Defective Discoidin Domain Structure, Subunit Assembly, and Endoplasmic Reticulum Processing of Retinoschisin are Primary Mechanisms Responsible for X-linked Retinoschisis*

Received for publication, March 10, 2003, and in revised form, April 15, 2003
Published, JBC Papers in Press, May 13, 2003, DOI 10.1074/jbc.M302464200

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Retinoschisin is a 24-kDa discoidin domain-containing protein that is secreted from photoreceptor and bipolar cells as a large disulfide-linked multisubunit complex. It functions as a cell adhesion protein to maintain the cellular organization and synaptic structure of the retina. Over 125 different mutations in the RS1 gene are associated with X-linked juvenile retinoschisis, the most common form of early onset macular degeneration in males. To identify molecular determinants important for retinoschisin structure and function and elucidate molecular and cellular mechanisms responsible for X-linked juvenile retinoschisis, we have analyzed the expression, protein folding, disulfide-linked subunit assembly, intracellular localization, and secretion of wild-type retinoschisin, 15 Cys-to-Ser variants and 12 disease-linked mutants. Our studies, together with molecular modeling of the discoidin domain, identify Cys residues involved in intramolecular and intermolecular disulfide bonds essential for protein folding and subunit assembly. We show that misfolding of the discoidin domain, defective disulfide-linked subunit assembly, and inability of retinoschisin to insert into the endoplasmic reticulum membrane as part of the protein secretion process are three primary mechanisms responsible for the loss in function of retinoschisin as a cell adhesion protein and the pathogenesis of X-linked juvenile retinoschisis.

X-linked juvenile retinoschisis (XLRS)† is the most common form of early onset macular degeneration in males (1, 2). It is characterized by a mild to severe decrease in visual acuity, radial streaks extending from the central retina due to a splitting of the inner retina, progressive macular atrophy, and reduction in the electroretinogram b-wave (2–5). Lesions in the peripheral retina associated with impairment in peripheral vision are observed in half the cases. During the course of the disease, complications can arise, which include retinal detachment, vitreal hemorrhaging, and neovascular glaucoma leading to a poor outcome.

The RS1 gene responsible for XLRS was identified by positional cloning and found to encode a 24-kDa protein called retinoschisin, or RS1 (6), that is secreted from photoreceptor and bipolar cells as a disulfide-linked oligomeric complex (7, 8). The polypeptide consists of a leader sequence with a putative signal peptidase cleavage site and a discoidin domain spanning most of the protein (6). Discoidin domains, first identified in the discoidin I protein of Dictyostelium discoideum (9, 10), have now been found in many secreted and transmembrane proteins, including blood coagulation factors, tyrosine kinase receptors, and proteins involved in neural development (11–13). The function of the discoidin domain is not well understood, but in some proteins it has been implicated in cell adhesion and cell signaling through protein-protein, protein-carbohydrate, or protein-lipid interactions. Recently, the three-dimensional crystal structures of the C2 discoidin domain of blood coagulation Factors V and VIII have been determined, providing insight into the molecular interactions that contribute to its structure and function (13–15).

The role of retinoschisin in retinal cell adhesion is supported by recent studies on WT and RS1 knockout mice. Retinoschisin, most abundantly expressed in photoreceptor cells (16), is localized along the extracellular surfaces of rod and cone photoreceptors and bipolar cells including the synapses (7). Mice heterozygous for the disrupted RS1 gene show general retinal tissue disorganization with irregular displacement of cells in various retinal layers, splitting of the inner retina with gaps between bipolar cells, and disruption of the synapses between the photoreceptors and bipolar cells (17). Progressive rod and cone photoreceptor degeneration and a preferential loss in the electroretinogram b-wave are additional characteristic features of these knockout mice.

Over 125 different missense, nonsense, insertions, deletions, and splice-site mutations associated with XLRS have now been catalogued (18) (www.dmd.nl/rs/index). Most missense mutations are in the discoidin domain of retinoschisin, with over 25% involving the loss or gain of a Cys residue. Disease-causing missense mutations are also found in regions flanking the discoidin domain as well as within the leader sequence (4, 19).

