BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 905–911 905
DOI: 10.1021/bi901622w

Biochemical Analysis of Phenotypic Diversity Associated with Mutations in Codon 244 of the Retinal Degeneration Slow Gene†

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Received September 16, 2009; Revised Manuscript Received January 6, 2010

ABSTRACT: Mutations in the protein product of the retinal degeneration slow (RDS) gene cause both rod-dominant retinitis pigmentosa and different forms of cone-dominant macular dystrophies. In particular, mutations in codon 244 can cause either of these types of disease. In this study, we examine the biochemical effects of N244H and N244K in an effort to understand the mechanism underlying rod- and cone-dominant defects, respectively. COS-1 cells were cotransfected with either wild-type (WT) RDS or RDS containing an N244H or N244K mutation along with its binding partner, ROM-1 (rod outer segment membrane protein 1). Cell extracts were analyzed for mutant protein stability by Western blot, and localization was examined by immunocytochemistry. Interactions between transfected proteins were assessed by reciprocal co-immunoprecipitation, and nonreducing velocity sedimentation was used to identify the pattern of RDS complex assembly. Interactions were confirmed using GST fusion constructs of WT and mutant RDS in GST pull-down assays from WT mouse retinal extract. In COS-1 cells, recombinant N244H RDS had a weakened ability to assemble into higher-order complexes but retained the ability to co-immunoprecipitate with ROM-1 as well as localize properly throughout the cells. In contrast, recombinant N244K protein did not associate with ROM-1, showed signs of protein aggregation, and colocalized with an ER marker. These experiments support the hypothesis that RDS mutations that interrupt higher-order oligomer formation but still interact with ROM-1 and fold properly in membranes may cause dominant, gain-of-function disease phenotypes while mutations that cause RDS misfolding (and thus incorrect trafficking and assembly) may be associated with a loss-of-function haploinsufficiency phenotype.

Retinal degeneration slow (RDS)[1] is a tetraspanin glycoprotein which localizes to the disk rim regions of rod and cone photoreceptor outer segments (OSs). RDS is critical for photoreceptor OS morphogenesis and maintenance as well as disk shedding and membrane fusion (*1*−*3*). More than 100 different disease-causing mutations in the *RDS* gene have been identified and cause a wide array of diseases, from autosomal dominant retinitis pigmentosa (ADRP), which preferentially affects rods, to various cone-dominant disease phenotypes [cone dystrophy, adult vitelliform macular dystrophy (MD), and butterfly-shaped pigment dystrophy (http://www.retina-international.org/sci-news/rdsmut.htm)]. This diversity in disease phenotypes caused by a single gene is rare, and studying multiple disease-causing mutations in *RDS* enables us to better understand not only the disease pathophysiology but also the underlying molecular and cellular features of the RDS protein that make it required for both rod and cone OS biogenesis.

RDS is known to assemble into homo- and heterotetramers with ROM-1 (rod outer segment membrane protein 1) in the photoreceptor inner segment (IS) before being trafficked to the OS (*4*). Once in the OS, multiple tetramers form higher-order oligomeric complexes via intermolecular disulfide bonds mediated by an unpaired cysteine at position 150 (C150). These higher-order oligomers are vital for OS viability and maintenance, and animals expressing RDS with mutations that impede intermolecular disulfide bonding (C150S) do not form OSs (*5*). It is not clear whether RDS−ROM-1 complexes are identical (in size and composition) in rods and cones, but we have clearly shown that rods and cones have a differential requirement for RDS (*6*, *7*). In the rod-dominant wild-type (WT) background, rods without RDS do not form OS or transmit visual signals while cones (in the cone-dominant $nrl^{-/-}$ background) retain significant capacity for phototransduction and some OSs (albeit dysmorphic ones) (*7*).

Consistent with other tetraspanins, RDS includes four conserved transmembrane domains, a small loop (D1) and a large loop (D2) found in the intradiscal space, and the amino- and carboxyl-terminal tails found in the OS cytosol. The large D2 loop contains >70% of the RDS disease-causing mutations (http://www.retina-international.org/sci-news/rdsmut.htm) and has been identified as the area of interaction between RDS and ROM-1 and the area where intermolecular disulfide bonding occurs (*8*, *9*). We have shown that the area between Y140 and N182 is crucial for RDS and ROM-1 association, while RDS−RDS homo-associations depend on the region between C165 and N182 (*8*). Because the area required for RDS−ROM-1 interactions is much larger, changes to the tertiary structure

†This work was supported by National Institutes of Health Grants EY010609 and EY018656 (M.I.N.) and EY018512 (S.M.C.), the Foundation Fighting Blindness, and the Oklahoma Center for the Advancement of Science and Technology.

