Proteolytic Shedding of the Extracellular Domain of Photoreceptor Cadherin
IMPLICATIONS FOR OUTER SEGMENT ASSEMBLY*

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Photoreceptor cadherin (prCAD) is a distinctive cadherin family member that is concentrated at the base of rod and cone outer segments and is required for their structural integrity. During retinal development, prCAD localizes to the site of the future outer segment before rhodopsin or other phototransduction proteins. In vivo, prCAD undergoes a single proteolytic cleavage that releases the ectodomain as a soluble fragment. The C-terminal fragment containing the transmembrane and cytosolic domains remains associated with the outer segment. In rds(−/−) retinas, in which outer segment assembly is severely disrupted because of the absence of retinal degeneration slow (RDS)/peripherin, an essential outer segment structural protein, the level of prCAD is increased, whereas the levels of other outer segment proteins are decreased relative to wild type retinas. Additionally, the ratio of intact:cleaved prCAD polypeptides is increased in rds(−/−) retinas. These data imply that prCAD ectodomain cleavage is an integral part of the outer segment assembly process, and they further suggest that outer segment assembly might be driven, at least in part, by the near irreversibility of proteolysis.

In the vertebrate retina, phototransduction occurs within the outer segments (OSs) of rod and cone photoreceptors. The OS is a modified cilium that projects from the apical face of the photoreceptor cell toward the overlying retinal pigment epithelium (RPE). In mammals, the typical rod OS is ∼1 μm in diameter and ∼30–50 μm in length. Each rod OS consists of a stack of ∼1,000 flattened membrane sacs, referred to as discs, surrounded by plasma membrane. The light-absorbing visual pigments reside within the disc and plasma membranes at millimolar concentrations; other less abundant phototransduction proteins reside within one or both of these membranes or within the cytosolic space between adjacent discs.

The OS of both rods and cones are subject to constant renewal throughout the life of the organism, with synthesis and assembly of new discs at the base of the OS and with RPE-mediated phagocytosis and degradation of the oldest discs at the tip of the OS (1). In mammals, each disc requires ∼10 days to move along the length of the OS, implying that each photoreceptor assembles ∼100 new OS discs/day. All of the OS protein and lipid constituents originate in the cell body proper and are funneled through the thin connecting cilium to the site of assembly in the OS. The most widely accepted model for OS disc assembly, based on electron microscopic analyses of primary photoreceptors, posits that nascent discs form by evagination at the base of the OS and that as they enlarge and move distally they become progressively enclosed in plasma membrane (2).

The precisely orchestrated synthesis, transport, and assembly of proteins and lipids into an almost crystalline array of OS discs raise numerous questions regarding underlying mechanisms. What controls the regular evagination of plasma membrane to form new discs? What determines the size and shape of each new disc? What guides the subsequent growth of plasma membrane around the newly forming discs, and why does this process go to completion in rods but not in cones? One insight into some of these processes has come from the observation that cytochalasin D-mediated disruption of the actin filaments within photoreceptor cilia leads to the production of aberrantly large discs (3–5). This observation implies the existence of a cytoskeletal system that controls the size of nascent discs.

The present experiments are focused on photoreceptor cadherin (prCAD), a recently discovered protein that is likely to play a role in OS assembly (6). prCAD is a single-pass transmembrane protein with six cadherin repeats within its extracellular domain. prCAD localizes to the nascent discs at the base of the OS in both rods and cones, and targeted disruption of the mouse prCAD gene leads to disorganization of photoreceptor OS (6). In this paper we show that prCAD is conserved across a wide range of vertebrates, that it is one of the first photoreceptor proteins to localize to the site of the developing OS, that the ectodomain of prCAD is released by proteolysis in the OS, and that proteolysis is coupled to OS assembly. These data suggest a model in which proteolysis of prCAD irreversibly drives assembly of the OS.

MATERIALS AND METHODS

Mice—The production and characterization of prCAD(−/−) mice have been described previously (6). rds(−/−) mice (C3A.BLIA-Pde6b−/−;Rdsflad/J) were obtained from the Jackson Laboratory.
Cloning of Zebrafish and Xenopus prCAD cDNAs—Using a probe derived from bovine prCAD, multiple full-length zebrafish prCAD cDNA clones were isolated by low stringency screening of an adult zebrafish retina cDNA library (a gift of Dr. James Hurley (University of Washington)). Three independent cDNA clones were sequenced. A *Xenopus laevis* expressed sequence tag clone (za26c10; GenBank™/EMBL accession AW199202) encompassing the 3' 55% of the prCAD coding region was obtained from Dr. Richard McCombie (Cold Spring Harbor Laboratory). The complete *Xenopus* prCAD coding region sequence was obtained by RACE-PCR amplification using the SMART RACE cDNA kit (BD Biosciences) and total RNA from *Xenopus* retina.

