Differences in RDS trafficking, assembly and function in cones versus rods: insights from studies of C150S-RDS

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Cysteine 150 of retinal degeneration slow protein (RDS) mediates the intermolecular disulfide bonding necessary for large RDS complex assembly and morphogenesis of the rim region of photoreceptor outer segments. Previously, we showed that cones have a different requirement for RDS than rods, but the nature of that difference was unclear. Here, we express oligomerization-incompetent RDS (C150S-RDS) in the cone-dominant nrl2/2 mouse. Expression of C150S-RDS leads to dominant functional abnormalities, ultrastructural changes, biochemical anomalies and protein mislocalization in cones. These data suggest that RDS complexes in cones are more susceptible to disruption than those in rods, possibly due to structural or microenvironmental differences in the two cell types. Furthermore, our results suggest that RDS intermolecular disulfide bonding may be part of RDS inner-segment assembly in cones but not in rods. These data highlight significant differences in assembly, trafficking and function of RDS in rods versus cones.

INTRODUCTION

The outer segments (OSs) of vertebrate cone photoreceptors (COSs) are composed of stacks of photopigment-containing membranous lamellae that are essential for normal vision. COSs are structurally distinct from their rod counterparts, being made up of lamellae instead of fully enclosed discs; however, the mechanisms underlying the formation of these two divergent structures are poorly understood. The retinal degeneration slow protein (RDS, also known as peripherin/Rds or peripherin-2) is found in the rims of cone lamellae and rod discs and is required for the proper development and maintenance of these structures (1,2). Interestingly, this protein appears to function differently in rods and cones (3). Retinal degeneration slow (rds−/−) mutant mice (in the rod-dominant wild-type background) fail to develop OSs and have no detectable retinal function (4–6). In contrast, we have reported that cones lacking RDS (in the cone-dominant neural retina leucine zipper knockout, nrl−/−, background) (7–9) retain some OS function and structure, albeit abnormal (3,10). This divergence in the role of RDS in rods versus cones is underscored by the observation that mutations in the human Rds gene are associated with a variety of rod- or cone-dominant blinding retinal diseases, including autosomal dominant retinitis pigmentosa and multiple classes of macular degeneration (11,12). The large variety and severity in RDS-related disease make evaluation of its role in OS biogenesis key to our understanding of photoreceptor cell biology and to the development of rational treatments for multiple forms of retinal degeneration.

Further insight into the differences in RDS function in rods and cones has come from our recent report evaluating the role of the cysteine at position C150 (13). RDS is a member of the tetraspanin superfamily. It has cytoplasmic N- and C-terminal regions, the requisite four transmembrane domains and small (D1) and large (D2) intradiscal loops. The D2 loop (~150 amino acids) harbors seven highly conserved cysteine residues, six of which form intramolecular disulfide bonds required for the maintenance of proper D2 loop structure. Mutagenesis studies in tissue culture (14) and our previous work in mice (13) demonstrate that the seventh cysteine (at position 150) is responsible for RDS–RDS intermolecular disulfide bonding and the formation of covalently linked multimeric RDS complexes. In the rod photoreceptor inner...
segment (IS), RDS forms non-covalently bound homo- and hetero-tetramers with its homolog ROM1 (3,10,15–17), another tetraspanin protein with similar localization and structure to RDS (18,19). After trafficking to the OS, homo- and hetero-tetrameric RDS complexes are linked to octomers while RDS homo-tetramers are linked to higher order oligomers through C150-mediated disulfide bonds. Ablation of C150 in C150S/rds−/− transgenic mice eliminates the formation of disulfide-bonded complexes and as a consequence, these mice lack OSs, demonstrating that higher order oligomers are crucial for disc rim formation in rods and cones (13,20). It is not clear what the precise role of ROM1 is in this process, but we have observed that ROM1 is only found in the tetrameric and intermediate complexes but not in the larger oligomers (15).

We have recently reported (13) that there are fundamental complex differences in the role of C150 and C150-mediated complex formation in rods versus cones. In the rod-dominant wild-type (rds+/+) retina, which contains endogenous RDS, expression of C150S-RDS in rods had little effect on rod structure or function. Furthermore, the transgenic protein was properly trafficked to OSs and formed tetrameric complexes with ROM1, although C150S-RDS alone was not capable of driving OS formation in the absence of endogenous RDS. On the contrary, when C150S-RDS was expressed in cones, we observed an early-onset dominant-negative phenotype; cone structure and function were severely compromised, even in the presence of endogenous RDS. Furthermore, the transgenic protein was not able to bind ROM1 in cones, and OS proteins including RDS and short- (S-) and medium- (M-) wavelength cone opsins were mislocalized throughout the photoreceptor (13). This study was the first to demonstrate that RDS processing, trafficking and complex regulation are different in rods and cones, but further study of this phenomenon in the wild-type background was uninformative since the presence of so many rods had the potential to mask biochemical changes in cones. Here, we study the role of C150 and C150-mediated complex formation in cones by utilizing our transgenic mice expressing C150S under the control of the M-cone opsin promoter (which drives expression in all cone types) in the cone-dominant nrl−/− background and in the presence of varying levels of endogenous RDS (rds+/+, rds+/−, rds−/−).

