Published in final edited form as:

Exp Eye Res. 2011 May ; 92(5): . doi:10.1016/j.exer.2011.03.010.

Peripherin/rds co-distributes with putative binding partners in basal rod outer segment disks

Thomas C. Edrington V,

Center for Membrane Biology Department of Molecular Physiology and Biological Physics
University of Virginia School of Medicine Charlottesville, VA 22908-0886 tcet3@virginia.edu

Maxim Sokolov, and

Departments of Ophthalmology and Biochemistry West Virginia University School of Medicine,
Morgantown, West Virginia , 26506,USA. sokolovm@rcbhsc.wvu.edu

Kathleen Boesze-Battaglia

Department of Biochemistry University of Pennsylvania SDM Philadelphia, PA 19104
battagli@upenn.edu

The retinal degeneration slow protein peripherin/rds, (a.k.a. RDS), encoded by the *rds* gene, is essential for the formation, organization and maintenance of outer segments (Arikawa et al., 1992; Connell et al., 1991; Connell and Molday, 1990; Kedzierski et al., 2001; Molday, 1998). In humans, any one of over 160 mutations within this gene results in a broad variety of late onset progressive retinal dystrophies characterized by abnormal photoreceptor structure (Dryja et al., 1997; Keen and Inglehearn, 1996; Kohl et al., 1998). The pathogenic mechanism(s) underlying peripherin/rds mediated retinal dystrophies are unknown, however new insight has been gained in our understanding of peripherin/rds function based upon recent structural studies identifying the C-terminal domain of this protein as homologous to structures called intrinsically disordered domains (IDD) (Dyson, 2005; Edrington, 2007b; Ritter, 2005). IDDs are unstructured domains that become structurally stabilized by interacting with one or with several binding partners (Uversky et al., 2008). Structural flexibility imparts multi-functionality to the protein and binding partners act as regulators of its function (Lakoucheva et al., 2002; Vucetic et al., 2007). In humans, numerous mutations in the *rds* C-terminus result in rod/cone or cone/rod dystrophies. In a murine model of retinitis pigmentosa, deletion of codon 307 in the *rds* gene results in severely dysmorphic outer segments (OSs) in mice (McNally et al., 2002). The *rds* 307 del mouse provides the first *in vivo* evidence for a correlation between intrinsic disorder and retinopathy. In these mice disruption of the peripherin/rds C-terminus results in a gene product with a more intrinsically ordered C-terminal domain characterized by an increase in α -helical content.

A propensity of peripherin/rds to assume an ordered confirmation in the absence of known binding partners is critical if one considers that regions within the C-terminus are involved in targeting of peripherin/rds containing vesicles to the OS as well as with the formation and maintenance of OS structure (Tam et al., 2002; Tam, 2004). As the peripherin/rds protein traffics up the transition zone to aid in the formation of new disks it has numerous binding partners, including a cargo sorting protein melanoregulin (MREG) as well as Calcium/Calmodulin (Ca/CaM) (Boesze-Battaglia, 2007; Edrington, 2007a). These functional and

© 2011 Elsevier Ltd. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

binding regions of peripherin/rds are illustrated in Fig.1. In this short communication we document the association of Ca/CaM with peripherin /rds, analyze its distribution profile in photoreceptor cells and map its' binding site on the C-terminal domain of peripherin /rds.

In our previous studies peripherin /rds binding to Ca/CaM was documented in GST pull-down assays, in those studies GST-peripherin/rds C-terminus was incubated with purified calmodulin as a function of increasing calcium concentration as well as in the presence of peptide inhibitors (Edrington, 2007a). In these studies we asked if calmodulin binds peripherin/rds in solubilized retinal extracts using an experimental strategy outlined by Warren and Molday, 2002 (Warren and Molday, 2002) to follow Ca/CaM binding to the cGMP gated channel. In brief, bovine rod outer segments (ROSs) prepared under dim red light were washed in hypotonic buffer (10mM HEPES, pH 7.4, 1mM DTT) containing either 1uM CaCl₂ or 2mM EGTA. After solubilization of membrane proteins in 10mM OG, peripherin/rds protein complexes were immunoprecipitated with anti-peripherin/rds mAb 2B6 as described in our previous studies (Boesze-Battaglia, 2007). Samples were then subjected to SDS-PAGE and Western blots probed with mAb2B6 (anti-peripherin/rds), or anti-calmodulin Ab (Zymed) (Fig. 2A and B.). Based on previous studies (Warren and Molday, 2002) the cytosolic extract in the absence of solubilized membrane is expected to contain CaM when isolated in the presence of EGTA and less CaM or no CaM when isolated in the presence of calcium. Therefore, aliquots of the OS cytosolic extract prior to solubilization were reserved to confirm the presence of cytosolic calmodulin (Fig. 2A) in the presence of EGTA. When the ROS were treated with EGTA no detectable calmodulin was associated with peripherin/rds. In contrast, treatment of the OS with 1uM CaCl₂ resulted in association of calmodulin with peripherin/rds. As shown in Fig.2B, a 17 kDa band immunoreactive with anti-CaM was observed as well as the expected 32 kDa peripherin /rds band. No detectable binding was observed in the isotype control.

