The photoreceptor rim protein is an ABC transporter encoded by the gene for recessive Stargardt's disease (*ABCR*)

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Abstract Rim protein (RmP) is a high-M_r membrane glycoprotein that has been localized to the rims of photoreceptor outer segment discs, but its molecular identity is unknown. Here, we describe the purification of RmP and present the sequence of its mRNA. RmP is a new member of the ATP-binding cassette (ABC) transporter superfamily. We show that RmP is expressed specifically in photoreceptors and predominantly in outer segments. Further, RmP is identical to the protein recently shown to be affected in recessive Stargardt's disease. RmP is the first ABC transporter observed in photoreceptors and may play a role in the photoresponse.

Key words: Rim protein; Stargardt's disease; Outer segment; Macular degeneration; Transporter; Retinal degeneration

1. Introduction

Retinal photoreceptors contain a light-sensitive structure called the outer segment, comprising a dense stack of membranous discs. Rim protein (RmP) is an abundant membrane glycoprotein of approximately 240 kDa in frog retina that is present in the rims of rod and cone outer segment discs [1–3]. A smaller protein (210 kDa) in bovine rod outer segments (ROS) has been assumed to be the mammalian homolog of frog RmP [1–6], although immunocytochemical localization of mammalian RmP has never been described. The function of RmP is unknown. It has been suggested that RmP may represent the electron-dense filaments at the disc rim that span the cytoplasmic space between adjacent discs [7,8], or between disc rim and plasma membrane [9]. RmP has also been shown to be phosphorylated in a light-dependent fashion [10], suggesting a role in the photoresponse.

The ABC transporters effect ATP-dependent translocation of specific substrates across cellular membranes. One member of the ABC family, the cystic fibrosis transmembrane conductance regulator (CFTR), functions as a cAMP-regulated chloride channel instead of an active transporter [11]. The ABC proteins are present in species ranging from *Escherichia coli* to humans [12–14], and are expressed in many cell types. The substrates are best defined for the *E. coli* transporters, and include sugars, metal ions, amino acids, and polypeptides [13]. The substrates are unknown for many of the eukaryotic ABC transporters. The genes for ABC transporters have also been implicated in several human inherited diseases. Recessive Stargardt's is an inherited disease that causes progressive loss of central vision in children [15]. Recently, the gene for recessive Stargardt's was cloned and found to encode a novel ABC transporter expressed in photoreceptors [16]. In this report, we show that RmP is a new member of the ATP-binding cassette (ABC) transporter superfamily, and is identical to the predicted product of the Stargardt's disease gene (*ABCR*).

2. Materials and methods

2.1. Biochemical methods

ROS were purified from frozen bovine retinae (J. and A. Lawson, Lincoln, NE) as described [17], except a 25%, 35% sucrose step gradient [18] was used. Dark-adapted ROS had an A280/A500 ratio of 2.2–2.4 [17]. ROS were prepared from frozen frog (*Rana catesbeiana*) retinae by the same procedure. Protein quantitation was performed with the BCA kit (Pierce, Rockford, IL). Proteins were separated on Laemmli or porous [19] SDS-PAGE gels. Standards for protein electrophoresis consisted of the 10 kDa ladder (BRL, Gaithersburg, MD) supplemented with filamin (Sigma, St. Louis, MO). For non-reducing SDS-PAGE, proteins were solubilized in Laemmli sample buffer without 2-mercaptoethanol (2-ME) and containing 1 mM oxidized glutathione (GSSG) (Sigma, St. Louis, MO) [10]. Gels were stained and immunoblots were prepared as described [17]. Antibodies were diluted 1000 to 1500-fold in TBS plus 1% BSA. Immunoreactive polypeptides were visualized with NBT/BCIP or with the ECL kit (Amersham, Arlington Heights, IL). For detecting concanavalin A (ConA)-binding proteins, blots were probed 1 h at 25°C with 1 μg/ml ConA–HRP (Sigma, St. Louis, MO) in 0.1% BSA, 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$, in TBS, and stained with the 4CN kit (KPL, Gaithersburg, MD).