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[1]Abbreviations: RDS, retinal degeneration slow; ADRP, autosomal dominant retinitis pigmentosa; N244H, asparagine to histidine mutation at position 244 in RDS; N244K, asparagine to lysine mutation at position 244 in RDS.

Published on Web 01/07/2010

induced by mutations to other areas of the D2 loop may be capable of inhibiting RDS−ROM-1 binding without interfering with RDS−RDS interactions.

Two RDS disease-causing mutations are found at position 244 in the D2 loop. These mutations are of particular interest to us because of their divergent disease phenotypes. Usually mutations in the same amino acid yield similar disease phenotypes; for example, one of the arginines in the D2 loop of RDS (R172) can be mutated to tryptophan, glycine, or glutamine, but patients always present with a cone-dominant macular degeneration (*10*, *11*). This is not the case in patients with mutations at N244. Those carrying the N244H (asparagine 244 → histidine) mutation in RDS present with autosomal dominant cone−rod dystrophy, a disease that causes severe cone degeneration followed by a late-stage progressive rod degeneration (*12*). In contrast, patients with the N244K (asparagine 244 → lysine) mutation acquire RP, a progressive rod degenerative disease with cone defects (bull's eye maculopathy and macular degeneration MD) appearing only in advanced stages (*13*).

In this study, we investigated cellular and biochemical mechanisms by which these two mutations at codon 244 in RDS confer different disease phenotypes. We took advantage of a heterologous COS-1 cell expression system to monitor the properties of these two mutants along with the previously described R172W (*10*, *11*) and C214S (*14*−*16*) mutants for the sake of comparison. We demonstrate that the N244K protein leads to biochemical changes consistent with a loss-of-function phenotype while the N244H mutation causes a much more subtle defect.

MATERIALS AND METHODS

COS-1 Cell Transfection. The pcDNA3.1 (Invitrogen, Carlsbad, CA) construct containing murine WT *Rds* cDNA corresponding to nucleotides 1−1820 was used as a template for site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primer sequences 5′-CT GAG GAG CTC **CAC** TCT GGC TGC G-3′ and 5′-CG CAG CCA GAG **GTG** GAG CTC CCA G-3′ were used to introduce the AAC → CAC change to create the N244H mutation, while primers 5′-G ACT GAG GAG CTC **AAA** CTC TGG CTG CGG-3′ and 5′-CCG CAG CCA GAG **TTT** GAG CTC CTC AGT C-3′ were used to introduce the AAC → AAA change to create the N244K mutation in *Rds*. In addition, a pcDNA3.1 construct containing murine *Rom-1* cDNA (nucleotides 1−1082) was generated. All constructs were confirmed by sequence analysis from both strands.

COS-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum and a penicillin/streptomycin mixture (all from Invitrogen/Gibco, Carlsbad, CA). Cells were maintained in humidified air at 37 °C with 5% CO_2. For experiments, cells were grown on 100 mm dishes to 90% confluence before being transfected with 8 μg of either the WT, N244H, N244K, C214S, or R172W along with 8 μg of ROM-1 (where indicated). The transfection procedure was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Cells were harvested 48 h post-transfection; one portion of cells was seeded onto coverslips, while the other portion was used for protein extraction.

Antibodies. Anti-RDS-CT, anti-RDS-D2, and anti-ROM-1-CT antibodies were generated in house and described previously (*4*, *8*). These three antibodies were used at a 1:1000 dilution for both immunocytochemistry and Western blotting. Anti-GST goat polyclonal antibody was obtained from Amersham/GE and used at a 1:2000 dilution. Chicken polyclonal antibody against the endoplasmic reticulum (ER) marker Calreticulin was obtained from abCam (Cambridge, MA) and used at a 1:250 dilution. Mouse monoclonal antibody (mAb) 2B7 against the C-terminus of murine RDS was generated for us by Precision Antibody (Columbia, MD) using standard techniques. Antibody specificity is shown in Figure 1 of the Supporting Information. Hybridoma supernatants from clone 2B7 were used at a dilution of 1:1000 for Western blotting and 1:500 for immunocytochemistry.

Expression and Purification of the GST Fusion Proteins. Expression and purification of the GST fusion proteins used in this study were conducted as described previously (*8*, *17*). In brief, *Escherichia coli* BL21 cells were transformed with the pGEX-4T-2 plasmid which contained either the WT, N244H, or N244K RDS D2 loop fused to GST (GST−RDS-WT, GST−RDS-H, or GST−RDS-K, respectively). The protein was affinity purified by glutathione agarose resin per the manufacturer's protocol (Amersham Biosciences/GE Healthcare, Piscataway, NJ).