To determine the structure-function relationships of retinoschisin and define molecular and cellular mechanisms responsible for XLRS, we have examined the effect of 15 Cys-to-Ser mutations and 12 disease-linked missense mutations on the expression, structural properties, intracellular localization, and secretion of retinoschisin. Here, we report on 1) the identification of Cys that mediate intramolecular and intramolecular...
lar disulfide linkages critical for the structure, function, and subunit assembly of retinoschisin, 2) the generation of a structural model of the discoidin domain of retinoschisin, and 3) the elucidation of molecular and cellular mechanisms underlying XLRs.

EXPERIMENTAL PROCEDURES

Retinoschisin Mutants and RS1 3R10 mAb—Human RS1 cDNA was subcloned into the pCPE4 vector (Invitrogen) using the XhoI and HindIII restriction sites. The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce 10 single Cys mutations (C38S, C40S, C42S, C48S, C59S, C61S, C72S, C109S, C110S, and C223S), 12 disease-linked mutations (L13P, C59S, E72K, G110Y, R141C, C142W, D143V, R182C, P203L, C219R, C223R), and 5 double Cys mutations (C59S/C219S, C63S/C219S, C59S/C223S, C63S/C223S, C110S/C142S). All constructs were sequenced to verify the presence of the desired mutations and absence of random mutations. The RS1 3R10 mAb (17) was purified and coupled to CNBr-activated Sepharose 2B as previously described (20).

Cell Transfections and Protein Preparation—EBNA 293 cells (American Type Culture Collection) were transfected in one 10 cm dish with 20 μg of DNA using the calcium phosphate transfection procedure. Briefly, 62 μl of a 1 M calcium chloride solution was added to a 188 μl solution of DNA (prior to the addition of 250 μl of NNN-β-hydroxyethyl)-2-aminoethane-buffered saline, pH 7.95. The 293 cells were resuspended in the DNA solution and added to a 10-cm dish containing media. This media containing the transfection solution was replaced with regular media on day 2, and the cells were harvested on day 4. The cellular fraction was obtained by washing the cells twice in PBS (157 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4), by low-speed centrifugation. The final pellet was resuspended in 200 μl of PBS containing 20 mM NEM and added to an equal volume of PBS containing 2% Triton X-100, 20 mM NEM, and Complete Protease Inhibitor (Roche Applied Science). After 1 h at 4 °C, the solution was centrifuged at 300,000 × g in a Beckman TLA-100.4 rotor for 20 min and the supernatant was retained for SDS gel electrophoresis. The secreted fraction was obtained by centrifuging 10 ml of the media at 300,000 × g in a Beckman TLA-75 rotor at 4 °C for 20 min to remove any membrane material. The samples were then incubated for 2 h with the RS1 3R10-Sepharose 2B affinity matrix. After washing with column buffer (20 ml Tris, 0.02% Triton X-100, and 20 mM NEM), bound retinoschisin was eluted with 4% SDS in column buffer.

SDS Gel Electrophoresis and Western Blotting—Proteins were denatured in an SDS mixture (10 mM Tris, pH 6.8, 1% SDS, 10% glycerol) in the absence or presence of 4% β-mercaptoethanol and separated on 12% SDS gels. Proteins were transferred to an Immobilon-P membrane and electrophoretically transferred to an Immobilon-P membrane. The discoidin domain, which is flanked on the N-terminal side by a 39-amino acid RS1 domain, not found in other proteins, and on the C-terminal side by a 5-amino acid segment (Fig. 1). Of the 10 Cys, 5 are located in the discoidin domain, 4 in the Rs1 domain, and 1 in the C-terminal segment. To determine the importance of individual Cys on the structural properties of retinoschisin, we individually replaced each Cys with a Ser and analyzed protein expression in the cellular and secreted fractions of 293 cells on reducing and nonreducing SDS gels.