Crucial for understanding the role of Ca/CaM in modulating peripherin/rds function is knowledge of its subcellular localization in rod cells. In this next series of studies, we analyzed the subcellular distribution of Ca/CaM using serial tangential sectioning of flat-mounted frozen retinas coupled with Western blot analysis. In this approach, the subcellular localization of proteins of interest in the rod is determined by comparing its distribution in the sections to the distribution of protein markers confined to specific subcellular compartments (Sokolov et al., 2002; Song H et al., 2007). A representative experiment using this technique is shown Figure 2C. The distribution of peripherin /rds and Ca/CaM throughout the serial sections obtained from the retina of a dark-adapted rat was compared with the distribution of the rod ROS marker, rhodopsin, and the rod inner segment (IS) marker, subunit I of cytochrome c oxidase, COX I, revealing the mitochondria-rich ellipsoid domain of the IS immediately adjacent to the OS. Tubulin abundant in all cellular compartments, except for the distal OS, was used as a loading control showing that each section contained similar amount of tissue. The distribution of CaM was compared to MREG a known inhibitor of peripherin/rds fusion function (Edrington, 2007a). Our data indicate that Ca/CaM is present throughout the entire IS (sections 8-19), and mostly excluded from the OS (sections 2-5) with the exception of the basal part of this compartment (sections 6-7). The profile of peripherin/rds subcellular distribution closely resembles that of rhodopsin in being predominantly targeted to the OS (sections 2-7), however minute amounts of both proteins were also detectable in the IS (sections 8-10) where both of these proteins are synthesized. Thus peripherin /rds and Ca/CaM co-localize along the trafficking path of peripherin /rds in the IS (sections 8-9) and, importantly, at the base of the OS (sections 6-7) where new disks are formed.

Binding of the peripherin/rds C-terminal peptide (PerCter) to Ca/CaM was previously established using a combination of fluorescence spectroscopy and pull-down assays

(Edrington, 2007a). Sequence homology between other Ca/CaM binding proteins, CaM binding site prediction, and mutagenesis of the peripherin/rds C-terminal peptide identified the most likely Ca/CaM binding site to reside between residues W316-G329. In the present study, a synthesized peptide containing this predicted region, PP-C, was utilized to verify the Ca/CaM binding domain on peripherin /rds. We determined the K_D of the PP-C-Ca/CaM complex to confirm that the predicted Ca/CaM binding site is the actual Ca/CaM binding site. Comparison of the PP-C-Ca/CaM K_D with the PerCter-Ca/CaM complex K_D will also reveal whether the full-length peripherin /rds C-terminus contains sequence elements outside this region that are necessary for Ca/CaM association. Our previous studies showed that the blue-shift and quantum yield increase of the peripherin /rds C-terminal domain Tryptophan (Trp) fluorescence emission upon Ca/CaM binding was decreased relative to other previously reported blue-shifts and quantum yield increases for Trp-bearing calmodulin binding domain (CBD) peptides. We interpreted those results as being due to an unequal sequestration of the PerCter Trp residues in the PerCter-Ca/CaM complex. The PP-C peptide only contains one of the peripherin /rds C-terminal domain tryptophan residues, W316, and was thus used to test that hypothesis.