To purify RmP, ROS proteins (5 mg/gel) were subjected to SDS-PAGE on 10% porous gels and stained in Coomassie. The RmP band was excised and electroeluted using Centricon 30 cartridges with the Centrilutor (Amicon, Beverly, MA). The eluted protein was concentrated in the Centricon cartridge. For amino acid sequencing, electroeluted protein was subjected to SDS-PAGE, transblotted onto Immobilon P (Millipore, Bedford, MA), and sent to a local facility for Edman degradation. For deglycosylation experiments, electroeluted RmP was treated with 5 U/ml endoglycosidase F (EnF) (Boehringer, Indianapolis, IN) in 0.25 M sodium acetate (pH 4.5), 0.1 mM EDTA, 2.5% TX-100; or with 2 U/ml *N*-glycosidase F (NgF) (Boehringer, Indianapolis, IN) in 100 mM Tris-HCl, pH 6.9, 30 mM EDTA, 1% TX-100, for 4 h at 37°C. Samples were precipitated with acetone for SDS-PAGE analysis.

2.2. Molecular techniques

Total RNA was isolated from mouse and bovine retina using Trizol (BRL, Gaithersburg, MD). Poly(A)$^+$ RNA was purified from total RNA with the polyA Tract system (Promega, Madison, WI). Random and oligo(dT)-primed retinal cDNA libraries were generated in lambda ZAP II (Stratagene, La Jolla, CA), according the manufacturer's procedure. Sequence analysis was performed on an ABI 377 automated DNA sequencer, and by using the Radiolabeled Terminator Cycle Sequencing kit (Amersham, Arlington Heights, IL). Both strands were sequenced in entirety. DNA and predicted protein sequences were analyzed with the DNAsis and PC Gene software packages. For Northern analysis, poly(A)$^+$ RNA from mouse retina was separated by electrophoresis on 1.0% agarose formaldehyde gels, and transferred to nitrocellulose membranes. Blots were hybridized with 10^6 cpm/ml of ^{32}P-labeled cDNA insert fragments from RmP clone pRmP9A or *rds* clone B9A [20]. Final stringency-wash conditions

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were 0.2×SSC, 0.1% SDS at 65°C. Labeled mRNA bands were visualized on a Molecular Dynamics Phosphor Imager after overnight exposures.

To map the human *RmP* gene, oligonucleotide primers derived from the 5′ untranslated region of a human RmP cDNA (Azarian and Travis, unpublished) were used to amplify DNA prepared from 93 human–hamster radiation hybrid cell lines of the Genebridge 4 panel (Research Genetics, Huntsville, AL) by PCR. The score vehicle was submitted via the internet to the Whitehead Institute for a map assignment.

3. Results

3.1. Biochemical identification of RmP

We tentatively identified bovine and frog RmP as the 210- and 240-kDa bands, visible by Coomassie staining after SDS-PAGE of purified ROS, based on their size and abundance (Fig. 1a). On an immunoblot, these bands were reactive with the same antiserum against frog RmP (α-fRmP) that showed disc-rim localization by immunocytochemistry [1,2]. In bovine ROS, the 210-kDa protein was also immunoreactive with a polyclonal antiserum (α-bRmP$_1$) raised against a synthetic peptide corresponding to amino acids #2-16 in bovine RmP, derived from the sequence analysis (see below). This reactivity was blocked by pre-incubating the antiserum with the synthetic peptide. Bovine RmP could be extracted from ROS membranes with Triton X-100 and CHAPS detergents, but not urea, confirming that it is a membrane protein. By Coomassie staining, the ratio of RmP/rhodopsin appeared significantly higher in frog compared to that in bovine ROS (Fig. 1a). We confirmed our identification of bovine and frog RmP by demonstrating that the biochemical properties of the 210- and 240-kDa proteins were identical to those previously reported. When ROS were solubilized in Laemmli sample buffer containing GSSG instead of 2-ME, we observed an upward-shift in apparent M_r of both the bovine and frog proteins, as previously described [1,10] (Fig. 1b). The oxidized and reduced forms of bovine and frog RmP reacted with ConA [4] and with α-fRmP. Treatment of purified bovine RmP with the glycosidases, EnF or NgF, caused a small reduction in apparent M_r (Fig. 1c), suggesting that RmP is N-glycosylated, and that the N-linked glycans contribute little to its total mass. Collectively, these data indicate that we have correctly identified bovine and frog RmP, and show it to be an abundant high-M_r membrane glycoprotein that is present in photoreceptor outer segments.

