Peripherin/rds Influences Membrane Vesicle Morphology

IMPLICATIONS FOR RETINOPATHIES*

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Peripherin/rds is an integral membrane glycoprotein found in the rim regions of vertebrate photoreceptor cell discs. Natural mutations of the encoding gene result in degenerative retinal disorders, such as retinitis pigmentosa. The retinal degeneration slow (rds) phenotype, observed in mice, is considered to be an appropriate model for peripherin/rds-mediated retinitis pigmentosa. Associated abnormalities in the outer segment of photoreceptor cells have implicated peripherin/rds in some aspect of disc morphology, yet it remains unclear whether such morphological effects are the cause or the result of this condition. Here we present the first direct evidence to support a role for peripherin/rds in maintaining the flattened vesicle morphology characteristic of photoreceptor outer segments. In vitro expression yields a 36-kDa immunoreactive species, which is inserted into membranes and undergoes N-glycosylation, inter- and intramolecular disulfide bonding, and dimerization. Electron microscopy reveals that peripherin/rds flattens microsomal vesicles. This effect appears to be dependent on disulfide bond formation but not N-glycosylation. The inability of two pathogenic peripherin/rds mutants (P216L and C165Y) to flatten membrane vesicles implicates such mutations as the primary cause of the retinal degeneration observed in retinitis pigmentosa.

The outer segment of the vertebrate rod photoreceptor cell exhibits a highly specialized structure, comprised of a stack of 1000 or more flattened vesicles or discs (1). This organization is vital to the visual process, as it maximizes the area available for photoreception and allows efficient renewal of the photoreceptor outer segments. Any elements involved in the formation and maintenance of this structure are, therefore, of vital importance to the visual process. One protein that has been implicated to have such an involvement is peripherin/rds. Peripherin/rds is a 39-kDa integral membrane glycoprotein localized exclusively to the rim regions of photoreceptor cell discs (2, 3). A topological model has been proposed (4) in which the protein possesses four transmembrane helices and cytosolically oriented N and C termini. The protein has been shown to form disulfide-linked homodimers (2). These peripherin/rds dimers and homodimers of the related disc rim protein ROM-1 (5) are believed to non-covalently associate to form a functional heterotetrameric complex (6, 7).

The clinical significance of the human peripherin/rds gene is illustrated by its involvement in a wide range of degenerative retinal disorders, including retinitis pigmentosa (8). To date, over 50 pathogenic mutations within the human gene have been identified. In addition there is a mutation in the murine gene that causes the related disorder, retinal degeneration slow (rds) (9). Phenotypically rds mice exhibit distinctive photoreceptor degeneration (10, 11), and hence, murine rds is considered an important animal model for the inherited human retinal degenerative condition. Mice homozygous for the rds condition have no detectable peripherin/rds and fail to form outer segments. In contrast, heterozygous mice possess reduced levels of peripherin/rds and form highly abnormal outer segments, which are much shorter in length, with the disc membranes aligned in irregular whorls that appear swollen and vacuolated (11). This evidence has led to the proposition that peripherin/rds plays a role in maintenance of the curvature of the rim region and the flattened vesicle morphology (3, 4, 12).

In this study we provide the first direct experimental evidence in support of this hypothesis. The in vitro expression of peripherin/rds and accompanying membrane insertion of the protein have allowed examination of the effects of peripherin/rds on vesicle morphology. Electron microscopical analysis demonstrates that under non-reducing conditions peripherin/rds expression leads to a widespread flattening of microsomal vesicles. Site-directed mutagenesis was then used to demonstrate that pathogenic mutants of peripherin/rds are unable to elicit this effect.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The construct denoted pRDS was produced by subcloning the murine peripherin/rds cDNA (13) into the pBlue-script-KS expression vector under the control of the T3 promoter. A second construct containing the murine peripherin/rds cDNA under the control of the T7 promoter was also produced. In this construct the FLAG antigenic tag was incorporated by polymerase chain reaction mutagenesis downstream of the peripherin/rds cDNA prior to the stop codon. This construct was denoted pRDSF.

