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RESEARCH ARTICLE

Conservation and divergence of Grb7 family of Ras-binding domains

Raju V. S. Rajala[1,2,3] ✉, Ammaji Rajala[1,3], Vivek K. Gupta[1,3]

[1] Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
[2] Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
[3] Dean A. McGee Eye Institute, Oklahoma City, OK 73104, USA
✉ Correspondence: raju-rajala@ouhsc.edu
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ABSTRACT

Ras proteins are signal-transducing GTPases that cycle between inactive GDP-bound and active GTP-bound forms. Ras is a prolific signaling molecule interacting with a spectrum of effector molecules and acting through more than one signaling pathway. The Ras-effector proteins contain a Ras-associating (RA) domain through which these associate with Ras in a GTP-dependent manner. The RA domain is highly conserved among the members of the growth factor receptor-bound (Grb) 7 family of proteins which includes Grb7, Grb10 and Grb14. Our laboratory has reported an unusual observation that RA domain of Grb14 binds to the C-terminal nucleotide binding site of cyclic nucleotide gated channel (CTR-CNGA1) and inhibits the channel activity. Molecular modeling of the CTR-CNGA1 displays 50%—70% tertiary structural similarity towards Ras proteins. We named this region as Ras-like domain (RLD). The interaction between RA-Grb14 and RLD-CNGA1 is mediated through a simple protein-protein interaction temporally and spatially regulated by light and cGMP. It is interesting to note that Grb14 binds to GTPase-mutant Rab5, a Ras-related small GTPase whereas Grb10 binds only to GTP-bound form of active Rab5 but not to GTPase-defective mutant Rab5. These results suggest that Grb14 might have been evolved later in the evolution that binds to both Ras and nucleotide binding proteins such as CNGA1. Our studies also suggest that eukaryotic CNG channels could be evolved through a gene fusion between prokaryotic ion channels and cyclic nucleotide binding proteins, both of which might have undergone several sequence variations for functional adaptation during evolution.

KEYWORDS growth factor receptor-bound protein 14, Ras-associating domain, cyclic nucleotide gated channel, rod outer segments, tyrosine kinase signaling, Ras proteins

INTRODUCTION

The Ras proteins belong to a low-molecular weight class of G-proteins with an approximate molecular mass of around 20 kDa (Bourne et al., 1990; Karnoub and Weinberg, 2008). The Ras oncoproteins are encoded by three genes: Ki-*ras*, Ha-*ras*, and N-*ras* (Karnoub and Weinberg, 2008). These proteins bind di- or tri-phosphate containing guanine nucleotides (GTP and GDP) for their activation and downstream effects (Karnoub and Weinberg, 2008). These small GTPases cycle between inactive GDP-bound and active GTP-bound forms (Ponting and Benjamin, 1996). In their GTP-bound state, Ras proteins physically interact with their downstream effector proteins, triggering activation of multiple signaling pathways with complex and divergent effects (Kyriakis, 2009). Most of the proteins that interact with Ras proteins possess unique Ras association (RA) or Ras binding domains (RBD) (Ponting and Benjamin, 1996). Ras proteins are prenylated and membrane-associated and Ras binding recruits Ras effectors via RA or RBD (Ras binding domain) to the plasma membrane (McCormick and Wittinghofer, 1996; Magee and Marshall, 1999). In 2000, a new Ras effector was identified, RAS-association domain family 1 (RASSF1), and there are currently 8 members in this family (RASSF1–8) (van der Weyden and Adams, 2007).

In contrast to Ras-like GTPase sequences, RA-domain sequences are divergent and it has been suggested that these RA-domains may bind to small GTPases, but not to Ras (Ponting and Benjamin, 1996). Recently a novel function of an

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RA domain containing protein in photoreceptors has emerged from our studies (Gupta et al., 2010). We found that RA-domain of Grb14 interacts with the nucleotide binding pocket of a cyclic nucleotide gated channel alpha subunit (CNGA1) and inhibits its activity (Gupta et al., 2010, 2011). It is interesting to note that CNGA1 is not a Ras protein; however, both Ras and CNGA1 are nucleotide binding proteins.

