys40, Cys42, and Cys83) with a serine in a C59S/C223S octamer-defective mutant, and analyzed the cellular and secreted protein fractions from transfected HEK 293 cells. These four cysteines were selected because previous studies had suggested that the remaining cysteines (Cys63, Cys110, Cys142, and Cys219) are involved in intramolecular disulfide bonds within the discoidin domain (20). Under disulfide-reducing conditions, all four mutants exhibited similar levels of cellular expression and secretion on SDS gels and migrated as 24-kDa monomers (Fig. 2). Under nonreducing conditions, the parent C59S/C223S mutant from the secreted fraction ran solely as a 47-kDa dimer, whereas the three C59S/C223S mutants containing an additional C38S, C42S, or C83S mutation migrated as a mixture of dimers and monomers. Only the C59S/C223S mutant containing a C40S mutation ran as the monomer under nonreducing conditions in both the cellular and secreted fractions without detectable dimer. These studies suggest that Cys^{40} residues of individual subunits are directly involved in dimer formation. Partial loss in dimer formation present in the C38S and C42S mutants, and to a lesser extent the C83S mutant, most likely result from structural perturbations, which partially restrict the formation of Cys^{40}-mediated intermolecular disulfide bonds.

All of these cysteine mutants exhibit an additional protein band just above the 24-kDa monomer in the cellular fraction of nonreduced gels (Fig. 2, lower left). This upper band, not found in the secreted fraction, most likely corresponds to a misfolded monomer that migrates anomalously on nonreducing SDS due to the presence of abnormal intramolecular disulfide bonds. This species is recognized by the quality control system of the ER as a misfolded protein and therefore is not secreted from the cell.

RS1 Contains Disulfide-linked Dimers within the Disulfide-linked Octamer—The finding that intermolecular disulfide bonds are formed between Cys^{59} and Cys^{223}, and between individual Cys^{40} residues of different subunits, suggests two models. In one model (Fig. 3A, left), the Cys^{63}-Cys^{219} disulfide bonds are solely responsible for octamer formation, and additional Cys^{40} disulfide bonds form between two adjacent or opposing subunits within the octamer. In another model (Fig. 3A, right) the Cys^{59}-Cys^{223} intermolecular disulfide bonds result in the formation of tetramers. Two tetramers further link together in a head-to-head arrangement through Cys^{40} disulfide bonds to form the octamer. For the first model, one predicts that RS1 containing a C40S dimer-defective mutation should still form an octamer, whereas for the second model, this mutation should result in tetramers. To distinguish between these models, we expressed a C38S/C40S/C42S/C83S mutant (termed Δ4Cys), which is defective in disulfide-linked dimer formation, for analysis on reducing and nonreducing SDS gels. Fig. 3B...
**Table I**

Tryptic peptides of reduced RS1

| Position | Sequence | Mis-cuts | Empirical mass, MALDI | Theoretical mass, average | Empirical mass, LC/MS/MS | Theoretical mass, monoisotopic |
|----------|----------|----------|-----------------------|---------------------------|--------------------------|-------------------------------|
| A        | 24–36    | STEDEGEDPWYHK | 0                     | 1592.89                   | 1592.60                  |                               |
| B        | 103–115  | LNSQGFCACWLS | 0                     | 1467.66                   |                           |                               |
| C        | 116–128  | FQDSQQLQLQDLK | 0                     | 1607.68                   | 1607.78                  |                               |
| D        | 132–141  | VISGILTQGR   | 0                     | 1043.22                   | 1043.23                  |                               |
| E        | 142–150  | CDIDEWMTK    | 0                     |                           | 1196.43                   | 1196.48                       |
| F        | 142–150  | (Met oxidized) | 0                     | 1212.38                   |                           | 1212.48                       |
| G        | 151–156  | DQTNQNR      | 0                     | 814.97                    | 814.90                   | 814.40                        |
| H        | 157–167  | TDESLNWIYYK  | 0                     | 1431.45                   | 1431.56                  |                               |
| I        | 175–182  | VFYGNNSDR    | 0                     | 957.05                    | 957.01                   | 956.37                        |
| J        | 183–197  | TSTVQNLRRPIISR | 0                     | 1695.08                   | 1695.00                  | 1693.86                       |
| K        | 201–209  | LIPLGHWYRV   | 0                     | 1090.15                   | 1090.34                  |                               |
| L        | 214–222  | MELLCVSK     | 0                     | 1107.44                   | 1107.53                  |                               |
| M        | 214–222  | (Met oxidized) | 0                     | 1123.42                   | 1123.53                  |                               |
| N        | 157–174  | TDESLNWIYYKDKQTGNNR | 0 | 2127.27                   | 2127.34                  |                               |
| O        | 68–100   | PLGFESGEVTDPDTCTSNVEQVGYWSSWTANK | 0 | 3819.11                   | 3819.12                  |                               |
| P        | 79–100   | DQITCNVEQVGYWSSWTANK | 0 | 2704.92                   | 2704.91                  |                               |