RESULTS

Expression of C150S-RDS leads to cone OS defects

In the rod-dominant wild-type background, the COP-C150S-RDS transgene (COP-T) drives expression of transgenic protein (also COP-T) only in cone photoreceptors (13). Owing to the limited number of cones in the murine wild-type retina, we generated mice expressing COP-T in the cone-dominant rds+/+/nrl−/− background. To confirm that these mice stably expressed the transgene in the appropriate cells, paraffin-embedded retinal sections taken from eyes collected at postnatal (P) day 30 were co-labeled with monoclonal antibody (mAB) 3B6 and our well-characterized RDS-CT polyclonal antibody. mAB 3B6 recognizes only transgenic protein (Materials and Methods) (13,21,22) while RDS-CT recognizes both endogenous and transgenic protein (13,23). As shown in Supplementary Material, Figure S1, the COP-T protein is not ectopically expressed, and is detected panretinally in the photoreceptor cell layers of the COP-T/ rds+/+/nrl−/−. No mAB 3B6 staining is detected in the rds+/+/nrl−/− animals.

To study the effects of the transgenic protein on retinas with varying amounts of endogenous RDS, COP-T animals were crossed onto the rds+/+/nrl−/−, rds+/−/nrl−/− and rds−/−/ nrl−/− backgrounds. Eyes were collected at P30 and examined histologically. On the light level, COP-T animals (compared with non-transgenic controls) do not exhibit thinning of the photoreceptor layer (Supplementary Material, Fig. S2). However, we do observe a decrease in the number of infoldings (termed rosettes) in the outer nuclear layer (ONL) in COP-T/rds+/+/nrl−/− animals compared with non-transgenic counterparts. To quantify this observation, retinal sections were fluorescently labeled with S-opsin to visualize rosettes, and rosettes were counted across retinal sections. As shown in Supplementary Material, Figure S3, the number of rosettes in COP-T transgenic animals is significantly reduced in all rds backgrounds, although this reduction is most severe in the COP-T/rds+/+/nrl−/−. It is not clear why the reduction in rosette number should be most pronounced in the presence of endogenous RDS (i.e. in the rds+/+/nrl−/−) background; in previous studies, we have observed that significant reduction in rosette number occurs in the absence of RDS (rds−/−/nrl−/−) (3,10). In contrast to this apparent COP-T-mediated improvement in retinal lamination, electron microscopic (EM) examination of sections reveals that expression of COP-T leads to ultrastructural abnormalities in cone OSs (Fig. 1A). In the presence of two endogenous Rds alleles (COP-T/rds+/+/nrl−/−), expression of COP-T leads to OS with poorly organized lamellae and the presence of many open, vacuole-like structures within the OS, in contrast to the flattened and well-organized lamellae in rds+/+/+/nrl−/−. In the presence of a single endogenous Rds allele (COP-T/ rds+/+ /nrl−/−), virtually no normal lamellae are seen. OSs lack regular structure and are filled with open, vacuole-like membranes, in contrast to the rds+/+/nrl−/− retina which has flattened, albeit grossly abnormal lamellae. Previously, we have demonstrated that the rds−/−/nrl−/− does not have OSs with lamellae, instead OSs are open membranous sacks (3). Similarly, OSs of COP-T mice on the rds−/−/nrl−/− background completely lack lamellae and form balloon-like membranes. To confirm that the abnormal OS structures are in fact OS in origin and not just membranous detritus in the OS layer, immunogold labeling with polyclonal S-opsin antibody was undertaken. As shown in Figure 1B, the abnormal OS membranes are labeled with S-opsin, confirming their identity.

Expression of COP-T leads to a dominant-negative effect on cone function

To determine whether these structural abnormalities correlated with functional deficits, we undertook photopic electroretinography (ERG) testing. At P30 (Fig. 2A), photopic b-wave amplitudes were decreased by ~40% (P < 0.05) in COP-T/ rds+/+ /nrl−/− animals compared with the non-transgenic controls on the same background. Amplitudes in COP-T animals
in the \textit{rds}^{+/−}/\textit{nrl}^{−/−} background were decreased by 30%. In the double-knockout background, transgenic and non-transgenic animals had equivalent photopic ERG amplitudes. Interestingly, ERG values for COP-T animals on the \textit{rds}^{+/−}/\textit{nrl}^{−/−} or the \textit{rds}^{−/−}/\textit{nrl}^{−/−} background were the same, suggesting that the adverse functional effects of the COP-T protein on cones may be independent of the quantity of endogenous RDS present. As shown in Figure 2B, photopic ERG function in all animals continued to decline with time, consistent with ongoing retinal degeneration in the \textit{nrl}^{−/−} background.