In *Situ* Hybridization to Zebrafish and Xenopus—*In situ* hybridization was performed essentially as described previously (8) using digoxigenin-labeled riboprobes transcribed from zebrafish and *Xenopus* prCAD cDNAs and encompassing codons 1–449 and 1–857, respectively.

In *Vitro* Transcription and Translation—A mouse prCAD cDNA template was amplified by PCR using eight different forward primers, each of which contained (from 5' to 3') a T7 promoter, an initiator methionine codon within an optimal consensus (‘Kozak’) sequence for translation initiation, and a segment of mouse prCAD sequence to direct the site of priming. A single reverse primer located downstream of the mouse prCAD stop codon was used for each PCR reaction. The resulting PCR products were gel purified and used for *in vitro* transcription/translation using the T7 Kit (Promega).

Enzymatic Treatments to Detect Posttranslational Modification of prCAD—Mouse retina extracts were treated with glycosidase F (PNGase F; New England Biolabs) to remove N-linked oligosaccharides, with endo-0-glycosidase (ProZyme) to remove O-linked disaccharides, or with bacteriophage λ protein phosphatase (New England Biolabs) according to the manufacturers’ recommendations. The treated extracts were analyzed by immunoblotting to test for shifts in the mobility of prCAD and its proteolytic cleavage products.

Antibodies—The anti-mouse prCAD C-terminal and anti-mouse prCAD N-terminal antibodies are described elsewhere (6). Rabbit polyclonal antibodies against the C-terminal third of bovine prCAD (amino acids 488–867) were generated by immunization with a T7 gene 10 fusion protein expressed in *Escherichia coli* and affinity-purified using a maltose-binding protein fusion carrying the same prCAD C-terminal segment. Other antibodies were from the following sources: anti-rod transducin (Santa Cruz Biotechnology), anti-ABCR (Ref. 9), anti-RP1 (Drs. Qin Liu and Eric Pierce), anti-Na,K-ATPase α subunit (Abcam) (Developmental Studies Hybridoma Bank), anti-arrestin (Dr. Toshi Shinohara), and anti-rodopsin mAb 1D4, anti-ROM1 mAb 1D5, and anti-rod CNGC α mAb 1D1 (Dr. Robert Molday).

Immunostaining and Immunoelectron Microscopy—Immunostaining of fresh frozen retinal sections and postembedding immunoelectron microscopy were performed as described by Rattner et al. (6). Protein Extracts from Mouse Retinas and Bovine OSes—Dissected retinas were extracted in buffer containing 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% Nonidet P-40, and complete protease inhibitor mixture (Roche Applied Science). Following a 5-min incubation on ice, the lysate was cleared of nuclei and insoluble material by centrifugation at 1,600 × g for 5 min at 4 °C. For the comparison of WT and rd(−/−) retinas, extracts were prepared both from dissected retinas and from whole eyes, in the latter case to eliminate the possibility that OS fragments might be lost from the dissected rd(−/−) retina. Comparisons of these two preparations showed little difference in the recovery of OS proteins.

To isolate the Triton X-100-insoluble fraction from bovine rod OSs, purified dark adapted OSs prepared under dim red light as described previously (10) were incubated with excess 11-cis retinal to maximally regenerate rhodopsin and then fractionated as shown in Fig. 4E. All procedures were carried out in darkness or under dim red light to minimize opsin aggregation.

**RESULTS**

**Evolutionary Conservation of prCAD Structure and Expression**—In earlier work, we determined the sequences of mouse, bovine, and chicken prCAD by sequencing retina-derived cDNAs. To explore the structure of prCAD in more distant species, we determined the sequences of *X. laevis* and *Danio rerio* (zebrafish) prCAD homologues by sequencing overlapping cDNA clones and RACE-PCR products derived from *Xenopus* and zebrafish retinas (Fig. 1A). The *Xenopus* and zebrafish prCAD orthologues share ~50% amino acid identity with mouse prCAD, and the corresponding zebrafish gene, as well as two predicted *Fugu rubripes* (pufferfish) genes, shows the same intron-exon arrangement as found among mammalian prCAD genes (6). This intron-exon arrangement differs from that of classical cadherin genes; for example, the cytosolic domain is coded by a single 3'–exon in each prCAD gene and by four exons in classical cadherin genes.