Steady-state Trp fluorescence emission and anisotropy were used to both assess the binding of the PP-C to Ca/CaM and to determine the K_D of the PP-C-Ca/CaM complex. Trp fluorescence emission spectra were recorded for the PP-C peptide in the absence and presence of purified Ca/CaM from bovine brain (Figure 3A). Upon the addition of Ca/CaM, there was a 62% increase in PP-C Trp fluorescence emission intensity at 340 nm and a λ_{max} blue-shift from approximately 352 nm to 332 nm. The observed intensity increase and the λ_{max} blue-shift are essentially two-fold greater than the 35% intensity increase and 8 nm blue-shift observed when Ca/CaM bound to the peripherin /rds C-terminal domain. They are also more consistent with previously reported blue-shifts and quantum yield increases for Ca/CaM binding to Trp-bearing CBD peptides (Chabbert et al., 1995; DeGrado et al., 1985; Murase and Lio, 2002). Emission spectra were recorded for the PP-C peptide in the presence of Ca/CaM and 6 mM EDTA. In the presence of EDTA there was no significant blue-shift or quantum yield increase.

The K_D of the PP-C-Ca/CaM complex was determined using fluorescence anisotropy (Figure 3B). The average anisotropy of the PP-C peptide increased in a hyperbolic manner as the Ca/CaM concentration increased. Non-linear curve fitting of the anisotropy data yielded a K_D of 190 ± 55 nM. The K_D of the PP-C-Ca/CaM complex is comparable to the K_D of the PerCter -Ca/CaM complex (320 ± 150 nM). Taken together these fluorescence spectroscopy results indicate that the PP-C does contain the Ca/CaM binding motif present in the peripherin/rds C-terminal domain, that Ca/CaM has a similar affinity for both peptides, and that CaM binding to both peptides is calcium-dependent. Additionally, these confirm the previous interpretation of the fluorescence results from Ca/CaM binding to the peripherin /rds C-terminal domain. In that, the decreased quantum yield and relatively minor blue shift in Trp fluorescence emission, as well as the incomplete iodide quenching of Trp fluorescence, when Ca/CaM was bound to the PerCter was interpreted to be caused by residue W316 being sequestered in the Ca/CaM binding channel while residue W306 remained exposed to solvent. In the PP-C peptide, only W316 is contributing to the Trp fluorescence, thus the blue shift and quantum yield increase reflect complete protection from solvent quenching of Trp fluorescence.

Previous studies in the laboratory predicted that the Ca/CaM binding domain on the C-terminus of peripherin /rds was localized to a region in the vicinity of residues 314-329 (Edrington, 2007a). The present studies verify this binding domain and show that no other sequence elements in the peripherin /rds -C-terminus are required for Ca/CaM association. We previously reported that Ca/CaM binding inhibited peripherin /rds C-terminal domain

mediated membrane fusion. The increased concentration of Ca/CaM at base of the ROS (Fig. 2C, sections 6-7) could provide a control mechanism for inhibiting premature peripherin /rds -mediate fusion between the disk membrane and ROS outer membrane, thus ensuring disk membrane integrity in a region of the ROS critical for photoreceptor activity. Alternatively, the peripherin/rds on the disk rim may act as a reservoir for calmodulin for subsequent binding of the plasma membrane associated cGMP gated channel.

In summary, the intrinsically disordered peripherin /rds C-terminal domain exhibits multifunctionality (Boesze-Battaglia et al., 1997; Boesze-Battaglia et al., 1998; Boesze-Battaglia, 2007; Damek-Poprawa et al., 2005; Edrington, 2007a; Edrington, 2007b; Tam et al., 2002; Tam, 2004) with critical regions involved in peripherin /rds trafficking, stabilization of disk rim and membrane perturbations as it transits from site of synthesis to the outer segment (Fig. 1). We hypothesize that multifunctionality is conferred in part through the association of several binding partners, including Ca/CaM.