Although a 40% reduction in photopic ERG amplitude is certainly biologically significant, it is of much lesser magnitude than the \textasciitilde 75% decrease in photopic ERG amplitude we previously observed when the COP-T protein was expressed in the wild-type \textit{rds}^{+/+} background (13). In the \textit{rds}^{+/+} background, morphometric analysis suggested that short-wavelength cones (S-cones) were less affected by COP-T than medium-wavelength cones (M-cones): in COP-T/\textit{rds}^{+/+} mice (compared with \textit{rds}^{+/+}), S- and M-cone numbers were reduced by 52 and 71%, respectively. To further study this phenomenon, spectral ERG was undertaken on \textit{rds}^{+/−}/\textit{nrl}^{−/−} and COP-T/\textit{rds}^{+/−}/\textit{nrl}^{−/−} animals. In the \textit{rds}^{+/−}/\textit{nrl}^{−/−} retina, rods are converted to S-cone-like photoreceptors, so the S-cone responsive UV b-wave is significantly larger than the green b-wave (which measures M-cone responses). As shown in Figure 2C and D, in the \textit{rds}^{+/−}/\textit{nrl}^{−/−} background, M-cone ERG amplitudes are more severely affected by the presence of the COP-T protein than S-cone ERG amplitudes (94 and 49% reduction, respectively, compared with non-transgenic counterparts, \(P < 0.0001\)).
Expression of critical OS and phototransduction proteins is decreased in COP-T

Retinal extracts were prepared from P30 mice and protein levels were assayed by reducing SDS/PAGE coupled with western blotting. Protein levels were normalized to actin; for comparisons, levels in the rds+/nrl−/− were set to 100%. Total (endogenous and transgenic) RDS protein was decreased in all genotypes. By 12 months of age, amplitudes were not significantly different in all genetic backgrounds. A significant functional deficit was associated with the expression of COP-T in rds+/−, COP-T/+/nrl−/−, rds−/−/nrl−/−, rds−/−/nrl−/−, and COP-T/rds−/−/nrl−/− are shown. A significant functional deficit was associated with the expression of COP-T in rds+/−, and rds−/−/nrl−/− backgrounds. *P < 0.05.

Expression of critical OS and phototransduction proteins is decreased in COP-T

COP-T does not associate with ROM1 in the cone-dominant retina

RDS and ROM1 together form different sizes of heteromeric complexes. Previously, we observed that C150S-RDS protein binds to ROM1 in rods but not in cones (13). To further evaluate the role of this association in the cone-dominant retina, co-immunoprecipitation (co-IP) with RDS-CT and ROM1-CT antibodies was performed on retinal extract from COP-T animals in the rds−/−/nrl−/− background. Wild-type (rds+/−), rds−/− and rds−/−/nrl−/− were included as positive and negative controls (respectively, Fig. 4A and B). IP with RDS-CT from rds+/−/nrl−/− was performed on retinal extract from COP-T and non-transgenic controls (respectively, Fig. 4A and B). IP with RDS-CT from rds+/−/nrl−/− failed to bring down ROM1 (Fig. 4A-lane 1, & 4B-lane 3). On the contrary, IP from COP-T/rds+/−/nrl−/− failed to bring down ROM1 (Fig. 4A, lanes 3 and 4). Reciprocal IP with ROM1-CT confirmed that ROM1 is present in COP-T animals (Fig. 4B, lanes 1 and 5), but it does not associate with the COP-T protein. To determine whether the presence of COP-T in cones interfered with the ability of normal RDS to interact with ROM1, we conducted co-IP on rds+/−/nrl−/− and COP-T/rds+/−/nrl−/− using RDS-CT and ROM1-CT antibodies. As shown in Figure 4C, RDS binds less ROM1 when COP-T is present (lane 2). Similarly, less RDS comes down with ROM1 in the COP-T/rds+/−/nrl−/− than in the rds+/−/nrl−/− (Fig. 4D, lane 2).

Expression of COP-T hinders the formation of higher order complexes in cones

Non-reducing, continuous sucrose gradient velocity sedimentation on P30 COP-T and non-transgenic retinal extracts was...
used to examine total (covalent and non-covalent) RDS and ROM1 complex composition in cones. Twelve fractions were collected and subjected to reducing SDS–PAGE/western blotting (Fig. 5). We have previously demonstrated that under these conditions, higher order homo-oligomeric complexes are detected mainly in fractions 1–3, intermediate complexes in fractions 4–5 and core tetrameric complexes in fractions 6–9 (15). Although we detect higher order oligomers in COP-T retinas in the presence of endogenous RDS, (Fig. 5C and D, fractions 1–3, solid line, and E), a smaller percent of total RDS is found in that form (compare with fractions 1–3 in Fig. 5A and B, solid line, and E). COP-T/rds+/−/nrl−/− retinas appear to have a smaller fraction of RDS in the intermediate fractions as well, compared with rds+/−/nrl−/− (Fig. 5A, lanes 4 and 5, solid line, versus C, lanes 4 and 5, solid line). ROM1 distribution is also altered in the COP-T/rds+/−/nrl−/− compared with the rds+/−/nrl−/−; in the presence of COP-T, a smaller percentage of ROM1 is found in the intermediate complexes in favor of the tetrameric form (Fig. 5A and C, lanes 6–9, dotted line, and F). These results suggest that the presence of COP-T partially inhibits the endogenous protein from forming large RDS or RDS-ROM1 complexes, by either adversely affecting their stability and/or their assembly.

