

# Coexpression and Interaction of Wild-type and Missense RS1 Mutants Associated with X-Linked Retinoschisis: Its Relevance to Gene Therapy

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**PURPOSE.** X-linked retinoschisis (XLRS) is an early-onset retinal disease caused by mutations in retinoschisin (RS1), a multisubunit, extracellular protein implicated in retinal cell adhesion. Delivery of the normal *RS1* gene to photoreceptors of retinoschisin-deficient mice results in prolonged protein expression and rescue of retinal structure and function. However, most persons with XLRS harbor a missense mutation in the *RS1* gene leading to expression of a nonfunctional protein. The purpose of this study was to examine the effect that coexpression of wild-type RS1 with disease-causing mutants has on RS1 expression, oligomerization, and secretion to further evaluate gene therapy as a possible treatment for XLRS.

**METHODS.** RS1 mutants (C59S, D158N, C142W, C142S, T185K, R141H, R141G) were individually expressed or coexpressed with myc-tagged wild-type RS1 (myc-RS1) in EBNA293 cells. Protein expression, secretion, and subunit assembly of wild-type and mutant RS1 were analyzed by Western blotting and coimmunoprecipitation. Immunofluorescence was used to examine the cellular distribution of RS1.

**RESULTS.** Myc-RS1 was identical to untagged, wild-type RS1 with respect to cellular localization, disulfide-linked octamer formation, and secretion. In coexpression studies, myc-RS1 assembled into a disulfide-linked octameric complex and was secreted from cells independent of all disease-linked RS1 mutants studied except the R141H mutant.

**CONCLUSIONS.** When wild-type RS1 is expressed in the same cells as disease-causing mutants, the wild-type protein undergoes protein folding, subunit assembly, and secretion independent of all disease-causing RS1 mutants studied except R141H. These studies suggest that gene therapy may be an effective treatment for most persons with XLRS. (*Invest Ophthalmol Vis Sci.* 2007;48:2491–2497) DOI:10.1167/iovs.06-1465

X-linked retinoschisis (XLRS) is a leading cause of early-onset macular degeneration in males. Affected persons typically display cystic streaks projecting from the parafoveal region of the retina, a decrease in visual acuity, and a reduction

in the b-wave amplitude of the full-field electroretinogram (ERG).<sup>1,2</sup> Optical coherence tomography (OCT) further reveals a splitting of the outer plexiform and adjacent retinal layers.<sup>3,4</sup> During the course of the disease, retinal detachment, vitreal hemorrhage, and neovascular glaucoma can arise as secondary complications, leading to a poor visual outcome. Female carriers are clinically unaffected.

The *RS1* gene associated with XLRS encodes a 224-amino acid protein known as retinoschisin, or RS1,<sup>5</sup> which is expressed and secreted primarily from photoreceptor cells.<sup>6–8</sup> Retinoschisin consists of a 23-amino acid N-terminal signal or leader peptide, a 39-amino acid Rs1 domain, a 157-amino acid discoidin domain, and a 5-amino acid C-terminal segment.<sup>5,9,10</sup> The signal peptide directs the nascent polypeptide chain across the endoplasmic reticulum (ER) membrane. A signal peptidase in the lumen of the ER removes the signal peptide, enabling the processed polypeptide to assemble into a disulfide-linked octameric complex for secretion from cells.<sup>9</sup> Disulfide-linked octamer formation is mediated by two cysteine residues, one in the Rs1 domain (C59) and one in the C-terminal segment (C223). The discoidin domain is the main structural feature of retinoschisin. Discoidin domains, first discovered in discoidin proteins of the slime mold *Dictyostelium discoideum*, have been found in a variety of extracellular and cell surface membrane proteins, including blood coagulation factors 5 and 8, milk fat globule protein, neuropilins 1 and 2, neurexin IV, and discoidin domain receptor protein tyrosine kinases.<sup>11,12</sup> The function of discoidin domains is not understood, though they have been implicated in cell adhesion, signaling, and development.

To date, more than 130 different mutations in the *RS1* gene have been associated with XLRS.<sup>13</sup> The effects of a number of disease-causing missense mutations on retinoschisin expression, structure, subunit assembly, and secretion have been investigated in culture cells.<sup>9,10,14,15</sup> Missense mutations in the signal peptide prevent translocation of the polypeptide across the ER membrane, resulting in rapid protein degradation. Disease-causing mutations in the Rs1 domain and C-terminal segment prevent the normal assembly of retinoschisin into a homo-octamer. Most mutations in the discoidin domain cause protein misfolding and retention in the cell by the quality control system of the ER. Interestingly, at least one disease-causing mutation in the discoidin domain (R141H) results in normal expression and secretion from cells.<sup>15</sup> The mechanism by which this mutation causes XLRS is unknown.