*a* Peptide O was generated from a double digest of Trypsin/LysC, and Peptide P was generated from a double digest of Trypsin/AspN. The empirical masses are for the NEM-alkylated peptide. Both reduced and nonreduced samples gave the same peptide masses.

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**Fig. 6. Locations of the peptides used.** LS, leader sequence; Rs1, Rs1 domain; Discoidin, discoidin domain.

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shows that the secreted C38S/C40S/C42S/C83S mutant, like WT RS1, migrated as a 24-kDa monomer under reducing conditions and a 185-kDa octamer under nonreducing conditions. Similar results were obtained for the C40S single mutant (data not shown). These results support the first model in which an octamer is formed via Cys55-Cys223 disulfide bonds even in the absence of Cys40 mediated dimer formation.

**RS1 from Retina Tissue Is Heterogeneous**—To determine if RS1 from retina tissue exhibits a similar disulfide-linked octameric structure as recombinant protein, we compared the migration behavior of these proteins, along with the C59S/C223S mutant, on SDS-polyacrylamide gels under reducing and nonreducing conditions. As shown in Fig. 4, a fraction of RS1 from the retina migrated as a 24-kDa monomer under reducing conditions and a 185-kDa octamer under nonreducing conditions similar to recombinant WT RS1. However, a significant fraction of retinal RS1 migrated more slowly. Under reducing conditions a portion of RS1 migrated as a 24-kDa monomer and another fraction ran as a diffuse band between the monomer and dimer. Heating at 60 or 95 °C for 5 min in the presence of reducing agent (β-mercaptoethanol) and SDS did not abolish the slow migrating bands in the retinal RS1 sample. Under nonreducing conditions, a diffuse band was observed above the 185-kDa octamer. A similar diffuse RS1-labeled band was reported by Reid et al. (7) for mouse retinal extracts under nonreducing conditions. These additional species were not observed for the recombinant WT or C59S/C223S mutant protein expressed in HEK 293 cells (Fig. 4), nor were they seen in RS1 derived from Weri RB1 retinoblastoma cells (data not shown). The banding pattern of RS1 from frozen bovine retina was also observed for freshly dissected bovine and mouse retina tissue (data not shown) indicating that the diffuse bands observed in the retina sample are not due to artifacts generated from aged tissue or from the use of bovine tissue. These data suggest that a portion of RS1 expressed in photoreceptor and/or bipolar cells undergoes a heterogeneous type of posttranslational modification.

**Velocity Sedimentation of RS1 under Nondenaturating Conditions**—Velocity sedimentation studies were carried out to determine whether RS1 exists as an octamer or a higher ordered oligomer under nondenaturating conditions. Fig. 5 shows sedimentation profiles for retinal and recombinant RS1 generated from Western blots of fractions collected from a 5–20% sucrose gradient. The WT recombinant protein (rWT) sedimented just ahead of retinal RS1 and mouse IgG (~160 kDa) used as a standard. This indicates that both retinal and WT RS1 exist as a disulfide-linked octamer under nondenaturating conditions, as well as under denaturing (nonreducing) conditions, and does not form higher ordered oligomers through noncovalent interactions. Heterogeneous post-translational modification may account for the small decrease in sedimentation rate and broader peak profile observed for retinal RS1 relative to the recombinant WT protein (Fig. 5). The octamer-defective C59S/C223S mutant migrated much more slowly, consistent with its smaller size, presumably a dimer as observed under nonreducing, denaturing conditions. This suggests that noncovalent interactions do not play a significant role in octamer formation or stabilization.