3.2. Purification of RmP and isolation of cDNAs

We purified bovine and frog RmP from ROS by preparative SDS-PAGE under both oxidizing and reducing conditions, followed by electroelution. These samples were submitted to peptide microsequence analysis. We reasoned that if the RmP band were contaminated with another comigrating species, it is very unlikely that this contaminating protein would demonstrate the identical redox-shift as that of RmP. The amino-terminal sequence obtained for bovine RmP was 'MGFARQIKLLLXKNXT', and was identical between the oxidized and reduced forms. The sequence obtained for frog RmP was 'MRFSAKVKLLLXXNNT', which was 60% iden-

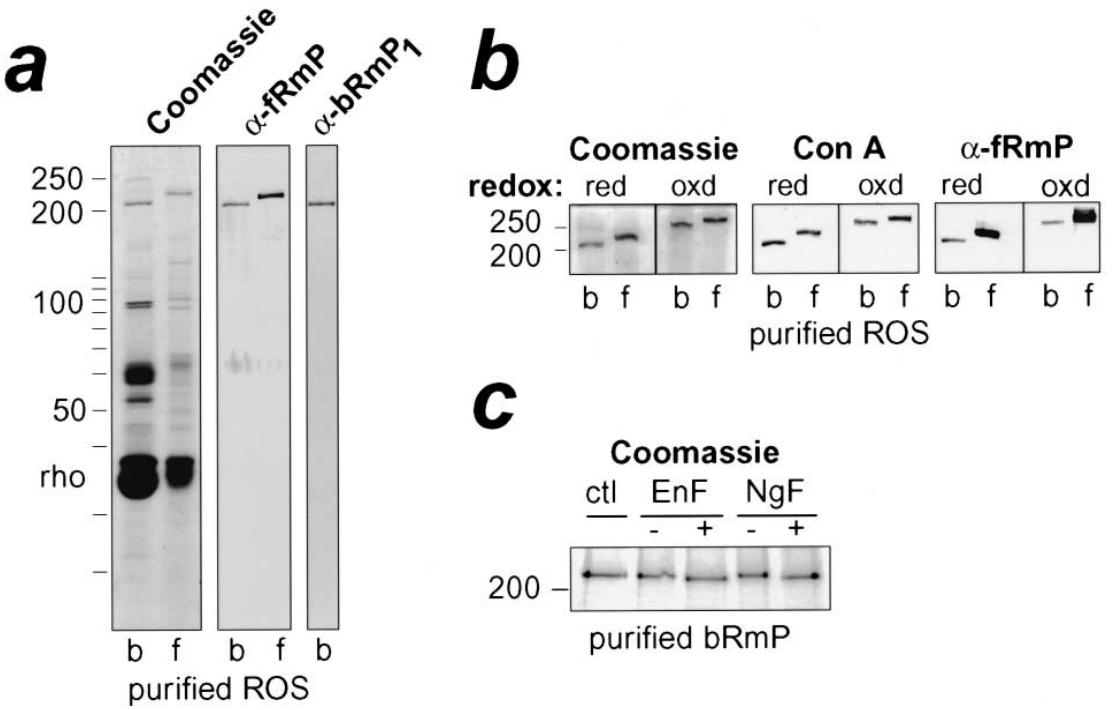

Fig. 1. Biochemical and immunological characterization of RmP. a: SDS-PAGE and immunoblot analysis of RmP in bovine (b) and frog (f) ROS. The first panel is a 10% porous gel stained with Coomassie. The major band labeled 'rho' is rhodopsin. ROS corresponding to 0.1 bovine and 0.5 frog retinae were loaded in each lane. The second and third panels are transblots of equivalent gels reacted with α-fRmP, and α-bRmP$_1$. b: Oxidation–reduction (redox) behavior of RmP. First panel shows a Coomassie-stained 6% Laemmli gel containing 0.2 retina-equivalents of bovine or 1.0 retina-equivalent of frog ROS per lane, electrophoresed in the presence of 2-ME (red) or 1 mM GSSG (oxd). Second panel shows transblot of similar gels reacted with ConA–HRP. Third panel shows transblot of similar gels reacted with α-fRmP. c: Enzymatic deglycosylation of purified RmP. 6% Laemmli gel of bovine RmP digested with or without EnF or NgF. Control (ctl) lane contains RmP kept on ice. Positions of the M_r standards are indicated on the left in kDa.

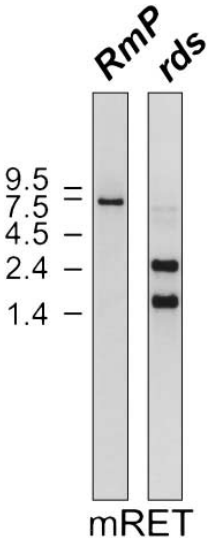

Fig. 2. Northern blot analysis of retinal RNA. First panel shows a blot containing 5 μg of mouse retinal (mRET) poly(A)$^+$ RNA hybridized with a 1.6-kb fragment of pRmP9A. Second panel shows the same blot stripped and rehybridized with *rds*-B9A. The two *rds* transcripts of 2.7 and 1.6 kb arise by alternative polyadenylation [20]. Mobilities of RNA size standards are indicated on the left in kb.