RESULTS

The Leader Sequence of Retinoschisin Is Cleaved after Ser-23—Retinoschisin contains a highly conserved, hydrophobic N-terminal leader sequence predicted to be cleaved by a signal peptide as part of the protein secretion process (6, 16, 19). To confirm that the leader signal peptide (Fig. 1) is cleaved in native retinoschisin and identify the site of cleavage, the N-terminal sequence of immunoaffinity-purified retinoschisin from retina tissue was determined by standard Edman degradation methods. The first five amino acid residues were identified as Ser-Thr-Glu-Asp-Glu (28), indicating that the signal peptide had removed the N-terminal 23-amino acid leader sequence to produce the mature secreted protein.

The Effect of Cys Mutagenesis on Expression, Subunit Assembly, and Secretion—Retinoschisin contains a 157-amino acid discoidin domain, which is flanked on the N-terminal side by a 39-amino acid RS1 domain, not found in other proteins, and on the C-terminal side by a 5-amino acid segment (Fig. 1). Of the 10 Cys, 5 are located in the discoidin domain, 4 in the Rs1 domain, and 1 in the C-terminal segment. To determine the importance of individual Cys on the structural properties of retinoschisin, we individually replaced each Cys with a Ser and analyzed protein expression in the cellular and secreted fractions of 293 cells on reducing and nonreducing SDS gels.

Under disulfide-reducing conditions, WT retinoschisin and all Cys variants were present in the cellular fraction at similar amounts migrating as a 24-kDa monomer (Fig. 2). Under nonreducing conditions, WT retinoschisin and 4 mutants (C38S, C40S, C42S, and C59S) migrated as a ~180-kDa disulfide-linked oligomer. The C83S mutant, however, was atypical in that it ran as a broader band. The remaining 6 Cys mutants appeared primarily as aggregates at the top of the gel, a pattern indicative of protein misfolding and aberrant intermolecular disulfide bond formation.

In the secreted fraction, 5 mutants (C38S, C40S, C42S, C59S, and C83S) were present at levels comparable with WT retinoschisin, 3 mutants (C63S, C219S, and C223S) showed moderate levels of secretion, and 2 mutants (C110S and C142S) displayed very limited secretion when analyzed under reducing conditions (Fig. 2). The relative levels of secretion were consistent between experiments, indicating that the differences were not due to variations in transfection efficiency. Under nonreducing conditions, WT retinoschisin and 4 mutants (C38S, C40S, C42S, and C59S) were secreted as 180-kDa oligomers. In contrast, C59S, which secreted at a high level, and 3 mutants (C63S, C219S, and C223S) that were secreted at moderate levels migrated at the dye front of a 6.5% gel. This suggests that selected Cys in this subset participate in disulfide-linked oligomerization of retinoschisin.

Identification of Cysteines Responsible for Disulfide-mediated Oligomerization—To further identify the residues responsible for disulfide-linked oligomerization, 4 Cys double mutants were characterized (Fig. 3). Only the C59S/C223S mutant was secreted at levels comparable with WT retinoschisin when analyzed under reducing conditions. When the secreted mutants were analyzed under nonreducing conditions, the C59S/C223S mutant, unlike WT retinoschisin, migrated as a low molecular weight species at the dye front of a 6.5% gel. The size of the secreted nonreduced C59S/C223S mutant was further ana-
Identified Cys Critical for Retinoschisin Structure and Oligomerization

The 10 Cys are conserved between mammalian and the evolutionarily distant Fugu rubripes pufferfish retinoschisin (6, 23, 24). This conservation, together with the high percentage of disease-linked missense mutations involving Cys, led us to examine the role of Cys in protein folding,

Discussion

Although retinoschisin plays a crucial role in retinal cell adhesion and XLRS, little is known about molecular determinants that contribute to its structure and function or molecular mechanisms responsible for the pathogenesis of XLRS. To address this issue, we compared the expression, cellular localization, molecular properties, and secretion of WT retinoschisin with Cys and disease-linked variants. Our studies identify Cys residues that are involved in intramolecular disulfide bonds critical for proper folding of the discoidin domain structure and intermolecular disulfide bonds that mediate the assembly of retinoschisin subunits into a large multimeric complex. We also show that defective disulfide-linked subunit assembly, discoidin domain misfolding, and ineffective insertion of the nascent polypeptide chain into the ER membrane are the primary molecular mechanisms underlying XLRS associated with missense mutations in the RS1 gene.