A distinctive feature of the set of prCAD sequences is the relatively high conservation within the N-terminal extracellular cadherin repeat domains and the relatively low conservation within the C-terminal cytosolic domain, with only a few small regions of the cytosolic domain showing substantial amino acid identity across all orthologues (Fig. 1B). E-cadherin and N-cadherin sequences from the same three species show the reciprocal pattern of sequence conservation, with the highest conservation in the cytosolic domain. For these two cadherins, the interaction with cytoskeletal linker proteins such as β-catenin and plakoglobin could provide the selective pressure for sequence conservation within the cytosolic domain (11). For prCAD, the pattern of sequence conservation suggests a conserved function for the extracellular domain and a limited role for much of the cytosolic domain.

By *in situ* hybridization to *Xenopus* and zebrafish retinas, we observe prCAD transcripts exclusively in the photoreceptor layer (Fig. 1C). In both species, prCAD transcripts are also present in distinct regions in the brain, most prominently the pineal gland. Pineal expression of prCAD also occurs in mammals as determined by the abundance of prCAD sequences among expressed sequence tags in mammalian pineal libraries.

These data indicate that prCAD orthologues are present in diverse vertebrates, and in each species thus far examined, these orthologues are expressed in photoreceptor cells.

**Subcellular Localization of prCAD**—In earlier work, we showed by pre-embedding immunoelectron microscopy that antibodies directed to the C terminus of prCAD preferentially stain the base of the OS in transverse sections of adult mouse retina (6). This analysis further showed that prCAD immunostaining is concentrated on the face of the OS opposite the connecting cilium/axoneme, a region corresponding to the free edges of nascent OS discs where the mature disc rim structure has not yet formed (2). As this immunostaining pattern has not been described for any other OS protein and as it has significant implications for hypotheses regarding prCAD function and OS disc biogenesis, we sought to further define the localization of prCAD by serial reconstruction of immunostained photoreceptors after sectioning parallel to the plane of the retina.

Fig. 2, A–J, shows five consecutive sections encompassing part of the IS-OS junction of two photoreceptors, the locations of which are shown schematically in Fig. 2K. The silver-enhanced immunogold particles are visible as *small black dots* in the electron micrographs in the left-hand set of panels and as *red dots* in the graphical representations of one of the cells in the right-hand set of panels. As suggested by the earlier analysis of transverse retinal sections, the highest concentration of prCAD immunoreactivity is found in those regions of the OS opposite the connecting cilium and axoneme. Proceeding from the most proximal section (Fig. 2A) to the most distal section (Fig. 2I), prCAD immunoreactivity is seen to cluster more tightly along this face of the OS. Under the mild fixation conditions employed (4% paraformaldehyde, 0.1% glutaraldehyde for 1 h at room temperature), the immunostained regions, which likely correspond to the free edges of nascent discs, show less complete preservation of ultrastructure. In regions of the
OS that contain mature and fully enclosed discs, prCAD immunostaining is minimal. The prCAD immunolocalization results described above and by Rattner et al. (6) could conceivably reflect a uniform distribution of prCAD throughout the OS but a selective accessibility of prCAD to antibody probes only at the OS base. Arguing against this possibility is the observation that polyclonal antibodies directed against either the N-terminal pair of cadherin domains or the extreme C terminus give the same pattern of immunostaining predominantly at the OS base (Fig. 2, L and M). Moreover, pretreating unfixed frozen sections of mouse retina with acetone, acetic acid, or Bouin's fixative (picric acid and Formalin) did not alter the immunostaining pattern for either of these anti-prCAD antibodies. In all of these experiments, the specificity of the immunostaining signal was confirmed by its absence from prCAD(−/−) retinas. A similar pattern of immunostaining was seen in the macaque retina using antibodies against the N terminus of mouse prCAD. Although not evident in the images shown in Fig. 2, L and M, both anti-N- and anti-C-terminal prCAD antibodies also reveal low levels of prCAD immunoreactivity throughout the OS layer.