Retinoschisin knockout mice exhibit many features found in persons with XLRS.<sup>16</sup> These include disorganization of the retinal cell layers with gaps in the inner retina, reduction in the b-wave amplitude of the full-field ERG, marked decrease in the photopic response, and progressive rod and cone photoreceptor degeneration. More recently, retinoschisin-deficient mice have been used as a model system to test the proof-of-principle concept that adeno-associated viral (AAV)-mediated *RS1* gene delivery can serve as

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**FIGURE 1.** Schematic of retinoschisin showing the location of the signal or leader sequence (LS; amino acids 1–23), Rs1 domain (Rs1; amino acids 24–62), discoidin domain (amino acids 63–219), and the carboxyl terminal (CT) segment (amino acids 220–224). The location of the 9-amino acid myc-tag (EQKLISEED) and the disease-causing missense mutation studied are shown.

a potential treatment for XLRS.<sup>17,18</sup> A single subretinal injection of recombinant AAV containing human *RS1* cDNA under the control of a mouse opsin promoter was shown to result in prolonged retinoschisin expression and normal localization in the retina. This correlated with increases in scotopic and photopic ERGs, significant improvement in retinal cell and synaptic organization, absence of inner retina splitting, and increased rod and cone survival.<sup>18</sup>

Although these studies suggest that delivery of the normal *RS1* gene to photoreceptors deficient in retinoschisin expression can rescue the structure and function of the retina, it remains to be determined whether delivery of the normal *RS1* gene to cells expressing mutant protein is equally effective. Because retinoschisin is a multisubunit protein, assembly of the normal protein subunits with mutant subunits within the cell could result in the expression and secretion of a nonfunctional retinoschisin complex, thereby limiting the application of gene therapy for XLRS. In this article, we have examined the interaction of wild-type (WT) retinoschisin with various disease-causing mutants to further evaluate the potential application of gene therapy as a general treatment for XLRS.

## MATERIALS AND METHODS

### Generation of cDNA Constructs

Human wild-type (WT)-*RS1* cDNA and C59S, R141C, C142W, and C142S mutants in pCEP4 (Invitrogen, Carlsbad, CA) have been described.<sup>10</sup> Quick change site-directed mutagenesis (Stratagene, La Jolla, CA) was used to introduce the following amino acid substitutions into *RS1* (T185K, R141H, R141G, R141A, R141K, R141E, R141Q, R141V, R141S, D158N). The myc tag was inserted into WT-*RS1* cDNA following the leader sequence (after aa 23) by PCR amplification of *RS1* fragments containing a 5' untranslated region, the N-terminal part of *RS1* from position 1 to 69 (aa 1–23), the N-terminal half of the myc tag sequence, and an *AseI* restriction site using primers P1 and P3 and amplification of a fragment consisting of an *AseI* site, the C-terminal half of the myc tag and the C-terminal part of *RS1* position 70 to 675 (aa 24–224), and a 3' untranslated region using primers P2 and P4. Purified fragments were digested with *AseI* (New England Biolabs, Pickering, ON, Canada) and ligated with T4 ligase (NEB). The resultant product was reamplified by PCR and cloned in pCEP4 using *XbaI* and *HindIII*. Primers used were as follows, with the *AseI* restriction site marked in bold letters: P1, AGCAGAGCTCGTTTAGTGAACCG; P2, GTGGTTTGCCAAACTCATC; P3, GAAATTAATCAAGTGAAGATCTGTCTACCGAGGATGAAGGCG; P4, CTGATTAATTTCTGCTCCGATAATCCCAATGTGGCTTC. All constructs were sequenced to verify the desired mutation and the absence of random mutations.

### Cell Culture and Transfection

EBNA 293 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco modified Eagle medium (DMEM) with l-glutamine, 10% fetal calf serum, and G418 (Invitrogen). Single transfections were carried out in 10-cm dishes with 20  $\mu$ g DNA per dish

using the calcium phosphate transfection procedure previously described.<sup>10</sup> For cotransfection, 10  $\mu$ g myc-tagged *RS1* (myc-*RS1*) and 10  $\mu$ g other plasmids were used. Briefly, 500  $\mu$ L BES-buffered saline (50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethane, 280 mM NaCl, 1.4 mM Na<sub>2</sub>PO<sub>4</sub>, pH 6.95) was added dropwise to a DNA solution containing 250 mM calcium chloride and was incubated for 20 minutes at room temperature. DNA was then added to exponentially growing EBNA 293 cells at 37°C under 5% CO<sub>2</sub>. The DNA-containing medium was replaced with regular medium the next day, and the cells were harvested 2 days later.

