Interaction of 4.1G and cGMP-gated channels in rod photoreceptor outer segments

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SUMMARY

In photoreceptors, the assembly of signaling molecules into macromolecular complexes is important for phototransduction and maintaining the structural integrity of rod outer segments (ROS). However, the molecular composition and formation of these complexes are poorly understood. Using immunoprecipitation and mass spectrometry, 4.1G was identified as a new interacting partner for the cyclic-nucleotide gated (CNG) channels in ROS. 4.1G is a widely expressed multifunctional protein that plays a role in the assembly and stability of membrane protein complexes. Multiple splice variants of 4.1G were cloned from bovine retina. A smaller splice variant of 4.1G selectively interacted with CNG channels not associated with peripherin-2-CNG channel complex. A combination of truncation studies and domain binding assays demonstrated that CNG channels selectively interacted with 4.1G through its FERM and CTD domains. By immunofluorescence, punctate labeling of 4.1G partially co-localized with CNG channels in the ROS. Our studies indicate that 4.1G interact with a subset of CNG channels in ROS and implicate this protein-protein interaction in organizing the spatial arrangement of CNG channels in the plasma membrane of outer segments.

INTRODUCTION

Assembly of proteins into macromolecular complexes within cellular compartments facilitates signal transduction by providing speed and specificity. In rod photoreceptor cells, the special arrangement of protein complexes is partly achieved through the highly ordered structure of the outer segment consisting of a stack of discs enclosed by a plasma membrane. Each disc is made of double membranes embedded with the light-sensitive visual pigment rhodopsin and circumscribed by a specialized hairpin margin referred to as the disc rim. The disc rim and the plasma membrane of the outer segment, each having a distinct protein composition (Molday and Molday, 1987), are connected through dynamic complexes formed by proteins within these structures. Immunoprecipitation and cross-linking studies have shown that the rod photoreceptor cyclic nucleotide-gated (CNG) channel forms a complex with the Na/Ca-K exchanger in the plasma membrane (Molday and Molday, 1998; Schwarzer et al., 2000) and the peripherin-2/rom-1 complex on the disc rim (Poetsch et al., 2001). The latter interaction involving the glutamic acid-
rich protein (GARP) domain of the channel is supported by a recent in situ fluorescence complementation studies in *Xenopus laevis* rod photoreceptors (Ritter et al., 2011). These protein interactions not only facilitate phototransduction, but also help to stabilize the highly ordered structure of the outer segment (Zhang et al., 2009). To date, little is known about additional proteins that may comprise these complexes.

Members of the protein 4.1 family play a critical role in the assembly and stability of protein complexes in plasma membrane. The first member to be discovered 4.1R was shown to be essential for maintaining normal cell shape by connecting plasma membrane proteins to the spectrin-actin cytoskeleton in erythrocytes (Tyler et al., 1979; Ungewickell et al., 1979). To date, four additional 4.1 family members have been described: 4.1G (general (Parra et al., 1998; Walensky et al., 1998)), 4.1N (neural (Walensky et al., 1999)), 4.1B (brain (Parra et al., 2000)), and 4.1O (ovary (Ni et al., 2003)). All members in the 4.1 family share the membrane-binding (FERM) domain, the spectrin-actin binding (SAB) domain, the C-terminal domain (CTD), and non-conserved regions at the N-terminal (U1) and between domains (U2, U3).

The most widely expressed homolog, 4.1G, is abundantly expressed in many tissues including the nervous system (brain, spinal cord, Schwann cells, microglia, and retina) (Ohno et al., 2005; Ohno et al., 2006; Rose et al., 2008), heart (Pinder et al., 2012), testis (Ohno et al., 2005; Terada et al., 2010) and adrenal gland (Wang et al., 2010). The gene encoding 4.1G has one translation initiation site but undergoes extensive, tissue-specific alternative splicing giving rise to multiple isoforms, some of which lack the SAB domain (Wang et al., 2010; Yang et al.).

Various physiological functions have been assigned to 4.1G due to its ubiquitous expression and its increasing number of interacting partners, a majority of which are ion channels and receptors including SERC2 (Pinder et al., 2012), GluR1 and GluR4 (Coleman et al., 2003), parathyroid hormone receptor (Saito et al., 2005), metabotropic glutamate receptor (Tateyama and Kubo, 2007) and adenosine receptor (Lu et al., 2004a; Lu et al., 2004b). 4.1G has been implicated in increasing the surface membrane localization of these channels and receptors, and more specifically, directing proteins to lipid rafts where specific networks of signaling proteins congregate in the plasma membrane (Gibson et al., 2012). In the nervous system, 4.1G is required for the precise
localization of glial adhesion molecules and axonal proteins in the internodes (Ivanovic et al., 2012). 4.1G is also essential for the assembly of tight junction protein complexes in neuroglia (Xia and Liang, 2012) and the assembly of extracellular matrix adhesion sites in astrocytes (Jung and McCarty, 2012).

In the retina, 4.1G has been found in the neuronal synaptic layers, as well as in the photoreceptor layer (Rose et al., 2008) by immunofluorescence microscopy. An earlier proteomics study also detected the presence of 4.1G in bovine rod photoreceptor outer segment (ROS) preparations (Kwok et al., 2008). However, the role of 4.1G in photoreceptors remains elusive. In this study, we have identified 4.1G as a binding partner of the CNG channel in photoreceptor rod outer segments and examined its mode of interaction.