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lyzed on a 10% SDS-polyacylamide gel. As shown in Fig. 3 (lower right panel), the C59S/C223S mutant migrated as a mixture of monomers and dimers. The C63S/C223S mutant also migrated at the dye front on a 6.5% gel, but this variant was present at extremely low levels. The C59S/C219S mutant was present as a mixture of oligomers and low molecular weight components, and the C63S/C219S variant migrated anomalously slow. The results of the single and double mutants together with molecular modeling (see “Discussion”) indicate that the Cys-59 and Cys-223 residues are solely responsible for the generation of the 180-kDa disulfide-linked multimeric retinoschisin complex. One or more other cysteine residues, however, may be involved in dimer formation within the 180-kDa multimeric complex.

We also expressed the C110S/C142S double mutant. As shown in Fig. 3, this variant was present at low levels in the secreted fraction and migrated more slowly than WT retinoschisin under nonreducing conditions. This suggests that replacement of both these Cys leads to an alteration in the structure that causes a reduction in secretion of retinoschisin and anomalous migration behavior.

Analysis of Disease-linked Mutants—To assess the effect of disease-linked mutations on the expression, folding, oligomerization, and secretion of retinoschisin, we analyzed 12 missense mutations linked to XLRS (Fig. 1). All mutants, with the exception of the L13P variant, were present in the cellular fraction at levels comparable with WT retinoschisin when analyzed under reducing conditions (Fig. 4). However, the E72K and P203L mutants migrated more rapidly than the WT protein, possibly due to limited proteolysis. Under nonreducing conditions, most mutants in the cellular fraction were retained at the top of the gel as protein aggregates. Only the C223R and C59S disease-linked mutants were detected in the secreted fraction. In contrast to WT retinoschisin, these cysteine variants migrated as a low molecular weight species under nonreducing conditions, consistent with the role of Cys-223 and Cys-59 in disulfide-linked oligomerization.

Localization of WT and Mutant Retinoschisin to the ER—The subcellular localization of WT and mutant retinoschisin was examined by immunofluorescence microscopy. WT, Cys mutants, and disease-linked variants with the exception of L13P partially co-localized with the GRP 94 chaperone protein to the ER of COS-1 cells as shown in Fig. 5A for WT, C223S, and P203L mutants. In contrast, the L13P variant with the mutation in the leader sequence did not co-localize with GRP 94, but instead showed a less intense, diffuse labeling pattern indicative of cytoplasmic localization. The number of cells expressing detectable amounts of L13P mutant was also low. Only 10% of the transfected cells expressed the L13P mutant compared with over 30% for WT and the other mutants. WT and the secreted C223S mutant also co-localized with golgin-97, a Golgi marker that labels all of the cis, medial, and trans Golgi (Fig. 5B), consistent with the view that these proteins translocate from the ER to the Golgi as part of the secretion process. In contrast the L13P, P203L, and other nonsecreted mutants did not colocalize with the Golgi marker. Wang et al. (27) have recently observed broadly similar labeling patterns for a different set of retinoschisin disease-linked mutants.

Discussion

Although retinoschisin plays a crucial role in retinal cell adhesion and XLRS, little is known about molecular determinants that contribute to its structure and function or molecular mechanisms responsible for the pathogenesis of XLRS. To address this issue, we compared the expression, cellular localization, molecular properties, and secretion of WT retinoschisin with Cys and disease-linked variants. Our studies identify Cys residues that are involved in intramolecular disulfide bonds critical for proper folding of the discoidin domain structure and intermolecular disulfide bonds that mediate the assembly of retinoschisin subunits into a large multimeric complex. We also show that defective disulfide-linked subunit assembly, discoidin domain misfolding, and ineffective insertion of the nascent polypeptide chain into the ER membrane are the primary molecular mechanisms underlying XLRS associated with missense mutations in the RS1 gene.
subunit assembly, and secretion of retinoschisin.