In Vitro Transcription/Translation—In vitro transcription and translation were performed using a TNT-coupled reticulocyte lysate system (Promega). Reactions were performed for 90 min at 30 °C in a final volume of 25 μl, using T7 RNA polymerase, 1 μg of template DNA, and 2 μl of [35S]methionine (10 Ci/mM, ICN), according to the manufacturer’s instructions. Where appropriate, reactions were supplemented by the addition of 2.5 μl (5 eq) of canine pancreatic microsomal membranes (Promega; 1 eq is the amount of membranes required to cleave the signal sequence of preprolactin by 50%). For electron microscopy, [35S]methionine was omitted, and reactions were supplemented with the full complement of unlabeled amino acids. The expression of peripherin/rds in these samples was assessed by immunoblotting for the FLAG antigenic tag using the M2-anti-FLAG monoclonal antibody (EASTman Kodak Co.). N-Glycosylation was competitively inhibited by the inclusion of 32 μM Ac-Asn-Tyr-Thr-NH2 (10). Non-reducing conditions were ensured by the inclusion of 2 mM oxidized glutathione (Sigma) (14).

Isolation of Microsomal Membranes—20 μl of the translation mixture was diluted with 4 volumes of 10 mM Tris-Cl, 100 mM NaCl, pH 7.9 (buffer A). The microsomal vesicles were pelleted by centrifugation

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FIG. 1. Peripherin/rds expression and N-glycosylation. a, immunoblot detection of peripherin/rds expressed in the absence of membranes with the M2 anti-FLAG antibody. A 38-kDa product of pRDS expression was detected (closed arrowhead). b, autoradiograph of a 10% SDS-PAGE gel used to analyze the products of in vitro transcription/translation of pRDS in the presence of [35S]methionine. Expression of peripherin/rds in the absence of membranes (lane 1), in the presence of membranes (lane 2), and in the presence of both membranes and Ac-Asn-Tyr-Thr-NH₂ (lane 3) illustrates membrane insertion and glycosylation of peripherin/rds. The non-glycosylated (open arrowhead) and the glycosylated (closed arrowhead) forms of peripherin/rds are indicated. c, autoradiograph of a 10% SDS-PAGE gel used to analyze the products from the in vitro expression of the N229S mutant peripherin/rds. Samples are shown from expression reactions of this mutant in the absence of membranes (lane 1), in the presence of membranes (lane 2), and in the presence of membranes and Ac-Asn-Tyr-Thr-NH₂ (lane 3). A 38-kDa product was detected in all samples (closed arrowhead). Numbers to the left of gels indicate relative molecular mass (in kDa).

(190,000 × g, 1 h, 4 °C), resuspended in 100 μl of buffer A, repelleted, and resuspended in 50 μl of buffer A for electron microscopy.

Electron Microscopy—3.05-mm, 400-mesh copper grids were either prepared with a simple carbon film (14) or were collodion-coated with a thin layer of carbon being applied prior to glow discharge treatment (15). Samples to be viewed were applied to the grids and negatively stained by application of an aqueous solution of 4% uranyl acetate. Grids were examined in a Phillips CM10 transmission electron microscope, operated at an accelerating voltage of 100 kV.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange™ mutagenesis kit (Stratagene Ltd.) according to the manufacturer’s instructions. All mutant constructs were produced using the pRDSF construct as thetemplate. Mutations were confirmed by double-stranded automated DNA sequencing (A.B.I.). Mutant constructs were prepared containing the C150S, C165Y, P216L, and N229S mutations.

RESULTS

To facilitate in vitro expression and identification of the protein two constructs were produced containing the murine peripherin/rds cDNA. The construct denoted pRDS comprised the murine cDNA subcloned into the pBluescript-KS expression vector. pRDSF also comprised the murine peripherin/rds cDNA within pBluescript-KS but possessed an additional FLAG antigenic tag at the C terminus of the protein. The recombinant proteins were expressed in an in vitro rabbit reticulocyte lysate system supplemented, where indicated, with canine pancreatic microsomal membranes. The expression of pRDS and pRDSF in the absence of membranes yielded products of 36 and 38 kDa respectively (Fig. 1). In addition, pRDSF displayed immunoreactivity toward the anti-FLAG antibody (Fig. 1a). When expressed in the presence of microsomal membranes, both products exhibited an increase in apparent molecular mass of approximately 2.5 kDa (Fig. 1b). However, upon inclusion of Ac-Asn-Tyr-Thr-NH₂, the intensity of this band shift was reduced. In contrast, expression of the mutant form of peripherin/rds containing the N229S mutation yielded a product of 38 kDa in both the absence and presence of microsomal membranes (Fig. 1c). The inclusion of Ac-Asn-Tyr-Thr-NH₂ was observed to have no effect on this product.