The CNG gated channels have a C-terminal cGMP nucleotide binding site. This region is around 200 residues long and depicts sequence similarity to Ras proteins. Biologically, this resemblance translates into the Ras-like domain (RLD) of CNG channels interacting with the cGMP. Grb14 protein is shown to interact with the nucleotide binding region of CNGA1 through its RA domain. This is probably because RA domain recognizes the cyclic nucleotide binding region of CNGA1 as having structural similarities with the Ras proteins. This protein region is approximately of the same size as Ras proteins. In this study we have examined the conservation and divergence of Grb7 family of RA domains.

RESULTS

RA domain of Grb14 interacts with the Ras-like domain of CNG channel

Protein sequence analysis of the C-terminus of CNGA1 (residues 489–653) revealed a 30%–35% similarity with various Ras proteins (Fig. 1A). In the absence of a three dimensional structure of CNGA1, a molecular model of its C-terminal domain (residues 489–653) based on the closely homologous sequences was constructed (Fig. 1B). The homology model was analyzed by programs based on Ramchandaran plot to ensure that most of the residues lie in permissible regions. Analysis of the distortion of the carbon backbone, angles of side-chains, energy minimization and molecular packing yielded scoring analogous to allowed conformations, and distributions of bond lengths and angles were within accepted values for protein structures. The model showed that the C-terminal RLD domain of CNGA1 displays 50%–70% tertiary structural similarity towards Ras proteins (Fig. 1B). The FLAG tagged RLD-CNGA1 was co-expressed in HEK-293T cells with Myc-RA-Grb14. The immunoprecipitation of the FLAG RLD-CNGA1 (Fig. 1C) resulted in the co-immunoprecipitation of Myc-RA-Grb14 (Fig. 1D). Similarly, Myc-RA-Grb14 was able to reciprocally co-immunoprecipitate FLAG RLD-CNGA1 (data not shown). To determine the specificity of FLAG and Myc antibodies, we expressed FLAG-tagged PHLPPL, a serine/threonine phosphatase (Kanan et al., 2010) and Myc-tagged PP2C phosphatase region of PHLPPL in HEK-293T cells. The expressed proteins were subjected to immunoblot analysis with anti-FLAG antibody and the results indicate that FLAG antibody did not cross react with Myc-tagged protein and *vice versa* (Fig. 1E and 1F).

RA domain of Grb14 binds to inactive form of Rab5

Our experiments suggest that CNGA1 has a putative Ras-like domain, and we sought to determine whether Grb14 exhibits the Ras protein binding properties. The Ras protein binding characteristics of Grb7, Grb10 and Grb14 were evaluated by GST pull-down assays. HEK-293T cells were transiently expressed either with pCDNA3 vector or Myc-tagged Grb7, Grb10 or Grb14. The expressed proteins were subjected to GST pull-down assays with GTP-bound form of active Rab5, a small GTPase or GTPase-defective mutant Rab5 (Q79L). The expressed proteins were subjected to GST pull-down assays with Rab5 or mutant Rab5 (Q79L). The results suggest that RA domain of Grb14 is able to bind to both GTP-bound (active Rab5) and mutant Rab5 (Fig. 2A). The Grb10 binds only to Rab5 but not to mutant Rab5 (Fig. 2A) suggesting that Grb10 binds to activated or GTP-bound form of Rab5. We failed to observe any binding interaction between Grb7 and Rab5 or mutant Rab5 (Fig. 2A). Similar results were observed when we incubated the *in vitro* translated Myc-Grb14, Myc-Grb10 and Myc-Grb7 with GST-RLD-CNGA1 and subjected to GST pull-down assay followed by immunoblot analysis with anti-Myc antibody (Fig. 2C). These studies suggest that RA-domains within Grb7 family are divergent and exhibited different binding properties towards Ras related small GTPases.

CTR-CNGA1 does not possess any GTPase activity

CNGA1 is a cGMP-binding protein (Kaupp and Seifert, 2002) and the Ras proteins are small GTPases (Symons and Takai, 2001). In this experiment we determined whether the RLD-CNGA1 has any GTP binding or GTP hydrolysis property, and we expressed RLD-CNGA1 as GST fusion protein and carried out the GTP binding/hydrolysis assay (Liang et al., 2000). GST, GST-Rab5 and GST-mutant Rab5 (Q79L) proteins were used as a positive and negative control. The results indicate that RLD-CNGA1 has no associated GTP binding or hydrolysis properties (Fig. 2D). GST-Rab5 is able to hydrolyze the GTP to GDP whereas mutant Rab5 binds to GTP but fails to hydrolyze the bound GTP (Fig. 2D).