tical to the bovine sequence over the common 16-residue stretch, with mainly conservative substitutions. These data confirm that the 210-kDa bovine and 240-kDa frog proteins are homologous.

Degenerate antisense oligonucleotides (5′-A(A/G)NGTCC-A(A/G)TT(G/T)TTCCA and 5′-AG(C/T)TT(A/G)ATCTG(C/T)C(G/T)(A/G/T)GC) were designed by reverse-translation of the RmP peptide sequence and used pairwise in combination with T3 or T7 oligonucleotides as primers for nested PCR on DNA from a randomly primed bovine retinal library. Direct sequence analysis of the resulting amplification products gave a predicted amino acid sequence that was 100% identical to that obtained by the Edman degradation of purified RmP. The bovine cDNA fragment from near the 5′ terminus was used to screen a randomly primed mouse retinal cDNA library. To obtain full-length cDNA representation, it was necessary to re-screen an oligo(dT)-primed mouse library with a 3′ fragment of the initial mouse cDNA isolate. We assembled the composite presumed full-length mouse RmP cDNA, pRmP9A, from two overlapping cDNA subclones.

3.3. *RmP is an ATP-binding cassette transporter*

Clone pRmP9A contained an insert of 7290 bp. Sequence analysis revealed a single long open reading frame of 6930 bp, with an initiator codon in the Kozak consensus sequence [21], after a 5′ untranslated region (UTR) of 84 bp. The 3′-UTR contained 210 bp and a consensus polyadenylation signal (AAUAAA), followed by a poly(A) tail. By Northern blot analysis of mouse retina, this cDNA detected a single mRNA of 7.3 kb (Fig. 2), suggesting that clone pRmP9A is full-length. This blot was re-hybridized with a probe, of comparable length and specific activity, corresponding to the well-characterized photoreceptor-specific mRNA, *rds* [20] (Fig. 2). Based upon the isolation-frequency of RmP clones from our retinal libraries, we estimate that the RmP mRNA is present in retina at an abundance of approximately 0.01%.