**COP-T protein is mislocalized throughout the cone photoreceptor**

Previously, we observed that the C150S-RDS protein was mislocalized throughout the membranes of the cone photoreceptor while no mislocalization was detected in rods. To confirm that this phenomenon also occurs in the cone-dominant background, sections from paraffin-embedded eyes taken from transgenic and non-transgenic mice on the rds+/−/nrl−/− background were stained for COP-T using mAB 3B6 (Fig. 6A). No mAB 3B6 immunoreactivity is detected in the rds+/−/nrl−/− retina, while mislocalization of COP-T is seen in transgenic retinas. To confirm that this abnormal phenotype was not due simply to the C-terminal modification we incorporated in the transgene, mice expressing a normal mouse peripherin/RDS transgene (NMP) with the same modification (22) in the rds+/−/nrl−/− were also labeled, but no mislocalization of the transgenic protein was observed (Fig. 6A). mAB 3B6 works better on frozen sections than paraffin-embedded ones, and mAB 3B6 labeling of frozen sections (Fig. 6B) demonstrates that the mislocalization of COP-T is quite extensive and is observed within and outside of rosettes. Arrows mark mislocalization within the ONL, while arrowheads mark mislocalization in the OPL. Previously, we were unable to determine whether the COP-T protein was mislocalized alone or together with endogenous RDS since the RDS-CT antibody recognizes both endogenous and transgenic RDS (13). As shown in Figure 6C (left three panels), RDS-CT immunoreactivity is detected in both rds+/−/nrl−/− and COP-T/rds+/−/nrl−/−. In the transgenic retinas (Fig. 6C, upper three panels), RDS-CT immunostaining is mislocalized and co-localizes with mAB 3B6. Recently, we have generated and characterized a new mAB against the C-terminus of
mouse RDS (mAB 2B7) (24). To confirm that mAB 2B7 does not recognize transgenic RDS (i.e. COP-T), we used retinal sections from rds+/−/nrl−/− animals carrying one of our other RDS transgenes (R172W). The R172W transgene has the same P341Q mAB 3B6 epitope as COP-T (23), and Supplementary Material, Figure S4 demonstrates that mAB 2B7 does not recognize the transgenic protein. When COP-T/rds+/−/nrl−/− retinas are stained with mAB 2B7, no mislocalization is observed (Fig. 6C, right panels), indicating that only COP-T, not endogenous RDS, is abnormally distributed. Co-labeling with mAB 3B6 and ROM1-CT demonstrated that ROM1 is also normally distributed (Fig. 6D).

Expression of COP-T causes mislocalization of M-cone opsin

In the rds+/+background, C150S-RDS mislocalization in cones is accompanied by mislocalization of S- and M-cone opsins (13). Here we have demonstrated that in the rds+/+/nrl−/− background, effects of COP-T on M-cones are more severe than its effects on S-cones. Figure 7A shows that M- but not S-opsin is grossly mislocalized throughout the photoreceptor cell membranes of the COP-T/rds+/+/nrl−/− retina. M-opsin mislocalization is observed in normally oriented photoreceptors (Fig. 7A) and also within rosettes (Fig. 7B). Small amounts of mislocalized S-opsin are seen in the COP-T/rds+/+/nrl−/− retina, but only when micrographs are collected with long exposure times (Supplementary Material, Fig. S5); in the nrl−/− background, the vast majority of S-opsin is properly localized to the COS. Normal M-opsin distribution in NMP/rds+/+/nrl−/− eyes (Supplementary Material, Fig. S6) indicates that M-opsin mislocalization is not due to the presence of excess RDS but to the presence of the COP-T mutant protein. Furthermore, M-opsin mislocalization in the presence of the COP-T occurs regardless of the quantity of endogenous RDS present; M-opsin is also abnormally distributed in the COP-T/rds−/−/nrl−/− (data not shown).

The COP promoter drives expression in both S- and M-cones, but its origin is the M-opsin gene. To confirm that both S- and M-cones express the transgene, retinal sections were labeled with S-opsin, M-opsin and mAB 3B6. As shown in Figure 8A, COP-T (mAB 3B6-red) transgene is expressed in M-cones (blue) and partially co-localizes with M-opsin (purple). Similarly, S-opsin (green) co-localizes with mAB 3B6 (red) in the OS layer, as indicated by yellow labeling (Fig. 8A). Higher magnification images of M- and S-opsin co-labeling with mAB 3B6 are shown in Figure 8B and C, respectively, indicating that both cone types express COP-T.

At P30, we do not observe significant thinning of the ONL in COP-T animals (Supplementary Material, Fig. S1), indicating that significant cell death has not yet begun, but the functional defects we observe are consistent with degeneration. Since some cone opsin mislocalization has been associated with degeneration, we endeavored to determine whether the mislocalization we observe is of this type. An early indicator of retinal stress is the upregulation of glial fibrillary acidic protein (GFAP), a Müller cell marker. Modest induction of intermediate Müller cell fibers is observed in COP-T/rds+/+ /nrl−/− transgenic animals at P30 (Fig. 9A, arrowheads), indicating mild gliosis. To determine whether opsin mislocalization and gliosis are observed at earlier timepoints, retinal sections from COP-T animals and non-transgenic controls in the wild-type (rds+/+) background were collected at P15 and cone opsins were labeled (Fig. 9B). Both M- and S-opsin are mislocalized, even at this early timepoint. However, GFAP induction is not seen at P15 (Fig. 9C).

These results suggest that cone opsin mislocalization precedes the onset of retinal stress and degeneration. To determine whether the presence of the COP-T protein might have an effect on the localization of other OS or phototransduction proteins, we examined the localization of the cone cyclic nucleotide gated channel (CNGB3), cone transducin (GNAT2) and cone arrestin (mCAR) and found that all are properly localized in the presence of COP-T (data not shown).