Light-dependent binding of Grb14 to rod outer segment membranes

To determine the binding of Grb14 to rod outer segment (ROS) membranes, dark-adapted wild-type rats were exposed to 300 lux light, sacrificed at 0, 5, 10, 15, 20, 30, 60 and 120 min thereafter, and the retinas were harvested and prepared for the ROS membranes. Equal amount of ROS proteins was subjected to immunoblot analysis with anti-Grb14 and anti-rhodopsin (internal and loading control) antibodies. Densitometirc analysis of Grb14 was carried out and expressed as percentages of Grb14 binding to ROS. The

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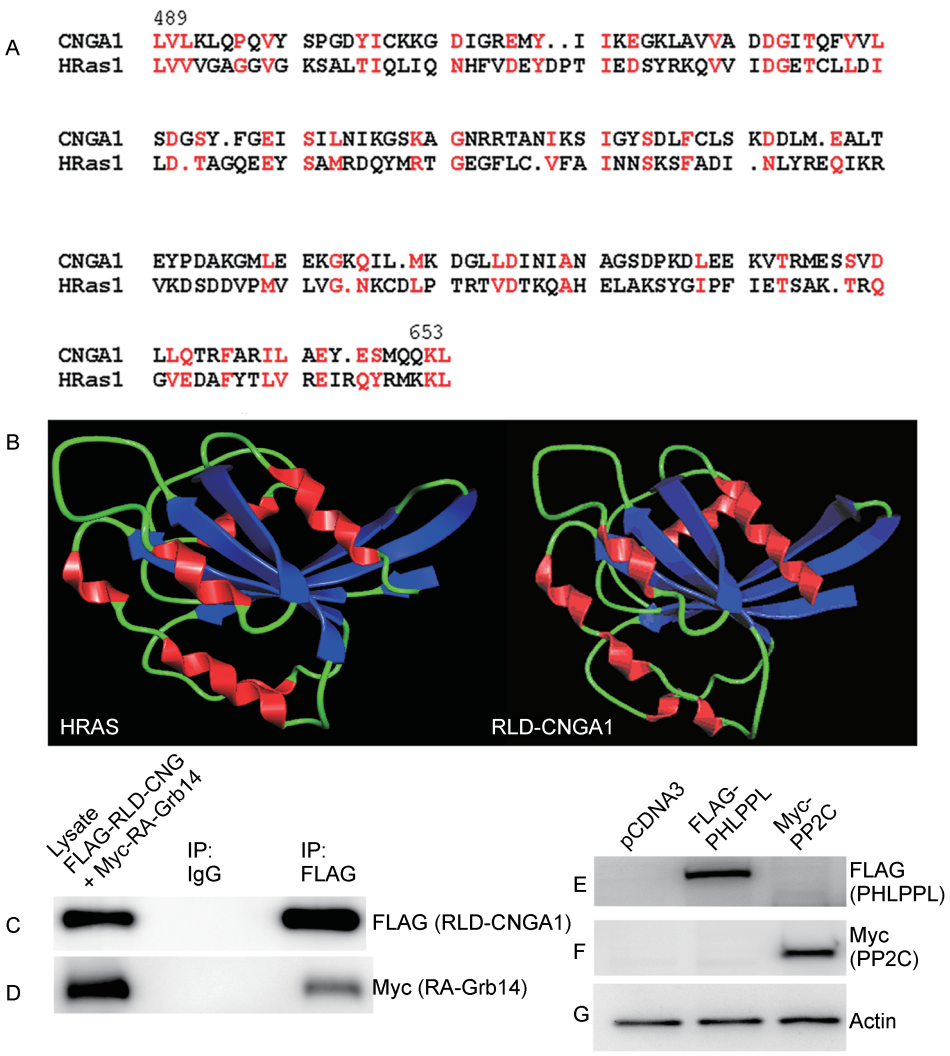