The single large open reading frame encodes a putative protein of 2310 residues (Fig. 3a), with significant identity to members of the ABC transporter family. Within the region of overlap, the predicted mouse protein is 93% identical to the N-terminus of the bovine rim protein, determined by peptide and cDNA sequence analysis. Primary structural analysis of the mouse protein showed 12 predicted membrane-spanning segments and three potential sites for N-linked glycosylation, all three within the first large extracellular (intradiscal) loop (Fig. 3a,b). RmP also contains two consensus ATP/GTP nucleotide-binding sites (Walker A motifs) [22] and two ABC transporter signature motifs [23] (Fig. 3a,b). Both transporter motifs contain a potential site of phosphorylation by protein kinases A or G (PKA/PKG). A Walker B motif ($RX_{6-8}J_4D$) [22] overlaps with transporter motif 1. Within the large cytoplasmic loop, containing the first nucleotide binding fold (NBF) and transporter motifs, we also observed a 22-residue highly hydrophobic domain (HD), containing three proline residues. A dot matrix similarity plot of mouse RmP against itself showed two stretches of incomplete duplication between residues 940–1160 and 1940–2150 within the NBF1 and NBF2 cytoplasmic domains (Fig. 3c). The sequence of mouse RmP is 86% identical (92% with substitution of similar amino acids) to the human ABCR protein, encoded by the gene affected in recessive Stargardt's disease [16].

3.4. *Tissue distribution of RmP expression*

A polyclonal antibody raised against the complete bovine protein (α-bRmP$_4$) reacted specifically with RmP on immunoblots containing bovine ROS and mouse retina homogenates (Fig. 4a). On an immunoblot containing equal protein amounts from various mouse tissues, α-bRmP$_4$ showed reactivity only in retina (Fig. 4b). To localize RmP within retina, we examined the level of immunoreactivity in samples containing different cellular components. No RmP immunoreactivity was observed in eyecups from 6-month-old *retinal degeneration* (*rd*) mutant mice, which lack photoreceptors but contain all other retinal cell types [24] (Fig. 4c). We observed weak immunoreactivity in eyecups from 3-week-old *retinal degeneration slow* (*rds*) mutant mice, which lack outer segment structures, but contain all retinal cell types including photoreceptor cell bodies [25]. When the same immunoblot was stripped and reacted again with an antibody (α-bRho) against the outer segment protein, rhodopsin, a similar pattern was observed (Fig. 4c). These data suggest that RmP is present only in retina, where it is expressed specifically in photoreceptors, and predominantly if not exclusively in outer segments.

3.5. *Chromosomal localization of the RmP gene*

We determined the chromosomal localization of the human *RmP* gene using a panel of 93 human–hamster radiation hybrid cell lines. Matrix analysis of the hybrids containing the human *RmP* gene showed that it maps with a lod score of 21 to human chromosome 1p, 6.9 cR from the WI-3099 marker. This localization placed the *RmP* gene between the markers D1S424 and D1S236. Besides recessive Stargardt this is also within the critical region for a new form (RP19) of autosomal dominant retinitis pigmentosa [26,45]. By synteny, the mouse *RmP* gene maps to chromosome 3 between the loci *Ly-37* [27]