DISCUSSION

Here we have shown that expression of oligomerization-incompetent RDS results in significant adverse structural, functional and biochemical changes in cone photoreceptors. In cones, COP-T-associated ultrastructural changes are dominant and characterized by defects in lamella formation and accumulation of large, unflattened membranous structures inside the OSs. Cone function is severely decreased in the presence of COP-T, and M-cones are more affected than S-cones. M-opsin is mislocalized throughout the photoreceptor prior to the onset of degeneration, as is COP-T, while other
Figure 5. Complex formation in cones is altered in the presence of COP-T. Non-reducing velocity sedimentation coupled with reducing SDS/PAGE and western blotting was used to examine subunit assembly of RDS and ROM1 in P30 retinas from different genotypes. Whole retinal extracts (200 µg of protein each) from rds+/−/nrl−/− (A), rds−/−/nrl−/− (B), COP-T/rds+/−/nrl−/− (C) and COP-T/rds+/−/nrl−/− (D) were used. Blots were probed with RDS-CT and ROM1-CT. Three blots per genotype/protein were assayed using densitometry and the average percentage (± SD) of total immunoreactivity found in each fraction is plotted in each graph (solid line, RDS; dashed line, ROM1). RDS homo-oligomers (fractions 1–3) are less abundant in COP-T retinas than non-transgenic controls regardless of rds background. In the COP-T/rds+/−/nrl−/−, less ROM1 is found in intermediate complexes (fractions 4–6) than in rds+/−/nrl−/−. Numerical values in the left-hand margin (Mr, in kDa) of each panel indicate the migration positions of protein molecular weight markers in the SDS–PAGE. Peak fractions for molecular weight markers in the gradient are shown with arrows; apoferritin (443 kDa), beta-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). (E and F) Total percentage of RDS (E) or ROM1 (F) found as tetramers (fractions 6–9), octomers (fractions 5–6) or higher order oligomers (fractions 1–3) is summarized from velocity sedimentations in (A–D).
proteins are normally localized, including endogenous RDS, ROM1, Gnat2, mCAR and CNGB3. In stark contrast to the situation in rods, C150S-RDS in cones cannot interact with ROM1, and inhibits the ability of endogenous RDS to do so. Furthermore, unlike in rods, cones expressing COP-T along with endogenous RDS have fewer higher order oligomers than their non-transgenic counterparts.

We observe that M-cones are more affected by the presence of COP-T than S-cones are. These results are unexpected; from a cell biological standpoint, it has been thought that the major difference between M-cones and S-cones is the pigment and that otherwise the cells are identical. Indeed, in mice, many cones express both S- and M-opsin pigments. The differential response of these two cone cell types to the presence of COP-T along with endogenous RDS have fewer higher order oligomers than their non-transgenic counterparts.

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RDS and ROM1 appear to be involved in two separate processes: (i) formation and flattening of the rim region, and (ii) disc and lamella sizing (in cones and rods) and fusion (in rods). Previous experiments support the role of C150 and higher order RDS oligomers in the disc-flattening process: in the absence of C150, vesicles lose flattened morphology and do not pinch into the characteristic rim structure (26). Consistent with the absence of ROM1 in RDS higher order oligomers, mice lacking ROM1 (rom1<sup>−/−</sup>) exhibit no defects in flattening of the rim region (27). In vivo, we demonstrated that the expression of C150S-RDS in rods does not affect this process (13). That is, provided some native RDS is expressed in rods (enabling the formation of the requisite higher order oligomers), flattening is not affected by the presence of RDS and ROM1 appear to be involved in two separate processes: (i) formation and flattening of the rim region, and (ii) disc and lamella sizing (in cones and rods) and fusion (in rods). Previous experiments support the role of C150 and higher order RDS oligomers in the disc-flattening process: in the absence of C150, vesicles lose flattened morphology and do not pinch into the characteristic rim structure (26). Consistent with the absence of ROM1 in RDS higher order oligomers, mice lacking ROM1 (rom1<sup>−/−</sup>) exhibit no defects in flattening of the rim region (27). In vivo, we demonstrated that the expression of C150S-RDS in rods does not affect this process (13). That is, provided some native RDS is expressed in rods (enabling the formation of the requisite higher order oligomers), flattening is not affected by the presence of