RESULTS

4.1G Interacts with the Rod Cyclic Nucleotide-gated (CNG) Channel

To identify interacting partners of the rod CNG channel, an antibody against the beta (CNGB1) subunit was used to immunoprecipitate the channel and associated proteins from bovine rod outer segment (ROS) for analysis by mass spectrometry. Beside the two CNG channel subunits (CNGA1 and CNGB1), 4.1G (also known as 112 kDa protein) was detected with a high level of confidence (Table 1A). A total of 14 peptides of 4.1G were found of which 11 were unique. In addition, peripherin-2 (peripherin/Rds) and sodium/potassium-calcium exchanger (SLC24A1) were also among the top proteins identified confirming the previously established interaction of these proteins as a complex (Molday and Molday, 1998; Poetsch et al., 2001; Schwarzer et al., 2000).

Western blotting of the immunoprecipitates using the polyclonal antibody against the carboxyl-terminal of 4.1G confirmed the interaction and further revealed that the 4.1G polyclonal antibody recognized two dominant variants in ROS (Fig. 1A, Input). Only the smaller 4.1 G variant co-immunoprecipitated with CNGB1 (Fig. 1A, Elute). Other weaker bands were detected by the 4.1G antibody, but whether these bands are additional variants of 4.1G or degraded products remains to be determined. Immunoprecipitation with the anti-CNGA1 antibodies also pulled down 4.1G from bovine ROS supporting their interaction as a complex (Fig. 1A).
To further establish their interaction, the 4.1G polyclonal antibody was used to immunoprecipitate 4.1G and associated proteins from bovine ROS. The top proteins identified with a high level of confidence were 4.1G (Epb41l2) and the CNGA1 and CNGB1 channel subunits (Table 1B). At the time of the analysis, the complete sequence of bovine 4.1G was not published. Hence, only peptides of the mouse and human 4.1G were identified. However, these peptides are completely conserved with those from bovine 4.1G as revealed by later sequence analysis. Western blotting of the 4.1G immunoprecipitation complex using anti-CNGA1 and anti-CNGB1 antibodies supported their interaction (Fig. 1B). 4.1G was also detected by mass spectrometry as one of the main proteins in immunoprecipitates of CNGA1 from membranes prepared from mouse retinal extracts prepared by centrifugation (Fig. 1C, Table 1C). The interaction of 4.1G and CNGA1/CNGB1 observed in both the bovine and mouse retina supports the significance of this binding. Intriguingly, as observed in bovine ROS, western blotting with the 4.1G antibody revealed that only the shorter 4.1G variant of ~100 kDa (Fig. 1C) co-precipitated with CNGA1 and CNGB1 from mouse retinal membranes. These observations suggest that each variant may have specific roles even within the same cellular environment conforming to the known complexity and functional diversity of splice variants in the 4.1 protein family.

The CNG channel located on the plasma membrane of photoreceptor ROS has been shown to interact with the peripherin-2-rom-1 complex on the rim region of the disc membrane (Poetsch et al., 2001), thereby forming a physical linkage that is important for the structural organization and stability of the outer segment. However, 4.1G does not appear to participate in this channel-peripherin-2 complex as immunoprecipitation with an anti-peripherin-2 antibody failed to pull down any 4.1G (Fig. 1D). This indicates that 4.1G selectively binds to a population of the channels that is free of peripherin-2.

**Splice Variants of 4.1G in Retina**

Since the complete mRNA sequence of the bovine 4.1G was not available at the initiation of this project, the novel observation of a 4.1G splice variant interacting with the CNG channel motivated the cloning of the retinal 4.1G variants and the generation of a highly
specific 4.1G antibody in order to gain further understanding of the mechanism of this interaction.

The 4.1G protein in bovine retina was originally detected in one of the first proteomics studies of photoreceptors (Kwok et al., 2008). Twelve peptides from a protein called 112 kDa protein with the accession number IPI00697691 were among the peptides detected in this proteomic study. Bioinformatics search of IPI00697691 resulted in a match in the EMBL database as the bovine protein sequence UPI0000F3237F. To find the corresponding nucleotide sequence, Blast search (tBlastn) of UPI0000F3237F against the National Center for Biotechnology Information (NCBI) database yielded the predicted bovine miscellaneous RNA, LOC538959, which is comprised of 4387 nucleotides and two tandem open reading frames (ORF), interrupted with a stop codon after the first 159 amino acids. Pairwise alignment of the ORFs of LOC538959 and UPI0000F3237F showed almost perfect identity other than the premature stop codon (Fig. 2). The EMBL protein sequence encodes a protein of 1001 amino acid (aa) residues with a predicted molecular weight of 112 kDa. It was also predicted to have conserved domains shared among the 4.1 protein family including the FERM domain, the SAB domain and the C-terminal domain (CTD). The RNA sequence of LOC538959 surrounding the start codon and the final stop codon were used to design primers to clone the full-length 4.1G from bovine retinal tissue.