Immunoprecipitation, GST Pull Down, and Western Blot Analysis. We extracted protein from cells or retinas by homogenizing them in buffer [50 mM Tris (pH 7.8), 100 mM NaCl, 5 mM EDTA, 0.05% SDS, 1% TX-100, 2.5% glycerol, and 1 mM PMSF], solubilizing them for 1 h at 4 °C, and removing the insoluble material by centrifugation. Standard procedures for immunoprecipitation, GST pull-down assay, and Western blotting were used as described previously (*2*). For immunoprecipitation, beads without the primary antibodies and cell extracts from mock transfected cells were used as negative controls. Immunoprecipitation with mouse control IgG was also included (data not shown). After adsorption, the beads were washed several times, and bound proteins were eluted with Laemmli sample buffer for Western blotting analysis.

Velocity Sedimentation. Velocity sedimentation was performed as previously reported (*4*, *11*, *18*). In brief, transfected COS-1 cell extracts (300 μg) were overlaid on a nonreducing, 5 to 20% sucrose gradient (w/v) consisting (from bottom to top) of 500 μL each of 20, 15, 10, and 5% sucrose in PBS containing 1.0% (v/v) Triton X-100 and 0.1 mM *N*-ethylmaleimide. Gradients were allowed to become continuous by a 1 h diffusion period at room temperature. After centrifugation at 109000*g* for 16 h, a 26.5 gauge needle was used to puncture the bottom of the tube for collection of six-drop fractions (~120 μL). Fractions were resolved by SDS−PAGE and transferred for immunoblotting with anti-RDS-CT. As a control, standard proteins from the Gel Filtration Molecular Weight Marker Kit (Sigma) were individually overlaid in the same manner as the COS-1 extracts as described in ref *4*. The molecular masses of the standard proteins correlated with those of RDS monomers, dimers, tetramers, and higher-order oligomers.

Immunocytochemistry. After transfection, COS-1 cells were seeded onto coverslips and allowed to adhere overnight. The cells were washed for 2 × 5 min with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, washed for 2 × 5 min with PBS, permeablized with 0.3% (v/v) Tween 20 in PBS for 15 min at room temperature, and then blocked for 1 h at room temperature in 5% BSA in PBS. Cells were incubated overnight in primary antibody (in blocking buffer). After the mixtures had been washed for 3 × 10 min with PBS, antigens were detected with Alexa 488-, 555-, or 647-conjugated secondary antibodies (Invitrogen/Molecular Probes). Cells were then washed, and the

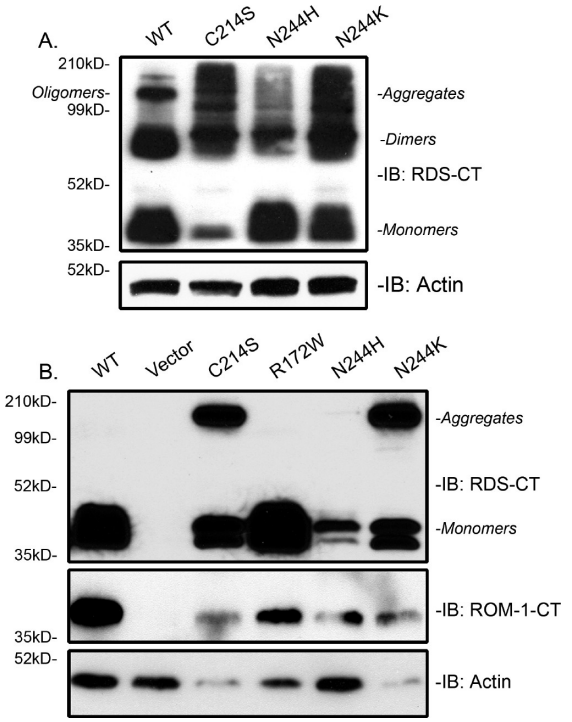

FIGURE 1: N244H RDS and N244K RDS are stably expressed in transfected COS-1 cells. COS-1 cells were transiently transfected with RDS (WT, C214S, R172W, N244H, and N244K), and WT ROM-1 constructs and proteins were extracted for nonreducing (A) or reducing (B) SDS−PAGE and Western blotting. (A) WT RDS is present in oligomeric, dimeric, and monomeric forms, while C214S RDS and N244K RDS are present as monomers and large aggregates. N244H RDS is present in monomeric and dimeric form, but little to no higher-order oligomer is detected. (B) Under reducing conditions, WT RDS, N244H RDS, and R172W RDS are present in monomeric form, while C214S RDS and N244K RDS display significant evidence of aggregation. ROM-1 is shown to confirm cotransfection.

coverslips were mounted in ProlongGold with Dapi (Invitrogen/Molecular Probes). Image stacks were collected using a spinning disk confocal microscope (BX62, Olympus, Tokyo, Japan). Image deconvolution was completed using the nearest-neighbor algorithm and Slidebook software (Intelligent Imaging Innovations, Denver, CO). Single planes are shown.

