An Intramembrane Glutamic Acid Governs Peripherin/rds Function for Photoreceptor Disk Morphogenesis

Andrew F. X. Goldberg, Linda M. Ritter, Nidbi Khattree, Neal S. Peachey, Robert N. Fariss, Loan Dang, Minzhong Yu, and Alyssa R. Bottrell

PURPOSE. Peripherin/rds (P/rds), the product of the retinal degeneration slow (rds) gene, is a tetraspan protein that plays a pivotal role for photoreceptor outer segment (OS) structure and is involved in a broad spectrum of inherited retinal degenerations. P/rds interacts with the homologous protein rom-1, previously proposed to regulate P/rds function. The authors examined the significance of an intramembrane glutamic acid conserved in all P/rds proteins (and many other tetraspanins) but absent in all rom-1 orthologs.

METHODS. The authors performed isosteric glutamine substitution of the conserved glutamate at position 276, in the fourth transmembrane domain of bovine P/rds, and expressed E276Q P/rds in COS-1 cells and in transgenic mouse photoreceptors of rds+/−, −/−, and −/+ backgrounds. Western blot, immunoprecipitation, and sedimentation analyses were used to assess protein structure and interactions. Microscopy and electroretinography were used to characterize transgenic protein localization and retinal photoreceptor structure and function.

RESULTS. E276Q P/rds was expressed, assembled, and properly localized in photoreceptor OSs of transgenic mice. In contrast to wild-type (WT) P/rds, however, this mutant did not rescue the OS structural defects observed in rds−/− and −/+ mice. Moreover, E276Q expression did not prevent the retinal degeneration that occurred as a consequence of OS disruption.

CONCLUSIONS. E276Q plays a critical role in P/rds support of photoreceptor OS structure. This finding provides a molecular rationale for asymmetry in P/rds and rom-1 function and for rom-1 regulation of P/rds activity. These findings also suggest that ionizable intramembrane residues may serve regulatory roles for tetraspan proteins more generally. (Invest Ophtalmol Vis Sci. 2007;48:2975–2986) DOI:10.1167/iows.07-00049

Vision in humans and other vertebrates is initiated within rod and cone photoreceptor outer segments (OSs). In normal OSs, hundreds of photopigment-containing membranous disks are neatly stacked and aligned along the axis of incoming light. These organelles function as the initial transducers for the encoding of visible light into neural signals. OSs undergo a daily renewal process, thought to play a restorative role by replacing damaged proteins and lipids. Shedding of mature disks from OS distal tips is coordinated with new disc morphogenesis at OS basal regions to maintain a constant average OS length. Perturbations in the renewal process have been implicated frequently in progressive retinal diseases.

Although the molecular mechanisms that underlie OS disc structure and renewal are not well understood, the integral membrane protein P/rds is known to play a key role. A null defect in the retinal degeneration slow (rds) gene causes loss of P/rds and results in the disorganization and complete absence of OSs in heterozygous (rds−/+1) and homozygous (rds−/−) null mice, respectively. Although these phenotypes make clear that P/rds is required for OS morphogenesis, its mechanism of action at the protein level is incompletely understood. We have suggested that this molecule acts as a multifunctional scaffolding element for OS architecture and renewal and that it functions through interactions with membranes and other proteins. P/rds is well documented to form stable complexes with the homologous integral membrane protein rom-1. These polypeptides associate noncovalently and through disulfide bonds to form heterotetramers and higher-order polymers. Interestingly, a complete loss of rom-1 produces a relatively mild phenotype; a rom-1 knockout mouse model generates largely intact OSs with slightly enlarged discs and mild retinal degeneration. These findings reveal functional asymmetry between P/rds and rom-1 because complete loss of P/rds but not rom-1, is catastrophic for the elaboration of OSs. Similarly, genetic defects in humans that affect rom-1 only become pathogenic if a defect in P/rds is also present. Although a disparity in expression levels might provide a partial explanation of this asymmetry, it is clear that P/rds has at least one essential activity not present in rom-1. This point was demonstrated directly by an investigation of a chimeric rom-1 protein in transgenic mouse photoreceptors. That study found that regions in addition to the second extracellular loop (EC2) of P/rds are required for rescue of the rds (−/−) phenotype.

The notion that full P/rds functionality requires multiple activities has gained additional support, with more recent findings that subunit assembly, protein targeting, and membrane fusion activities are encoded within distinct protein domains and that the latter two properties are associated with P/rds but not with rom-1. Through a bioinformatics approach, we recently noted a fundamental structural difference between P/rds and rom-1—only the former has a conserved glutamic acid residue buried within its fourth transmembrane domain. This residue is particularly noteworthy because of its conservation within all known P/rds orthologs and its absence in all known rom-1 orthologs. Moreover, acidic intramembrane residues are highly conserved across the tetraspanin protein family. Tetraspanins are integral membrane proteins observed in all metazoa examined to date and are important for a broad spectrum of biological processes. Their mechanisms of action remain largely undefined, and in no instance is
the role of ionizable residues within their transmembrane domains understood. Here, we use a variety of molecular genetic, biochemical, biophysical, and morphologic approaches to examine the possibility that an intramembrane ionizable residue regulates P/rds functional activity.