Amplification of full-length 4.1G coding region in the bovine retina resulted in three PCR products with sizes of 2205 basepairs (bp) (short), 2775 bp (middle) and 2922 bp (long). Sequence analysis revealed that the short transcript lacks exons 12, 13 and 14; while the middle transcript lacks exons 12 and 13, and the long transcript lacks only exon 13 (Fig. 3A). In the EMBL database, there is supporting evidence for 4.1G cDNAs without exons 12-14, such cDNAs have been sequenced in human (Q68DV2.1), mouse (Q80UE5.1) and orangutan (Q5RC68.1). The cloned bovine 4.1G cDNAs have deduced ORF encoding proteins of 734, 924 and 973 aa, respectively. Pairwise alignment revealed the absence of aa residues 607 to 866, 607 to 676, and 607 to 627 of the predicted UPI0000F3237F in the short, middle and long cDNA, respectively, corresponding to the SAB domain and the U3 region of the predicted 4.1G. The adrenal gland 4.1G is another isoform that does not contain the SAB domain (Wang et al., 2010),
though this domain is observed in most of the 4.1G proteins characterized to date (Ramez et al., 2003; Taylor-Harris et al., 2005).

Expression of Cloned 4.1G Variants

To investigate the relative expression levels of the various variants, the 4.1G cDNAs encoding a 1D4-tag at the carboxyl terminal were cloned into pcDNA3 and transfected into HEK-293 cells. The expressed 4.1G proteins were analyzed by immunoblotting with the tag-specific Rho-1D4 antibody. The apparent sizes of the bovine 4.1G variants (125, 180 and 200 kDa) in HEK-293 cells (Fig. 3B) were larger than their predicted molecular weight. Similar observations were noted in 4.1G proteins expressed in other tissues (Parra et al., 1998; Peters et al., 1998), as well as other members of the 4.1 family: 4.1R (Conboy et al., 1991; Tang et al., 1990), 4.1N (Walensky et al., 1999) and 4.1B (Parra et al., 2000). In the negative control, no protein was detected with the Rho 1D4 antibody in non-transfected cells.

Specificity of 4.1G Antibodies

The specificity of 4.1G monoclonal (Epb41l2-13B2) and polyclonal antibodies was confirmed by immunoblotting the endogenous 4.1G from bovine and mouse retina (Fig. 4A). The monoclonal antibody recognized a single band migrating with an apparent molecular weight of 125 kDa in the bovine ROS and a band of ~140 kDa in the mouse retinal membrane lysate. In contrast, the polyclonal antibody recognized two dominant bands (125 and 150 kDa) in the bovine ROS and multiple bands in the mouse retina with a prominent band of ~140 kDa. The presence of multiple 4.1G variants detected in the retina is in general agreement with that observed in the mouse brain (Parra et al., 2000; Pinder et al., 2012), testis (Yang et al., 2011) and muscle (Okumura et al., 2010). The difference in labeling pattern observed with the two antibodies may be attributed to their immunological strength with the polyclonal antibody being stronger allowing the detection of weakly expressed variants. Furthermore, the polyclonal antibody was generated against a 20 aa region in the C-terminal domain that is conserved between human, mouse and bovine. This region shares an overall 60% similarity with other 4.1 homologs, specifically 90% with 4.1B, 80% with 4.1R and 70% with 4.1N. The
sequence similarity suggests the possibility of cross-reactivity with other 4.1 members as observed for antibodies generated against similar region (Scott et al., 2001). Due to the nature of the anti-4.1G polyclonal antibody, it was used only for immunoprecipitation studies in which the identities of the proteins could be confirmed by mass spectrometry.

To determine the epitope of the Epb41l2-13B2 monoclonal antibody, truncation constructs of 4.1G were generated. Subsequently, smaller constructs of the U1 region were produced with an increment of 50 aa residues to refine the antibody binding site. The epitope of antibody was mapped to the region spanning 62 – 112 aa in the U1 region of 4.1G. According to pairwise alignment, this region shares high similarity (74%) between the bovine and the mouse protein sequence. In terms of homolog specificity, it has been widely noted that there is limited homology among the U1 domain in the 4.1 family members. Moreover, the region of the antibody epitope (62 – 112 aa) in 4.1G shares only 28% similarity with 4.1R and is absent in 4.1B and 4.1N, attesting to the homolog specificity of the monoclonal antibody.

The physiological importance of the various 4.1G clones was assessed by a visual comparison of 4.1G in the retina and the heterologously-expressed protein by gel electrophoresis. Figure 4B showed that the dominant variant found in bovine ROS (125 kDa) is of similar molecular weight as the short bovine 4.1G (734 aa). The middle variant of bovine 4.1G (924 aa) is of similar size to a faint band migrating at ~180 kDa in the bovine ROS and is of the same apparent size as one of the endogenous 4.1G variants found lysates from mock-transfected HEK-293 cells. Since the major short bovine 4.1G clone appears to be the most physiological relevant variant in the retina, this clone was selected for domain interaction studies.

**FERM Domain of 4.1G is Required for Interaction with the CNG Channel**

The bovine 4.1G clone was used to further confirm the association between 4.1G and the rod CNG channel. Transient double-transfections of HEK-293 cells with cDNAs encoding 3F4-tagged 4.1G and CNGA1 or CNGB1 were performed. The 4.1G-3F4 co-immunoprecipitated with CNGA1 and CNGB1 subunits (Fig. 5A). This amount, however, was less than that observed in immunoprecipitation studies of bovine and mouse retinal tissues suggesting that additional photoreceptor proteins or photoreceptor-
specific post-translational modifications may be needed to enhance the interaction of 4.1G and the channel subunits.