When expressed in the presence of membranes and 5 mM oxidized glutathione, wild-type peripherin/rds displayed an altered mobility upon SDS-PAGE analysis (Fig. 2). In contrast to the 41-kDa species produced under reducing conditions, pRDS expression in the presence of 5 mM glutathione yielded a product with an apparent molecular mass of 38 kDa. In addition, a second product of approximately 66 kDa was also visualized.

To investigate the role of peripherin/rds in maintaining the morphology of photoreceptor discs, electron microscopy was used to detect whether in vitro peripherin/rds expression alters microsomal vesicle morphology (Fig. 3). Notably, over 60% of the microsomal vesicles expressing peripherin/rds under non-reducing conditions exhibited a highly unusual flattened morphology (Fig. 3a). In contrast, under reducing conditions all microsomes from peripherin/rds expression displayed a typical rounded morphology (Fig. 3b). Vesicular flattening was, however, observed in microsomes from the expression of peripherin/rds in the presence of membranes and Ac-Asn-Tyr-Thr-NH₂ (Fig. 3c). A flattened vesicle morphology was also visualized upon expression of the N229S mutant form of peripherin/rds under non-reducing conditions in the presence of microsomal membranes (Fig. 3h).

As an additional control, the four-transmembrane 16-kDa proteolipid of the vacuolar ATPase (15) and the seven-transmembrane vesicle protein squid rhodopsin (16) were also expressed under reducing and non-reducing conditions. These integral membrane proteins were both unable to elicit any vesicular flattening (Fig. 3d).

Following site-directed mutagenesis, electron microscopical visualization of microsomal vesicles incorporating the C165Y (Fig. 3e), P216L (Fig. 3f), or C150S (Fig. 3g) peripherin/rds mutants demonstrated a rounded appearance.

The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
The increase in apparent molecular mass of peripherin/rds observed in the presence of microsomal membranes is indicative of the N-glycosylation of the protein (Fig. 1b). This was confirmed as the inclusion of the competitive N-glycosylation inhibitor Ac-Asn-Tyr-Thr-NH₂ abolished this change in molecular mass (16). The magnitude of this shift in apparent molecular mass implies that only one N-glycosylation site within the protein was utilized (16). This is further substantiated by the inability of the N229S mutant peripherin/rds to exhibit any shift in molecular mass in the presence of membranes (Fig. 1c). The removal of the Asn²²⁹ N-glycosylation sequon therefore appears to completely abolish N-glycosylation of the protein, and this implies that the Asn²²⁹ sequon is entirely responsible for the N-glycosylation of native peripherin/rds.

As N-glycosylation occurs on the luminal side of microsomal membranes (16) and all of the putative N-glycosylation sites within peripherin/rds are predicted to occur in an extracytosolic loop, the N-glycosylation of peripherin/rds in this system also indicates that the protein is oriented in the vesicles in accordance with the current topological model. Therefore it would seem that microsomal expression resembles peripherin/rds expression in photoreceptor outer segments, with the protein traversing the membrane and having its two intradiscal loops in the lumen of the sealed vesicles and its N and C termini exposed to the extracellular environment.