### Immunoprecipitation and Protein Analysis

*RS1* 3R10<sup>16</sup> and the myc 9E10 (Developmental Studies Hybridoma Bank, Iowa City, IA) monoclonal antibodies were purified from hybridoma cell supernatants by protein G affinity chromatography and coupled to CNBr-activated Sepharose 2B.<sup>19</sup> Immunoprecipitation, SDS gel electrophoresis, and Western blotting were performed as described previously.<sup>9,10</sup>

### Immunofluorescence Microscopy

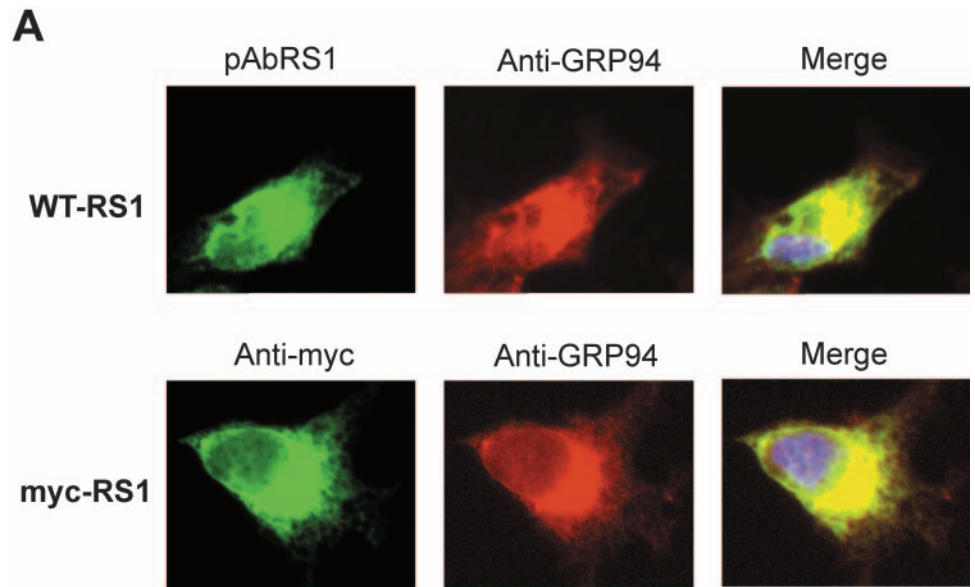
Transfected COS-7 cells were grown in DMEM with l-glutamine and 10% fetal calf serum on polylysine-coated coverslips at 37°C and fixed with 4% paraformaldehyde in phosphate buffer (PB; 0.1 M phosphate, pH 7.4) for 25 minutes. Coverslips were blocked in PB with 0.5% Triton X-100 and 10% goat serum for 30 minutes and labeled overnight with polyclonal *RS1* antiserum (1:1000 dilution) or an affinity-purified polyclonal antibody against the myc tag (1:500 dilution) and a monoclonal antibody against the ER marker GRP94 (1:300 dilution). After washing with PB, the samples were labeled with goat anti-mouse immunoglobulin conjugated with Alexa 568 and with goat anti-rabbit immunoglobulin conjugated with Alexa 488 followed by DAPI staining. Labeled cells were examined under a fluorescence microscope (Axio-plan 2; Zeiss, Oberkochen, Germany) equipped with a digital image analysis system.

## RESULTS

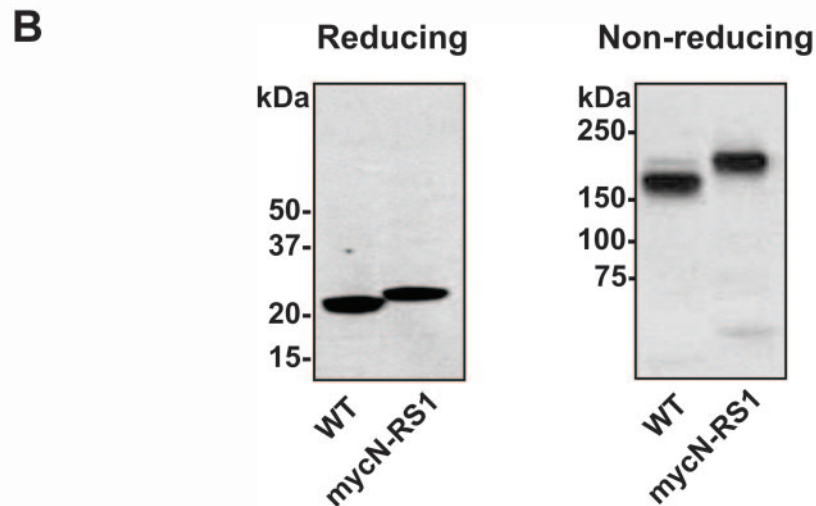
### Cellular Expression and Biochemical Analysis of Myc-Tagged *RS1*

To distinguish between WT and mutant retinoschisin in coexpression experiments, a 9-amino acid myc epitope was inserted just downstream of the N-terminal leader peptide cleavage site (Fig. 1). The effect of the myc tag on the subcellular localization of retinoschisin was examined in transfected COS-7 cells by immunofluorescence microscopy. Figure 2A shows that myc-tagged retinoschisin (myc-*RS1*), like the untagged WT retinoschisin protein (WT-*RS1*), had a perinuclear pattern that colocalized with the GRP-94 ER marker.