**Precocious Localization of prCAD to the Base of the OS in the Developing Retina**—In the mouse, rod photoreceptors begin to differentiate during the first postnatal week, and fully mature rods are seen during the third postnatal week. Numerous studies have examined the growth of the OS and the subcellular localization of OS proteins during this time (e.g. Refs. 12 and
prCAD C-terminal antibodies and 0.8-nm gold-conjugated secondary junction. Prior to embedding, the tissue was immunolabeled with anti-antigens through a WT mouse retina immediately distal to the IS-OS plane. Fig. 2. prCAD is localized to nascent disc membranes at the base of the OS. Shown are electron micrographs (A, C, E, G, and I) and graphical representations (B, D, F, H, and J) of serial horizontal sections through a WT mouse retina immediately distal to the IS-OS junction. Prior to embedding, the tissue was immunolabeled with anti-prCAD C-terminal antibodies and 0.8-nm gold-conjugated secondary antibodies. In the graphical representations, silver-enhanced immunogold particles are represented by red dots. The axoneme (AX) is yellow; the IS and its calycal processes are blue, and the OS is white. K, schematic of the outer retina (left) and the IS-OS junction (right) indicating the planes of section of the serial electron micrographs. ONL, outer nuclear layer; INL, inner nuclear layer; L and M, immunostaining of adult mouse retina with anti-prCAD C-terminal (L) or N-terminal (M) antibodies (green) and 4′,6-diamidino-2-phenylindole (blue). OPL, outer plexiform layer; IPL, inner plexiform layer. Scale bar, 25 μm.

In considering the function of different OS proteins, one would predict that those proteins involved in assembling and/or maintaining the structure of the OS should be the first to localize to the apical tip of the photoreceptor. OS proteins with no role in OS structure or assembly would be expected either to appear later or to be initially mislocalized; for this class of proteins, correct localization to the growing OS would only be expected after the OS transport and assembly apparatus is fully functional.

The disorganization of OS discs and the minimally perturbed electroretinogram responses seen in prCAD(−/−) retinas suggest that prCAD is involved in OS structure and/or assembly but not in phototransduction (6). To further test this idea, we immunostained developing mouse retinas at postnatal days 2, 3, 6, and 9 (P2, P3, etc.) with anti-prCAD and anti-rhodopsin antibodies (Fig. 3). At P2, when the first rods begin differentiating, rhodopsin immunoreactivity is seen throughout the cell body, but prCAD immunoreactivity is only detectable at the apical tip of the cell, the site of the developing OS (Fig. 3A). Moreover, for some developing rods there is little or no colocalization of rhodopsin and prCAD, suggesting that there may be a time window during the early development of each rod when rhodopsin (or opsin) accumulates but is not targeted to the site of the future OS. Between P2 and P9, as additional rods differentiate, the number of prCAD-stained cells increases, and progressively more rhodopsin localizes to the developing OS region. However, at each of these time points, large amounts of rhodopsin continue to accumulate in the outer nuclear layer (i.e. the rod cell bodies), whereas prCAD is only detectable at the base of the growing OS.

Analysis of arrestin immunolocalization at the same developmental times reveals a pattern and time course similar to those seen for rhodopsin. By contrast, the α subunit of the rod cyclic nucleotide-gated channel (14), rod outer segment membrane protein 1 (ROM1; Ref. 15), and the axoneme protein RP1 (16) are first clearly detectable at P9, at which time they are correctly localized to the growing OS. Liu et al. (16) observed a similar pattern of localization for RP1 in developing mouse photoreceptors but were able to detect immunostaining in the OS as early as P6. Taken together, these data show that prCAD is unusual among OS proteins in its early localization to the developing OS, a temporal pattern that is consistent with a role for prCAD in OS assembly.

Models to Explain Steady-state prCAD Localization at the Base of the OS—The localization of prCAD at the base of the OS leads to an apparent paradox. During OS biogenesis, integral membrane proteins such as rhodopsin and RDS/peripherin accumulate within nascent discs. Following the complete separation of the disc and plasma membranes, such integral membrane proteins remain embedded within the disc membrane and travel from the base to the tip of the OS over a period of ~10 days (1). A similar time course of synthesis and turnover is presumed to apply to other integral membrane OS proteins, such as the cyclic nucleotide-gated channel, which reside in the plasma membrane and are anchored via attachment to the adjacent disc rim (17). By analogy with these other OS membrane proteins, prCAD proteins that have been transported to
the base of the OS would be expected to move distally along the OS in association with the disc and/or plasma membranes. Therefore, at steady state, we would expect to see uniform staining of all integral membrane OS proteins, including prCAD, along the length of the OS. The low level of prCAD immunostaining beyond the base of the OS suggests that (a) the influx of newly transported prCAD to the base of the OS is matched by local degradation and/or that (b) unlike other integral membrane OS proteins, prCAD is selectively retained at the base of the OS.