RESULTS

Expression and Localization of N244H and N244K RDS. COS-1 cells were cotransfected with ROM-1 and one of the RDS constructs (WT, N244H, N244K, C214S, or R172W). Transfection efficiencies and expression levels varied between constructs and experiments, but banding patterns remained consistent in all experimental replicates. Total cellular extracts were collected and underwent Western blot analysis under nonreducing conditions. Both the WT and N244H recombinant RDS proteins appear as monomers and dimers, but N244H does not appear to form an appreciable quantity of higher-order oligomers (Figure 1A, lanes 1 and 3). N244K and C214S RDS proteins have less of the monomeric form (much less in the case of C214S), instead appearing mostly as large-sized aggregates (Figure 1A, lanes 2 and 4). Because of the increased magnitude of the signal from the aggregates in the N244K and C214S RDS extracts, it is difficult to determine whether higher-order oligomers are present. To help differentiate between oligomers and protein aggregates, samples were analyzed under reducing conditions (Figure 1B). As expected, oligomeric and dimeric forms of WT RDS were reduced to monomers; however, both N244K and C214S proteins still appeared in a high-molecular mass band at the top of the gel (~200 kDa) that was resistant to the reducing agent (Figure 1B, lanes 3 and 6), consistent with protein aggregation (*14*, *19*). Similar to WT, both N244H and R172W are reduced to their monomeric forms (Figure 1B, lanes 1, 4, and 5). RDS monomer doublets often, although not always, appear on Western blots using both our antibodies (see Figure 1 of the Supporting Information) and those of others (*20*, *21*). It has been hypothesized that the top bands of these doublets may be forms of RDS in which one or more of the intermolecular disulfide bonds is not completely reduced before SDS−PAGE (*21*). Differences in protein levels are due to variations in transfection efficiency. The same blot (Figure 1B) was probed with anti-ROM-1 to determine successful cotransfection of the recombinant proteins.

In the retina, RDS is localized to the disk rim region of rod and cone outer segments but is not found on the plasma membrane. Since there are no outer segments or disk rims in COS-1 cells, properly folded recombinant WT RDS is typically distributed throughout the cell on internal membranes (*16*, *20*). In contrast, improperly folded or aggregated protein tends to accumulate in the ER. To determine how the N244 mutations affect RDS distribution, co-immunocytochemistry on transfected COS-1 cells with different RDS constructs was undertaken using the ER marker calreticulin (green) and RDS-CT polyclonal antibody. As seen in Figure 2a (and Figure 2 of the Supporting Information), the transfected WT RDS protein is distributed throughout the cell and does not colocalize with the ER marker. Furthermore, the distribution of N244H RDS and R172W RDS is similar to that of the WT (throughout the cell) with little colocalization with the ER marker. On the other hand, N244K RDS colocalizes almost completely with calreticulin, consistent with the ER localization (*20*). As we previously reported, the C214S mutant protein is abnormally localized in COS-1 cells (*16*), and we here demonstrate that it too is exclusively found in the ER. In transgenic mice, we previously showed that the C214S protein was unstable, most likely due to improper protein folding (*16*), and the similarities between N244K RDS and C214S RDS in our experiments suggest that N244K may also be an improperly folded mutant protein. In addition, the normal distribution of the N244H protein suggests that it is likely to be properly assembled and localized in vivo.

To determine what effect expression of ROM-1 had on protein localization, cells were cotransfected and triply labeled with anti-ROM-1-CT, anti-calreticulin, and our newly generated RDS C-terminal monoclonal antibody, mAb 2B7. It does not cross-react with ROM-1 and recognizes both monomeric and multimeric forms of RDS (Figure 1 of the Supporting Information). Figure 2B and Figure 2 of the Supporting Information demonstrate that ROM-1 partially colocalizes with WT, R172W, and N244H (pink labeling in Figure 2B) but is also found in discrete punctuate structures that do not contain RDS. In cells expressing N244K or C214S, no colocalization with ROM-1 is apparent. Localization of all forms of RDS is not affected by coexpression of ROM-1.