Next, we determined whether myc-*RS1* is secreted from cells as a disulfide-linked oligomeric complex similar to WT-*RS1*. Figure 2B shows that myc-*RS1* was present in the secreted fraction of transfected cells at levels comparable to



**FIGURE 2.** Immunofluorescence and Western blotting of WT-RS1 and myc-RS1. **(A)** Immunofluorescence microscopy of COS-7 cells expressing WT-RS1 or myc-RS1. Cells were double labeled with a polyclonal antibody to RS1 (pAbRS1) or a polyclonal antibody to the myc tag (anti-myc) and a monoclonal antibody to the ER marker GRP94 (anti-GRP94) followed by goat anti-rabbit immunoglobulin Alexa 488 (green) and goat anti-mouse immunoglobulin Alexa 568 (red) and subsequently stained with the nuclear dye DAPI. Merged images are also shown. **(B)** Western blot analysis of the secreted fraction from cells expressing WT-RS1 and myc-RS1 under disulfide-reducing and -nonreducing conditions. Blots were labeled with the RS1 3R10 monoclonal antibody.



those of WT-RS1 and that it migrated on SDS gels as a 24-kDa monomer under disulfide-reducing conditions and a 180-kDa oligomer characteristic of an octamer under nonreducing conditions.<sup>9</sup> The decreased mobility of myc-RS1 relative to WT-RS1 is consistent with its increased size because of the myc epitope.

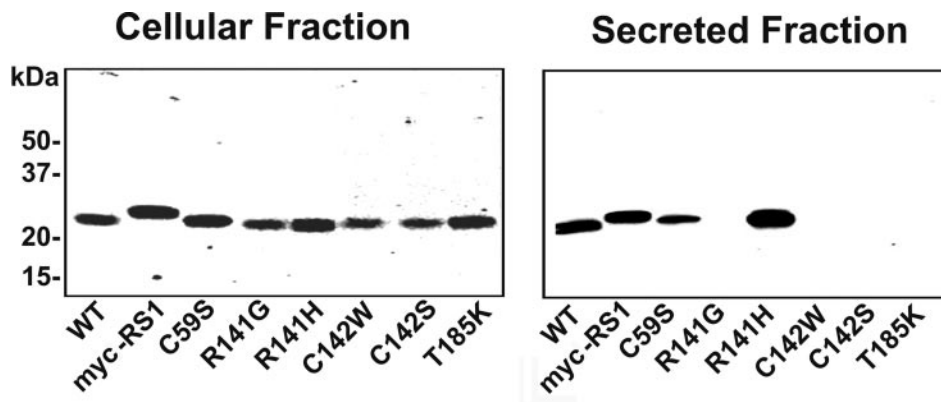
#### Expression of WT and Disease-Linked Mutants

The expression of WT-RS1 and a number of disease-linked mutants was examined in the cellular and secreted fractions of transfected cells. All mutants were detected in the cellular fraction of EBNA 293 cells at levels comparable to that of WT RS1; however, only the C59S and R141H mutants were observed in the secreted fractions (Fig. 3). The R141H mutant, like WT-RS1, assembled into a disulfide-linked octameric complex (see Fig. 7), whereas the C59S mutant did not, as previously reported.<sup>10,15</sup> R141G, C142W, C142S, and T185K mutants were not present in the secreted fraction,

indicating that these proteins were highly misfolded and were retained inside the cells by the quality control system of the ER.

#### Coexpression and Interaction of Myc-RS1 with WT-RS1

To determine whether myc-tagged RS1 associates with the WT protein to form a natively complex, the secreted fraction from cells coexpressing the myc-RS1 and WT-RS1 was compared with the secreted fractions from individually transfected cells under nonreducing conditions. As shown in Figure 4, a mixture of individually expressed myc-RS1 and WT-RS1 (lane b) resolved into two closely spaced bands corresponding to myc-RS1 (upper band) and WT RS1 (lower band). In contrast, the coexpression of myc-RS1 and WT-RS1 resulted in a broader band (lane c) that migrated between the WT RS1 (lane a) and myc-RS1 band (lane d). This suggests that disulfide-linked octameric complexes consisting



**FIGURE 3.** Expression of WT RS1, myc-RS1, and disease-causing RS1 mutants. Cellular and secreted fractions of individually transfected EBNA 293 cells were run on 10% reducing gels. Western blots were labeled with RS1 3R10 monoclonal antibody.

of the myc-RS1 and WT RS1 proteins form when these proteins are coexpressed and secreted from cells.