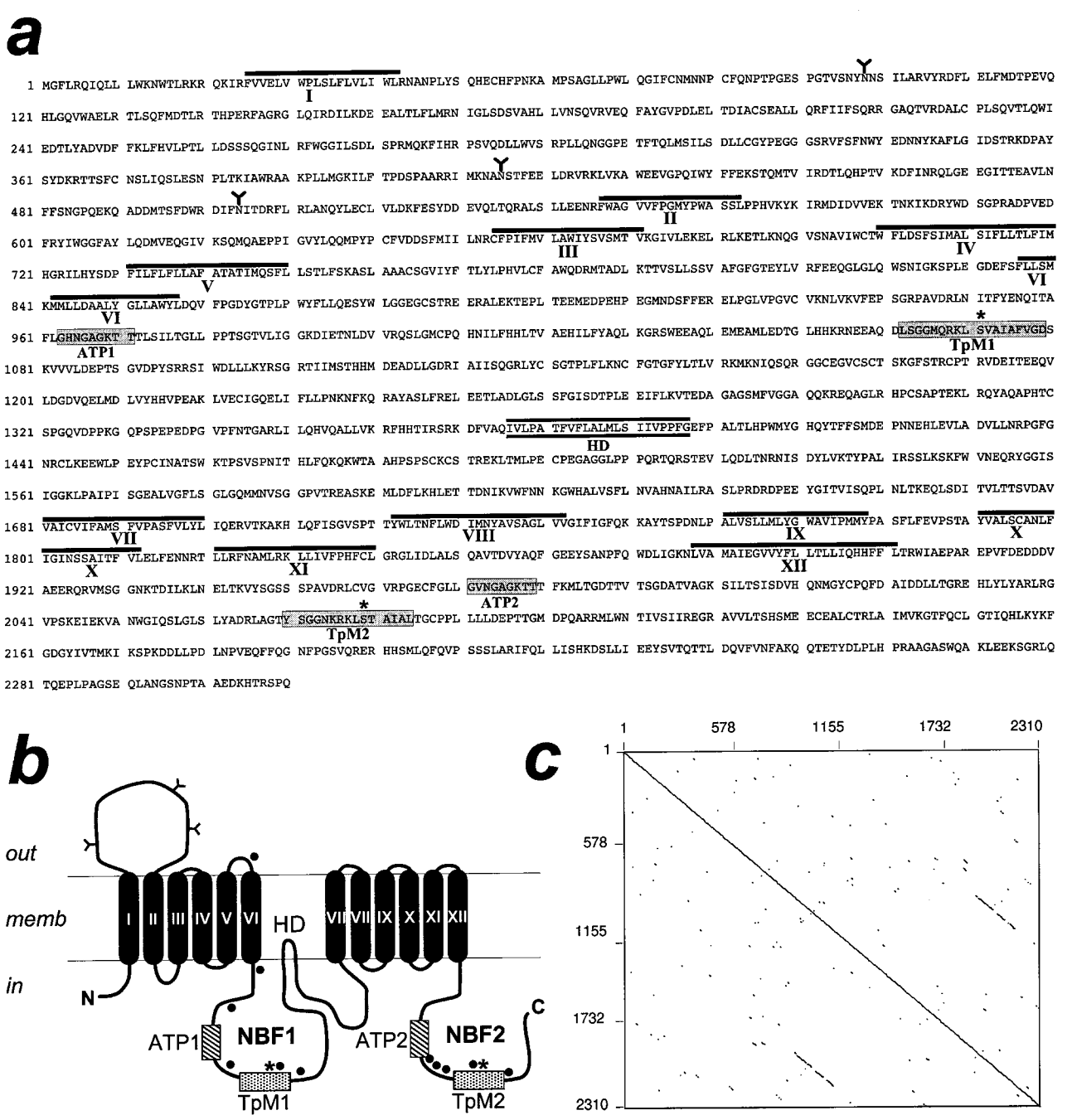

Fig. 3. Primary structure analysis of mouse RmP. a: Amino acid sequence predicted from the cDNA. The 12 predicted membrane-spanning α-helical segments are indicated with a heavy bar above the sequence and the corresponding Roman numeral below. The three predicted N-glycosylation sites on the extracellular surface are indicated by 'Y'. Residues comprising the nucleotide binding sites are boxed and indicated by 'ATP1' and 'ATP2'. The transporter signature-motifs (including Walker B motif) are boxed and labeled 'TpM1' and 'TpM2'. A proline-containing hydrophobic domain is indicated by double bars and 'HD'. The two PKA-phosphorylation sites are indicated by '*'. The nucleotide sequence of clone pRmP9A was deposited in *GenBank* under accession numbers AF000148 and AF000149. b: Schematic showing the predicted topology of RmP in the membrane. The extracellular (intradiscal) surface is on top and the cytoplasmic on bottom of the figure. Black dots indicate the approximate positions of point mutations causing single-residue substitutions associated with recessive Stargardt's [16]. c: Dot-matrix plot of mouse RmP against itself. Note the region of incomplete duplication between residues 940–1160 and 1940–2150 within NBF1 and NBF2. Analysis was performed with a check size of 20 and match size of 8. Numbers on the sides denote residue positions.

and *Tshb* [28]. No mouse mutants with a retinal phenotype have been mapped to this region of chromosome 3.