Figure 1. Cytoplasmic C-teminal domain of CNGA1 displays similarity to Ras proteins. The homology was established by multiple sequence alignment and molecular modeling. (A) Multiple sequence alignment of the C-terminal cytoplasmic domain (RLD) of bovine CNGA1 [UniProtKB Accession No. Q00194] with HRas1 [UniProtKB Accession No. P01111]. (B) The tertiary structure of GTPase H-Ras (PDB ID: 3DDC chain A) (left) is compared with the model of RLD of CNGA1 (Right). The corresponding secondary structural folds of the two proteins are highlighted in the same color. RA domain of Grb14 interacts with the RLD domain of CNGA1. (C) Myc-tagged RA-domain of Grb14 was transiently co-expressed with FLAG-tagged RLD-CNGA1 in HEK-293T cells. Protein extracts from cells expressing FlAG-RLD-CNGA1 was subjected to co-immunoprecipitation using IgG or anti-FLAG antibody. (D) The presence of Myc-Grb14-RA in the precipitate was tested by immunoblot analysis using anti-Myc antibody. The expression of proteins in the total lysate from cells is shown in each case. No co-immunoprecipitation was observed in IgG control immunoprecipitates. HEK-293T cells were transiently transfected with either pCDNA3 or wild type FLAG-tagged PHLPPL or Myc-tagged PP2C-phosphatase domain. Lysates were immunoblotted with (E) anti-FLAG, (F) anti-Myc, and (G) anti-actin antibodies.

results indicate the absence of Grb14 in dark-adapted ROS (0 min), and the peak binding of Grb14 to ROS was at 15 min, and the binding was progressively decreased as the time increased from 20 to 120 min (Fig. 3).

Grb14 is localized to ROS plasma membrane

Bovine ROSs were subjected to FICOLL gradient centrifugation to isolate ROS disks (Smith and Litman, 1982). ROS, plasma membrane, and isolated disks were subjected to immunoblot analysis with anti-Grb14, anti-Glut1, and anti-rhodopsin antibodies. The results indicate that Grb14, Glut1, and rhodopsin immunoreactivity was present in the ROS (Fig. 4). Grb14 and the plasma membrane marker Glut1 immunoreactivity was enriched in the plasma membrane fraction of the ROS (Fig. 4). Rhodopsin blot shows the enrichment of rhodopsin in the disk membranes (Fig. 4). These results suggest that Grb14 is localized to the ROS plasma

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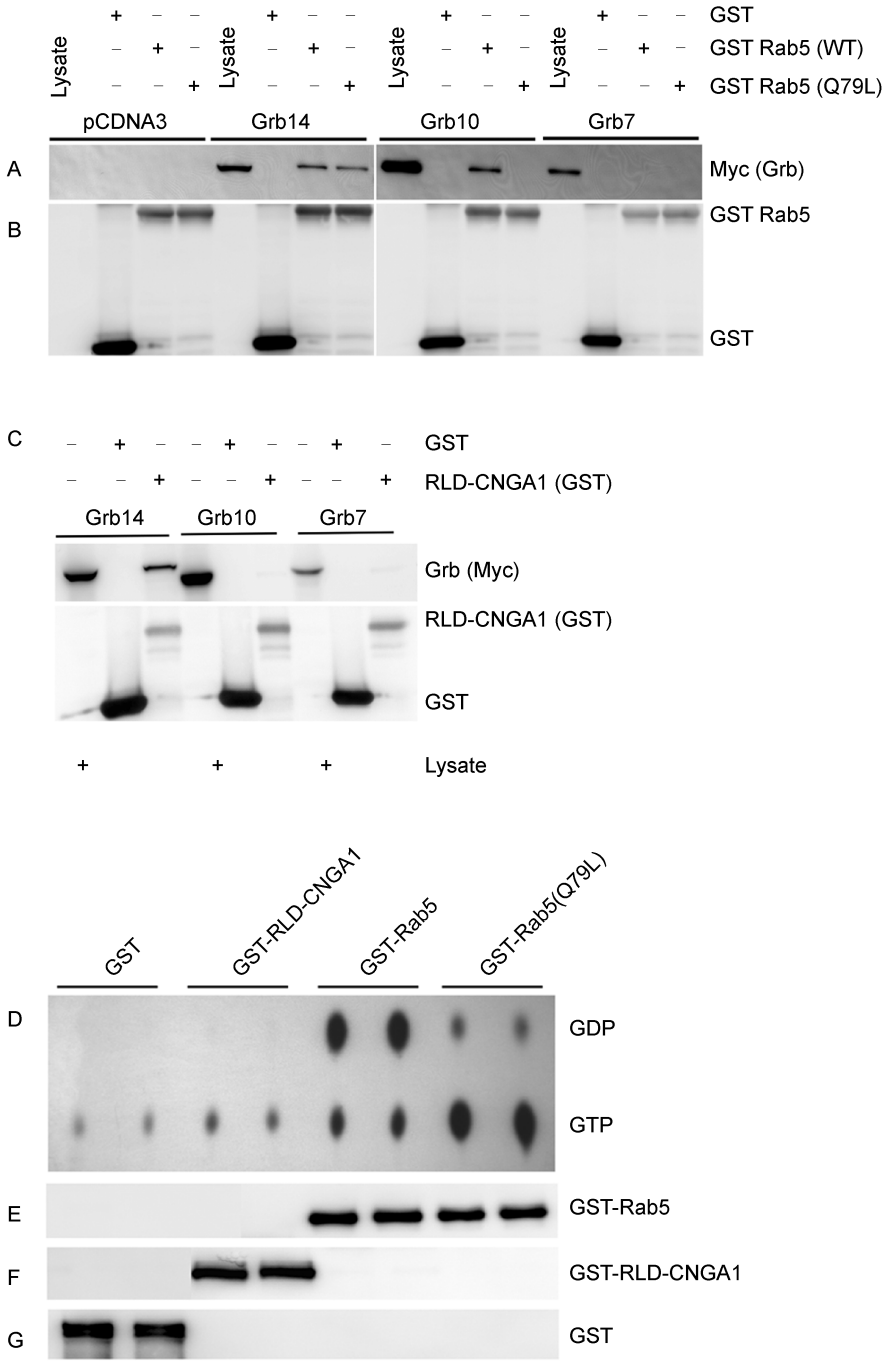