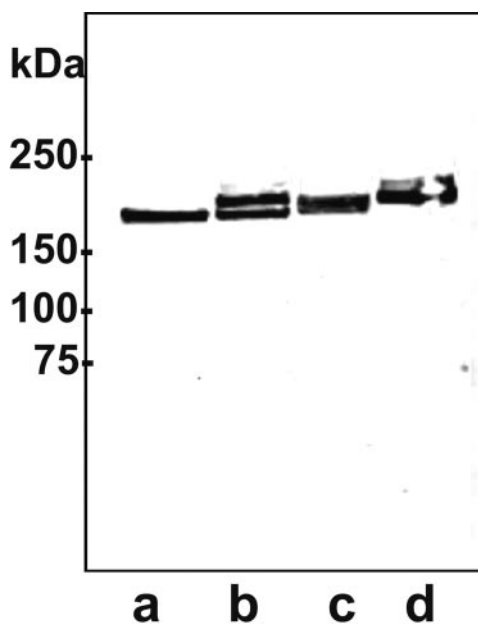
### Coexpression of Myc-RS1 with Disease-Linked Mutants

The interaction between myc-RS1 and disease-linked RS1 mutants was studied in coexpression and coimmunoprecipitation experiments. First, the ability of RS1 mutants to coexpress with myc-RS1 was examined. Figure 5 shows that the cellular fraction of cotransfected cells contained two closely spaced bands under disulfide-reducing conditions corresponding to myc-RS1 (upper band) and untagged WT or mutant RS1 (lower band).

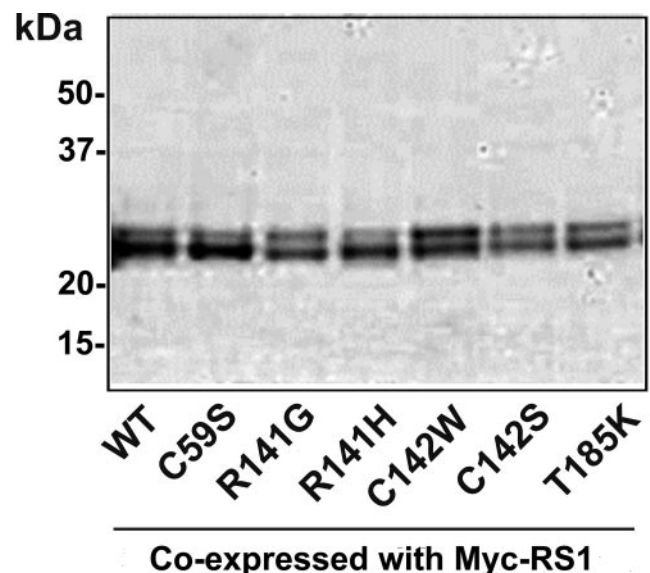
To determine whether myc-RS1 associates with mutant proteins to form a complex that is secreted from cells, coimmunoprecipitation experiments were carried out. The secreted fraction from cells coexpressing myc-RS1 and WT or mutant

RS1 was immunoprecipitated on either the anti-RS1 3R10-Sepharose or the anti-myc 9E10-Sepharose matrix, and the bound fraction was analyzed on Western blot analysis labeled with the anti-RS1 antibody, which recognizes both myc-RS1 and untagged RS1, or the anti-myc antibody, which recognizes only myc-RS1. The presence of two anti-RS1-labeled bands in the bound fraction of RS1 3R10-Sepharose indicates that the untagged WT or mutant RS1 (lower band) is present in the secreted fraction together with myc-RS1 (upper band), whereas two bands from myc 9E10-Sepharose show that a complex was formed between myc-RS1 and WT or mutant RS1.

