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The Function of Oligomerization-Incompetent RDS in Rods

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Abstract

The photoreceptor-specific tetraspanin glycoprotein RDS (retinal degeneration slow) is associated with many forms of inherited retinal disease. RDS shares features in common with other tetraspanin proteins, including the existence of a large intradiscal D2 loop containing several cysteines. While these cysteines are used only for intramolecular disulfide bonds in most tetraspanins, RDS expresses a seventh, unpaired cysteine (C150) used for intermolecular disulfide bonding in the formation of large RDS oligomers. To study oligomerization-dependent vs. oligomerization-independent RDS functions in rods, we generated a transgenic mouse line harboring a point mutation that replaces this Cys with Ser (C150S), leading to the expression of an RDS protein that cannot form intermolecular disulfide bonds. The mouse opsin promoter (MOP) was used to direct C150S RDS expression specifically in rods in these transgenic mice (MOP-T). Here we report improvement in scotopic ERGs in MOP-T/*rds*^{+/-} mice (compared to non-transgenic *rds*^{+/-} controls) and the appearance of malformed outer segments (OSs) in MOP-T mice that do not express native RDS (MOP-T/*rds*^{-/-}). These results suggest that while normal OS structure and function require RDS oligomerization, some RDS function is retained in the absence of C150. Since one of the functions of other tetraspanin proteins is to promote assembly of a membrane microdomain known as the “tetraspanin web”, future studies may investigate whether assembly of this web is one of RDS’s oligomerization-independent functions.

The photoreceptor (PR)-specific glycoprotein RDS (retinal degeneration slow) is a member of the tetraspanin protein family. This family contains proteins that share a similar structural arrangement: four transmembrane domains containing some polar residues, a consistent loop arrangement, and the presence of cysteines in the so-called D2 loop. These proteins are expressed in almost every cell of the body and are involved in a diverse range of biological processes, including modulating the immune system, enhancing fertilization, cell motility/adhesion, the formation of neuromuscular junctions, and, of course, retinal function (Hemler 2003). In spite of the variety of processes linked to tetraspanins, there are similarities in their roles in many tissues (Hemler 2003; Stipp et al. 2003). For example, RDS is a structural protein known to be required for the formation and maintenance of the PR outer segment (OS), a role with many parallels to that of the tetraspanin CD151, which has been shown to regulate cytoskeletal remodeling in epithelial and mesenchymal cells by interacting with integrins, protein kinase C, actin, and E-cadherin (Shigeta et al. 2003).

On the molecular level, tetraspanins are known to interact directly with themselves and other tetraspanins, with cell-matrix adhesion receptors, such as integrin $\alpha\beta1$ and $\alpha4\beta1$ (Serru et al. 1999; Yauch et al. 1998), with other non-tetraspanin membrane proteins (Wu et al. 1995), and with cytoskeletal components (Delagauillaumie et al. 2002; Lagaudriere-Gesbert et al.

1998). Furthermore, via palmitoylation and other secondary interactions, tetraspanins form large functional complexes known as the “tetraspanin web”. This typically contains a core of directly interacting tetraspanins linked covalently or non-covalently to other complex members and commonly defines a membrane microdomain similar to, but distinct from, lipid rafts (Hemler 2003; Levy and Shoham 2005; Stipp et al. 2003). Taken together, these findings support the notion that tetraspanins such as RDS may play a significant modulatory role in maintaining cell structure.

The *RDS* gene encodes a PR-specific glycoprotein found in both rods and cones (Connell and Molday 1990; Travis et al. 1991) that exhibits evolutionary conservation all the way from skates to humans (Li et al. 2003; Naash et al. 2003). RDS is restricted to the rims of PR discs, as well as the basal regions of rod and cone outer segments (OSs) adjacent to the cilia where disc morphogenesis occurs (Arikawa et al. 1992; Moritz et al. 2002). In the PR inner segment, RDS assembles into non-covalently bound tetramers, which are then trafficked to the OS where they are further assembled into disulfide bonded higher-order oligomers (Chakraborty et al. 2008b; Loewen and Molday 2000). RDS is necessary for disc assembly, orientation, and physical stability (Molday et al. 1987; Wrigley et al. 2000), and although most research on RDS has focused on its role in the PR and vision, recent insights into its other potential functions have come from research on other members of the tetraspanin family.

Over 80 mutations in the *RDS* gene have been identified in multiple forms of both rod- and cone-dominant hereditary retinal degeneration (Farjo and Naash 2006), <http://www.retina-international.org/sci-news/rdsmut.htm>. Both the phenotypic variability seen in patients with *RDS* mutations and animal/cell biological studies of *RDS* mutations support the hypothesis that RDS behaves differently in rod vs. cone PRs.