**Proteolytic Cleavage of the prCAD Ectodomain in Vivo**—To test the possibility that prCAD might be subject to proteolysis in vivo, immunoblots of mouse retina were probed with affinity-purified prCAD antibodies directed against either the N-terminal pair of cadherin domains or the extreme C terminus (Fig. 4C). Fig. 4A shows that mouse retinas contain the expected ~120-kDa full-length prCAD polypeptide as well as a ~95-kDa N-terminal fragment and a ~25-kDa C-terminal fragment. The specificity of each of these bands is demonstrated by their absence in protein samples prepared from prCAD−/− retinas (Fig. 4A). An identical cleavage into N- and C-terminal fragments was observed in the rat retina. This cleavage is unlikely to be an in vitro artifact or the result of nonspecific proteolysis because immunoblot patterns indistinguishable from the ones shown in Fig. 4A were obtained both with mouse retinas that were immediately dissected into SDS sample buffer and with retinas that were allowed to stand for 30 min at room temperature. Moreover, expression of prCAD in transfected 293 cells results in the accumulation of full-length prCAD without detectable cleavage.

Based on the apparent molecular masses of the N- and C-terminal fragments, the point of cleavage is predicted to reside on the extracellular face of prCAD, and therefore this cleavage should release the extracellular domain from the membrane. To test this idea, freshly isolated mouse retinas were incubated in hypotonic medium for 30 min at room temperature, and then the retinas and medium were analyzed separately by immunoblotting for prCAD and its fragments (Fig. 4B). Prior to immunoblotting, the medium was centrifuged at 14,000 × g for 10 min to separate soluble proteins from detached OS. A comparison of the anti-N-terminal immunoblots in Fig. 4, A and B, shows that the N-terminal fragment is efficiently released into the medium during the 30-min incubation in hypotonic medium. A very similar result was obtained when this experiment was conducted with isotonic medium (Dulbecco’s modified Eagle’s medium/F12). Fig. 4B further shows that following centrifugation of the incubation medium, full-length prCAD and the C-terminal fragment are found in the OS pellet and that the N-terminal fragment is found in the supernatant. These experiments demonstrate that proteolysis of prCAD produces a soluble N-terminal fragment and a cell-associated C-terminal fragment (Fig. 4C). Although the discovery of prCAD ectodomain cleavage suggests that in situ proteolysis plays an important role in the elimination of full-length prCAD, the subsequent fates of the N- and C-terminal fragments, including their half-lives, remain to be determined.

prCAD proteolysis most likely occurs in the OS rather than the IS because both anti-N-terminal and anti-C-terminal antibodies produce essentially the same staining pattern at the base of the OS (Fig. 2, L and M), from which we infer that substantial quantities of full-length prCAD are present at the base of the OS prior to proteolytic cleavage. We note, however, that this inference rests on the assumption that the cleaved ectodomain diffuses away from the base of the OS, as suggested by its efficient release in vitro.

To more precisely define the site of proteolytic cleavage in prCAD, the electrophoretic mobility of the C-terminal fragment obtained from mouse retinas was compared with the mobilities of a series of in vitro translated polypeptides encompassing the same C-terminal sequences but differing in length at their N termini (Fig. 4D). For this experiment, a series of eight DNA templates were constructed with initiator methionine codons at positions 659, 661, 663, ... 673 (in Fig. 4D these are labeled according to the identity and location of the first prCAD-derived amino acid within each encoded polypeptide, i.e. Lys-660 (K660), Arg-662 (R662), Ser-664 (S664), ... Lys-674 (K674)). Electrophoretic separation and immunoblotting of a mixture of each in vitro translated polypeptide with an aliquot of mouse retina suggest that in vivo cleavage occurs near Thr-669 or Thr-670. A potential complication in interpreting this experiment arises from the possibility that the retina-derived C-terminal fragment of prCAD might carry one or more posttranslational modifications that alter its electrophoretic mobility. Although treatment of the retina sample with λ phosphatase, endoglycosidase F, or endo-β-galactosidase produced no shift in the electrophoretic mobility of the C-terminal fragment,
we cannot rule out the possibility that this fragment carries modifications other than phosphorylation or glycosylation that might affect its electrophoretic mobility.