Homo and Hetero Interactions of N244H and N244K RDS. Since proper assembly of OSs depends on the ability of RDS to interact with itself and with ROM-1, our next step was to determine whether the N244 mutant proteins retained that ability. Protein was extracted from cotransfected COS-1 cells, and WT and mutant RDS and ROM-1 were co-immunoprecipitated with

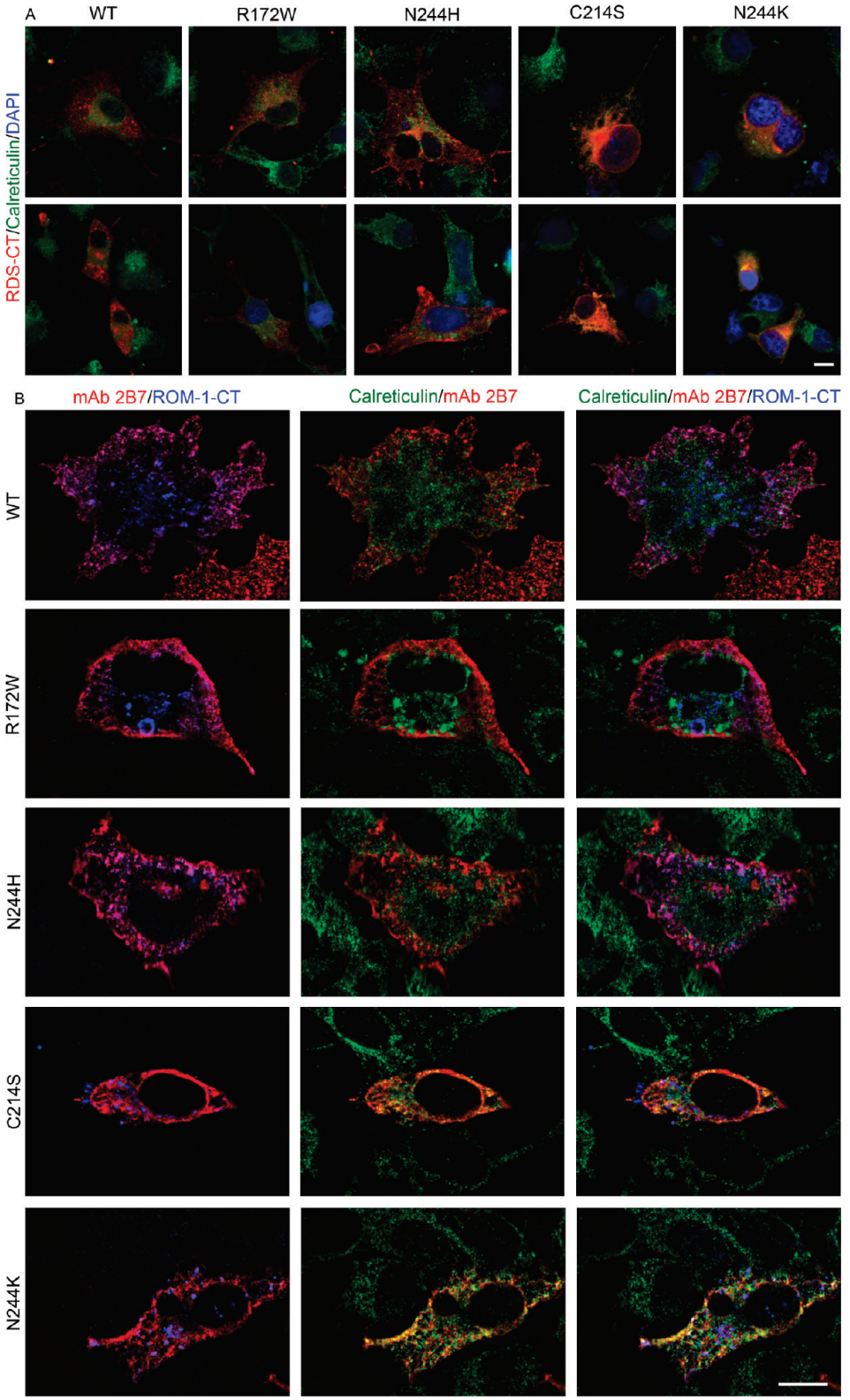

FIGURE 2: N244H RDS is distributed throughout the cell, while N244K RDS is restricted to the ER. COS-1 cells were transfected with the indicated RDS vector (A) or the indicated RDS vector and ROM-1 (B). After 48 h, cells were fixed and stained with (A) rabbit polyclonal antibody against RDS (red) and chicken polyclonal antibody against the ER marker calreticulin (green) or (B) rabbit polyclonal antibody against ROM-1 (blue), mouse monoclonal antibody 2B7 against RDS (red), and chicken polyclonal antibody against calreticulin (green). (A) N244K and C214S RDS colocalize with the ER marker, while WT, R172W, and N244H RDS are distributed throughout the internal membranes of the cell. (B) ROM-1 partially colocalizes with WT, R172W, and N244H RDS (pink) but not with C214S or N244K RDS. Coexpression of ROM-1 with RDS does not affect RDS localization. The scale bar is 10 μm. Images are single planes from spinning disk confocal stacks.