The Cys can be divided into 3 classes based on their potential to form intramolecular or intermolecular disulfide bonds and their effect on retinoschisin structure. One class consists of 4 Cys (Cys-63, Cys-110, Cys-142, and Cys-219) in the discoidin domain that are essential for proper folding and efficient protein secretion. When any one of these is substituted with a Ser, a major portion of expressed protein is retained in the ER as misfolded, disulfide-linked protein aggregates. The small fraction that is secreted exhibits two distinct behaviors on nonreducing gels. The C63S and C219S mutants migrate as low molecular weight species, whereas the C110S and C142S mutants migrate as oligomers. This suggests that Cys-63 and Cys-219 constitute one intramolecular disulfide-linked pair and Cys-110 and Cys-142 make up another. If any one of these Cys is replaced with a Ser, protein misfolding and aberrant disulfide-mediated aggregation occurs resulting in retention in the ER.

Support for these two intramolecular disulfide assignments comes from analysis of other discoidin family members and molecular modeling of the retinoschisin discoidin domain. Cys residues at the beginning and end of the discoidin domain, corresponding to Cys-63 and Cys-219 in retinoschisin, are conserved in the discoidin domains of blood coagulation proteins, Factors V and VIII, as well as all other discoidin family members (Fig. 6A). Crystal structures of the C2 discoidin domain of Factor V and Factor VIII blood proteins reveal that the side chains of these Cys form an intramolecular disulfide bond, clamping together the N and C termini of the discoidin domain (14, 15). Likewise, in our molecular modeling of retinoschisin, the Cys-63 and Cys-219 residues come in close proximity to one another, suggesting that these two residues form an intramolecular disulfide bond (Fig. 6C).

The Cys-110 and Cys-142 residues, not found in other discoidin family members, reside in two different loops or spikes (Fig. 6, A and C). The side chain of Cys-110 in spike 2 comes sufficiently close to the side chain of Cys-142 in spike 3 to allow for the formation of another intramolecular disulfide bond within the retinoschisin discoidin domain. This disulfide bond appears to link these spikes together into a more rigid structure (Fig. 6C).

The importance of the Cys-63-Cys-219 and Cys-110-Cys-142 intramolecular disulfide bonds is further highlighted by the finding that mutations in 3 of the 4 Cys (C110Y, C142W, and C219R) result in XLRS (4, 18) and, as indicated in this study, cause protein misfolding, aggregation, and retention in the ER.
It is likely that a missense mutation in Cys-63 would also be responsible for XLRS. Indeed, missense mutations in the analogous residue of Factor VIII are associated with hemophilia A (29).

A second class consists of Cys-59 and Cys-223 lying just outside the discoidin domain. A large fraction of both single and double cysteine mutants fold properly as indicated by their ability to effectively pass through the ER quality control system.
Fig. 6. Sequence alignment, structural model for the retinoschisin discoidin domain, and localization of disease-linked mutations. A, alignment of the discoidin domain of retinoschisin with the C2 discoidin domain of coagulation factors FV and FVIII. Numbering is for retinoschisin amino acids. Conserved residues are highlighted in yellow and the two conserved cysteines (C63 and C219) are highlighted in black. Disease mutants examined in this paper and by Wang et al. (27) and shown to be defective in secretion are indicated by v. Other disease missense mutations are marked by + (18). The 8 core β strands and 3 spikes are underlined as initially described by Fuentes-Prior et al. (13) for factors FV and FVIII. B, intramolecular and intermolecular disulfide-bonding pattern of Cys residues within and flanking the discoidin domain. It is not known if Cys-83 forms an intramolecular or intermolecular disulfide bond. C, ribbon diagram of the retinoschisin discoidin domain modeled from the Factor V and Factor VIII C2 discoidin domain structures. The model shows the locations of the Cys-63-Cys-219 and Cys-110-Cys-142 intramolecular disulfide bonds (arrows), Cys-83, and the locations of several amino acids mutated in X-linked Juvenile Retinoschisis. D, stick model of the retinoschisin discoidin domain in which blue represents backbone and side chain nitrogens, and red represents side chain oxygens.
system (25), but unlike WT retinoschisin, they are unable to form the large disulfide-linked oligomeric complex. From these results, we conclude that Cys-59 and Cys-223 are not critical for the folding of the retinoschisin subunit, but instead are responsible for intermolecular disulfide bonds that mediate retinoschisin oligomerization (Fig. 6B). The size of the oligomer remains to be determined. However, on nonreduced SDS gels, the complex migrates with an apparent molecular mass of ~180 kDa, suggesting that the subunits assemble as a disulfide-linked octamer.