**Peptide Mapping of RS1 and Identification of the Cys110, Cys142 Disulfide Bond by Mass Spectrometry**—To further characterize the disulfide bonding pattern of RS1, the reduced 24-kDa monomer and nonreduced 185-kDa octamer of RS1 from bovine retina were digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF MS and LC/MS/MS...
MS. As listed in Table I and illustrated in Figs. 6 and 7, 14 tryptic peptides were identified from various regions of RS1. Peptide A (m/z of 1592.6 for bovine RS1 and m/z of 1583.6 for human RS1 (not shown)) corresponds to the N-terminal tryptic peptide of the processed RS1 polypeptide, confirming that signal peptidase cleavage had occurred at Ser23 as part of the protein secretion pathway (Fig. 7). The remaining peptides were derived from the RS1 discoidin domain (Table I). In addition a broad peak corresponding to a peptide mass of 11011 kDa was observed (data not shown). This peptide was in agreement with the mass of the large tryptic fragment extending from position 40 to 100. The broad nature of the peak may reflect, in part, the heterogeneous nature of retinal RS1.

The mass spectrum of tryptic peptides from nonreduced RS1 is shown in Fig. 7. In addition to peptides identified in reduced RS1, a peak at m/z of 2548.8 was observed. This peak corresponded to a tryptic peptide (1410.6 Da) containing Cys110 disulfide bonded to a tryptic peptide (1140.3 Da) containing Cys142. (To calculate the theoretical mass of the nonreduced peptide, a mass of 2 Da is subtracted from the sum of the masses of these two cysteine-containing peptides due to removal of two hydrogens upon disulfide bond formation.) Another peak at m/z of 2564.7 most likely corresponds to the same disulfide-linked peptide, but with the single methionine (located on the Cys142 peptide) in an oxidized state. To confirm that these peptides indeed contain a disulfide bond, we reduced this sample with DTT and alkylated the free cysteines with IAM. Under these conditions, the peaks at m/z of 2548.8 and 2564.7 disappeared and a new peak at 1467.6 appeared (Fig. 7). The mass of this new peptide is consistent with the tryptic peptide containing an IAM-modified Cys110 residue. The tryptic peptide containing the alkylated Cys142 residue was not observed by MALDI-TOF mass spectrometry, but was detected by LC/MS/MS (Table I).

Cys83 of RS1 Exists in Its Reduced State—To determine the redox state of Cys83, nonreduced RS1 was alkylated with NEM and double-digested with trypsin/LysC, or trypsin/AspN. The trypsin/LysC sample contained a peptide with an m/z of 3819, and the trypsin/AspN sample contained a peptide with an m/z of 2705 (Fig. 8 and Table I). These masses correspond to peptides containing Cys83 that have been alkylated with NEM. This indicates that the Cys83 within the discoidin domain of RS1 exists in its reduced state. Additional peaks that have slightly higher masses appear to correspond to the same peptide containing one or more oxidized amino acids.

DISCUSSION

In this study, we have analyzed the oligomeric structure of RS1 as an important step in understanding how this extracellular protein functions in retinal cell adhesion and how mutations in RS1 cause XLRS. RS1 is composed of eight identical subunits that are linked together through intermolecular disulfide bonds between Cys59 and Cys223 residues on adjacent subunits. These disulfide bonds are required for octamer assembly, because substitution of Cys59 and Cys223 with serine abolishes octamerization of RS1 under nonreducing conditions as observed by SDS-gel electrophoresis and native conditions as analyzed by velocity sedimentation. In addition to the Cys59-Cys223 intermolecular disulfide bond, RS1 contains a Cys40-Cys40 disulfide bond responsible for dimer formation (Fig. 9A). Dimerization is independent of octamerization, and octamerization is independent of dimerization, because each multimeric species forms in the absence of the other (Fig. 9B). Accordingly, RS1 is composed of four disulfide-linked dimers.
within a disulfide-linked octameric structure. The exact arrangement of the subunits, however, awaits determination of the high-resolution structure of RS1.