Figure 6. COP-T transgenic protein is mislocalized but not endogenous RDS or ROM-1. Immunohistochemical labeling was performed on paraffin (A) or frozen sections (B–D) from P30 eyes of the indicated genotypes. (A and B) mAB 3B6 (red) labeling documents mislocalization in the COP-T/rds<sup>+/−</sup> nrl<sup>−/−</sup> but not in non-transgenic or NMP/nrl<sup>−/−</sup> transgenics (A). In COP-T/rds<sup>+/−</sup> nrl<sup>−/−</sup>, mislocalization is observed both in rosettes and along the outer edge of the retina (B). (C) Sections were labeled with mAB 3B6 (red) and RDS-CT (green, left three panels) or mAB 2B7 (red, right panel). Only transgenic RDS is mislocalized. (D) Co-labeling with ROM1-CT (green) and mAB 3B6 (red) indicates that endogenous ROM1 is not mislocalized in the presence of the COP-T protein. Arrowheads, mislocalized protein in the OPL; arrows, mislocalized protein in the ONL; OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; R, rosette. Scale bar, 20 μm.
C150S-RDS. Here we demonstrate this is patently not the case in cones. Regardless of the amount of endogenous RDS present, expression of COP-T in cones (COP-T/rds+/+/nrl−/−) leads to the appearance of un-flattened and rounded COS membranous structures. This observation suggests that in cones, expression of COP-T either destabilizes higher order oligomers or adversely affects their initial formation, resulting in a net loss of higher order complexes. This hypothesis is supported by velocity sedimentation data showing that in COP-T/rds+/+/nrl−/− retinas (compared with non-transgenic controls), a much lower percentage of total RDS is found in fractions associated with higher order oligomers. In contrast, in rods, expression of C150S-RDS had no effect on the formation of RDS complexes (13). Additional support for this hypothesis comes from observations of other RDS mutants. For example, RDS carrying the macular dystrophy mutation R172W can form higher order oligomers, but tryptic digestion experiments demonstrated that the transgenic protein is less stable than WT. Expression of this protein in the rds+/− background has no affect on rods, but causes a severe, dominant cone degeneration. Thus, these data combined suggest that while RDS higher order oligomers are found in both rods and cones, those in cones are less stable and more sensitive to disruptive influences than those in rods.

This may be tied to the different microenvironment of cone lamellae rims versus rod disc rims. In rods, discs and their rims are fully inside the cell, surrounded completely by the plasma membrane, and thus buffered from the mechanical stresses that may be present in the extracellular space. In contrast, cone lamellae and their rims are exposed to the extracellular space. In addition, in rods, RDS in the OS binds to the GARP portion of the β-subunit of the CNG channel, possibly tethering disc rims to the plasma membrane (28). In cones, this stabilizing interaction is absent; the cone CNG channel does not have a GARP portion, and RDS does not interact with the channel (29). Cone rims may be therefore more susceptible to disruption than rod rims, and require additional structural support from disulfide-bonded RDS complexes to keep them intact.

Disc sizing appears to be a more complicated process requiring the presence of normal amounts of both RDS and ROM1. In fact, the primary defect of rom1−/− rods is a dysregulation of disc sizing (27). In rom1−/− mice, disc rims and pinching are normal, but discs are larger in size than in normal controls. However, sizing clearly involves RDS as well as ROM1; in rds−/− retinas with <50% of the normal amount of RDS, rod OSs are characterized by large whorl formations instead of discs. In these mice, the OS membranes are still packed quite densely into the OS space, i.e. no large open vesicles are observed, but the membranes just grow in large flattened circles. Our evidence here suggests that interaction between RDS and ROM1 is required for proper disc sizing. In rods, C150S-RDS (MOP-T) retains the ability to bind ROM1, and therefore its expression helps alleviate the defects in disc sizing associated with RDS deficiency: disc size and structure in MOP-T/rds+/− mice are significantly better than in rds+/− mice (13). In contrast, COP-T in cones cannot bind to ROM1, and COP-T protein cannot alleviate the abnormal disc sizing seen in the rds+/+/nrl−/−.

An additional observation arising from this study is that COP-T and cone opsins are mistrafficked and partially co-localized in transgenic retinas, providing insight into both RDS complex assembly and OS-trafficking pathways in cones. Defects in other photoreceptor genes have been...
shown to cause a pattern of cone opsin mislocalization similar to that which we report. In some cases, this process appears to be part of the degenerative process (as in the case of CNGA3<sup>-/-</sup>) (30); however, in COP-T mice, mislocalization precedes degeneration. Consistent with our results, in the majority of the models exhibiting cone opsin mislocalization (such as Kif3a<sup>-/-</sup> and BBS4<sup>-/-</sup>), normal RDS is not mislocalized (31–36). Data from this body of work suggest that rods have different trafficking pathways for membrane proteins than cones do, but that as in rods (37), cones traffic RDS and opsin by different pathways.

Our data suggest that COP-T is co-opting and interfering with normal cone opsin transport. The key question is why this occurs in cones but not in rods. We hypothesize that differences in RDS assembly in rods versus cones may account for this difference. Our results suggest that any vesicle containing normal RDS (even if it also contains COP-T) is properly trafficked to COS since all endogenous RDS and some of COP-T gets to COS. By default, only vesicles containing COP-T alone (and their cargo) will mislocalize. However, C150S-RDS expressed in rods does not mislocalize, indicating that disulfide bonding plays a different role in complex assembly in the IS of cones than in rods.

In rods, RDS traffics to the OS as a non-covalently bound tetramer. Thus, C150S-RDS is normally trafficked in rods. The abnormal localization of COP-T in cones suggests that in contrast to rods, cones may form some covalently bound disulfide-linked RDS tetromers before trafficking to the OS. As a result of this, oligomerization-incompetent RDS does not always assemble properly in the cone IS. Since only tetrameric RDS/ROM1 is trafficked to the OS, monomeric COP-T may accumulate and become abnormally packaged into cone opsin-containing vesicles causing aberrant trafficking and contributing to the observed decreases in retinal function. Differential RDS complex assembly in cones versus rods may also help explain why C150S-RDS can bind ROM1 in rods but not in cones. Future studies on RDS complex assembly in cones will enable us to test these hypotheses.