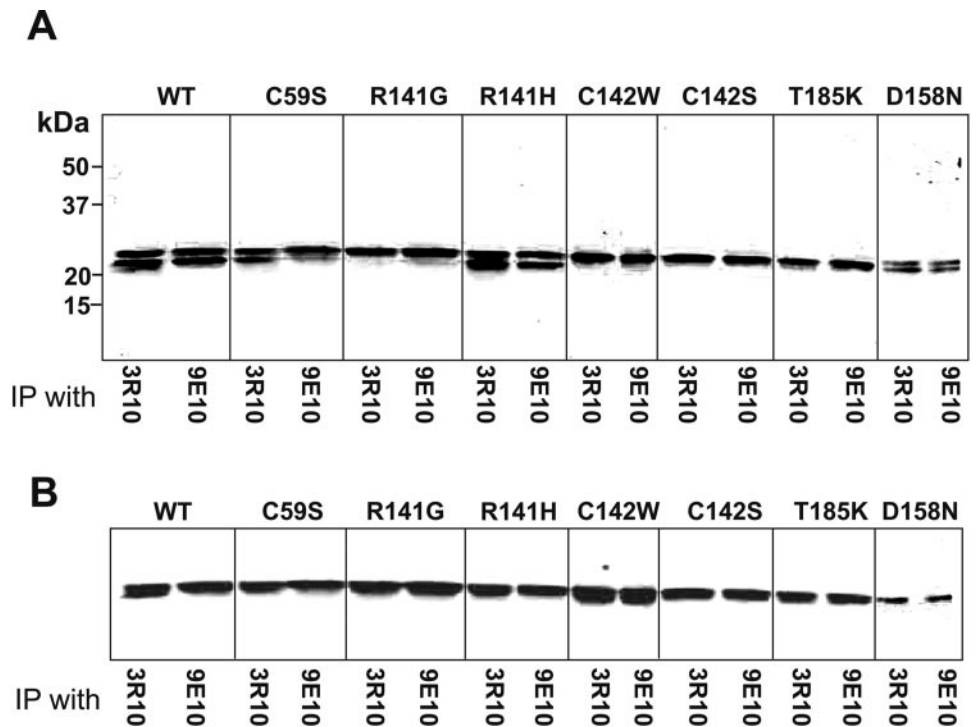
The results of these studies are shown in Figure 6A and Table 1.<sup>13-15,20-23</sup> Immunoprecipitation with RS1 3R10-Sepharose showed two bands in the secreted fraction from cells expressing myc-RS1 with WT-RS1, the polymorphic D158N variant, and the disease-causing mutants, C59S and R141H, but only a single myc-RS1 band for coexpression with the R141G, C142W, C142S, and T185K mutants, indicating that WT, D158N, C59S, and R141H proteins are present with myc-RS1 in the secreted fraction, whereas the R141G, C142W, C142S, and T185K mutants are retained inside the cell when expressed individually or with myc-RS1. Immunoprecipitation with myc



**FIGURE 4.** Western blot analysis of the secreted fraction from cells individually expressing WT-RS1 and myc-RS1 and coexpressing these proteins. *Lane a:* secreted fraction from cells expressing only WT-RS1. *Lane b:* mixture of secreted fraction of cells individually expressing WT-RS1 and myc-RS1. *Lane c:* secreted fraction from cells coexpressing WT-RS1 and myc-RS1. *Lane d:* secreted fraction from cells expressing only myc-RS1. SDS gels were run under nonreducing conditions, and Western blots were labeled with the RS1 3R10 monoclonal antibody.



**FIGURE 5.** Western blot analysis of the cellular fraction from cells coexpressing myc-RS1 with WT-RS1 or RS1 mutants. Western blots were labeled with the RS1 3R10 monoclonal antibody. The *upper band* represents myc-RS1, and the *lower band* represents WT or mutant RS1.



**FIGURE 6.** Coexpression and coimmunoprecipitation of myc-RS1 with WT-RS1 or mutant RS1. Secreted fractions from cells coexpressing myc-RS1 and WT-RS1 or mutant RS1 were coimmunoprecipitated with anti-RS1 (3R10) or with anti-myc (9E10) Sepharose. Bound proteins were run on a 15% SDS-polyacrylamide gels under reducing conditions, and Western blots were labeled with the RS1 3R10 monoclonal antibody (A) or a polyclonal antibody to the myc epitope (B).

9E10-Sepharose showed the presence of two bands for the WT, D158N variant, and R141H mutant but only a single band for C59S mutant, indicating that WT, D158N, and R141H associate with myc-RS1 to form a complex, whereas the C59S mutant does not assemble with myc-RS1. Western blot analysis labeled with an anti-myc antibody showed the presence of myc-RS1 in the secreted fractions of all mutants (Fig. 6B).

### Expression, Oligomerization, and Secretion of RS1 Mutants with Various Amino Acid Substitutions at Position 141

The R141H mutant is unique among the disease mutants studied here in that, like WT-RS1, it is secreted from cells as a disulfide-linked octameric complex and is capable of interacting with WT-RS1 to form a native-like complex. It was of interest to determine what effect replacement of arginine at

position 141 with other amino acids has on protein expression, oligomerization, and secretion. As shown in Figure 7, all RS1 141 mutants studied were expressed at levels similar to those of WT-RS1, as analyzed for the cellular fraction. However, only three mutants, R141H, R141A, and R141S, were secreted from cells as octamers at levels similar to those of WT-RS1.