References

- Arikawa K, Molday LL, Molday RS, Williams DS. Localization of peripherin/rds in the disk membranes of cone and rod photoreceptors: relationship to disk membrane morphogenesis and retinal degeneration. *J. Cell Bio.* 1992; 116:659–667. [PubMed: 1730772]
- Boesze-Battaglia K, Kong F, Lamba OP, Stefano FP, Williams DS. Purification and light dependant phosphorylation of a candidate fusion protein, photoreceptor peripherin/rds. *Biochemistry.* 1997; 22:6835–6846. [PubMed: 9184167]
- Boesze-Battaglia K, Lamba OP, Napoli A, Sinha S, Guo Y. Fusion between retinal rod outer segment membranes and model membranes; a role for photoreceptor peripherin/rds. *Biochemistry.* 1998; 37:9477–9487. [PubMed: 9649331]
- Boesze-Battaglia KHS, Sokolov M, Lillo C, Pankoski-Walker L, Gretzula C, Gallagher B, Rachel RA, Jenkins NA, Copeland NG, Morris F, Jacob J, Yeagle P, Williams DS, Damek-Poprawa M. The Tetraspanin protein peripherin-2 forms a complex with melanoregulin, a putative membrane fusion regulator. *Biochemistry.* 2007; 46:1256. [PubMed: 17260955]
- Chabbert M, Piemont E, Prendergast FG, Lami H. Fluorescence of a tryptophan bearing peptide from smooth muscle myosin light chain kinase upon binding to two closely related calmodulins. *Arch. Biochem. Biophys.* 1995; 332:429–436. [PubMed: 7574718]
- Connell GJ, Bascom R, Molday L, Reid D, McInnes R, Molday RS. Photoreceptor peripherin is the normal product of the gene responsible for retinal degeneration in the rds mouse. *Proc. Natl. Acad. Sci. USA.* 1991; 88:723–726. [PubMed: 1992463]
- Connell GJ, Molday RS. Molecular cloning , primary structure, and orientation of the vertebrate photoreceptor cell protein peripherin in the rod outer segment disk membrane. *Biochemistry.* 1990; 29:4691–4698. [PubMed: 2372552]
- Damek-Poprawa M, Krouse J, Gretzula C, Boesze-Battaglia K. A Novel Tetraspanin Fusion Protein, Peripherin-2, Requires a Region Upstream of the Fusion Domain for Activity. *J. Biol. Chem.* 2005; 280:9217–9224. [PubMed: 15591062]
- DeGrado W, Prendergast F, Wolfe HJ, Cox J. The design, synthesis, and characterization of tight-binding inhibitors of calmodulin. *J. Cell Biol.* 1985; 29:83–93.
- Dryja TP, Hahn LB, Kajiwarra K, Berson E. Dominant and digenic mutations in the peripherin/rds and ROM-1 genes in retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 1997; 38:1972–1982. [PubMed: 9331261]
- Dyson HJ, Wright PE. Intrinsically unstructured proteins and their functions. *Nature Reviews Molecular Cell Biology.* 2005; 6:197.
- Edrington TC, Yeagle PL, Gretzula CL, Boesze-Battaglia K. Calcium-dependent association of calmodulin with the C-Terminal domain of the tetraspanin protein peripherin/rds. *Biochemistry.* 2007a; 46:3862. [PubMed: 17323925]
- Edrington TC, LaPointe R, Yeagle PL, Gretzula CL, Boesze-Battaglia K. Peripherin-2: an intracellular analogy to viral fusion proteins. *Biochemistry.* 2007b; 46:3605. [PubMed: 17323921]