**METHODS**

**Expression of E276Q P/rds in COS-1 Cells**

Expression and analyses of recombinant P/rds in cultured cells was conducted essentially as described.17,24

**Generation, Genotyping, and Expression Level Analysis of Transgenic Mice**

Bovine P/rds was expressed in transgenic mice under control of a 4.2-kbp fragment of the mouse opsin gene promoter region active in rods and cones.25 The transgene plasmid construct was derived from that described by Olshevskaya et al.26 and includes a splice site and a codon optimized for the endogenous murine WT rds gene and the E276Q P/rds bovine transgene in cDNA samples prepared from individual mice (n = 2). Transgene expression levels are reported as a copy number ratio, relative to the expression measured from a single copy of the WT endogenous murine rds gene.

**Biochemical Analyses of P/rds in Murine OSs**

OSs were purified from freshly dissected murine retinas essentially as described31 and stored at ~70°C until use. Western blot and velocity sedimentation analyses of P/rds were performed as reported previously.52 Immunoprecipitation analyses were conducted using an anti-P/rds monoclonal antibody (mAbC6) covalently coupled to Sepharose beads at approximately 2 mg purified IgG/mL beads (SulfoLink Coupling Gel; Pierce, Rockford, IL). Rod outer segments (ROSs) from transgenic retinas (25 μg) were solubilized for 40 minutes at room temperature (RT) in solubilization buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT, and 1% Triton X-100) and then were centrifuged at 70,000g for 30 minutes at 4°C in a rotor (TLA-55; Beckman, Palo Alto, CA). The supernatant was incubated with the mAbC6 affinity matrix at RT for 1 hour. Unbound material was washed, beads were washed, and bound proteins were eluted with C6 peptide (5 mg/mL) or were boiled for 10 minutes in 3× LSB/5% β-mercaptoethanol (βME), or both.

**Retinal Functional Analysis by Electroretinography**

After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). Eyedrops were used to anesthetize the cornea (1% proparacaine HC1) and to dilate the pupil (1% tropicamide, 2.5% phenylephrine HCl, 1% cyclopentolate HCl). Mice remained on a temperature-regulated heating pad throughout the recording session. ERGs were recorded with the use of a stainless steel loop that made contact with the corneal surface through a thin layer of 0.7% methylcellulose. Needle electrodes placed in the cheek and the tail served as reference and ground leads, respectively. Responses were differentially amplified (0.05–1500 Hz), averaged, and stored using a signal averaging system (UTAS E-3000; LKC Technologies, Gaithersburg, MD).

Two recording sessions were conducted for each mouse. The first examined standard ERG intensity-response functions.35 Dark-adapted responses were recorded first using flash intensity that ranged from ~3.6 to 2.1 log cd · s/m2. Stimuli were presented in order of increasing intensity, and the number of successive responses averaged together decreased from 20 for low-intensity flashes to 2 for the highest intensity stimuli. Duration of the interstimulus interval (ISI) increased from 7–0.8 to 1.9 log cd · s/m2. Stimuli were dark adapted between stimulus pairs for 5 minutes. Amplitude of the b-wave was measured from the a-wave baseline. Amplitude of the a-wave was measured 8 ms after flash onset from the prestimulus baseline. Amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave or, if no a-wave was present, from the prestimulus baseline.

**Retinal Histology and Immunohistochemistry**

Mice were euthanatized by asphyxiation with CO2 under normal room illumination. The superior hemisphere of each eye was marked, and the eyes were enucleated and placed into 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Each cornea and lens was re-
moved after an initial fixation of 30 minutes, and the eyecups were fixed further for 4 hours. Eyecups were washed in 0.1 M sodium phosphate buffer and then were embedded in low-gelling temperature agarose.59 A vibrating microtome (Leica Microsystems, Deerfield, IL) was used to cut 100-μm sections, which were blocked in 5% normal goat serum in 0.1 M PBS, 0.5% BSA, 0.2% Tween-20, 0.05% sodium azide, pH 7.3, for 1 hour at RT. Sections were labeled with primary antibodies (anti-bovine P/rds mAbC6 and anti-mus P/rds pAbMPCT) for 12 hours at 4°C, washed, and labeled with fluorescence-conjugated secondary antibodies (goat anti-mouse; Alexa-Fluor 488; Invitrogen) and goat anti-rabbit Cy5 (Jackson ImmunoResearch, Bar Harbor, ME) and propidium iodide (2 μg/mL; Invitrogen) for 4 hours at 4°C. Sections were washed, transferred to slides containing mounting medium (Gel/Mount; Biomeda, Foster City, CA), and coverslipped. Samples were evaluated, and images were collected on a laser scanning confocal microscope (SP2; Leica Microsystems) under standardized instrument parameters.

Analysis of Photoreceptor Degeneration

Mice were euthanatized by asphyxiation with CO2 under normal room illumination. The superior hemisphere of each eye was marked, and the eyes were enucleated and placed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Each cornea and lens was removed after initial fixation of 30 minutes, and the eyecups were fixed further for 1.5 to 2 hours. Eyecups were cryoprotected in 20% sucrose/0.1 M sodium phosphate buffer overnight at 4°C, then frozen in a 2:1 solution of 20% sucrose/PBS and optimal cutting temperature (OCT) compound, essentially as described.56 Cryosections (8 μm) cut through the optic nerve head along the vertical meridian were collected on silane-coated glass slides and were stored at −20°C until use.