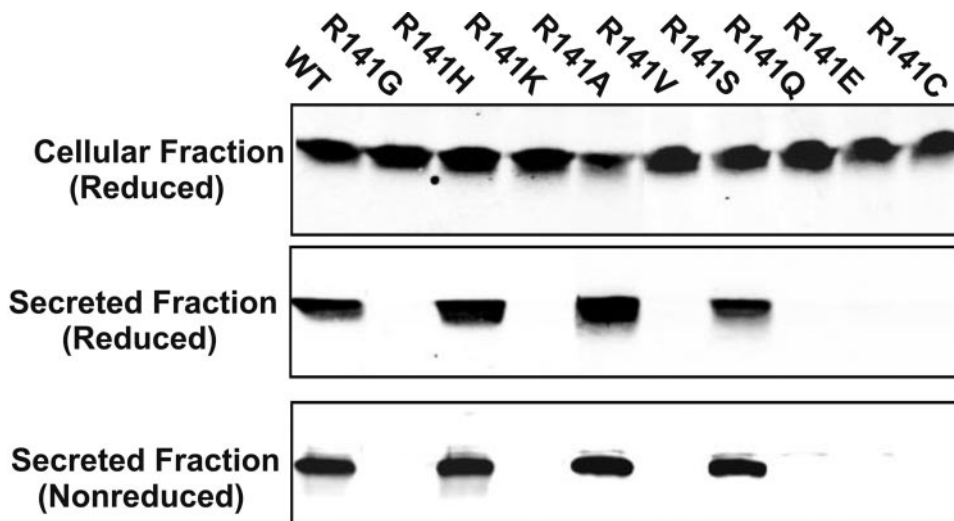
### DISCUSSION

In previous studies, transfected EBNA 293 and COS-7 cells were used to study the expression, secretion, and subunit assembly of WT and disease-causing retinoschisin mutants.<sup>9,10,14,15</sup> Most mutations in the discoidin domain resulted in protein misfolding, aggregation, and complete retention in cells by the quality control system of the ER (Table 1). A few proteins with disease-causing mutations in the discoidin do-

**TABLE 1.** Characteristics of the Retinoschisin Mutants

Mutation	Intracellular Expression	Secretion	Octamerization	Disease Relationship	Interaction with WT
WT	Yes	Yes	Yes	No	—
C59S	Yes	Yes	No	Yes <sup>13,14</sup>	No
R141G	Yes	No*	No	Yes <sup>13,15,20</sup>	No
R141H	Yes	Yes	Yes	Yes <sup>13,21</sup>	Yes
R141A	Yes	Yes	Yes	NR	ND
R141V	Yes	No	No	NR	ND
R141S	Yes	Yes	Yes	NR	ND
R141Q	Yes	No	No	NR	ND
R141C	Yes	No	No	Yes <sup>13,20</sup>	ND
R141E	Yes	No	No	NR	ND
R141K	Yes	No	No	NR	ND
C142W	Yes	No	No	Yes <sup>15,22</sup>	No
C142S	Yes	No	No	NR	No
D158N	Yes	Yes	Yes	No <sup>13</sup>	Yes
T185K	Yes	No	No	Yes <sup>23</sup>	No

\*Wang et al.<sup>14</sup> reported the secretion for the R141G mutant from COS-7 cells but at low levels. NR, none reported to date; ND, not determined.



**FIGURE 7.** Expression and secretion of WT RS1 and R141 RS1 mutants. Cellular and secreted fractions from individually transfected EBNA 293 cells were analyzed under reducing or nonreducing conditions on Western blots labeled with the RS1 3R10 monoclonal antibody. All proteins expressed were detected as 24-kDa monomeric proteins in the cellular fraction under reducing conditions, but only WT RS1 and the R141H, R141A, and R141S mutants were detected in the secreted fraction as monomers under reducing conditions and octamers under nonreducing conditions.

main were reported to be secreted from cells, but at a significantly reduced level.<sup>15</sup> In such cases, a small fraction of the proteins apparently adopt a conformation sufficiently similar to that of the WT protein to pass through the ER quality control system as part of the secretion process.

The R141H disease-linked mutant, as recently reported by Wang et al.<sup>15</sup> and confirmed in this report, is unique among the disease-causing mutations studied to date. Like WT retinoschisin, this mutant is expressed and secreted at normal levels as an octamer, suggesting that this protein preferentially folds into a natively conformation. The fact that this mutant causes XLRs,<sup>21</sup> however, suggests that it may be defective in its function as an extracellular retinal protein. The C59S and C223R disease-causing mutations just outside the discoidin domain result in proteins that are also secreted from cells at essentially normal levels, but they are defective in their ability to form disulfide-linked octamers characteristic of WT retinoschisin, indicating that the octameric structure is required for retinoschisin function.<sup>9,10</sup>

Despite the different effect various disease mutations, including R141H, have on the structure and secretion of retinoschisin, to date no significant correlation has been found between the severity of the disease phenotype and the underlying genotype.<sup>20,21</sup>