RDS-CT and ROM-1-CT antibodies, respectively. Transfected WT and N244H RDS are clearly capable of interacting with ROM-1 (Figure 3A, lanes 2 and 4). In contrast, N244K RDS did not bind to ROM-1 in immunoprecipitations using ROM-1-CT antibody or RDS-CT antibody even though a substantial amount of ROM-1/N244K protein was present.

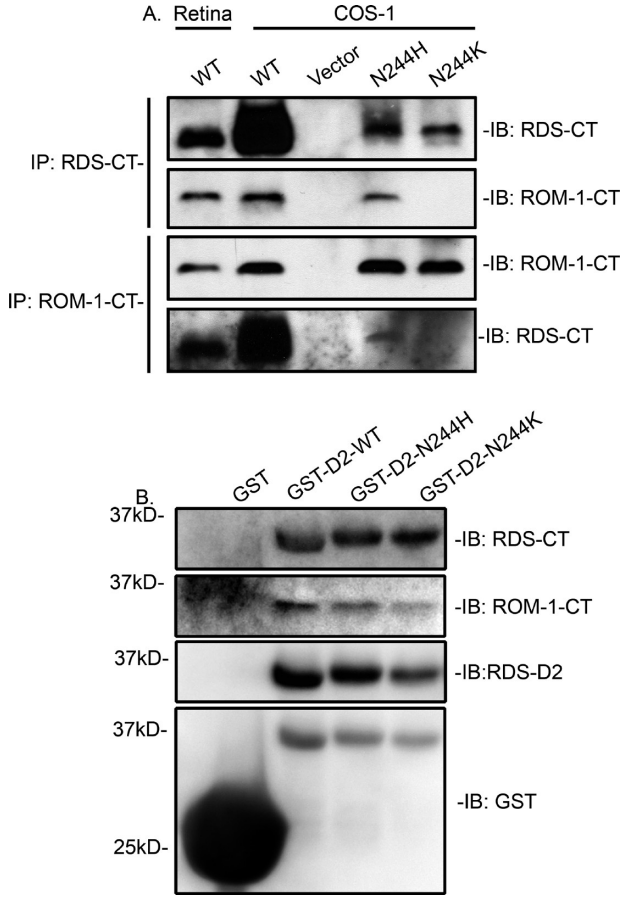

FIGURE 3: N244H RDS retains the ability to bind ROM-1, while N244K RDS does not. (A) Protein was extracted from COS-1 cells transiently cotransfected with RDS constructs, and ROM-1 and RDS−ROM-1 complexes were reciprocally co-immunoprecipitated using polyclonal anti-RDS-CT or polyclonal anti-ROM-1-CT. Immunoprecipitants were processed for reducing SDS−PAGE and Western blotting with antibodies as shown. As expected, WT retinal extracts and WT COS-1 extracts bound ROM-1. N244H RDS transfected COS-1 extracts also bound ROM-1, but N244K RDS extracts did not regardless of whether ROM-1-CT or RDS-CT was used for the IP. ROM-1 expression in all cotransfected cells demonstrates that the lack of ROM-1 expression does not account for the observed lack of association between N244K RDS and ROM-1. (B) GST fusion constructs carrying the RDS D2 loop (with or without additional mutation) were immobilized on a glutathione column and incubated with WT retinal extracts. Column eluents were processed for reducing SDS−PAGE and Western blotting as shown. All constructs retained the ability to bind retinal RDS as shown by immunoreactivity with anti-RDS-CT (which does not recognize the D2 fusion proteins), and all constructs brought down ROM-1. RDS-D2 and GST immunoreactivity are shown to confirm the presence of the fusion proteins.

Because our IP antibody recognizes the C-terminus of RDS, it is not possible to use IP to determine whether mutant forms of recombinant RDS can form RDS homo interactions with each other, To test whether the N244H and N244K RDS can bind to native RDS, GST pull-down assays on WT retinal extracts were performed. The RDS D2 loop is responsible for RDS−ROM-1 interactions (*8*), and we have previously shown that a fusion protein containing GST and the WT RDS D2 loop is capable of binding retinal RDS and ROM-1 (*8*). For this study, GST−D2 loop mutant constructs were generated containing the N244H or -K mutation, and WT retinal extracts were passed over a column containing the purified GST−D2-N244H or GST−D2-N244K fusion proteins. Using the RDS-CT antibody (which does not recognize the D2 fusion proteins), we show that all three D2 constructs bind native RDS (Figure 3B, top panel). We also demonstrate that a pull-down assay with any of the three constructs also brings down ROM-1 from retinal extract (Figure 3B, second panel) since it is bound to native RDS. The presence of the fusion proteins is confirmed by probing with the anti-GST and anti RDS-D2 antibodies (Figure 3B, bottom panels). An excess of GST protein was used as our negative control (Figure 3B, lane 1) to confirm that all observed associations were specific to the D2 loop.