Figure 2. Interaction of Grb7 family members with RLD-CNGA1. (A) Members of Grb7 family members (Grb14, 10 and 7) were expressed individually in HEK-293T cells and lysates subjected to either GST or GST-Rab5 or GST-Rab5 (Q79L) pull-downs followed by immunoblot analysis with (A) anti-Myc and (B) reprobed with anti-GST antibodies. pCDNA3 alone was used as a negative control. (C) The *in vitro* translation products of Myc-Grb14 or Myc-Grb10 or Myc-Grb7 were incubated with either GST or GST-RLD-CNGA1 and subjected to GST pull down assay. The bound proteins were run on SDS-PAGE followed by immunoblot analysis with (C) anti-Myc and reprobed with anti-GST antibodies. (D) Autoradiography of the GTP hydrolysis products as analyzed by thin layer chromatography. Single-step GTP hydrolysis assay by the GST fusion proteins of RLD-CNGA1, Rab5 and mutant Rab5 (Q79L) was carried out as described (Liang et al., 2000). (E–G) The expression of the fusion proteins were examined by immunoblot analysis with anti-GST antibody.

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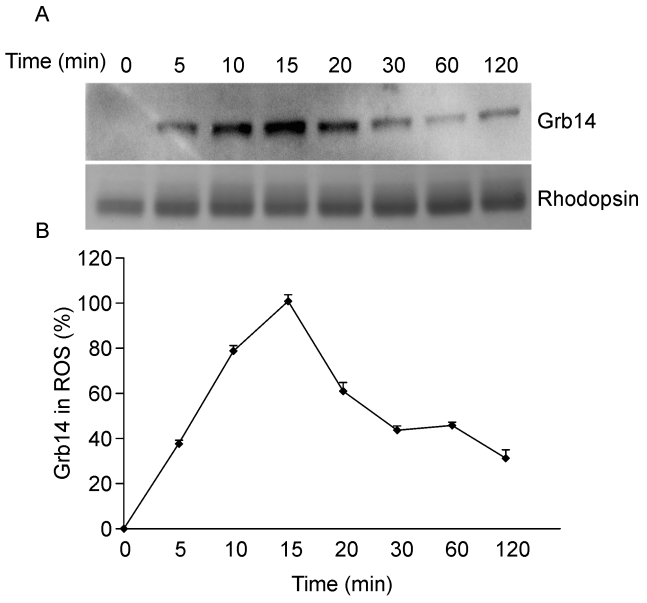

Figure 3. Light-dependent binding of Grb14 to ROS membranes. (A) ROS membranes were prepared from dark- and light-adapted (0–120 min) rats and the ROS proteins were subjected to immunoblot analysis with anti-Grb14 and anti-rhodopsin antibodies. (B) Densitometric analysis of Grb14 was carried out and expressed as percentages of Grb14 binding to ROS. Data are mean ± SD, n = 3. ROS, rod outer segment.