The cysteine residues involved in these intermolecular disulfide bonds are located in segments that flank the discoidin domain, i.e., the 39-amino acid Rs1 domain upstream and a 5-amino acid segment downstream of the discoidin domain (Fig. 9A). Therefore, a principal function of these flanking regions is to assemble RS1 into a disulfide-linked octamer. Octamerization appears to be essential for the function of RS1 in retinal cell adhesion, because the C59S and C223R mutations, which do not significantly affect the folding and secretion of dimeric RS1, are known to cause XLRS (20, 24, 25). However, it is unclear if Cys40-mediated dimerization is critical for RS1 function, because substitution of Cys40 with serine does not affect folding, secretion, or octamerization of RS1 (20), and to date no disease-linked missense mutations at position 40 have been found. It is possible that Cys40-linked dimerization may simply contribute to the stability of the octameric complex but not be critical for its function. A biochemical assay for retinal cell adhesion would help to resolve this issue.

In addition to cysteine at position 40, the RS1 domain contains two additional cysteine residues at positions 38 and 42. Substitution of these residues with serine does not significantly affect octamerization (20) and only partially affects dimerization, as shown here. We were not able to determine the redox state of Cys38 and Cys42 by mass spectrometry. It is possible that these residues form an intramolecular disulfide bond or additional intermolecular disulfide bonds that facilitate dimer formation. Alternatively, the Cys38 and Cys42 cysteine residues may exist in their reduced state. Interestingly, this -CKCDC-
motif is highly conserved in all vertebrate RS1 proteins that have been sequenced to date.

The discoidin domain is the main functional part of the RS1 protein. Like the discoidin domains of other proteins, RS1 contains conserved cysteine residues at the beginning (Cys63) and the end (Cys219) of its discoidin domain. High resolution structures of the discoidin domains of Factor V, Factor VIII, and neuropilin 1 indicate that these cysteines marking the beginning and end of the discoidin domain form an intramolecular disulfide bond. Because Cys63 and Cys219 have been shown to come in close proximity to each other in modeling studies of RS1 and are required for proper protein folding (20), it is likely that these cysteines form an intramolecular disulfide bond in the RS1 polypeptide. In addition to Cys63 and Cys219, the RS1 discoidin domain contains 3 additional cysteine residues. The Cys110 and Cys142 cysteines, located in spike 2 and spike 3, respectively, had been suggested to form another intramolecular disulfide bond based on molecular modeling and site-directed mutagenesis (20). This has been confirmed directly in the present study through the mass spectrometric identification of a Cys110-Cys142 containing proteolytic fragment generated from nonreduced RS1. The function of the spike regions in the RS1 discoidin domain has yet to be determined. However, analogous spikes in the C2 discoidin domain of Factors V and VIII are known to insert into the lipid bilayer of phosphatidylserine-rich platelet membranes as part of the blood coagulation process. Partial sequence conservation of RS1 with the C2 domain of Factors V and VIII in the vicinity of the spike regions (12), together with molecular dynamic simulation, have led to the suggestion that RS1 may interact with membranes in a similar fashion (21). On this basis, the Cys110-Cys142 disulfide bond may be important in stabilizing the spike regions of RS1 for insertion into the lipid bilayer.

An additional cysteine residue, not found in the discoidin domain of other proteins, is present at position 83 of the RS1 discoidin domain. Mass spectrometric analysis of nonreduced and alkylated proteolytic fragments indicates that this cysteine is in its reduced state. This is consistent with molecular mod-

![Image](image_url)
Disulfide-linked Octameric Structure of RS1

Fig. 10. Pathway for RS1 ER insertion, disulfide-mediated oligomeric assembly, and secretion. RS1 inserts into the ER membrane through the leader sequence enabling the nascent polypeptide chain to be translocated into the ER lumen. Signal peptidase cleaves off the 23-amino acid leader sequence and folding of the RS1 subunit occurs with the formation of intramolecular disulfide bonds (Cys63-Cys219 and Cys110-Cys142). WT RS1 assembles into a dimer mediated by Cys40-Cys40 and an octamer by Cys59-Cys223 intermolecular disulfide bonds. The WT protein is subsequently targeted for secretion into the extracellular space. Dimer and octamer formation can occur independently. Monomers (C40S/C59S/C223S mutant) and dimers (C59S/C223S mutant) can be secreted when octamer formation is defective.