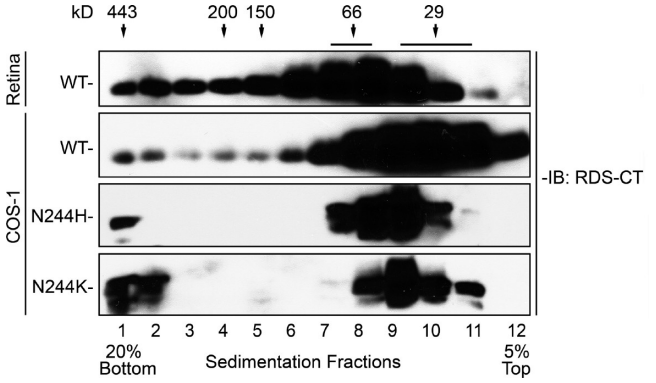

FIGURE 4: Complex formation in N244H and N244K mutant RDS is altered. Protein extracts from WT retinas or COS-1 cells transfected with WT RDS, N244H RDS, or N244K RDS underwent nonreducing velocity sedimentation on a 5 to 20% (as labeled) continuous sucrose gradient. Collected fractions were processed for reducing SDS−PAGE and Western blotting. The normal fractionation pattern (oligomer fractions 1−3, intermediate complex fractions 4−6, and tetramer fractions 7−10) was detected in WT retinal extracts. Peak fractions for molecular mass markers are shown with arrows: apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). As expected, the tetrameric fractions from WT RDS-transfected COS-1 cells shifted slightly to the right. N244H RDS and N244K RDS were present mostly as tetramers, although some immunoreactivity was detected in high-molecular mass fractions.

Complex Assembly of N244H and N244K RDS. Finally, to more thoroughly investigate the ability of N244H RDS and N244K RDS to form complexes, velocity sedimentation analysis was conducted on single transfected COS-1 cell extracts under nonreducing conditions (with WT retinal extract as a control). Cotransfection with ROM-1 was not used in these experiments since we have no way to biochemically confirm that individual transiently transfected cells express both proteins. Following sedimentation on a continuous 5 to 20% sucrose gradient, 12 fractions were collected and subjected to reducing SDS−PAGE and Western blotting with anti-RDS-CT antibody. To avoid biasing against detection of fractions with only small amounts of RDS, long exposure times were used. Using nonreducing velocity sedimentation combined with reducing SDS−PAGE and blotting coupled with molecular mass standards, we have previously shown that RDS oligomers are found in fractions 1−3 while intermediate-sized complexes appear in fractions 3−6 and tetrameric complexes appear in fractions 6−10. In COS-1 cells, Molday's group has shown that this profile is mostly maintained, although the peak corresponding to tetrameric fractions shifts slightly to the right (fractions 7−11) and the peak corresponding to oligomeric fractions is smaller (perhaps due to the lack of OS-type disks in COS-1 cells) (*22*). The pattern of sedimentation with WT retinal extract and recombinant RDS from COS-1 extracts is consistent with those reported previously (*4*, *22*) (Figure 4, top two panels). Both N244H

and N244K proteins seem to be present mainly in tetrameric form (Figure 4, bottom panels), with very little of the larger intermediate and oligomeric complexes that would normally appear in fractions 1−6. There is strong immunoreactivity in fractions 1 and 2 for N244K and fraction 1 for N244H. Given the results presented in Figure 1, immunoreactivity in fractions 1 and 2 of the N244K panel likely represents protein aggregation.

DISCUSSION

This study is the first to investigate the inconsistency in disease phenotype associated with mutations of the same amino acid of RDS. We report that, when expressed in COS-1 cells, N244H and N244K RDS exhibit many of the biochemical hallmarks of traditional loss-of-function (N244K) and gain-of-function (N244H) mutations. N244K protein does not form normal higher-order RDS oligomers or intermediate complexes and aggregates near the nucleus of the cell (consistent with protein misfolding and retention in the ER). Furthermore, this mutant protein does not interact with or colocalize with ROM-1. On the contrary, N244H RDS retains the ability to bind ROM-1 and is distributed throughout the COS-1 cell in a pattern indistinguishable from that of WT RDS. While it does not appear to form significant aggregates, it also appears to form fewer higher-order RDS oligomers than WT RDS.