Photoreceptor degeneration was analyzed in sections with intact morphology, in which the optic nerve head was visible. Retinal cryosections were labeled with 1 μg/ml 4’6-diamidino-2-phenylindole dihydrochloride (DAPI) nuclear stain in PBS for 5 minutes, then were mounted (Fluoromount-G, Southern Biotechnology Associates, Birmingham, AL) and coverslipped. Digital images were acquired with a microscope (Optiphot-2; Nikon, Tokyo, Japan) equipped with epifluorescent illumination and a digital imaging system (SPOT RT; Diagnostic Instruments, Sterling Heights, MI). Software (SPOT; Diagnostic Instruments) was used to measure ONL thickness in the superior central retina 200 μm from the edge of the optic nerve head. Plotted points represent measurements on individual animals.

Analysis of Photoreceptor Ultrastructure

Mice were euthanatized by asphyxiation with CO2 under normal room illumination. Eyes were enucleated and placed in 4% paraformaldehyde (2% paraformaldehyde, 2.5% glutaraldehyde, 100 mM cacodylate, pH 7.4) for 4 hours. Cornneas and lenses were subsequently removed, and eyecups were sectioned into six to eight pieces and fixed overnight. Tissues were postfixed for 1 hour with 1% OsO4 in 1% blocked stained for 30 minutes with 2% uranyl acetate, dehydrated in a graded ethanol series, and infiltrated and embedded in medium (Polysbed 812; Polysciences, Inc., Warrington, PA). Thin sections cut with an ultramicrotome (MT 7000; Ventana, Tucson, AZ) were collected onto nickel grids, stained with 2% uranyl acetate and lead citrate, and imaged in a transmission electron microscope (Morgagni; FEI Company, Hillsboro, OR) at 80 kV. Photoreceptor ultrastructure was examined in at least four animals of each genotype.

Postembedding Immunogold Labeling

Mice were euthanatized by asphyxiation with CO2 under normal room illumination. Eyes were enucleated and placed into freshly made fixative (4% paraformaldehyde, 0.1% glutaraldehyde, 100 mM sodium phosphate, pH 7.5) for 30 minutes. Cornneas and lenses were subsequently removed, and eyecups were sectioned into six to eight pieces that were fixed for an additional 1 to 4 hours and then embedded in resin (LR White or Lowicryl K4M; Electron Microscopy Sciences, Hatfield, PA). Silver sections cut using an ultramicrotome (MT 7000; Ventana) were collected onto nickel grids. Sections were blocked for 1 hour using 5% goat serum in 20 mM Tris buffer, pH 7.5. Sections were incubated with primary antibodies overnight at 4°C. Grids were washed in buffer and then were incubated with gold-conjugated secondary antibodies (Accurate Chemical & Scientific Corp., Westbury, NY) for 1 hour at 37°C. Grids were again washed, subjected to silver enhancement (IntenSE kit; Amersham, Piscataway, NJ), stained with uranyl acetate/lead citrate, and imaged in an transmission electron microscope (Morgagni; FEI Company) at 80 kV.

Immunohistochemical Reagents

Antibodies used in this study, including mAbC6,17 pAbMutt,17 and mAbB36,37 have been described previously. All other antibodies used, including pAbBPCT, pAbMPCT, pAbEC2, and pAbRHO, were generated by immunization of New Zealand White rabbits with synthetic peptides (CVEAEGEDAGQPAAAG, CVEAEGDAGAPEAG, CKEVK-DRIKSNVGDGR, and CS(T/A)V3KTETSQVAPA, respectively), covalently coupled to keyhole limpet hemocyanin. Anti-peripherin/rds antibodies pAbMPCT and pAbBPCT, directed against C-terminal peptides corresponding to mouse and bovine proteins, respectively, were purified from sera using affinity column chromatography. pAbMPCT and pAbBPCT synthetic peptides were covalently coupled to Sepharose beads through their N-terminal cysteines with a coupling gel kit (SulfoLink; Pierce) essentially as recommended by the manufacturer. All sera were subsequently purified using a serial two-step (positive and negative) chromatographic procedure. Antibody specificity was confirmed by Western blot analyses with purified murine and bovine OS membranes.

RESULTS

We sought to investigate the significance of an intramembrane glutamic acid at position 276 for P/rds structure and function. This residue is particularly noteworthy because E276 is absolutely conserved among all known P/rds orthologs, but this position is substituted with a nonionizable glutamine in all known rom-1 orthologs (Fig. 1A). Moreover, strongly polar amino acids occur relatively infrequently within transmembrane helices, yet intramembrane acidic residues are highly conserved features of tetratetraspans (including P/rds), and their significance remains to be explained.20–22 We used site-directed mutagenesis to create an E276Q missense mutation in the coding region of a bovine P/rds cDNA and expressed the recombinant protein in cultured cells and in the photoreceptors of transgenic mice. Analysis was directed toward testing the hypothesis that E276 represents a significant determinant for P/rds structure or function.