- Lakoucheva LM, Brown CJ, Lawson JD, Obradovic Z, Dunker AK. Intrinsic disorder in cell-signaling and cancer-associated proteins. *J Mol Biol.* 2002; 323:573–84. [PubMed: 12381310]
- Kedzierski W, Nusinowitz S, Birch D, Clarke G, McInnes RR, Bok D, Travis GH. Deficiency of rds/peripherin causes photoreceptor death in mouse models of digenic and dominant retinitis pigmentosa. *Proc. Natl. Acad. of Sci.* 2001; 98:7718–7723. [PubMed: 11427722]
- Keen TJ, Inglehearn CF. Mutations and polymorphisms in the human peripherin-RDS gene and their involvement in inherited retinal degeneration. *Human Mutation.* 1996; 8:297–303. [PubMed: 8956033]
- Kohl S, Giddings I, Besch D, Apfelstedt-Sylla E, Zrenner E, Wissinger B. The role of the peripherin/RDS gene in retinal dystrophies. *Acta Anat. (Basel).* 1998; 162:75–84. [PubMed: 9831753]
- McNally N, Kenna PF, Rancourt D, Ahmed T, Stitt A, Colledge WH, Lloyd DG, Palfi A, O'Neill B, Humphries MM, Humphries P, Farrar GJ. Murine model of autosomal dominant retinitis pigmentosa generated by targeted deletion at codon 307 of the rds-peripherin gene. *Human Molecular Genetics.* 2002; 11:1005–16. [PubMed: 11978760]
- Molday RS. Photoreceptor membrane proteins, phototransduction, and retinal degenerative diseases. The Friedenwald lecture. *Invest. Ophthalmol. Vis. Sci.* 1998; 39:2491–2513. [PubMed: 9856758]
- Murase T, Lio T. Static and kinetic studies of complex formations between calmodulin and MastoparanX. *Biochemistry.* 2002; 41:1618–1629. [PubMed: 11814356]
- Ritter LM, Arakawa T, Goldberg AF. Predicted and measured disorder in peripherin/rds, a retinal tetraspanin. *Protein and Peptide Letters.* 2005; 12:677. [PubMed: 16522184]
- Sokolov M, Lyubarsky AL, Strissel KJ, Savchenko AB, Govardovskii VI, Pugh EN Jr, Arshavsky VY. Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. *Neuron.* 2002; 34:95–106. [PubMed: 11931744]
- Song H, M B, EJ Y, M S. Compartment-specific phosphorylation of phosducin in rod underlies adaptation to various levels of illumination. *J. Biol. Chem.* 2007; 282:23613. [PubMed: 17569665]
- Tam BM, Moritz OL, Hurd LB, Papermaster DS. Characterization of the Functional Properties of the C-terminus of Xenopus Peripherin. *Investigative Ophthalmology & Visual Science.* 2002;43.
- Tam BM, Moritz OL, Papermaster DS. The C terminus of peripherin/rds participates in rod outer segment targeting and alignment of disk incisures. *Molecular Biology Cell.* 2004; 15:2027.
- Uversky VN, Oldfield CJ, Dunker AK. Intrinsically Disordered Proteins in Human Diseases: Introducing the D(2) Concept. *Annu Rev Biophys.* 2008; 37:215–246. [PubMed: 18573080]
- Vucetic S, Xie H, Iakoucheva LM, Oldfield CJ, Dunker AK, Obradovic Z, Uversky VN. Functional anthology of intrinsic disorder. 2. Cellular components, domains, technical terms, developmental processes, and coding sequence diversities correlated with long disordered regions. *J Proteome Res.* 2007; 6:1899–916. [PubMed: 17391015]
- Warren R, Molday RS. Regulation of the rod cyclic nucleotide gated channel. *Advances in Experimental Medicine and Biology.* 2002; 514:205. [PubMed: 12596923]

Research Highlights

- We delineate the spatial localization of peripherin/rds binding partners in photoreceptor rod cells.
- Changes in proteins localization were examined by serial tangential sectioning.
- Peripherin/rds was found to co-distribute with its binding partner, Calmodulin in basal disks.

Figure 1.
Schematic representation of various functional and protein binding domains of peripherin/
rds C-Terminus.

Figure 2.

Immunoprecipitation of CaM-Peripherin/rds complex from solubilized bovine retinal extracts. A. Ca/CaM levels in calcium or high EGTA wash. B. Anti-peripherin/rds mAb 2B6 was used to immunoprecipitate protein from ROS extracts, and the IP products were detected with anti-Ca/CaM or anti-peripherin/rds. mAb 2B6 was a generous gift from Dr. R. Molday. C. Western blots of fractions from serial tangential sections of dark-adapted retinas, probed for proteins indicated.

Figure 3.

Steady-state tryptophan fluorescence emission and fluorescence anisotropy of PP-C upon binding Ca²⁺/CaM. (A) Representative emission spectra of 10 mM PP-C (black) and 10 mM PP-C after the addition of 20 mM CaM in the presence of 2 mM Ca²⁺ (blue). Addition of 20 mM CaM in the presence of 2 mM Ca²⁺ and 6 mM EDTA (red) resulted in no significant blue shift or quantum yield increase of PP-C tryptophan fluorescence. Spectra represent an average of three independent measurements. (B) Fluorescence anisotropy of 2 mM CaM upon titration of CaM in the presence of 2 mM Ca²⁺. Error bars represent the standard deviation of at least five measurements of the anisotropy. The curve was fit assuming 1:1 stoichiometry to determine the $K_D = 190 \pm 55$ nM.