The second model noted above for the localization of prCAD at the base of the OS, selective retention, could be most easily explained if prCAD associated with OS cytoskeletal elements. Consistent with this possibility, full-length prCAD from total mouse retina or purified bovine rod OS can be pelleted by high speed centrifugation (200,000 × g, 45 min) in the presence of 1% Triton X-100. Previous work has shown that this Triton X-100 high speed pellet is also enriched for axonemal and other cytoskeletal proteins (18). In purified bovine OS, the prCAD C-terminal antibody (H9251) was washed away from the retina and was detected in the soluble fraction (P, compare R and S, S fractions were analyzed by immunoblotting with anti-prCAD C-terminal (left) or N-terminal (right) antibodies. Most of the N-terminal fragment was washed away from the retina and was detected in the soluble fraction (compare R and S); none was detected in the particulate fraction (right, P). The C-terminal fragment was associated either with the retina (left, R) or with the particulate fraction (left, P); none was detected in the soluble fraction (left, S). C, schematic depiction of prCAD structure and processing. prCAD is a single-pass membrane protein with six extracellular cadherin repeats. The cleavage site, as predicted from the size of N- and C-terminal polypeptides, is within the sixth extracellular cadherin domain. The immunogens used for production of anti-N- and anti-C-terminal antibodies are indicated at the right, TM, transmembrane domain; N, N terminus; C, C terminus. D, mapping of the cleavage site in prCAD. Top, the sequence of mouse prCAD between amino acids 657 and 677 is shown above. Eight proteins, forming a nested deletion series encompassing the mouse prCAD C terminus and including an additional initiator methionine (red), were synthesized in vitro using a rabbit reticulocyte lysate. For each polypeptide, the first prCAD-derived amino acid and its position in the prCAD sequence are indicated on the right. Bottom, the in vitro translated polypeptides were mixed with a mouse retina protein extract, resolved by SDS-PAGE, and immunoblotted. The retina-derived C-terminal fragment (green dots and arrows) and the in vitro translated polypeptides (red dots) were detected with anti-mouse prCAD C-terminal antibody. E, the C-terminal fragment of bovine prCAD is associated with the Triton X-100-insoluble fraction of bovine rod OS. Left, schematic representation of OS fractionation into Triton X-100-soluble (S1 and S2) and -insoluble (P1 and P2) fractions; all manipulations were performed under dim red light to minimize bleaching and aggregation of rhodopsin. Right, SDS-PAGE analysis of the four fractions. Identical sets of samples were either stained with Coomassie Blue (CBB, left), showing mainly the distribution of rhodopsin (rho, arrow on the left), or immunoblotted (right) with an anti-bovine prCAD C-terminal antibody (aBC), which recognizes the bovine prCAD C-terminal fragment (arrow on the right). The band at ~80 kDa is presumed to represent a cross-reacting protein; a faint band at ~125 kDa is likely to represent full-length prCAD. In bovine OS, the ratio of C-terminal fragment:full-length prCAD is higher than in mouse retina. ROS, rod OS.
structural protein that resides at the OS disc rim and plays an essential role in disc morphogenesis (19, 20). In the absence of RDS, the OS fails to form, and instead, large numbers of photoreceptor-derived membrane vesicles accumulate in the space between the photoreceptors and the RPE (21). Progressive photoreceptor degeneration occurs over the first several months of life.

Immunoblot analysis of 3–4-week-old WT and rds(−/−) retinas using the anti-C-terminal prCAD antibody shows a ~4-fold higher level of full-length prCAD in the rds(−/−) sample and a ~2-fold increase in the ratio of intact to cleaved prCAD polypeptides (Fig. 5, A and B). A similar analysis with the anti-N-terminal antibody shows a ~2-fold increase in both the absolute level and the proportion of full-length prCAD in the rds(−/−) sample. By contrast, immunoblot and/or immunohistochemical analysis of six representative rod OS proteins (rhodopsin, ABCR, the α subunit of the cGMP-gated channel, ROM1, arrestin, and the α subunit of transducin) shows decreased protein levels in the rds(−/−) retina in each case. By immunoblotting, rhodopsin, transducin, and ROM1 each show at least a 10-fold decrease at this age, consistent with an earlier analysis of rhodopsin levels (22). (The decrease in steady-state levels of ROM1 may be a special case, as ROM1 normally associates with RDS (23).) ABCR and the α subunit of the cGMP-gated channel show at least a severalfold decrease as judged by immunoblotting and immunocytochemistry, respectively. Fig. 5A shows WT versus rds(−/−) immunoblots for arrestin, which shows the smallest decrease (~2-fold) among the six OS proteins tested, and for the α subunit of rod transducin.