4. Discussion

In this paper, we present a molecular characterization of RmP. Our identification of RmP in mammalian retina is based upon three observations: (i) it cross-reacts with the same antibody that was originally used to identify RmP in frog discs; (ii) it has biochemical properties similar to those of frog RmP; and (iii) it is homologous to frog RmP by microsequence analysis. Based upon the sequence of a full-

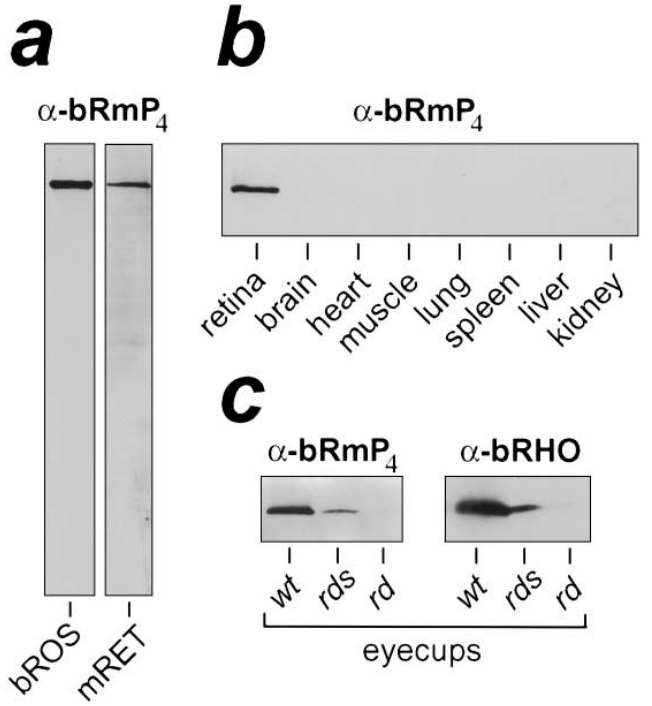

Fig. 4. Tissue and subcellular distribution of RmP. a:) Immunoblots containing homogenates of purified bovine ROS (bROS, 40 μg protein) and mouse retina (mRET, 400 μg protein) reacted with α-bRmP$_4$. b: Immunoblot containing homogenates of indicated mouse tissues (400 μg protein) reacted with α-bRmP$_4$. c: Immunoblots containing eyecup homogenates (400 μg protein) of C57BL/6 wild-type (wt), pre-degenerate *rds* homozygous mutant (*rds*), and fully degenerate *rd* homozygous mutant (*rd*) mice, reacted with α-bRmP$_4$. The same blot was stripped and reacted again with a polyclonal antibody against bovine rhodopsin (α-bRho). Blots were generated from 10% porous gels.

length cDNA, RmP is a new member of the ABC transporter superfamily, identical to the protein encoded by the gene for recessive Stargardt's disease [16].

In common with most eukaryotic ABC transporters, RmP contains 12 transmembrane segments with cytoplasmic amino and carboxy termini. RmP also contains two nucleotide binding sites [22], two transporter motifs [23], and a single Walker B motif [22], the latter in cytoplasmic loop NBF1. The two predicted PKA/PKG phosphorylation sites are located within the transporter motifs, both exactly 100 residues downstream of the nucleotide binding sites. The spacing between nucleotide binding and transporter motifs is conserved in many ABC transporters [29], suggesting that this 100-residue segment is an important structural element. By dot matrix analysis, this segment is part of an imperfect duplication in cytoplasmic domains NBF1 and NBF2, similar to the other eukaryotic ABC transporters [29]. Interestingly, the preponderance of point mutations in recessive Stargardt's affect codons for residues within these two nucleotide-binding folds [16] (Fig. 3). This distribution of mutations corroborates the importance of the NBF1 and NBF2 domains in RmP activity.