This work significantly advances our understanding of the structural role and function of RDS in cones. Higher order RDS oligomers are more sensitive in cones than in rods; the way RDS interacts with ROM1 may be different in cones versus rods; and excitingly, RDS complexes may assemble and traffic differently in cones versus rods. The data presented herein provide insight into divergent rod and cone trafficking pathways and the cellular requirements for rod versus cone OS formation and the processes which support rod versus cone vision.
MATERIALS AND METHODS

Generation and characterization of C150S transgenic mice

The cone-expressing C150S-RDS (COP-T) transgenic mice were generated and characterized previously (13,38). Briefly, the C150S mutation was introduced into the 1.6 kb full-length mouse Rds cDNA carrying the P341Q modification. The P341Q modification does not affect the function or structure of the protein and enables specific antibody recognition (see antibodies section) (22). Expression of the transgene was directed to S- and M-cones by the inclusion of the previously characterized 6.5 kb fragment of the human red/green opsin promoter (COP-generously shared with us by Dr Jeremy Nathans, John Hopkins University, MD, USA) (39,40) while an SV40 small t-intron poly A signal (0.9 kb) was included to stabilize the message (13). This promoter has been shown to direct robust and uniform expression of the transgene (40). Injection of the transgene, screening and characterization of the founders and maintenance of the lines were described previously (13,41). Mice were bred into alternate backgrounds using nrl+/+ (generously shared by Dr Anand Swaroop, NEI) and rds+/+ (generously shared by Dr Neeraj Agarwal) lines. All experiments and animal protocols were approved by the local Institutional Animal Care and Use Committee (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA) and conformed to the guidelines on the care and use of animals adopted by the Society for Neuroscience and the Association for Research in Vision and Ophthalmology (Rockville, MD, USA).

Antibodies

Antibodies used in this study are as follows. Rabbit polyclonal RDS-CT, ROM1-CT and S-opsin antibodies were generated in house, characterized previously (3,13,23) and used at dilutions of 1 : 1000 for western blot (WB) and immunofluorescence (IF) and 1 : 10 for EM/immunogold. RDS-CT recognizes both endogenous and, to a lesser extent, transgenic RDS (21,22). Mouse mAB 3B6 against bovine RDS was generously shared by Dr Robert Molday (University of British Columbia) and was used for IF at 1 : 100. We have previously shown that mAB 3B6 recognizes the P341Q modification (21,22). Mouse mAB 2B7 raised against murine RDS was generated for us by Precision Antibodies and was recently characterized (24). mAB 2B7 was used at 1 : 1000 for IF and recognizes only endogenous RDS (Supplementary Material, Fig. S4). Rabbit polyclonal antibodies against M-opsin and cone arrestin (mCAR) were generously shared by Dr Cheryl Craft (Keck School of Medicine, University of Southern California) (42–45). M-opsin was used at 1 : 20 000 for IF and 1 : 10 000 for WB while mCAR was used at 1 : 2000 for IF. Mouse mAB against GFAP was purchased from Chemicon International (MAB302, Temecula, CA, USA) and was used at 1 : 1000 for IF. Rabbit polyclonal antibody against the β-subunit of the cone cyclic nucleotide-gated channel (CNGB3) was generously shared by Dr Xi-Qin Ding (University of Oklahoma Health Sciences Center) (46,47) and was used at 1 : 250 for IF. Rabbit polyclonal antibody against cone transducin (GNAT2, 1 : 1000 IF) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (10) as was goat polyclonal antibody against S-opsin (1 : 500 IF). HRP-conjugated mouse mAB against beta-actin was purchased from Sigma-Aldrich (St Louis, MO, USA) and used at 1 : 5000 for WB.

WB and IP

Frozen retinas were homogenized on ice in solubilization buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS, 2.5% glycerol and 1.0 mM phenylmethylsulphonyl fluoride. Insoluble
protein was removed by a 25 min centrifugation at 54,000 rpm in a Sorvall Discovery M150 ultracentrifuge using a fixed angle rotor after 1 h incubation at 4°C. Reducing and non-reducing SDS–PAGE/WB (20 μg protein for detection of RDS, ROM1 and S-opsin or 75 μg for detection of M-opsin, which is present at lower levels in the nrl−/− than RDS, ROM1 and S-opsin) and IP (300 μg protein) were undertaken as described previously (13,23). WB densitometry was completed on at least three blots per protein imaged using a Kodak Image Station 4000r WB densitometry was completed on at least three blots

Velocity sedimentation analysis
Non-reducing velocity sedimentation was performed on whole retinal extract as described previously (13,15). Continuous density gradients of 5–20% sucrose were prepared by sequentially layering 0.5 ml each of PBS-buffered 20, 15, 10 and 5% sucrose, containing 0.1% Triton X-100 and 10 mM N-ethylmaleimide (Sigma-Aldrich). Gradients were allowed to become continuous by diffusion at room temperature for 1 h and then chilled on ice for at least 30 min prior to sample loading. An amount of 200 μl of whole retinal extract (200 μg protein) in solubilization buffer was layered onto gradients and centrifuged at 109,000g (40,000 rpm) for 16 h at 4°C in a Sorval Discovery M150 centrifuge with a swinging bucket rotor (Sorval no. S55S-1009). The centrifuge tube was punctured at the bottom with a 26.5 gauge needle and gradients were fractionated by dropwise collection (six drops/tube). The samples were then analyzed by SDS–PAGE and western blotting. Molecular weight markers for the determination of tetrameric, octomeric and oligomeric fractions were used and have been published previously (15,24). Summary graphs for velocity sedimentation experiments were prepared by summing the percent of total RDS found in each fraction corresponding to the relevant complex type—oligomeric (fractions 1–3), intermediate (fractions 4–5) and tetrameric (fractions 6–9). Experiments were repeated three to six times.