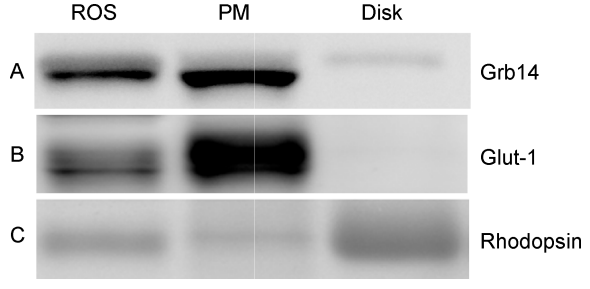

Figure 4. Grb14 localization to ROS plasma membrane. Bovine ROS, plasma membrane and disk proteins were subjected to immunoblot analysis with (A) anti-Grb14, (B) anti-Glut1, and (C) anti-rhodopsin antibodies.

membrane. Consistent with these results, the immunocytochemical analysis further confirmed the ROS localization of Grb14 (Fig. 5).

Expression of Grb7, Grb10 and Grb14 in ROS

Equal amounts of protein from dark- and light-adapted rat ROS and HEK-293T cell lysate were subjected to immunoblot analysis with anti-Grb7, anti-Grb10, anti-Grb14 antibodies. Our results indicate the presence of increased levels of Grb14 in light-adapted compared to dark-adapted ROS (Fig. 6). Grb7 and Grb10 immunoreactivity was absent in both dark- and light-adapted ROS (Fig. 6). HEK-293T cells expressed all members of Grb7 family (Fig. 6). These results suggest the localization of Grb14 in ROS but not Grb7 and Grb10.

Evolutionary conservation of CNG channels

It is interesting to note that in microbial genome we failed to observe homologous sequences of CNG channel; however, we observed that prokaryotic potassium/ion channels show 35% similarity to eukaryotic CNG channels (Fig. 7). These primitive ion channels in microbes do not have the C-terminus region of eukaryotic CNG channel, the so called RLD domain. On the other hand, prokaryotic catabolite gene activator proteins (CAP) exhibit 40% similarity to eukaryotic CNG channel C-terminus; and we also observed a 35% similarity between prokaryotic CAP and human-Ras (Fig. 7).

DISCUSSION

The RA-domain containing proteins are able to bind to their target proteins (Ras/small GTPases) in three different combinations. (1) RA domain containing proteins are known to bind to one or more of Ras proteins in a GTP-dependent manner (McCormick and Wittinghofer, 1996). (2) Active Ras (oncogenic Ras mutants) can also bind the RBDs present in class I phosphoinositide-3 kinase (PI3K); and this interaction serves to bring PI3K to the membrane facilitating contact with substrate lipids (Kyriakis, 2009). In the case of oncogenic Ras, the recruitment of PI3K to the plasma membrane occurs in the absence of upstream stimuli (Kyriakis, 2009). (3) Some RA domain proteins bind to small GTPases, but not to Ras (Raaijmakers and Bos, 2009).

Our current study demonstrated a novel regulatory mechanism of interaction between RA-Grb14 and RLD-domain of CNGA1 in rod photoreceptor cells. The interaction we identified in this study is neither dependent on GTP-binding nor GTPase activity and it is mediated through a simple protein-protein interaction. The interaction between RA-Grb14 and RLD-CNGA1 and the inhibition of channel activity by Grb14 is facilitated by light and lower concentrations of cGMP (Gupta et al., 2010). We have recently show that channel sensitivity to cGMP is controlled by Grb14 which is localized in the rod inner segment and is translocated to outer segments by bright light stimulus (Fig. 8) (Rajala et al., 2009). The channel closure is dictated by the light-mediated activation of rhodopsin/G-protein transducin/cGMP-phosphodiesterase which results in the hydrolysis of cGMP to GMP (Fig. 8) (Hargrave and McDowell, 1992a, 1992b; Hargrave, 2001). Our earlier studies revealed that RA domain of Grb14 is necessary and sufficient to be able to interact with the RLD domain of CNGA1 and inhibit its activity by competing with the cGMP-binding site. These studies also suggest that Grb14 could be competing for the same binding site to which cGMP