References
1. George, N. D., Yates, J. R., and Moore, A. T. (1995) Br. J. Ophthalmol. 79, 697–702
2. Sieving, P. A. (1996) in Genetic Diseases of the Eye (Traboulsi, E. I., ed), Oxford University Press, New York
3. Tantri, A., Vrabec, T. R., Cu-Unjieng, A., Frost, A., Annesley, W. H., Jr., and Donoso, L. A. (2004) Surv. Ophthalmol. 49, 214–230
4. Sauer, C. G., Gehrig, A., Warneke-Wittstock, R., Marquardt, A., Ewing, C. C., Gibson, A., Lorenz, B., Jurklies, B., and Weber, B. H. (1997) Nat. Genet. 17, 164–170
5. Reid, S. N., Akhmedov, N. B., Pirier, N. I., Kozak, C. A., Dancer, M., and Farber, D. B. (1999) Gene (Amst.) 227, 257–266
6. Molday, L. L., Hicks, D., Sauer, C. G., Weber, B. H., and Molday, R. S. (2001) Invest. Ophthalmol. Vis. Sci. 42, 816–825
7. Reid, S. N., Yamashita, C., and Farber, D. B. (2002) J. Neurosci. 23, 6030–6040
8. Grayson, C., Reid, S. N., Ellis, J. A., Rutherford, A., Sowden, J. C., Yates, J. R., Farber, D. B., and Trump, D. (2000) Hum. Mol. Genet. 9, 1673–1679
9. Weber, B. H., Schrewe, H., Molday, L. L., Gehrig, A., White, K. L., Seeliger, M. W., Jaisle, G. B., Friedburg, C., Tamm, E., and Molday, R. S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 6222–6227
10. Weber, B. H. (1999) PASER. Cambridge University Press, New York
11. Bauml, H., Sterzel, R. E. B., and Weber, B. H. (1996) Eur. J. Biochem. 240, 628–636
12. Puentes-Prior, P., Fujikawa, K., and Pratt, K. P. (2002) Curr. Protein Pept. Sci. 3, 313–339
13. Lee, C. S., Kreusch, A., McMullan, D., Ng, K., and Spragg, G. (2003) Structure. (Camb.) 11, 99–108
14. Yamakawa, M., and Tanaka, H. (1999) Dev. Comp. Immunol. 23, 281–289
15. Matsese, J. C., Black, S., and McClay, D. R. (1997) Dev. Biol. 186, 16–26
16. Shur, B. D., Ensslin, M. A., and Rodeheffer, C. (2004) Curr. Opin. Cell Biol. 16, 477–485
17. Hvarregaard, J., Andersen, M. H., Berglund, L., Rasmusson, J. T., and Petersen, T. E. (1996) Eur. J. Biochem. 240, 628–636
18. Macedo-Ibíribe, S., Bode, W., Huber, R., Quinn-Allen, M. A., Kim, S. W., Ortel, T. L., Burenkon, G. P., Bartunek, H. D., Stubbs, M. T., Kane, W. H., and Fuentes-Prior, P. (1999) Nature 402, 434–439
19. Pratt, K. P., Shen, B. W., Takeshima, K., Davie, E. W., Fujikawa, K., and Stoddard, B. L. (1999) Nature 402, 439–442
20. Wu, W. W. H., and Molday, R. S. (2003) J. Biol. Chem. 278, 28139–28146
21. Fraternali, F., Cavalo, L., and Museo, G. (2003) FEBS Lett. 544, 21–26
22. Wang, T., Waters, C. T., Rothman, A. M., Jakina, T. J., Romisch, K., and Trump, D. (2002) Hum. Mol. Genet. 11, 3097–3105
23. See, Y. P., and Jackowski, G. (1999) in Protein Structure: A Practical Approach (Creighton, T. E., ed) pp. 1–21, IRL Press, Oxford
24. Hiriyanna, K. T., Bingham, E. L., Yashar, B. M., Ayyagari, R., Fishman, G., Small, K. W., Weinberg, D. V., Weber, B. H., Lewis, R. A., Andreassen, S., Richards, J. F., and Sieving, P. A. (1999) Hum. Mutat. 14, 423–427
25. Retinoschisis Consortium (1998) Hum. Mol. Genet. 7, 1185–1192

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