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Letter to the Editor

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

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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 and Wright, 2005; Edrington et al., 2007b; Ritter et al., 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, 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 et al., 2007; Edrington et al., 2007a). These functional and 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 et al., 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) 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 (10 mM HEPES, pH 7.4, 1 mM DTT) containing either 1 uM $CaCl_2$ or 2 mM EGTA. After solubilization of membrane proteins in 10 mM OG, peripherin/rds protein complexes were immunoprecipitated with anti-peripherin/rds mAb 2B6 as described in our previous studies (Boesze-Battaglia et al., 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 solublized 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 solublization 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

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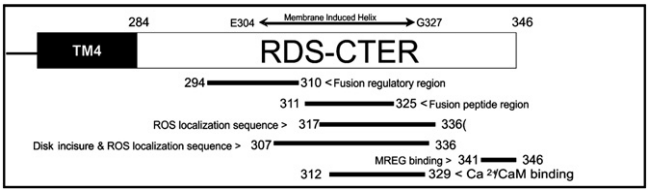

Fig. 1. Schematic representation of various functional and protein binding domains of peripherin/rds C-Terminus.

associated with peripherin/rds. In contrast, treatment of the OS with 1uM $CaCl_2$ 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 et al., 2007). A representative experiment using this technique is shown Fig. 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 et al., 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.

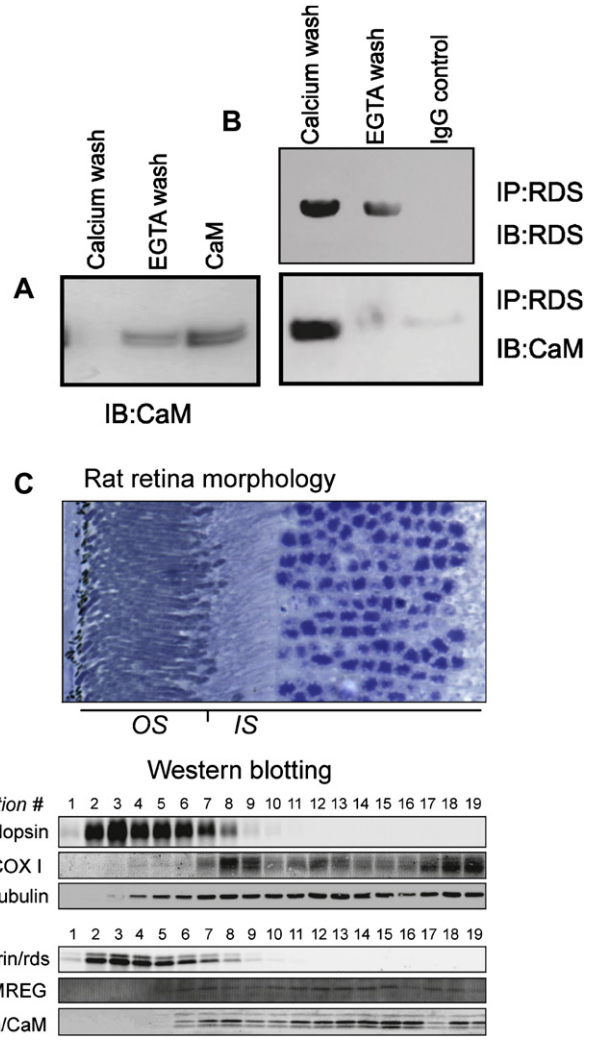

Fig. 2. Immunoprecipitation of CaM-Peripherin/rds complex from solublized bovine retinal extracts. A. Ca/CaM levels in calcium or high EGTA wash. B. Anti-peripherin/rds mAb 2B6 was used to immunoprecipitate protein from ROS extracts, and the IP products were detected with anti-Ca/CaM or anti-peripherin/rds. 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.

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 et al., 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 (Fig. 3A). Upon the addition of Ca/CaM, there was a 62% increase in PP-C Trp fluorescence emission intensity at 340 nm and a λmax blue-shift from approximately 352 nm to 332 nm. The observed intensity increase and the λmax 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

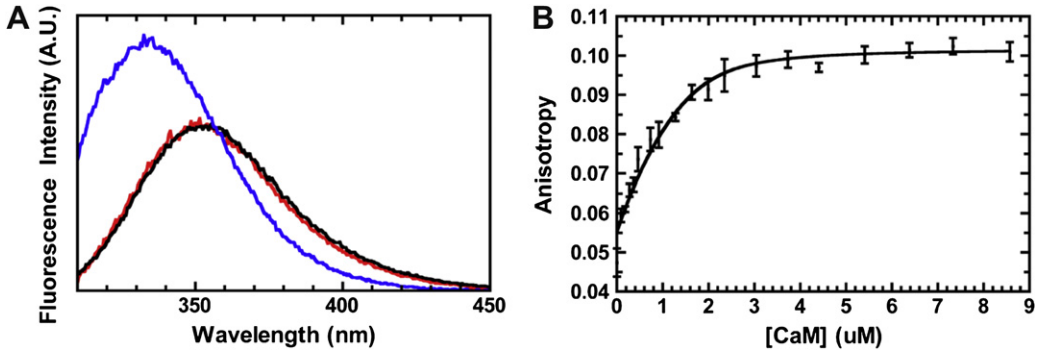

Fig. 3. Steady-state tryptophan fluorescence emission and fluorescence anisotropy of PP-C upon binding Ca2+/CaM. (A) Representative emission spectra of 10 mM PP-C (black) and 10 mM PP-Cafter the addition of 20 mM CaM in the presence of 2 mM Ca^{2+} (blue). Addition of 20 mM CaM in the presence of 2 mM Ca^{2+} 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 Ca^{2+}. 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 KD = 190 ± 55 nM (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

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 (Fig. 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. Alternatviely, the peripherin/rds on the disk rim may act as a reservoir for calmodulin for subsequent binding ot 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 et al., 2007; Damek-Poprawa et al., 2005; Edrington et al., 2007a,b; Tam et al., 2002, 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.

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