Expression and Localization of E276Q P/rds in COS-1 Cells

Because mutations in P/rds can cause protein misfolding24 and can reduce protein stability to negligible levels,38 we first expressed E276Q in COS-1 cells to examine the feasibility of investigating mutant protein structure and function. Figure 1B shows that E276Q P/rds is expressed in COS-1 and displays a subcellular distribution similar to that of WT. This pattern is consistent with a previously documented Golgi retention of normally folded and assembled tetrameric protein.9 Indeed, Western blot analysis (Fig. 1C) demonstrates that recombinant E276Q P/rds is expressed in COS-1 at the predicted molecular weight and that it displays normal posttranslational modifications, including an N-linked carbohydrate and intermolecular disulfide bonds. Moreover, velocity sedimentation analysis (Fig. 1D) demonstrated that the mutant protein was assembled into noncovalent tetramers indistinguishable from those previously documented for WT P/rds. In sum, these results demonstrate...
that the loss of E276 does not grossly alter P/rds structure or stability and that protein folding, disulfide bonding, and tetrameric subunit assembly do not require an acidic residue in this position.

**Expression and Localization of E276Q P/rds in Transgenic Mice**

E276Q bovine P/rds was expressed in transgenic mice under the control of a 4.2-kbp fragment of the mouse opsin gene promoter region, active in rods and cones. The bovine ortholog (93% AA identity vs. mouse) was used to allow its unique immunochemical identification against a background of endogenous mouse P/rds. We used a panel of monoclonal and polyclonal antibodies (mAbC6, mAb3B6, pAbMPCT, and pAbBPCT) directed against the P/rds C terminus to distinguish the transgenic E276Q (bovine) protein from the endogenous WT (murine) protein. The E276Q transgene was crossed onto mouse backgrounds that had varied levels of endogenous WT P/rds. We used the rds mutant mouse model for this purpose because it carries a null defect for rds and reduces P/rds expression levels to roughly 50% and 0% of normal in heterozygous and homozygous mice, respectively.

Figure 2B shows a typical analysis of E276Q transgene expression in retinas from two of the independent lines generated by pronuclear injection and backcrossed on the C57BL6/J background. Quantitative PCR analyses were performed using primer sets specific for the endogenous murine and transgenic bovine P/rds proteins. The two E276Q lines analyzed here were found to express the hemizygous E276Q transgene at 0.98-fold (line ES1, open circles) and 0.51-fold (line ES2, filled triangles) the level of a single endogenous WT murine rds gene. We conducted investigations primarily using the ES1 line and confirmed selected examples using ES2 mice. A line of control transgenic mice was also generated using a WT bovine transgene; the hemizygotes expressed at a level equivalent to 5.6-fold (filled circles) the level of a single endogenous WT murine rds gene.

We performed immunohistochemical analysis of retinas from E276Q transgenic mice and nontransgenic littermates to determine whether the E276Q P/rds protein was expressed. These experiments were performed on the WT [rds (+/+)] background to determine the fate of the E276Q mutant in photoreceptors expressing a normal complement of endogenous WT P/rds. Figure 2C shows that the E276Q bovine P/rds protein is expressed and readily detected in sections of fixed retinas from transgenic animals (Figs. 2C4, 2C6). In contrast, no expression was detected in WT nontransgenic littermates (Figs. 2C1, 2C3). These results simultaneously demonstrate that the E276Q variant was properly localized in photoreceptor...
FIGURE 2. Expression and subcellular localization of E276Q P/rds in transgenic murine photoreceptors. The bovine P/rds coding sequence was used to allow unique immunochemical identification of the transgenic protein against a background of endogenous WT murine P/rds. (A) Diagram of the transgene construct. A mouse opsin promoter region was used to drive expression of bovine P/rds in rod and cone photoreceptors; a mouse protamine 1 intron and polyadenylation signal were included to maintain mRNA stability. (B) Sybr Green quantitative PCR analysis of transgene expression on the WT mouse background. Analyses were performed on retinas from 6- to 8-week-old mice using primer pairs specific to the bovine transgene and endogenous murine gene. The two mutant lines analyzed, ES1 (open circles) and ES2 (filled triangles), were found to express the hemizygous E276Q transgene at 0.98-fold and 0.51-fold the level of a single endogenous WT murine P/rds. Similarly, neither hemizygous nor homozygous E276Q expression improved the morphology seen in the rds (−/+), E276Q murine retinas (on the rds (−/+)) background were indistinguishable from those seen in the retinas of nontransgenic littermates (Fig. 3A). Because E276Q P/rds, at 1× or 2× levels, was unable to provide a complete absence of OSs in rds (−/+), photoreceptors lacking normal levels of endogenous P/rds. It is well established that rds heterozygous mice generate highly disorganized OSs, whereas rds homozygous mice are completely unable to elaborate any structures recognizable as OSs. Expression of transgenic WT murine P/rds on the rds (−/−) and (−/+), as shown by an antibody directed against murine P/rds. In sum, these results demonstrate that E276 is not required for P/rds expression or targeting/localization into photoreceptor OSs.