The vast majority of *RDS* disease-causing mutations reside within the large intradiscal polypeptide loop (D2) of RDS. The D2 loop is a common tetraspanin feature and contains the conserved cysteines that are involved in intramolecular disulfide bonding (Hemler 2001). In addition to these six Cys residues, RDS contains a seventh, unpaired cysteine (C150), which is involved in intermolecular disulfide bonding and is thought to be required for the formation of RDS oligomers (Chakraborty et al. 2008; Goldberg et al. 1998). Interestingly, the lack of this cysteine in other tetraspanins highlights one of the differences between the role of RDS in the PR vs. the biological functions of other tetraspanins. While it is likely that RDS forms a tetraspanin web within the OS disc membrane, it is also responsible for the formation of the OS disc rim region, a function which requires that RDS complexes help to bridge adjacent membranes. This bridging function is not one usually attributed to tetraspanins. Given that a large portion of the function of most tetraspanins is due to their role in the assembly of the tetraspanin web (a function that does not rely on intermolecular disulfide bonds, since other tetraspanins do not form them), we wanted to see what functions (if any) of RDS are retained when the ability to form these intermolecular disulfide bonds is disrupted.

The role of RDS intermolecular disulfide bonds in formation of the flattened OS disc was first highlighted in in vitro studies. When wildtype (WT) RDS is incorporated into microsomal vesicles under non-reducing conditions, an abnormal, flattened morphology is produced, whereas vesicles incorporating RDS under reducing conditions possess a characteristically rounded appearance (Wrigley et al. 2000). However, when mutant C150S RDS is expressed, vesicular flattening is abolished. Subsequent studies in which GFP-tagged C150S RDS was co-expressed in *X. Laevis* rods with WT RDS showed no dominant-negative effect on rod photoreceptors (Loewen et al. 2003). Studies in COS cells have confirmed the role of C150 in the formation of intermolecular disulfide bonds; C150S RDS

expressed in COS cells folds properly and forms tetramers, but does not form higher-order oligomers (Goldberg et al. 1998). To further study the role of intermolecular disulfide bonding in the mammalian retina, we have generated two transgenic mouse models expressing C150S RDS in either rods (MOP-T) or cones (COP-T) by means of cell type-specific opsin promoters (Chakraborty et al. 2008). One of our goals is to differentiate between RDS functions that depend on intermolecular disulfide bonding (and, thus, higher-order oligomer formation) vs. those functions that do not, e.g., the formation of the RDS tetraspanin web.

Mice expressing C150S in cones exhibit a striking, dominant cone degeneration in which cone photoreceptors have extremely malformed OSs and die even in the presence of the WT RDS protein. Consistent with the structural defect, cone function is obliterated in these animals (Chakraborty et al. 2008). Interestingly, this dominant degenerative defect is not observed when C150S RDS is expressed in rods. While the different phenotype in the MOP-T vs. COP-T mice helps explain the differential role of RDS in rods and cones, the severity of the cone defect makes it difficult to study oligomerization-dependent vs. oligomerization-independent effects. Therefore, we have undertaken a further, more detailed examination of the milder rod phenotype of the MOP-T transgenic mice.

Previously, we showed that in rods and in the absence of native RDS, C150S RDS can form tetramers and retains the ability to bind ROM-1 (Chakraborty et al. 2008). However, velocity sedimentation studies showed that, as expected, C150S RDS does not form higher-order oligomers in the absence of WT RDS (Chakraborty et al. 2008). This observation is confirmed by the non-reducing Western blot shown in (Fig. 5.1a). Higher-order oligomers can be seen when C150S RDS (MOP-T) is expressed in the presence of WT RDS, but in its absence no oligomers or disulfide linked dimers are observed. Under reducing conditions (Fig. 5.1b) oligomers are reduced to monomers and low levels of dimers. However, no dimers are detected in MOP-T/*rds*^{-/-} retinas, although C150S monomer is stably expressed.

The results of functional full-field rod (scotopic a-wave) ERG studies undertaken at various ages are shown in (Fig. 5.2a). At early timepoints, there is no difference in retinal function between transgenic (MOP-T) and non-transgenic littermates (NT) in the WT RDS background confirming that there is no dominant negative effect on rods. Furthermore, although transgenic animals appear to lose rod function slightly faster than do non-transgenic controls, at 18 months of age both have similar rod function. This observation is confirmed on the structural level in (Fig. 5.2b). Light microscopic analysis undertaken at 18 months of age (Fig. 5.2b) shows that there is no structural difference in the retina between transgenic and non-transgenic mice on a WT background. These experiments clearly demonstrate that the presence of oligomerization-incompetent RDS (C150S RDS) is not harmful to rods in the presence of native RDS.