To determine the domain of 4.1G required for the binding of CNGA1 and CNGB1, deletion constructs of 4.1G were co-transfected with either CNGA1 or CNGB1 into HEK-293 cells and co-immunoprecipitation experiments were performed (Fig. 5B). 4.1G lacking the U1 domain co-immunoprecipitated with CNGA1 and CNGB1. However, further deletion of the FERM domain abolished the ability of 4.1G to interact with CNGA1 and CNGB1. Interestingly, deletion of the CTD domain also prevented this interaction. These studies suggest that both the FERM and CTD domains are required for optimal interaction of 4.1G with the CNG channel subunits.

To further delineate the 4.1G binding site for the channels, individual domains fused with either GST or MBP were incubated with bovine ROS. CNGA1 and CNGB1 co-precipitated with MBP-FERM, but failed to co-precipitate with the GST-U1 or GST-CTD (Fig. 5C). Control experiment using MBP alone did not pull-down any CNG channel subunits. These results indicate that the 4.1G-FERM domain is crucial for the binding of CNGA1 and CNGB1. The 4.1G-CTD may play a role in modulating the interaction.

**Comparison of 4.1G and CNGs Distribution in Photoreceptors**

To examine the localization of 4.1G in photoreceptors, mouse retinal sections were labeled with the 4.1G isoform-specific monoclonal antibody (Epb41l2-13B2). Punctate staining was detected throughout the length of the photoreceptor outer segments with additional labeling observed in the outer nuclear layer (Fig. 6). Double labeling with Epb41l2-13B2 and anti-CNGB1 polyclonal antibody showed partial co-localization of 4.1G and CNGB1 in the outer segment of photoreceptors (Fig. 6). The presence of 4.1G in the specialized photoreceptor outer segment compartment was further established by western blotting of highly pure ROS membranes prepared by hypotonic lysis of ROS followed by flotation on 5% Ficoll solution. The amount of 4.1G detected in these ROS membranes was dependent on the addition of 2 mM MgCl₂ (Fig. 7 left panel). In ROS membranes treated with EDTA used to chelate Mg ions, the majority of 4.1G was found in the soluble fraction (Fig. 7 right panel).
DISCUSSION

4.1 proteins exhibit diverse and complicated expression patterns attributed to alternative splicing with multiple splice variants often found within a certain tissue. Multiple splice variants of 4.1G were detected in the retina of both bovine and mouse with our anti-4.1G polyclonal antibody. An impressive molecular heterogeneity of 4.1G has been reported in various tissues with the apparent molecular weight of splice variants ranging from 70 to 200 kDa. Most studies have focused on human and rodent tissues, but alternative splicing of 4.1G is also common in the bovine retina as evident by the multiple cDNAs cloned in this study. Interestingly, only one of the cDNAs obtained from bovine retinal tissue encoded a protein with similar molecular weight as one of the endogenous 4.1G variants in the retina. Furthermore, an even longer variant in bovine retina is predicted by the peptide sequences obtained from the mass spectrometry. However, this hypothetical longer 4.1G is likely to be expressed at a low level since the peptide specific to this variant was only detected once out of the three mass spectrometry experiments performed on bovine retina.

It is conceivable that each 4.1G splice variant could play a unique role within the same cell as they differ in their structural motifs. Alternatively, the different splice variants may be expressed in distinct cells or cellular compartments. The smaller variant in the bovine retina identified in this study (125 kDa) lacks the spectrin-actin binding (SAB) domain suggesting that it has acquired a function outside of the conventional role of 4.1 as an adaptor protein linking membrane proteins to the spectrin-actin cytoskeleton. As shown by immunoprecipitation and western blotting, this specific variant interacts with the CNG channel in the plasma membrane of rod photoreceptor outer segments. The finding that only peptides located within the short variant was found by mass spectrometry supports the selective preference of this variant in outer segments. Binding to the CNG channel requires the FERM domain and to some extent the CTD domain of 4.1G. Both these domains have been shown to be responsible for protein-protein interaction of 4.1 proteins. In particular the CTD domain appears to be associated with the binding of membrane receptors more frequently (Gibson et al., 2012; Lu et al., 2004a; Lu et al., 2004b; Rose et al., 2008). In contrast to the selective interaction of the SAB-less 4.1G with CNG channels observed in the retina, all of the three 4.1G variants in the
mouse testis were co-immunoprecipitated with nectin-like 4 (NECL4) through binding of the FERM domain (Yang et al., 2011). The mechanism regulating the specific variant interaction is perplexing as the domain of interaction is present in all variants. Additional 4.1G domains, binding proteins or even ions that mediate this selection remain to be determined. There is recent evidence suggesting that calcium may play a role in regulating the activity of 4.1G. A conformational change is induced in the 4.1G protein when a calcium binding protein associates with the U1 domain of 4.1G (Nunomura et al., 2013). Moreover, our studies have found that 4.1G is detected in the Ficoll-prepared ROS only in the presence of MgCl₂, further supporting a degree of ion-dependence on the interaction of 4.1G with the CNG channel.

It is interesting to note that while the CNG channels are specific in binding to the SAB-less 4.1G variant, 4.1G itself also discriminates against CNG channels that are complexed with peripherin-2. There may be a functional purpose for having distinct populations of CNG channels. The 4.1G-bound population may serve a functional role in targeting and accumulating CNG channels to the plasma membrane in order to achieve the speed required for phototransduction. The peripherin-2-bound population of CNG channels has been implicated in playing a structural role in maintaining proper spatial relationship between the plasma membrane and the disc membrane within the outer segments (Poetsch et al., 2001; Zhang et al., 2009). However, whether CNG channels and peripherin-2 serve as the sole factors in providing the structure integrity of photoreceptor outer segments remains debatable as relatively limited photoreceptor structural defects were observed in mice lacking CNGB1 (Hüttl et al., 2005; Zhang et al., 2009).