The third class, comprised of Cys-38, Cys-40, Cys-42, and Cys-83, has a limited effect on retinoschisin structure and secretion as shown in Fig. 2. One Cys (Cys-83) in the β1 strand of the discoidin domain is occupied by Ala in most other discoidin family proteins (Fig. 6A). The 3 others (Cys-38, Cys-40, and Cys-42) are in the Rs1 domain as part of a conserved CXCZC motif (where X and Z are any amino acids) found in several extracellular proteins including most β-integrins and the silk proteins sp185 and sp220. Because mutagenesis of these Cys has no significant effect on protein expression, secretion, or oligomer formation, these residues do not appear to play a critical role in protein structure or subunit assembly. Although it is possible that these residues exist in their reduced state, more likely some or all the Cys side chains form intramolecular and/or intermolecular disulfide bonds in the oxidizing environment of the ER lumen and extracellular compartment, thereby contributing to protein stability and possibly function. In β-integrins, the two flanking Cys of the CXCZC motif form one disulfide bond and the central Cys forms another intramolecular disulfide bond with a more distal Cys (26). By analogy, it is possible that Cys-38 and Cys-42 form one intramolecular disulfide bond, and Cys-40 and Cys-83 of the discoidin domain form another disulfide bond. The secreted C59S/C223S mutant defective in oligomerization migrates as a mixture of monomers and dimers under nonreducing conditions (Fig. 3), suggesting that one or more of these Cysteines may be involved in intramolecular disulfide bonds that link monomers into dimers. Conclusive identification of the possible disulfide bonds involving these four cysteine residues awaits peptide-mapping studies. Interestingly, to date no missense mutations of these Cys residues have been linked to XLRs consistent with their more passive role in retinoschisin structure and function.

Disease-linked Mutations Affect Subunit Structure and Assembly—Missense mutations associated with XLRs can be divided into three groups based on their location in the sequence and effect on retinoschisin expression, structure, and cellular localization. One group comprises mutations in the hydrophobic leader sequence (4). As shown here, the L13P mutation results in a marked decrease in protein expression and irregular localization to the cytoplasmic compartment of the cell. The decrease in protein expression observed by SDS gel electrophoresis and Western blotting appears to be due in part to the lower number of cells expressing detectable levels of the L13P mutant and in part to the lower protein levels in the individual cells as visualized by immunofluorescence microscopy. Wang et al. (27) have recently reported a similar localization pattern for the L12H mutation. Their studies further showed that the mutated leader peptide is not cleaved by the signal peptidase, and the protein is readily degraded by proteosomes. Together, these studies suggest that substitution of hydrophobic residues in the leader sequence with Pro or hydrophilic/residues prevents the leader peptide from adopting an α-helical secondary structure required for insertion into the ER membrane. Instead, the newly synthesized polypeptide chain remains in the reducing environment of the cytoplasm as a misfolded protein, where it is rapidly degraded by proteosomes.

A second group consists of the C59S and C223R disease-linked mutations in the regions flanking the discoidin domain. A significant fraction of the protein passes through the Golgi and is secreted into the extracellular medium, indicating that these Cys substitutions have only a limited effect on protein folding. However, as discussed above, the C59S and C223R mutants, unlike WT retinoschisin, cannot assemble into disulfide-linked oligomers. In this instance, failure to form disulfide-linked oligomers and not defective secretion is responsible for the inability of retinoschisin to function as a cell adhesion protein, thereby resulting in the XRLS phenotype.