E276Q P/rds Expression Does Not Rescue rds Photoreceptors

We next tested whether expression and incorporation of the E276Q protein into OSs restores structure and viability to photoreceptors lacking normal levels of endogenous P/rds. The size and extent of the membranous whorls seen in the retinas of nontransgenic littermates (Fig. 3A). Because E276Q P/rds expression improved the morphology seen in the E276Q hemizygous transgenic retinas (on the rds (−/−)) background, we used transmission electron microscopy (TEM) to visualize photoreceptor ultrastructure in retinal cross-sections. We found a complete absence of OSs in rds (−/−) retinas, consistent with previous reports, inner segments lay directly apposed to the RPE cell layer, and retinal detachments were observed frequently. Hemizygous expression of a WT bovine transgene (at levels approximately fivefold normal endogenous) robustly rescued photoreceptor morphology in rds (−/+). The observation that WT bovine P/rds completely rescues the rds (−/−) structural phenotype demonstrated that the bovine ortholog functioned at least 40% as well as the WT murine protein in these cells. In contrast, hemizygous expression of the E276Q transgene, at a level essentially similar to that of endogenous P/rds, had no measurable effect on the ultrastructure of these photoreceptors. We obtained comparable results using E276Q homozygotes, which expressed E276Q at levels twice those of endogenous murine P/rds. Similarly, neither hemizygous nor homozygous E276Q expression improved the morphology seen in the rds (−/+), rds (−/+), and rds (−/−) retinas. The size and extent of the membranous whorls observed in the E276Q hemizygous transgenic retinas (on the rds (−/−) background) were indistinguishable from those seen in the retinas of nontransgenic littermates (Fig. 3A).
any rescue effect in rds (-/-) or (-/-) photoreceptors, we must conclude that this mutation inactivated protein function at least 50%. Finally, Figure 3A also demonstrates that the normal ultrastructure of rds (+/-) OSs remained unaffected by the expression of E276Q or WT transgenes.

Not surprisingly, we found that photoreceptor viability was also essentially unaffected by E276Q transgene expression. We evaluated photoreceptor degeneration by assaying ONL thickness, a direct measure of photoreceptor number,17 as a function of age. The E276Q transgene was crossed onto the rds (-/-) genetic background, and measurements of ONL thickness in the central superior retina were performed on DAPI-labeled fixed cryosections. Figure 3B illustrates that the presence of the E276Q transgene (triangles) did not significantly alter the time course for the slow retinal degeneration we observed for nontransgenic rds (-/-) animals (circles). This rate of photoreceptor loss was similar to that reported by other investigators for heterozygous rds mice.13 These results demon-

![Diagram of retinal photoreceptors](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933444/)
strated that E276Q inactivation of P/rds function for OS disc morphogenesis also abrogated its ability to restore photoreceptor viability to rds (−/+) photoreceptors.

**E276Q P/rds Is Correctly Localized to OS Disc Rims and Incisures**

We found that E276Q P/rds was expressed and properly trafficked to photoreceptor OSs yet could not support disc morphogenesis. Therefore, we wondered whether it was properly localized at the ultrastructural level and examined the localization of the E276Q protein in OS membranes of murine photoreceptors with a normal complement of WT P/rds with the use of postembedding immunogold labeling of retinal cross-sections. Figure 4A illustrates that the transgenically expressed E276Q bovine P/rds protein was detected solely in disc rim regions and incisures of OS membranes of transgenic retinas. This labeling pattern was identical to that originally documented for WT bovine P/rds.37 These data demonstrated that the E276Q mutation did not affect P/rds localization at the

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933444/)
ultrastructural level. No significant labeling was observed in OS membranes of nontransgenic littermates. Figure 4B shows a similar analysis performed using a mouse line expressing a WT bovine P\(\text{rds}\) transgene. The results are essentially identical. The absence of immunoreactivity in control animals (lacking the transgene) demonstrated the specificity of the antibody used to detect the transgenic protein. In sum, these results demonstrated that E276Q P\(\text{rds}\) was correctly localized at the ultrastructural level; like the endogenous protein, the mutant was restricted exclusively to OS disc rims and incisures. Furthermore, expression and incorporation of the E276Q mutant did not measurably alter the distribution of endogenous murine P\(\text{rds}\).

### Retinal Function and Viability Tolerate the Presence of Inactivated P\(\text{rds}\)

Genetic defects in P\(\text{rds}\) result in a broad range of retinal dystrophies. At least one instance of retinitis pigmentosa appears to act through a dominant negative mechanism on photoreceptor structure/function and viability,\(^48\), likely the result of WT protein inactivation through coassembly with mutant polypeptides. We therefore used ERG analyses to examine whether incorporation of E276Q P\(\text{rds}\) into murine photoreceptor OSs affected retinal function. Figure 5A plots representative ERGs obtained to strobe flash stimuli in the dark, which reflect primarily the activity of rod photoreceptors and the rod pathway.\(^49\) The overall waveform obtained from E276Q hemizygote mice (on the \(\text{rds}\) \(+/+)\) background) did not differ from that seen in WT littermates. The relationship between flash intensity and response amplitude was also comparable in the experimental and control groups, as shown by the summary intensity–response functions for the major ERG components (Fig. 5A). The fact that ERG a-waves obtained from WT and E276Q transgenic mice were comparable in amplitude indicated that the transgene did not impair the ability of rod photoreceptors to respond to light. The retention of normal ERG b-waves in E276Q mice indicated that the transgene did not alter communication between rods and rod bipolar cells, which generate this response component.\(^50\) Figure 5B plots representative cone ERGs obtained to strobe flash stimuli superimposed on a rod-desensitizing adapting field. The overall waveform did not differ between the transgenic and WT mice, nor did the relationship between cone ERG amplitude and flash intensity (Fig. 5B). These results indicated that function of cone photoreceptors and the cone pathway, which generate this response component,\(^51\) were not impaired by transgene