The main objective of this study was to determine whether WT RS1 can assemble with various disease-causing mutants and promote secretion of the mixed mutant proteins from cells. Normally, X-linked alleles are expressed in different cells because of X-linked gene inactivation. As a result, female carriers of XLRs express the WT gene in one cell and the mutated gene in another cell. Hence, the WT and mutant proteins do not have an opportunity to coassemble as a complex during protein synthesis and secretion. Indeed, in most instances, only the WT protein is secreted from cells because most disease-causing missense mutations result in protein misfolding and retention inside the cell, as discussed. Female carriers of XLRs studied to date do not exhibit the disease phenotype because sufficient WT protein is produced and secreted from cells expressing the normal gene. However, therapeutic gene delivery can, in principle, present a problem. The introduction of the normal *RS1* gene into cells expressing a mutated gene could result in the formation of a mixed complex that, even if secreted, could prove nonfunctional. This would nullify the benefits of gene therapy as a treatment for XLRs. Therefore, it is important to determine whether WT retinoschisin interacts with mutant retinoschisin when expressed in the same cell

and, if so, what effect this has on protein oligomerization, secretion, and ultimately function.

To distinguish between WT and mutant retinoschisin, we introduced a myc epitope immediately downstream of the signal peptidase cleavage site. The myc-RS1 showed properties similar to those of WT RS1 with regard to its level of expression, distribution within cells, and secretion as a disulfide-linked octamer. Myc-RS1 could be readily distinguished from WT or mutant RS1 on the basis of its small increase in size and its recognition by anti-myc antibodies. Importantly, coexpression of myc-RS1 with WT RS1 or RS1 containing the D158N polymorphism further showed that myc-RS1 could assemble with these proteins to form mixed octameric complexes that were efficiently secreted from cells.

The interaction of normal RS1 (myc-RS1) with selected disease-causing mutants was investigated using coexpression and coimmunoprecipitation. All mutants coexpressed with myc-RS1 in cells. However, though the myc-RS1 was secreted from cells, all discoidin domain mutants, with the exception of the R141H mutant, were retained inside cells. This indicates that the WT retinoschisin folded and assembled into a disulfide-linked octamer independent of the mutant misfolded proteins and was effectively secreted from cells. In contrast, the R141H mutant assembled with WT-RS1 and was secreted as a mixed octameric complex. Thus, the R141H is similar to WT retinoschisin not only in its ability to be secreted from cells as an octamer but also in its ability to interact with the WT subunits. When the C59S octamer-defective mutant was cotransfected with myc-RS1, these proteins did not associate with each other, though they were secreted from cells. Previous studies indicate the C59S mutant can form C40-mediated disulfide-linked dimers but not octamers.<sup>9,10</sup> The fact that C59S-myc-RS1 dimers were not observed in coimmunoprecipitation studies suggests that the presence of the myc tag within the RS1 domain may impede heterodimer formation.

Our studies indicate that the introduction of the normal *RS1* gene into cells expressing disease-causing mutants should not pose a problem in gene delivery studies because the normal retinoschisin protein expresses, assembles, and is secreted independently of the mutant RS1. The R141H mutant, however, is a possible exception because it assembles with WT-RS1. The function of RS1 or its interaction with other cell components is unknown at present. When these properties are known, it will be important to determine whether the octameric complex consisting of R141H, alone or with WT-RS1, is fully or at least partially functional.

Although the R141H mutant exhibits structural properties similar to those of WT-RS1, the R141G disease-causing mutant does not. Wang et al.<sup>14</sup> reported that R141G is secreted from COS-7 cells, though at reduced levels compared with R141H and WT proteins. We were unable to detect the R141G mutant in the secreted fraction from transfected EBNA 293 cells. It is possible that the differences in chaperone proteins in COS-7 and EBNA 293 cells account for the variation in R141G secretion from these cells. The differences in behavior of the R141H and R141G mutants first suggested to us that positively charged or polar residues may be required at codon 141 for effective folding and secretion of the retinoschisin protein. Therefore, we examined a number of mutants with different amino acid substitutions. In our cell system, only R141S, R141A, and R141H mutants were efficiently secreted from cells. These results indicate that an amino acid residue with a positively charged or even a polar residue at codon 141 is not required for effective folding, octamerization, or secretion of retinoschisin. Amino acid residues with small side chains, such as serine and alanine, appear to allow proper folding and octamerization. A high-resolution structure of retinoschisin is not available at present. Examination of a model of the retinoschisin discoidin domain derived from factor 5 and factor 8<sup>10</sup> suggests that arginine residue 141 is highly inaccessible to solvent and therefore likely plays a role in protein folding.

In summary, our studies indicate that WT-RS1 undergoes protein folding, oligomerization, and secretion independently of disease-causing misfolded RS1 mutants. Therefore, with the exception of R141H, delivery and expression of WT-RS1 into cells expressing disease-causing RS1 missense mutants should not be detrimental to patients with XLRS. However, it is possible that photoreceptors may process proteins differently than do the EBNA 293 cells used in this study. Therefore, it would be advisable to determine whether WT-RS1 expressed in photoreceptor cells of mice harboring disease-causing RS1 missense mutations results in the recovery of retinal structure and function before gene therapy trials.

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