In this study, mass spectrometry analysis failed to detect further confirmable interacting partners of 4.1G. Ankyrin G has been reported to co-immunoprecipitate with CNG channels in the mouse retina. These studies have implicated ankyrin G in the transport of CNG channels to rod photoreceptor cilia (Kizhatil et al., 2009). Ankyrin G is similar to 4.1 proteins in that it acts as an adaptor linking membrane proteins to the cytoskeleton. The mass spectrometry analysis performed on samples immunoprecipitated with the CNGA1 and CNGB1 antibodies failed to detect ankyrin G peptides. In fact, ankyrin G has not been found in mass spectrometric-based proteomic studies of
photoreceptor outer segments (Kiel et al., 2011; Kwok et al., 2008; Reidel et al., 2011; Skiba et al., 2013). Considering the size of this protein it is surprising that these proteomics studies have failed to detect peptides from ankyrin G in outer segment preparations. This discrepancy remains to be reconciled. Interestingly, another adaptor protein known as the cytoskeleton-associated protein 4 (Ckap4) co-immunoprecipitated with CNGA1 (Table 3). Ckap4 is a transmembrane protein anchoring the endoplasmic reticulum (ER) to microtubules (Klopfenstein et al., 1998) through which the proper spatial distribution of ER with respect to the nucleus is maintained (Vedrenne et al., 2005). It remains to be determined if Ckap4 is interacts with the CNGA1 within the ROS.

The Epb41l2-13B2 monoclonal antibody revealed a punctate staining pattern of 4.1G throughout the length of the photoreceptor outer segments where it partially co-localized with the CNG channels. This punctate labelling pattern has also been documented for 4.1R in the heart (Baines et al., 2009; Pinder et al., 2012), 4.1B at the region of cell-cell contact in the brain (Parra et al., 2000), and 4.1G at tight junctions of OLN-93 cells (Xia and Liang, 2012). This high local density of 4.1 proteins has been implicated in facilitating signal transduction by promoting cell surface expression and stable retention of transmembrane proteins, and accumulating signalling molecules at precise cellular compartments. Whether 4.1G has any effect on the activity and the surface expression level of CNG channels or on the structural integrity of photoreceptor outer segments remains to be investigated. Analysis of the retina from 4.1G-knockout mice would greatly enhance our understanding of the role of 4.1G in photoreceptor outer segment structure and function.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All protocols and procedures conformed to the University of British Columbia policy and were approved by the UBC Committee on Animal Care.

RT-PCR and Amplification of Full-Length cDNAs
Total RNA was isolated from bovine (Bos taurus) retinal tissue using RNeasy mini kit (QIAGEN, Maryland, MA) and was reverse transcribed using One-Step RT-PCR kit (QIAGEN) according to manufacturer instructions. The primers used for the amplification of 4.1G were: Bovine forward- 5’ ATA AGC TGT GAC CAT GAC TAC TGA AG 3’; Bovine reverse- 5’ CTC TTC CCC CTC CTC CAA CTC 3’. Cycling conditions were 30 min at 50°C, 15 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 62°C, and 3 min at 68°C, and a final elongation step of 10 min at 68°C. The resulting PCR products were cloned into pcDNA3 and all clones were sequenced at GENEWIZ, Inc (Seattle, WA). The GenBank accession numbers for the bovine short, middle and long 4.1G cDNAs are KF306276, KF306277, and KF306278 respectively.

**Generation of Antibodies to 4.1G**

The full-length bovine 4.1G with a 1D4 tag cloned in pcDNA3 was expressed in HEK-293 cells and purified with an anti-1D4 antibody column. Purified bovine 4.1G-1D4 was used to immunize mice and the spleen cells were fused with NS-1 mouse myeloma cells to generate hybridoma cell lines producing monoclonal antibodies against 4.1G. Culture fluid from hybridoma cells (Epb4112-13B2) was used at a dilution of 1:10 for Western blotting and undiluted for immunofluorescence labeling studies. The polyclonal antibody against 4.1G was generated by immunizing rabbits with a synthetic peptide containing HDQALAQAIREAREQHPDMS at YenZym Antibodies (South San Francisco, CA). The selected region of amino acid is conserved between human, bovine and mouse. For immunoprecipitation studies, purified 4.1G polyclonal antibodies were covalently coupled to CNBr-activated Sepharose 2B (GE Healthcare, Mississauga, ON) at 2 mg of protein/ml of packed beads.

**Antibodies**

The generation of anti-CNGA1 (PMc-1D1), anti-CNGB1 (Garp-8G8), and anti-peripherin-2 (Per-2B6) monoclonal antibodies has been described previously (Cook et al., 1989; Molday et al., 1987; Poetsch et al., 2001). For the detection of the 1D4 and 3F4 tags, Rho-1D4 and Rim-3F4 antibodies were used respectively (Illing et al., 1997; MacKenzie et al., 1984). Polyclonal antibody to the GARP part of CNGB1 has been
previously described (Colville and Molday, 1996). Anti-GST monoclonal antibody was purchased from Rockland Immunochemical (Gilbertsville, PA) and anti-MBP antibody was purchased from New England BioLabs (Ipswich, MA).