To further study the importance of RDS oligomerization for RDS function, ERG studies were undertaken at 2 months of age in multiple *RDS* backgrounds (Fig. 5.2c, *left panel*). In contrast to the situation in the WT background, when expressed in the *rds*^{+/-} background, the presence of C150S RDS improves rod function (even though it cannot form oligomers). However, in the absence of native RDS (*rds*^{-/-}) no ERG signals were detected. As expected, since C150S RDS in MOP-T mice is not expressed in cones, no alterations in photopic (cone) amplitudes were detected (Fig. 5.2a, *right panel*). The lack of functional improvement in the *rds*^{-/-} background confirms that RDS oligomerization is required for retinal function. However, the functional improvement seen when oligomerization-incompetent RDS (C150S) is present in the heterozygous background (MOP-T/*rds*^{+/-}) suggests that complete retinal function may depend on other RDS functions that do not necessarily require intermolecular disulfide bonding.

To see whether there is any structural evidence to support this hypothesis, we undertook detailed ultrastructural (electron microscopic, EM) analyses. Previously we have shown that in the absence of native RDS (MOP-T/*rds*^{-/-}) the OS layer is virtually gone and looks similar to that of *rds*^{-/-} retinas (Chakraborty et al. 2008). Consistent with the lack of function shown in Fig. 5.2c, the MOP-T/*rds*^{-/-} retina had no properly formed OSs. The OS areas of *rds*^{-/-} and MOP-T/*rds*^{-/-} mice were not identical however; MOP-T/*rds*^{-/-} mice had more membranous structures than their non-transgenic counterparts. To further study these structures, we performed EM immunogold analysis with monospecific antibodies against rhodopsin to determine whether these membranes are derived from the OS. In contrast to *rds*^{-/-} retinas, where no opsin immunoreactivity is detected (due to complete absence of an OS layer), membranous material in the OS area of MOP-T/*rds*^{-/-} did contain anti-opsin immunopositive staining. Furthermore, very rarely, rounded membranous structures were observed in MOP-T/*rds*^{-/-} retinas that were anti-opsin immunopositive (Fig. 5.3a). Interestingly, these structures are reminiscent of the malformed OSs seen in the *rds*^{+/-} mouse (*cf.* Fig. 5.3b), although they are much more infrequent. The appearance of these structures suggests that MOP-T/*rds*^{-/-} animals are trying, albeit unsuccessfully, to make OSs. Immunofluorescent labeling with anti-RDS antibody also was used to confirm that these attempted OSs contain the transgenic C150S RDS protein (data not shown).

Our structural and functional observations presented here support the idea that oligomerization-competent RDS is required for normal OS structure and function, but that RDS has some oligomerization-independent functions; hence, having a mutant (C150S) RDS is better than having no RDS at all. We hypothesize that one of these oligomerization-independent functions may be assembly of specialized membrane microdomains (*i.e.*, the tetraspanin web); however, at present, there is no direct evidence to support this hypothesis. Current studies are underway to identify other RDS binding partners and determine the composition of the RDS tetraspanin web, while future studies may investigate the role of oligomerization-incompetent (C150S) RDS in that process.

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Fig. 5.1.

Biochemical analysis of MOP-T; Retinas were harvested from transgenic (MOP-T) or non-transgenic (NT) animals at postnatal day (P) 30. Total retinal extracts were used for non-reducing (**a**) or reducing (**b**) SDS-PAGE followed by immunoblotting (IB) with the antibodies indicated (RDS-CT, ROM-1, rod outer segment membrane protein-1, an RDS homolog; and actin). Expression of C150S RDS does not interfere with the ability of WT RDS to form higher-order oligomers, but it cannot form oligomers alone

Fig. 5.2.

Functional analysis of MOP-T; **(a)** Non-transgenic (WT) and transgenic (MOP-T/WT) animals in the WT background underwent full-field scotopic ERG. Shown are maximum scotopic a-wave amplitudes at various ages as indicated. At both early (1–2 months) and late (18 months) ages, there is no difference in maximum scotopic ERG amplitude between transgenic and non-transgenic mice. **(b)** Toluidine blue stained, plastic embedded sections were collected from eyes harvested from transgenic and non-transgenic animals at 18 months of age. Scale bar, 20 μm. The C150S transgene has no structural effect on the retina in the WT background. **(c)** At P60, transgenic and non-transgenic animals in multiple *RDS* backgrounds underwent full-field scotopic and photopic ERG. Shown are averages (+/- S.E.M.) from 5 to 7 animals/category. In the absence of WT RDS, no retinal function is detected, however, the presence of the C150S transgene improves the maximum scotopic a-wave amplitude in the heterozygous *rds*^{+/-} mouse

Fig. 5.3.

Ultrastructural analysis of MOP-T; Eyes were harvested from P30 transgenic and non-transgenic eyes and processed for immunogold/EM (a) or EM (b). (a) mAB 1D4 against rhodopsin was used to label OSs in MOP-T/*rds*^{-/-} and WT (as a control) animals. No normal OSs were observed in MOP-T/*rds*^{-/-} animals, only rounded whorl-like membranous structures reminiscent of those seen in the *rds*^{+/-} (b), only larger. Scale bar, 2 μm