Double labeling of 3-week-old WT retinas with anti-C-terminal prCAD antibodies and antibodies to the Na,K-ATPase, an IS marker, RP1, an axoneme marker, or CNGC, an OS marker (16), shows intense prCAD immunostaining confined to a small region distal to the Na,K-ATPase and proximal to RP1 and CNGC (Fig. 5, C, E, and G). (We note that the resolution of the light microscope does not allow an unambiguous determination of the extent to which these double immunolabeling signals might partially overlap.) By contrast, in the rds(−/−) retina, the zone of intense prCAD immunostaining occupies a considerably larger region distal to the IS, consistent with the increased level of prCAD observed by immunoblotting (Fig. 5, D, F, and H). A nearly identical immunostaining pattern is seen with the anti-N-terminal prCAD antibody. The pattern of prCAD localization corresponds to the extracellular vesicles that accumulate in rds(−/−) retinas (21). Interestingly, in rds(−/−) photoreceptors, RP1 remains localized to discrete structures; these presumably correspond to the truncated axoneme or the connecting cilium (Fig. 5F).

The comparison of WT and rds(−/−) retinas shows that efficient proteolytic cleavage and turnover of prCAD require correct OS assembly. It is particularly striking that prCAD levels are elevated in the absence of RDS because RDS and prCAD show reciprocal patterns of OS localization; RDS occupies the mature disc rim region, from which prCAD is largely excluded, and prCAD is most concentrated at the free edges of nascent discs, where RDS is largely absent (24). The data presented here are consistent with a model in which the vesicles extruded from the apical face of rds(−/−) photoreceptors resemble nascent discs, and they support the idea that prCAD proteolysis is coupled to normal OS disc morphogenesis.

**DISCUSSION**

**A Conserved Role for prCAD in OS Assembly.—**The present work provides several lines of evidence that implicate prCAD in the assembly of rod and cone outer segments. First, prCAD orthologues have now been identified in widely divergent vertebrates (mammals, birds, fish, and amphibians), but they have not been found in invertebrates (Caenorhabditis elegans and Drosophila melanogaster), a distribution that matches the distribution of eyes with ciliary photoreceptors. Second, prCAD is concentrated at the base of the OS, as determined by immunostaining following tissue treatments with a variety of denaturing agents and using antibodies to two different regions of the prCAD C terminus (αC, top), rod arrestin (Arr, middle), or rod transducin α (Tα, bottom). FL, full-length; CT, C-terminal fragment. B, the levels of full-length and C-terminal prCAD polypeptides were compared using 30 μg of WT and either 15 or 7.5 μg of rds(−/−) total eye cup proteins. Full-length prCAD is present at a ~4-fold greater level, and the prCAD C-terminal fragment is present at a ~2-fold greater level in rds(−/−) retinas. C–H, immunohistochemical localization of prCAD in rds(−/−) retinas. 10-μm sections of fixed WT (C, E, and G) or rds(−/−) (D, F, and H) retinas were double-labeled with anti-prCAD C-terminal antibody (green) and anti-Na,K-ATPase antibody (red) to mark the IS, anti-RP1 antibody (red) to label the axoneme, or anti-CNGC antibody (red) to label OS membranes. In the rds(−/−) retina, prCAD accumulates distal to the shortened inner segments (D) and the deformed axoneme (F, arrowheads), presumably in OS-related vesicles released from the photoreceptors into the interphotoreceptor matrix (IPM), as indicated by its colocalization with CNGC (H, yellow indicates overlapping red and green signals). ONL, outer nuclear layer; DAPI, 4′,6-diamidino-2-phenylindole. Scale bar, 5 μm.

Fig. 5. Accumulation of full-length prCAD in the rds(−/−) retina. A, immunoblot analysis of total eye cup proteins from wild type and rds(−/−) mouse retinas. Equal quantities (30 μg) of total eye cup proteins from WT or rds(−/−) retinas were probed with antibodies to the prCAD C terminus (αC, top), rod arrestin (Arr, middle), or rod transducin α (Tα, bottom). FL, full-length; CT, C-terminal fragment. B, the levels of full-length and C-terminal prCAD polypeptides were compared using 30 μg of WT and either 15 or 7.5 μg of rds(−/−) total eye cup proteins. Full-length prCAD is present at a ~4-fold greater level, and the prCAD C-terminal fragment is present at a ~2-fold greater level in rds(−/−) retinas. C–H, immunohistochemical localization of prCAD in rds(−/−) retinas. 10-μm sections of fixed WT (C, E, and G) or rds(−/−) (D, F, and H) retinas were double-labeled with anti-prCAD C-terminal antibody (green) and anti-Na,K-ATPase antibody (red) to mark the IS, anti-RP1 antibody (red) to label the axoneme, or anti-CNGC antibody (red) to label OS membranes. In the rds(−/−) retina, prCAD accumulates distal to the shortened inner segments (D) and the deformed axoneme (F, arrowheads), presumably in OS-related vesicles released from the photoreceptors into the interphotoreceptor matrix (IPM), as indicated by its colocalization with CNGC (H, yellow indicates overlapping red and green signals). ONL, outer nuclear layer; DAPI, 4′,6-diamidino-2-phenylindole. Scale bar, 5 μm.
suggests that there is a specific stage in OS biogenesis when prCAD interacts with a variety of extracellular targets. E-remains to be determined.