**Truncation of 4.1G**

Deletion constructs of 4.1G with a 3F4 tag at the C-terminal were generated by PCR and subcloned into pcDNA3. Primers for ΔU1 were: forward- 5’ AAA ACT GTC CAG TGT AAA G 3’; reverse- 5’ CTC TTC CCC CTC CTC CAA CTC 3’. Primers for ΔFERM were: forward- 5’ CAG CCA CCA AAG GCC AA 3’; reverse- 5’ CTC TTC CCC CTC CTC CAA CTC 3’. Primers for ΔCTD were: forward- 5’ ATA AGC TGT GAC CAT GAC TAC TGA AG 3’; reverse- 5’ TTA GGA GAT TTC TGT CCT TTG 3’. All constructs were sequenced at GENEWIZ, Inc.

For mapping the epitope of the Epb41l2-13B2 monoclonal antibody, the U1 domain of 4.1G was further truncated into smaller fragments which were subcloned into pGEX-4T1 to make GST-fusion proteins. Primers used were: U1-210aa forward- 5’ AAA ACT GTC CAG TGT AAA G 3’; U1-148aa forward- 5’ CAG AAG AAA GAG AAA GAT 3’; U1-98aa forward- 5’ TTA GAC AAG GAG GAA TCT CT 3’; U1-48aa forward- 5’ GAA GAG AAG GTG AAG GAA AT 3’. Reverse primer 5’ TTA-TTT ACA CTG GAC AGT TTT GGT 3’ was used for all U1 domain truncated constructs. The identity of all constructs was confirmed by sequencing at GENEWIZ, Inc.

**Cell Culture Techniques**

HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Carlsbad, CA) containing 8% (v/v) bovine growth serum (Life Technologies) in 5% CO2. Cells were transfected with calcium phosphate and BES-buffered system. After 48 h, cells were harvested and experiments were performed.

**Preparation of Retinal Tissue**

Bovine ROS was isolated from frozen bovine retinas by sucrose gradient centrifugation as described previously (Molday and Molday 1993; Holopainen, Cheng et al. 2010). The procedure for isolating a sample of a more pure bovine ROS membranes by Ficoll
flotation method was described previously (Smith, Stubbs et al. 1975; Molday and Molday 1993). In brief, the crude ROS was subjected to hypotonic lysis in buffer with 10 mM Tris with or without 2 mM MgCl2 and subsequently purified by flotation on 5% Ficoll solution.

Preparation of mouse retinal membranes has been described previously (Molday, Djajadi et al. 2013). Briefly, 6 dissected mouse retinas were immersed in 400 ml Tris buffer saline (TBS: 20 mM Tris, pH 7.4, 0.1 M NaCl and cOmplete protease inhibitor (Roche)) for 20 min on ice. The suspension was then passed through a 22 g and 28 g needle, layered on top of 1.6 ml of 60% (w/w) sucrose/TBS, and centrifuged at 25,000 rpm for 30 min in a TLS-55 rotor using a Beckman TLX Optima centrifuge. The retinal membrane fraction which banded on top of the 60% sucrose was removed, diluted with 3 volumes of TBS, and pelleted by centrifugation at 30,000 rpm for 10 min in a TLA 100.4 rotor.

**Immunoprecipitation and Immunoblotting**

Co-immunoprecipitation experiments were performed with retinal tissue lysate and lysate from HEK-293 cells transfected with various combinations of 4.1G truncation and CNG constructs. Total protein from retinal tissues or transfected cells was extracted with lysis buffer (20 mM Tris, 150 mM NaCl, 1% TX-100), and lysate was incubated with either anti-4.1G, anti-CNGA1 or anti-CNGB1 antibody column for 2 h at 4°C. The column was washed extensively and bound proteins were eluted with 2% (w/v) SDS. The eluted proteins were analyzed by SDS-PAGE and immunoblotted with antibodies to 4.1G, CNGA1 and CNGB1. Detection was performed with anti-mouse Ig conjugated with Alexa 680 and anti-rabbit Ig conjugated with Alexa 800 (dilution 1:20,000, Life Technologies) and imaged by the LI-COR Odyssey system (Lincoln, NE).

**In-gel Digestion of Proteins for Proteomics Analysis**

Procedure for handling samples for proteomic analysis has been described previously (Kwok et al., 2008). In brief, each lane of an SDS gel containing the eluate from the
immunoprecipitation studies was cut into small pieces and digested with trypsin. Digested samples were submitted to Proteomics Core Facility of Center for High Throughput Biology (CHiBi) at the University of British Columbia. Peptide mass fingerprinting was performed by FTMS with a MASCOT database search for protein identification.

**Immunofluorescence Microscopy**

The procedures for the fixation and labeling of retinal tissue have been described previously (Cheng et al., 2013; Holopainen et al., 2010). In brief, whole mouse eye cups were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4 for 2 h and subsequently rinsed and cryoprotected in 0.1 M PB containing 20% sucrose (w/v). Cryosections were labeled overnight with primary antibody, rinsed, then labeled for 1 h with secondary antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 568 (Life Technologies) and counterstained with DAPI nuclear stain (Life Technologies). The stained sections were examined under a Zeiss LSM700 confocal microscope (Carl Zeiss, Toronto, ON) and processed with Zeiss Zen Image Browser.