Lysine (K) is a basic amino acid with a hydrophobic side chain, and N → K substitutions are known to adversely affect protein function and folding (*23*). Our work here on the mutant N244K RDS represents another example suggesting that RDS-associated retinitis pigmentosa (RP) is coupled with loss-of-function mutations. Previous evidence that supported this hypothesis came from extensive in vitro and in vivo characterization of another RDS RP mutant, C214S (*14*, *16*, *24*). In COS-1 cells, C214S mutant protein does not bind ROM-1 (*14*) and forms high-molecular mass aggregates (*14*, *16*). In the murine photoreceptor, this abnormal protein is unstable and quickly degraded, consistent with a loss-of-function allele (*16*). When ectopically expressed in the inner retina, C214S RDS accumulates in the perinuclear region, similar to what we show here (*16*). These findings led us to hypothesize that C214S-associated retinitis pigmentosa is caused by RDS haploinsufficiency, an idea further supported by the observation that the phenotype can be rescued by genetic supplementation with WT RDS (*15*). The well-characterized nature of C214S RDS makes it an excellent comparison for novel RDS mutations. Here we show that N244K RDS is biochemically similar to C214S. The N244K substitution likely results in misfolding of the D2 loop, albeit in a less severe fashion than that in C214S wherein critical intramolecular disulfide bonds are interrupted. This hypothesis is supported by the observation that even though homo and hetero interactions between RDS and ROM-1 occur on the side of the loop opposite position 244 (*8*), N244K RDS does not interact directly with ROM-1. Interestingly, the N244K-D2−GST protein retained the ability to bind endogenous RDS. This suggests that the D2 loop structural requirements for RDS−RDS interactions are less stringent than for RDS−ROM-1 interactions. However, in the case of the N244K mutation, this observation is likely irrelevant since our in vitro data suggest that in vivo N244K RDS mutant protein would likely remain in the ER and be degraded without being trafficked to the OS.

Histidine (H) is also a basic amino acid; however, the N → H change is not necessarily detrimental, and in some cases, N → H substitutions have been shown to increase enzymatic activity (*25*). The data we present here on the N244H mutation support the idea that, like R172W (*10*, *11*), N244H may also be a gain-of-function mutation. N244H appears to be processed normally and would likely be properly trafficked to the OS. We show that N244H behaves like WT with the exception of higher-order oligomer formation. After nonreducing velocity sedimentation, N244H had a significantly narrower distribution of RDS immunoreactivity in oligomeric fractions 1−3 (Figure 4) than WT RDS, suggesting some impairment in the maintenance of higher-order oligomers. This defect is not likely due to an inability to form higher-order oligomers, since oligomers form as a result of intermolecular disulfide bonding and N244H is clearly present in the disulfide-bonded dimeric form (Figure 1A) on the nonreducing Western blot. The most likely explanation is that, as in the case with R172W transgenic mice [wherein higher-order oligomers are slightly less compact than normal (*11*)], the N244H mutation causes a mild structural change in the D2 loop of RDS which is not sufficient to interrupt overall protein folding or ROM-1−RDS homo- or heterotetramer formation but causes a moderate change in complex conformation.

Combined, these results suggest that proper cone OS biogenesis and maintenance rely on having RDS with the proper conformation more than on total RDS quantity while rods (which are severely affected by haploinsufficiency mutations) may be more dependent on the total quantity of RDS present. When multiple mutations occur at the same position (in this case N244), the severity of the structural defect arising from the substituted amino acid (histidine vs lysine) likely determines which photoreceptor type is the most affected. Severe defects causing protein misfolding (N244K and C214S) lead to haploinsufficiency and rod-dominant disease, while milder structural defects (N244H and R172W) preferentially affect cones. Further study of the cone-dominant disease process will have to come from examination of cone-dominant animal models such as the $nrl^{-/-}$ mouse since neither in vitro systems nor the rod-dominant WT mouse facilitates the study of cones. It is likely that even though all cone-dominant RDS mutations may be classified as structurally imperfect gain-of-function mutations, they may not have the same disease process. It is not clear what the in vivo effects of N244H RDS would be on cones, but the multiplicity of RDS-associated disease phenotypes and variability in RDS behavior on the molecular and cellular level highlight the complexity of rod versus cone OS biogenesis and the need for further study.

ACKNOWLEDGMENT

We thank Drs. Muayyad Al-Ubaidi, Xi-Qin Ding, and Dibyendu Chakraborty and Mr. Michael Stuck for their excellent technical assistance.

SUPPORTING INFORMATION AVAILABLE

Characterization of mAB 2B7 and additional examples of cellular localization of various RDS mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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