prCAD functions in OS assembly, although its exact role organized OS but no defects in OS targeting of phototransduction proteins and only minimally perturbed electroretinogram responses (6). Taken together, these data imply that prCAD functions in OS assembly, although its exact role remains to be determined.

prCAD Ectodomain Shedding—Members of the cadherin superfAMILY interact with a variety of extracellular targets. E-cadherin mediates cell-cell adhesion through homophilic interactions (25). Protocadherins of the CNR class interact with Reelin (26), and cadherin-23 assembles its large extracellular domain into cables that constitute the tip links between adjacent stereociliary bundles in auditory hair cells (27). If the ectodomain of prCAD also interacts with binding partners on photoreceptors, the RPE, or the extracellular matrix, then its proteolytic release suggests that there is a specific stage in OS biogenesis when (a) the cell membrane disengages (via proteolysis) from an adhesive interaction and/or when (b) the prCAD ectodomain is released to diffuse to more distant binding targets.

Precedents exist in other systems for processes analogous to each of these two possibilities. The loss of cell-cell and cell-matrix attachment that accompanies apoptosis is associated with shedding of the ectodomains of E-cadherin and VE-cadherin (28, 29). In Drosophila, both the Notch receptor and its ligand Delta are subject to juxtamembrane cleavage, which has emerged as an important regulatory mechanism in the Notch pathway (30–32). In humans, the extracellular ligand binding domain of the growth hormone receptor is released from the cell surface via proteolysis, which decreases the responsiveness of target cells. Additionally, the presence of the released growth hormone receptor ectodomain in serum leads to a decrease in the concentration of free growth hormone (33). Analogous ectodomain shedding is observed with other single-span transmembrane receptors, including the tumor necrosis factor and the interleukin-6 receptors. Several growth factors and cytokines, including tumor necrosis factor-α and colony stimulating factor-1, which are initially synthesized as membrane-anchored precursors, are similarly released by proteolysis (34, 35). For these membrane-anchored ligands, proteolytic release is presumed to extend their spatial range of action.

Cadherin family members also interact with a variety of intracellular targets. The cytosolic domains of classical cadherins bind to β-catenin and organize cytoskeletal attachments to regions of plasma membrane adjacent to cell-cell contacts (11). The CNR protocadherins are presumably involved in signal transduction via their interaction with the cytosolic Fyn tyrosine kinase (36). The relative resistance of the prCAD C-terminal fragment to Triton X-100 extraction suggests that the cytosolic domain of prCAD may interact with cytoskeletal proteins, but the nature of these interactions and their response to ectodomain proteolysis remain to be determined.

A major goal for future work will be to identify the protease responsible for cleaving prCAD and to determine its subcellular localization and regulation. Work on ligand and receptor shedding in other systems suggests that this enzyme will be a member of the large family of metalloproteinases, and more specifically a member of either the ADAM (a disintegrin and metalloproteinase) or matrix metalloproteinase subfamilies (34, 35).

Implications for the Molecular Mechanism of OS Assembly—Although the exact role of prCAD in OS assembly remains uncertain, the discovery of prCAD ectodomain shedding suggests a model in which proteolysis of prCAD contributes to the thermodynamic driving force for OS assembly. In particular, if the correct stacking of nascent OS discs or the “zippering up” of the plasma membrane around them were coupled to proteolysis of prCAD, then these reactions would be rendered effectively irreversible. A number of precedents exist in which proteolysis ensures that a biochemical or cell biological process proceeds unidirectionally. These include destruction of cyclins to drive the eukaryotic cell cycle (37), cleavage of the cohesin protein to initiate chromosome segregation at mitosis (38), conversion of fibrogen to fibrin to initiate blood clotting (39), and the activation of transcription factors by proteolysis for sporulation in Bacillus subtilis (40) or for the transcription of cholesterol biosynthetic genes in vertebrates (41). It seems reasonable to speculate that OS assembly, like many other cell biological processes, might be driven, at least in part, by the near irreversibility of proteolysis.

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