**GST/MBP-Pulldown Technique**

U1 and CTD domain of 4.1G was cloned into pGEX-4T1 to generate GST-U1 and GST-CTD fusion proteins. The primers used to amplify the U1 domain were: forward- 5’ AAA ACT GTC CAG TGT AAA G 3’; reverse- 5’ TTA-TTT ACA CTG GAC AGT TTT GGT 3’. The primers used to amplify the CTD domain were: forward- 5’ CAG CCA CCA AAG GCC AA 3’; reverse- 5’ CTC TTC CCC CTC CTC CAA CTC 3’. The FERM domain was cloned into pMAL-C2 to generate maltose binding protein (MBP)-FERM fusion protein. The primers used to amplify the FERM domain were: forward- 5’ GTC CTG GCC AAA GTG ACC CTT 3’; reverse- 5’ TTA AGA CAC AAG CCT GTA GAA AGT 3’. The GST-U1, GST-CTD, MBP-FERM fusion proteins were expressed in BL21 bacterial cells, purified and immobilized on glutathione (GE Healthcare), or amylose (NEB) resin. Bovine retinal lysate was added to the resin and incubated for 3 h at 4°C. The resin was washed extensively and bound proteins were eluted with 2% (w/v) SDS. The eluted proteins were analyzed by SDS-PAGE and immunoblotted with
antibodies to GST/MBP, CNGA1 and CNGB1. Immunoreactive bands were visualized after incubation with secondary antibodies coupled to horseradish peroxidase (GE Healthcare) and with the chemiluminescence detection kit (GE Healthcare).

ACKNOWLEDGEMENTS

We thank Dr. Ming Zhong for help with initial cloning of 4.1G and Theresa Hii for technical assistance with 4.1G antibody production. We also thank Laurie Molday and Hidayat Djajadi for their expertise and assistance in the subretinal injection. This work was supported by a grant from the National Institutes of Health [grant no. EY002422]. RSM holds a Canada Research Chair in Vision and Macular Degeneration.

AUTHOR CONTRIBUTIONS

C.L.C designed and performed experiments, interpreted data and wrote the manuscript. R.S.M interpreted data and edited the manuscript.
REFERENCES


Functional Characterization of Protein 4.1B, a Novel Member of the Protein 4.1 Family with High Level, Focal Expression in Brain. *Journal of Biological Chemistry* **275**, 3247 - 3255.


**FIGURE LEGENDS**

**Fig. 1.** Association of 4.1G with CNG channels.

A) Co-immunoprecipitation of 4.1G with CNG channels. Bovine rod outer segments (ROS) were immunoprecipitated with antibodies against CNGA1 (PMc-1D1) (left panel) and CNGB1 (Garp-8G8) (right panel). The fractions (input, unbound and elute) were analyzed by separation on SDS-gel electrophoresis and immunoblot labeled with anti-4.1G antibody (top), anti-CNGA1 antibody (middle) or anti-CNGB1 antibody (bottom). Note that only the smaller 4.1G variants were pulled down with the CNG channels. The larger 4.1G variant that was absent from the bound fraction was present in diminished amount in the unbound fraction compared to the input fraction, possibly due to nonspecific adsorption to the immunaffinity matrix or proteolytic degradation.

B) Reverse co-immunoprecipitation of CNG channels with 4.1G. Bovine ROS were immunoprecipitated with antibody against 4.1G. Western blots were labeled with anti-4.1G antibody (top), anti-CNGA1 antibody (middle) or anti-CNGB1 antibody (bottom).

C) Co-immunoprecipitation of 4.1G with CNGA1. Mouse retinal membranes were immunoprecipitated with antibody against CNGA1. Western blots were labeled with anti-CNGA1 (top) or anti-4.1G (bottom) antibodies. Note that similar to the observation in bovine ROS, only the smaller 4.1G variant was pulled down with the CNGA1.

D) Co-immunoprecipitation of CNG channels with peripherin-2. Bovine ROS were immunoprecipitated by anti-peripherin antibody (Per-2B6). Western blots were labeled with anti-peripherin-2, anti-CNGA1, anti-CNGB1 or anti-4.1G antibody. Note that 4.1G was not pulled down with peripherin-2 or this population of CNG channels.

**Fig. 2.** Schematic representation of bovine 4.1G predicted RNA sequence and protein sequence.

The predicted bovine 4.1G RNA sequence (top, LOC538959) has two translation-initiation sites (ATG) as indicated at positions 91 and 649 basepair (bp) and two stop
codons (TGA) at positions 570 and 3114 bp. Its corresponding open reading frames (ORF) are identical to the protein sequence (1 – 159 aa; 181 – 1001 aa) from the EMBL database (bottom).

**Fig. 3.** Characterization of Retinal 4.1G.

A) Schematic representation of domain and exon structures of the predicted 4.1G protein in comparison with the cloned retinal 4.1G variants. The epitope of the monoclonal antibody against bovine 4.1G was mapped to amino acid residues 62 – 112 (#). Anti-4.1G polyclonal antibody was generated against peptide containing amino acid residues 965 – 984 (*).

B) Western blots of HEK-293 cell lysates labeled with Rho-1D4 antibody. HEK-293 cells were transfected with bovine 4.1G short (lane 2), middle (lane 3) and long (lane 4) variant each having a 1D4 tag at the C-terminal. Non-transfected HEK-293 cell lysate was loaded as control (lane 5). Molecular marker was loaded as reference (lane 1).