Physiologically, the expression of peripherin/rds is believed to lead to the formation of a functional heterotetrameric complex (6, 7) by the non-covalent association of disulfide-linked dimers of peripherin/rds and ROM-1 (13). It has previously been reported that the inclusion of 2–5 mM oxidized glutathione in a translation reaction promotes disulfide bond formation (17). The experiments reported here illustrate the ability of peripherin/rds alone to form a 66–69-kDa cross-linked dimer when expressed in vitro under non-reducing conditions (Fig. 2). In addition to dimerization, an increase in mobility of approximately 3 kDa was observed for both the glycosylated and non-glycosylated forms of the protein in the presence of 5 mM oxidized glutathione. This is indicative of the presence of at least one intramolecular disulfide linkage in addition to the intersubunit bonds within the protein. Together these findings suggest that the expressed protein is folded correctly and integrated into the microsomal membrane in the correct orientation.

Electron microscopical investigation of the ability of peripherin/rds to elicit any alterations in microsomal vesicle morphology produced a dramatic result. The finding that over 60% of microsomal vesicles from the expression of peripherin/rds displayed an uncharacteristic flattened morphology supports the proposed role of peripherin/rds in maintenance of a flattened photoreceptor disc morphology. This flattening results in regions where both sides of the vesicle can be seen to run parallel to each other, as well as tight turns within the membrane, reminiscent of a disc rim. In contrast, under reducing conditions the microsomes showed a typical rounded morphology (Fig. 3b). As vesicular flattening is only seen under conditions conducive to the formation of disulfide linkages, it would appear that disulfide bond formation and probably dimerization of peripherin/rds is required for the morphological effect, presumably by constraining a particular structural feature. We have also shown that N-glycosylation of peripherin/rds does not appear to be a prerequisite for vesicular flattening, as inhibition of this modification by Ac-Asn-Tyr-Thr-NH₂ does not alter the percentage of flattened vesicles seen under non-reducing conditions (Fig. 3c). This result was confirmed by analysis of the peripherin/rds mutant incorporating the N229S mutation (Fig. 3b). The inability of this mutant to display a band shift in the presence of microsomal membranes demonstrates that the removal of the Asn²²⁹ N-glycosylation sequon completely abolishes N-glycosylation of peripherin/rds (Fig. 1c). The vesicular flattening observed with this mutant form of peripherin/rds therefore further verifies that the N-glycosylation of peripherin/rds is not a prerequisite for this ability of the protein.
The inability of the 16-kDa proteolipid of the vacuolar ATPase (18) and squid rhodopsin to elicit such an effect demonstrates the specificity of this peripherin/rds-induced flattening. This also distinguishes the roles of peripherin/rds and rhodopsin in the pathogenesis of retinitis pigmentosa.

To determine whether the loss of this function may be responsible for the abnormal disc appearance in retinitis pigmentosa, several retinitis pigmentosa-inducing mutants of peripherin/rds were produced by site-directed mutagenesis of the pRDSF construct. The P216L and the C165Y mutants were chosen as they have been shown to cause retinitis pigmentosa in humans and in transgenic mice (19–21). The C150S mutation was selected as this side chain may be important in protein dimerization (22). The observation that peripherin/rds incorporating the C165Y or P216L mutations is unable to induce vesicular flattening is of particular clinical significance. These results imply strongly that retinitis pigmentosa-causing mutations may precipitate the disorder by abolishing the ability of peripherin/rds to flatten disc membranes. Another interesting observation was that the C150S peripherin/rds mutant also abolished vesicular flattening (Fig. 3g). This is consistent with recent proposals that this residue plays a crucial role in the dimerization of the protein (22) and that dimerization is necessary for the function of peripherin/rds. Although it has not yet been linked to a retinopathy, we postulate that any mutation of this residue will be associated with a pathogenic mutation in the future.

Almost a decade after the first suggestion of a structural role for peripherin/rds in stabilization of the photoreceptor outer segment discs (4), these data provide the first direct experimental evidence in support of this role. It has also been suggested that peripherin/rds may promote membrane fusion (23); however, no evidence for that activity was seen in these studies. The approach described in this study may provide a tool by which the involvement and mechanisms by which dysfunctional forms of peripherin/rds precipitate retinal degenerative disorders such as retinitis pigmentosa may be delineated. Further mutational analysis should allow the determination of the precise sites in the protein that are important to this critical function of peripherin/rds and ultimately may aid research into therapeutic approaches alleviating this form of retinal degeneration.

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