### FIGURE 5.

Although functionally inactivated, E276Q does not generate a dominant negative effect on photoreceptor function or viability. (A, left) ERG traces obtained from dark-adapted E276Q transgenic mice on the \(\text{rds}\) \((+/+)\) background, compared with WT littermate controls. Arrows: major a- and b-wave components. Scale bars, 100 ms and 200 \(\mu\)V. Right: intensity–response functions for dark-adapted ERG a- and b-wave amplitudes of E276Q transgenic mice (filled symbols) and nontransgenic littermates (open symbols). (B, left) Cone ERG traces obtained from dark-adapted E276Q transgenic mice on the \(\text{rds}\) \((+/+)\) background, compared with WT littermate controls. Arrows: major a- and b-wave components. Scale bars, 100 ms and 50 \(\mu\)V. Right: cone intensity–response functions for light-adapted ERG b-wave amplitudes of E276Q transgenic mice (filled symbols) and nontransgenic littermates (open symbols). (C) Recovery of the rod a-wave. For each trial, responses obtained to the probe flash (A2) are expressed relative to the response obtained to the conditioning flash (A1) and are plotted as a function of the duration of the ISI separating the conditioning and probe flashes. Data points (A–C) indicate mean \(\pm\) SD for measurements on six WT and three transgenic animals. (D) Analysis of retinal ONL thickness was used to assess retinal degeneration in E276Q transgenic and nontransgenic littermates on the \(\text{rds}\) \((+/+)\) background. ONL thickness measurements were performed on DAPI-stained cryosections (above) and are plotted as a function of age (below). The presence of the E276Q P\(\text{rds}\) in OSs (circles) had no measurable effect on the viability of \(\text{rds}\) \((+/+)\) photoreceptors compared with nontransgenic controls (triangles).
expression. Figure 5C compares recovery kinetics for rod ERG a-waves recorded in a two-flash paradigm. This assay monitors the recovery component of the rod photoresponse, which was not significantly different between the E276Q transgenic mice and WT mice. These results indicated that the presence of functionally inactive E276Q P/rds in OS disc rims did not measurably affect rod or cone photoresponses.

The essentially normal photoresponses observed in the E276Q transgenic mice suggested that little or no retinal degeneration occurred in young (6- to 10-week-old) animals. We also examined the potential effect of E276Q expression on photoreceptor viability in aging mice. This possibility was evaluated by assay of ONL thickness in the central superior retina of E276Q hemizygous transgenic (and nontransgenic littermate control) mice on the retinas of E276Q hemizygous transgenic (and nontransgenic littermate control) mice on the genetic background compared with OSs prepared from nontransgenic littermate controls. Figure 6A shows a Western blot, developed with the use of a monoclonal antibody (mAbC6) that specifically recognizes the transgenically expressed bovine proteins. E276Q bovine P/rds was expressed at its predicted molecular weight and displayed normal posttranslational modifications, including N-linked carbohydrate and intermolecular disulfide bonds. Identical results were obtained for the transgenically expressed WT bovine protein analyzed in a similar fashion. For example, disulfide-mediated dimerization of this protein was evident under non-reducing conditions (Fig. 6A, lane 5). The lack of reactivity in OSs purified from nontransgenic littermates (Fig. 6A, lane 1) demonstrated the specificity of the mAbC6 for the transgenically expressed proteins and its lack of cross-reactivity with the endogenous murine P/rds. In contrast, the Western blot analysis illustrated in Figure 6B was developed with pAbMPCT, an antibody directed against murine P/rds. This positive control demonstrated the presence of endogenous murine P/rds in OS preparations from transgenic (Fig. 6B, lanes 2–5) and nontransgenic (Fig. 6B, lane 1) mice.

**Biosynthesis, Assembly, and Polymerization of E276Q P/rds**

To further investigate the significance of E276 for P/rds, we examined the structural properties and interactions of the E276Q mutant at the protein level. Analyses were performed on photoreceptor OSs purified from E276Q and WT transgenic mice on the rds (+/+ ) genetic background compared with OSs prepared from nontransgenic littermate controls. Figure 6A illustrates that the presence of the E276Q transgene did not measurably affect rod or cone photoresponses.