The closest relative of RmP (besides its direct human ortholog) is ABC1 [29], at 49% amino acid identity. RmP and ABC1 share a structural feature that is not seen in the other ABC transporters: a highly hydrophobic domain with three proline residues in the NBF1 loop. It was suggested that this represents an integral-membrane hairpin structure in ABC1 [29]. RmP is longer, with 60 residues at the amino terminus that do not align with ABC1. In addition, the first predicted extracellular (intradiscal) loop of RmP, which contains the three sites for N-linked glycosylation, is much larger in RmP than that predicted for ABC1 [30]. RmP thus appears to have all the structural features that define a eukaryotic ABC transporter, plus a large extracellular loop of as yet undefined function. The carboxy terminus of mouse RmP contains 37 additional residues not present in the human protein (Azarian and Travis, unpublished) [16].

By immunoblot analysis, RmP was expressed specifically in retina, and predominantly but not exclusively in outer segments. The low level of RmP expression in *rds* eyecups may reflect RmP within the inner-segment secretory pathway. Weak reactivity was also observed with the outer-segment protein, rhodopsin. No signal was detected with α-bRmP$_1$ or α-bRmP$_4$ in immunocytochemical studies, probably due to epitope-masking. The ratio of RmP/rhodopsin was several-fold higher in frog than in bovine retina (assuming comparable Coomassie-staining of the corresponding frog and bovine proteins). Outer segment discs contain deep indentations, called incisures. The number of disc incisures is significantly higher in amphibians than mammals [7,31]. Since rhodopsin is present in the disc lamellae but excluded from the rims [9], a positive correlation between the ratios of RmP/rhodopsin and the disc circumference-to-disc area is consistent with disc–rim localization of RmP in mammalian retina. In one study of bovine ROS where outer segment plasma membranes were biochemically separated from disc membranes, the 210-kDa RmP band was present in the disc-membrane fraction, which lacked the plasma membrane cation channel [32]. These observations imply a similar distribution of RmP in mammalian and amphibian photoreceptors.

The function of RmP in photoreceptors remains a critical open question. As a recessive disorder, Stargardt's disease is presumably due to a loss of RmP function. The ophthalmological findings in patients with early disease is one of pure central-retinal (macular) degeneration [15,33–35]. The predominant histopathological changes in Stargardt's involves the retinal pigment epithelium [36,37]. Presumably, these changes are secondary to a cell-autonomous process occurring in photoreceptors, since RmP is expressed in photoreceptors but not in cells of the pigment epithelium (Fig. 4) [16]. The closest relative of RmP is ABC1 [29]. This widely expressed transporter may be required for the engulfment of cells undergoing apoptotic cell death [38]. A cellular process in retina related to the phagocytosis of cells undergoing apoptosis is the diurnal shedding and phagocytosis of distal outer segments by the pigment epithelium [39]. Although it is tempting to speculate that RmP, similar to ABC1, is playing a role in phagocytosis, the two systems are quite dissimilar. In the case of ABC1, it is the phagocytosing macrophage that expresses the transporter, while RmP is expressed in the phagocytosed outer segment, but not the phagocytosing pigment epithelial cell, since no RmP immunoreactivity was observed in *rd* eyecups. ABC1 has been shown to act as an electroneutral anion exchanger [30], although its physiological substrate has not been identified. A similar ion-transport function for RmP is plausible. The localization of RmP in discs places it adjacent to the plasma membrane, which contains the cGMP-gated cation channel. One hypothesis is that RmP functions to modulate the free Ca^{2+} concentration in outer segments, either

through ATP-dependent Ca^{2+} uptake into discs [40], or light-stimulated passive exchange of H^+ and Ca^{2+} across disc membranes [41,42]. RmP may also represent the light-activated Mg^{2+}-ATPase observed in ROS [43,44]. Allikmets et al. [16] suggest that ABCR may function as a retinoid transporter. This proposed function appears less likely with our identification of ABCR as the outer segment disc protein, RmP. Determining the function for RmP has become all the more urgent with the demonstration now that it is encoded by the gene for recessive Stargardt's.

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