Electroretinography
ERGs were performed as described previously (3,23,25). Briefly, following overnight dark-adaptation mice were anesthetized by intramuscular injection of 85 mg/kg ketamine and 14 mg/kg xylazine (Pharmaceutical Systems, Inc., Tulsa, OK, USA). Eyes were dilated with 1% Cyclogyl (Alcon, USA) and electrophysiological function was assessed using the UTAS system (LKC, Gaithersburg, MD, USA). ERGs were recorded with the use of a platinum wire loop electrode in contact with the corneal surface through a thin layer of 2.5% methylcellulose (Pharmaceutical Systems Inc.). Check and tail electrodes served as reference and ground, respectively. Assessment of rod photoreceptor function (scotopic ERG) was performed with a strobe flash stimulus of 157 cd s/m² intensity presented to the dark-adapted diluted eyes in a BigShot ganzfeld. To evaluate photopic response, animals were light adapted for 5 min under a light source of 29.03 cd/m² intensity in a BigShot ganzfeld. Cone photoreceptor function (photopic ERG) was assessed by averaging 25 strobe flash stimuli (79 cd s/m²) presented to the light-adapted animal. Measurement of photopic b-wave amplitude was made from the trough of the a-wave to the crest of the b-wave.

Spectral ERGs were undertaken using the same system. Animals were dark adapted and anesthetized and scotopic recordings were completed as described above for standard ERG. After 5 min of light adaptation, M-cone responses were measured by averaging 25 strobe flash stimuli of 530 nm (12.5 cd s/m²) using the BigShot green LED light source. After a recovery period of 1 min, S-cone responses were measured by averaging 25 strobe flash stimuli of 365 nm (0.79 cd s/m²) using the BigShot UV LED light source. At least eight mice per genotype were analyzed. Genotypes were compared using one-way ANOVA with Bonferroni’s post hoc test. All groups passed the Kolmogorov–Smirnov test for normality and P-values are from one-way ANOVA.

IF labeling
Tissue fixation and sectioning were performed as previously described for paraffin embedding (13). For frozen sections, eyes were enucleated, fixed and dissected as described (48). Eyecups were cryoprotected in 10, 20 and 30% sucrose before being embedded in M1 embedding medium (Thermo Scientific, Waltham, MA, USA). Ten micron frozen sections were cut on a Leica CM 3050 cryostat (Bannockburn, IL, USA). Six micron de-paraffinized or 10 μm frozen retinal sections were washed in Hanks PBS (Sigma-Aldrich) and water, then incubated for 2 min in 1% NaBH₄ (Sigma-Aldrich). Sections were blocked for 2 h at room temperature in HPBS containing 5% BSA (Sigma-Aldrich), 1% fish gelatin (Sigma-Aldrich), 20% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and 1% Triton X-100 (VWR). Sections were then incubated with primary antibodies diluted in blocking buffer overnight at 4°C, rinsed 3 × 10 min in HPBS and then incubated with AlexaFluor 488, 555 or 647 conjugated secondary antibodies (Invitrogen) at a dilution of 1 : 1000 for 1 h at room temperature. Sections were washed and mounted with ProlongGold containing DAPI (Invitrogen). Images were captured with a Zeiss universal microscope using Axiovision software (Supplementary Material, Fig. S3) or an Olympus BX-62 microscope equipped with a spinning disc confocal unit using either a 20× (air, 0.75 NA) or 60× (oil, 1.42 NA) objective (all other IF figures). Images were stored and deconvoluted (no neighbors paradigm) using Slidebook® version 4.2 and are either epifluorescent (Supplementary Material, Figs S1, S3 and S4) or single slices of a confocal stack (remaining fluorescent figures). Figure assembly was done in Adobe Photoshop CS. For rosette quantification, transverse (superior–inferior) retinal sections containing the optic nerve head were examined (at least three sections per eye, and three eyes per genotype) and rosettes were counted across the whole section. Student’s t-tests were used to compare transgenic with non-transgenic animals.
Histology, transmission electron microscopy and immunogold cytochemistry

The methods employed for tissue collection and processing for plastic-embbedment light and electron microscopy and immunogold labeling were as described previously (3,10,15). For light microscopy, 0.75 μm sections were viewed and photographed with an Olympus BH-2 photomicroscope with a Nikon digital camera system. Thin (600–800 Å) sections for TEM were collected on copper 75/300 mesh grids and stained with 2% (w/v) uranyl acetate and Reynolds’ lead citrate. Thin sections for immunogold were collected on nickel 75/300 mesh grids; primary antibodies were used as described above; secondary antibodies (AuroProbe 10 nm gold-conjugated goat anti-rabbit IgG; GE/Amersham, Piscataway, NJ, USA) were used at 1: 50 dilution. Sections were viewed with a JEOL 100CX electron microscope at an accelerating voltage of 60 kV.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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REFERENCES


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