The third, and largest, group of disease-linked missense mutations are those found in the discoidin domain. Insight into how these mutations cause protein misfolding leading to aggregation and defective secretion can be deduced from molecular modeling. Like Factors V and VIII, the retinoschisin discoidin domain model consists of a β-barrel core formed by 8 antiparallel strands (Fig. 6, C and D). Noncovalent interactions within the core structure, together with the Cys-63-Cys-219 and Cys-110-Cys-142 disulfide bonds, play essential roles in generating and maintaining this structure. Mutations in core, solvent inaccessible residues prevent proper protein packing resulting in protein misfolding and aggregation. This in turn results in the mutant protein being retained in the ER as visualized by immunofluorescence.

One subset of these mutations in the discoidin domain directly involves the loss of Cys residues. As mentioned above, C110Y, C142W, and C219R involve the replacement of key Cys required for intramolecular disulfide bonding and proper folding of the discoidin domain (Fig. 6C). The misfolded protein together with the formation of abnormal disulfide bonds in the oxidizing environment of the ER lumen results in protein aggregation and defective secretion. Interestingly, these disease mutants appear to be completely retained within the cell compared with the corresponding Cys to Ser variants. Evidently, bulky Tyr and Trp or charged Arg side chains have a more profound effect on the protein misfolding than Ser residues.

Another subset involves missense mutations in highly conserved, solvent inaccessible core residues not directly involved in disulfide bonds. For example, Pro-203 is a highly conserved, buried residue that interrupts the β strand (Fig. 6, A and C). Substitution with a leucine residue will cause a marked change in the secondary structure of the protein, which in turn affects protein packing. Other disease-linked mutations in core residues most likely cause the disruption in key noncovalent interactions crucial for the formation or stability of the discoidin domain. For instance, the G109E mutation involves substituting a charged residue for a highly buried Gly. Another example is the conserved Arg-182 residue, which in the retinoschisin model comes in close proximity to Glu-146. This suggests that a salt bridge may exist between these side chains that helps to fold and stabilize the protein structure. Disease-linked missense mutations will prevent ionic interactions from forming between these residues, thereby resulting in protein misfolding. In the case of R182C there is also the potential to form aberrant disulfide bonds with other Cys side chains. The E72K disease-linked mutant involves an inversion in charge, which leads to protein misfolding and ER retention suggesting that this Glu is involved in ionic interactions. Factor VIII contains a Glu in an analogous position, mutations in which have been linked to hemophilia. In this instance, it has been suggested that this residue plays a role in protein-protein interactions involving other blood proteins (28). In the case of retinoschisin,
this residue may be important in interactions with adjacent subunits.

In summary, our results indicate that intramolecular disulfide bonds between Cys-63 and Cys-219, and between Cys-110 and Cys-142 play critical roles in the structure of the retinoschisin discoidin domain. The Cys-59-Cys-223 intermolecular disulfide bond is not essential for subunit folding or secretion, but is crucial for retinoschisin oligomerization. Finally, we have identified three molecular mechanisms responsible for XLRS. Disease-linked mutations in the discoidin domain of retinoschisin cause protein misfolding and retention in the ER; mutations in Cys-59 and Cys-223 result in defective disulfide-linked subunit assembly and loss in function, but do not significantly affect secretion; and mutations in the leader sequence of retinoschisin prevent proper insertion of the polypeptide chain into the ER membrane, resulting in cellular mislocalization and defective secretion. In each case, there is a loss in the ability of retinoschisin to function as an extracellular retinal adhesion protein required to maintain the cellular architecture of the retina (17).

Acknowledgments—We thank Dr. Bernhard Weber for the human RS1 cDNA and helpful comments on the manuscript, and Laurie Molday and Jinhi Ahn for technical support.

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J. Biol. Chem. 2003, 278:28139-28146.
doi: 10.1074/jbc.M302464200 originally published online May 13, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302464200

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