**Figure 6.** Biosynthesis, assembly, and interactions of transgenic (E276Q and WT) P/rds in murine rds (+/+ ) photoreceptors. (A, B) Western blot analyses of photoreceptor OSs purified from WT hemizygous (lane 5), E276Q hemizygous (lanes 2–4), and nontransgenic (lane 1) murine retinas. Triton X-100 extracts were treated with Endo Hf, or were left untreated, and then were subjected to SDS-PAGE (10 µg total protein/lane) in the presence or absence of β-ME (as noted). Proteins electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore) were probed with mAbC6 (A) and pAbMPCT (B) to detect transgenic bovine and endogenous murine P/rds, respectively. These results demonstrate that the E276Q variant displays normal biosynthesis and posttranslational modifications and that its expression does not alter the properties of endogenous murine P/rds. (C) Subunit assembly assay of E276Q P/rds by velocity sedimentation. A Triton X-100 extract of OSs purified from transgenic retinas was sedimented in a 5% to 20% (wt/wt) sucrose gradient. The fractionated gradient and the particulate fraction (P) were assayed for transgenic E276Q P/rds by Western blot analysis with mAbC6 (upper) and for endogenous WT P/rds by Western blot analysis with pAbMPCT (lower). The E276Q sedimentation profile is essentially identical to that of the endogenous WT P/rds: these data indicate that the mutant protein adopts a normal tetrameric stoichiometry. Potential interactions of E276Q (and WT) bovine P/rds with endogenous murine proteins were assayed by immunoprecipitation followed by Western blotting. Blots compare detergent lysates (LYS), soluble supernatants (SUP), and precipitated (PTT) fractions. Upper: immunoprecipitation of E276Q bovine P/rds does not coprecipitate endogenous murine P/rds or rom-1; both proteins remain in the supernatant fraction. Lower: similarly, immunoprecipitation of WT bovine P/rds does not coprecipitate endogenous murine P/rds or rom-1; both proteins remain in the supernatant fraction. These data demonstrate that the transgenic P/rds variants do not coassemble with endogenous P/rds or rom-1.
Because P/rds is normally assembled in a tetrameric form and mutations can impair this process, we performed a velocity sedimentation analysis to assay the noncovalent assembly of the E276Q mutant. The sedimentation profile of E276Q P/rds shown in Figure 6C demonstrated that it had a mobility indistinguishable from that of the endogenous WT murine P/rds. Thus, like COS-1 cells (Fig. 1C), murine photoreceptors process E276Q into a normally assembled tetrameric form. In combination with the data presented in Figures 6A and 6B, these results demonstrated that substitution of a nonionizable glutamine for the conserved glutamic acid at position 276 did not inhibit P/rds biosynthesis or subunit assembly in murine photoreceptors.

Given that E276Q function, but not oligomeric assembly, was preserved, we were surprised that this variant did not produce dominant negative effects. Therefore, we directly examined whether the transgenically expressed proteins coassembled with the endogenous murine P/rds. Figure 6D shows a coprecipitation analysis using mAbC6 to collect transgenically expressed P/rds variants onto Sepharose beads. We performed immunoprecipitations on solubilized ROS membranes purified from E276Q hemizygotes and WT hemizygotes, each on rds (+/+) backgrounds. In each case, the transgenic protein was precipitated from the solubilized ROS membranes without any coprecipitation of the endogenous murine P/rds (or rds). In sum, these results demonstrated that bovine P/rds did not coassemble with endogenous murine P/rds when expressed in transgenic mouse photoreceptors.

**DISCUSSION**

Normal human vision requires a daily renewal of photoreceptor OSs. Although we know that P/rds plays a necessary role for this process and that it is involved in a broad spectrum of retinal degenerations, its mechanism of action remains uncertain. Defining P/rds molecular function by carefully characterizing this protein’s properties provides greater understanding of vertebrate photoreceptor cell biology and degenerative retinal diseases. New findings presented here identify E276 as an important determinant for P/rds activity and suggest that intramembrane acidic residues within other tetranspanin proteins may also have functional significance.

Although numerous studies have characterized P/rds hydrophilic regions, structure–function relationships within its transmembrane regions have not been documented to date. Primary sequence analysis identifies four strongly hydrophilic regions predicted to form transmembrane α-helices, and reports have linked instances of human retinal disease to mutations in three of the four membrane-spanning regions.3 We were struck by the presence of a buried glutamic acid within the fourth transmembrane domain (E276; Fig. 1A) because this is the only charged residue predicted to lie within a membrane-spanning segment. E276 is absolutely conserved across all identified P/rds orthologs3 and is also found in other members of the tetranspanin protein family.21,22 Notably, this position is substituted by a nonionizable glutamine in all rds-1 orthologs (Fig. 1A). Rom-1 is a homologous protein that, together with P/rds, acts at disc rings to support OS architecture and renewal. These two polypeptides coassemble to form heterotetramers and higher-order polymers,6,9,10,13 but they play distinct roles in vivo. The present study was designed to investigate whether the glutamic acid residue buried within the fourth transmembrane domain of P/rds contributes to its physiological activity.