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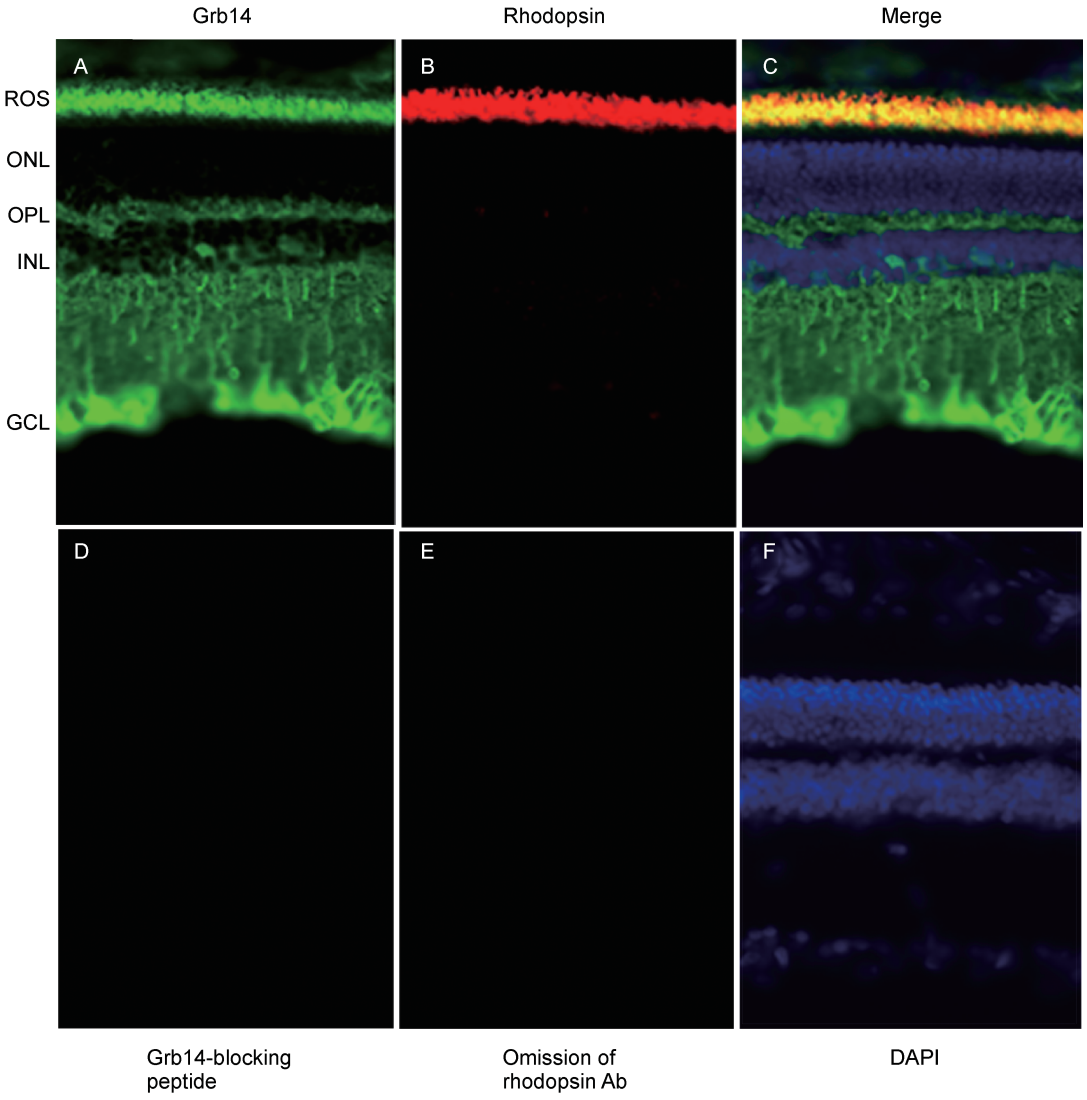

Figure 5. Immunocytochemical localization of Grb14 in light-adapted rat retina. Rat retina frozen sections were subjected to immunocytochemistry and the cryosections were incubated with (A) anti-Grb14, or (B) anti-rhodopsin, or (D) Grb14 blocking peptide or (E) omission of anti-rhodopsin antibody or (F) staining of nuclei with DAPI. (C) Merge image of anti-Grb14/rhodopsin antibodies with DAPI. ROS, rod outer segments; ONL, outer nuclear layer; OPL, outer plexiform cell layer; INL, inner nuclear layer; GCL, ganglion cell layer.