**Fig. 4.** Specificity of 4.1G polyclonal and monoclonal antibodies.

A) Western blots of bovine ROS and mouse retinal membranes. The left panel was labeled with the 4.1G polyclonal antibody (pAb) which detected two dominant variants (125 and 150 kDa) in the bovine ROS (lanes 2) and one dominant (140 kDa) in the mouse retina (lanes 3). The middle panel shows a Western blot labeled with the 4.1G monoclonal antibody (mAb) which detected only the dominant variant in the bovine (125 kDa) and mouse (140 kDa) retina. The coomassie blue-stained gel (right panel) is shown for the bovine and mouse samples. Molecular marker was loaded as reference (lane 1).

B) Western blots labeled with the 4.1G polyclonal antibody comparing the endogenous 4.1G variants in the bovine ROS with the cloned 4.1G exogenously expressed in HEK-293 cells. Lane 1, molecular marker; lane 2, lysate of bovine ROS; lane 3, cell lysate transfected with short bovine 4.1G; lane 4, cell lysate transfected with middle bovine 4.1G; lane 5, mock transfection. Note that the short bovine 4.1G (734aa, lane 3) was of similar molecular weight as the most dominant variant in the bovine retina (lane 2), which were both approximately 125 kDa. The 4.1G antibody also detected the endogenous 4.1G in all HEK-293 cell samples, including in the mock-transfected cell
lysate (~200 and 150 kDa, lane 5). An immunoblot labeled with Rho-1D4 antibody showed the expression of the 4.1G variants.

**Fig. 5.** Domains of 4.1G Required for Binding to CNG Channels.

A) Co-immunoprecipitation of 4.1G with CNG channels. Lysates of HEK-293 cells co-transfected with 4.1G-3F4 and CNGA1 (left panel) or CNGB1 (right panel) were incubated with anti-CNGA1 (PMc-1D1) or anti-CNGB1 (Garp-8G8) antibody column, respectively. Western blots of various fractions (input, unbound, wash and elute) were labeled with anti-CNGA1 (top left) or anti-CNGB1 (top right) antibody and Rim-3F4 antibody (bottom).

B) Co-immunoprecipitation of 4.1G deletion mutants with CNG channels. Lysates of HEK-293 cells co-transfected with deletion construct of 4.1G and CNGA1 (left panel) or CNGB1 (right panel) were incubated with anti-CNGA1 or anti-CNGB1 antibody column, respectively. Western blots were labeled with Rim-3F4 to detect 4.1G full length (top) or deletion mutants (bottom 3) all of which had a 3F4 tag at the C-terminal. Note that 4.1G lacking the FERM domain or the CTD domain failed to co-immunoprecipitate with CNGA1 and CNGB1.

C) Co-immunoprecipitation of CNG channels with MBP-FERM. The domains of 4.1G (U1, FERM, CTD) were fused to either MBP or GST and incubated with bovine ROS and immunoprecipitated with either amylose resin or glutathione resin. Western blots of the input and elute fractions were labeled with anti-CNGA1 (left panel), anti-CNGB1 antibody (middle panel) or an antibody against MBP or GST (right panel). Note that CNGA1 and CNGB1 were co-immunoprecipitated with MBP-FERM only. MBP and GST alone were used as control.

**Fig. 6.** Localization of 4.1G in photoreceptors in the mouse retina. Immunofluorescence labeling of mouse retinal cryosection with monoclonal antibody against 4.1G and anti-CNGB1 antibody. Punctate staining of 4.1G in the photoreceptor outer segments partially co-localized with CNGB1 labeling.

**Fig. 7.** Distribution of 4.1G in bovine photoreceptors. SDS-polyacrylamide gels and immunoblots of bovine ROS membrane preparations. Lanes 1, molecular weight makers; lanes 2, crude ROS prepared by sucrose gradient;
lanes 3, ROS membrane vesicles prepared by hypotonic lysis of ROS followed by flotation on Ficoll with the addition of 2 mM MgCl₂; lanes 4, respective ROS soluble fraction (MgCl₂); lanes 5, Ficoll ROS membrane vesicles prepared in the presence of 1 mM EDTA; lanes 6, respectively ROS soluble fraction (EDTA). The SDS-polyacrylamide gel was either stained with Coomassie blue or transferred to Immobilon membranes and labeled with anti-CNGA1 or anti-4.1G antibody. The soluble fraction was not contaminated with the membrane fraction as CNGA1 was not detected in this fraction (lane 4 and 6). 4.1G was detected in soluble fractions (lane 4 and 6) and it was only present in the ROS membrane fraction when MgCl₂ is added (lane 3).
**TABLE 1.** Proteins Identified by Mass Spectrometry Analysis from Co-immunoprecipitation Experiments.

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A) Proteins that were co-immunoprecipitated with CNGB1 from bovine rod outer segments. Database used was IPI_Bovine_BOVINE_v_315. Significant results (p<0.05) with a score > 70 is shown. Common contaminants such as keratin and trypsin have been omitted. Scoring is probability-based and is reported as -10*LOG10(P) where P is the absolute probability. The Exponentially Modified Protein Abundance Index (emPAI) is an approximate relative quantitation of the proteins in a mixture and is derived from the ratio of the number of experimentally observed peptides over the number of expected peptides for a protein.
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B) Top proteins that were co-immunoprecipitation with 4.1G from bovine rod outer segments. Database used was SwissProt 57.1.

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C) Proteins identified in mass spectrometry of co-immunoprecipitation with CNGA1 from mouse retinal membranes. Database used was SwissProt 57.1.