We used site-directed mutagenesis to create an E276Q missense mutation in the coding region of bovine P/rds cDNA and expressed the recombinant protein in cultured cells and in the photoreceptors of transgenic mice. The bovine ortholog was used to allow its unique immunochemical identification against a background of endogenous mouse P/rds. We used a WT bovine P/rds transgene to generate a mouse line to confirm that bovine P/rds is functionally active in the murine context. Indeed, the WT bovine P/rds transgene restored photoreceptor ultrastructure in rds (−/−) and (−/+), mice, generating OSs indistinguishable from those of WT murine photoreceptors. Given that this rescue was effected by P/rds expression at approximately 2.5-fold normal endogenous levels (in rds (−/−) animals), bovine P/rds must function in murine photoreceptors at least 40% as well as the endogenous protein. Consistent with these findings, the WT bovine protein had the expected posttranslational modifications and was assembled into a normal tetrameric form. Surprisingly, the bovine P/rds did not coassemble with the endogenous murine protein. This is a likely consequence of one of six amino acid changes in the EC2 domain because this domain mediates tetrameric subunit assembly. The rescue of the rds (−/+ and rds (−/−) phenotypes must therefore be attributed to tetramers consisting wholly of the bovine polypeptide. Technical difficulties prevented the generation of additional WT transgenic lines expressing at levels directly comparable to those of the endogenous genes.

The E276Q mutant P/rds also exhibited proper folding, disulfide bonding, subunit assembly, and stability in murine photoreceptors and cultured COS-1 cells. Moreover, E276Q mutant P/rds was properly targeted and localized to the rim regions (periphery and incisures) of OS disc membranes. Strikingly, however, E276Q expression, at up to twofold normal endogenous levels, did not provide any rescue effect in either rds (−/−) or rds (−/+ genetic backgrounds. Because intact murine photoreceptor OSs can be maintained by P/rds levels as low as approximately 60% of normal,54 twofold expression of a (minimally 40%) functional bovine P/rds variant on the rds (−/+ background should completely restore OS ultrastructure. Similarly, this level of functional protein should generate distinctive “whorls” of OS membranes in the rds (−/−) background. Instead, E276Q produced no improvement in OS ultrastructure in either background, indicating a profound functional deficit. Without a directly comparable line of control mice, it is not possible to determine whether the E276Q mutation completely abrogates P/rds function; however, at a minimum, the findings demonstrate that the E276Q mutation significantly (more than 50%) attenuates P/rds functional activity.

The finding that full P/rds functionality requires E276 is consistent with previous findings that rom-1 alone (which lacks E276) is insufficient to support OS morphogenesis. P/rds null3 and rom-1 knockout mouse models clearly demonstrate an absolute requirement for P/rds, but not rom-1, for the elaboration of photoreceptor OSs. In addition, an early transgenic mouse study, using a chimeric rom-1 protein, showed that P/rds has at least one essential activity (residing outside its EC2 domain) not present in rom-1.55 In fact, compared with P/rds, rom-1 lacks several properties thought critical for the support of OS morphogenesis, including E276, an OS targeting signal,18 and membrane fusion activity.55 It is possible that each of these properties is required for full P/rds functionality.

The precise manner in which E276 contributes to P/rds activity remains to be elucidated. P/rds and rom-1 are members of the tetranspanin family, a group (more than 100 members) of transmembrane glycoproteins that fulfill diverse biological roles but that share a common structural framework.20,21,23,56,57 They are anchored into their membranes by four transmembrane helices that form a closely packed bundle.56,57 The bundle is thought to be stabilized by “knob-into-holes” packing, with additional interactions provided by interhelical hydrogen bonds.22,58 The position identified in the present investigation (E276) corresponds to...
a conserved hydrogen bond donor/acceptor site, predicted to be buried within the bundle interior (Seigneuret M, personal communication, July 2006). Because substitution of glutamate by glutamine is isosteric and preserves this position as a hydrogen bond donor/acceptor, the major physicochemical consequence of the E276Q mutation is a loss of ionization potential. It is, therefore, interesting to consider the possibility that E276 functions as a pH sensor to regulate protein activity, particularly given that P/rds may be exposed to a significant change in pH during disc morphogenesis.80

Regardless of the precise mechanism, the finding that E276 acts to modulate P/rds activity is consistent with the previous proposal that rom-1, which contains the E/Q substitution, functions to regulate, rather than promote, disc morphogenesis.7 That study used rom-1 knockout mice to demonstrate that the rom-1 protein is not required for the elaboration of OSs. Instead, it found that photoreceptors completely lacking rom-1 produced OS discs with enlarged diameters. The enlarged disc phenotype was proposed to reflect an absence of rom-1 regulation of OS disc morphogenesis, potentially through direct interaction with P/rds.7 The findings reported here are consistent with this notion and suggest that the E/Q substitution in the fourth transmembrane domain of rom-1 attenuates its functional potential, producing a molecule that may negatively regulate disc morphogenesis through direct interaction with P/rds.

This report is the first to examine the significance of transmembrane domains for photoreceptor P/rds structure and function. It demonstrates that an intramembrane glutamic acid residue, conserved in P/rds and other tetraspanins but absent in rom-1, is critical for P/rds support of OS disc morphogenesis. The substitution of E276 by a nonionizable but strongly polar residue does not disrupt protein structure but does abrogate function in support of OS membrane architecture and suggests a molecular rationale for the regulation of P/rds activity by rom-1. These findings also offer the first evidence that ionizable intramembrane residues may serve regulatory roles for tetraspanin proteins more generally.

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