binds and its inhibitory effect can be alleviated by increasing the concentration of cGMP (Gupta et al., 2010). It is interesting to note that the peak binding of Grb14 to ROS is 15 min and the binding starts decreasing as the time progresses. These results suggest that the decreased binding of Grb14 in ROS could be due to the building up of the cGMP concentration in rod photoreceptors or due to some other post-translational modification that liberates Grb14 from ROS membranes. The plasma membrane marker Glut-1 is enriched in plasma membrane compared to ROS, but not Grb14. This discrepancy could be due to the soluble nature of Grb14 which might be due to the changes in the concentration of cGMP. The CNG channel is in the plasma membrane (Kaupp and Seifert, 2002) and accumulation of Grb14 in plasma membrane further suggests a functional interaction between these two proteins. Further studies are required to examine the release of bound Grb14 from ROS at different concentrations of cGMP and also to study the cGMP-mediated translocation of Grb14. Very recently we reported that Grb14 competes with cGMP for the CNGA1 binding pocket and electrostatically interacts with Arg^{559} through the negatively charged Glu residues (180–182) on β-turn at its RA domain which results in the inhibition of channel activity (Gupta et al., 2011). Our studies suggest that Grb14 is a novel

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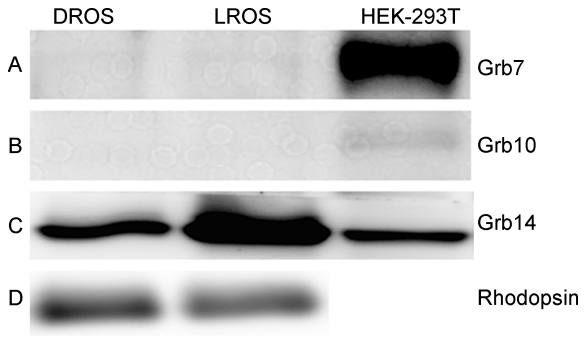

Figure 6. Expression of Grb7, Grb10 and Grb14 in ROS. Equal amounts of protein from dark- and light-adapted rat ROS and HEK-293T cell lysate were subjected to immunoblot analysis with (A) anti-Grb7, (B) anti-Grb10, (C) anti-Grb14, and (D) anti-rhodopsin antibodies. DROS, dark-adapted ROS; LROS, light-adapted ROS.

physiological modulator of CNG channel and we found that channel is more sensitive to cGMP or opens at a lower concentration of cGMP in Grb14$^{-/-}$mice (Gupta et al., 2010).

In this study we found that Grb14 binds to both GTPase-defective mutant Rab5 as well as CNGA1, whereas Grb10 binds only to GTP-bound form of active Rab5. Consistent with these findings, we recently reported that only Grb14 is able to modulate the channel activity, but not Grb7 or Grb10 (Gupta et al., 2011). Taken together, it suggests the existence of a divergence among the members of the Grb7 family, and that Grb14 might have been evolved later in the evolution that binds to Ras and nucleotide binding proteins. Evolutionary analysis of the CNG channels indicates that these channels may have eventually arisen from the fusion of prokaryotic ion channels with the prokaryotic CAP family of transcription regulators which show similarity with the nucleotide binding Ras proteins. The first characterized family containing a cyclic nucleotide binding-domain (CNB) in prokaryotes is the CAP family of transcriptional regulators (Weber et al., 1982) that contain a DNA binding helix-turn-helix (HTX) domain covalently linked to the CNB domain (McKay and Steitz, 1981). It has been suggested that CNB domains have evolved as a scaffold to sense a wide variety of second messenger signals (Kannan et al., 2007). These observations suggest that eukaryotic CNG channels could have evolved through a gene fusion between prokaryotic ion channels and cyclic nucleotide binding proteins, both of which might have undergone several sequence variations for functional adaptation during evolution.

MATERIALS AND METHODS

Animals

All animal work was in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals*, and the Association for Research in Vision and Ophthalmology on the Use of Animals in Vision Research. All the protocols were approved by the IACUC of the University of Oklahoma Health Sciences Center and the Dean McGee Eye Institute. A breeding colony of Albino sprague-dawley rats is maintained in our vivarium in cyclic light (12 h on; 12 h off; ~300 lux). Experiments were carried out on both male and female rats (150–200 g).

Cell lines and culture condition

HEK-293T cells were maintained in DMEM medium at 37°C. Approximately 2.5 × 10^5 cells were seeded in each culture dish 12–18 h before transfection. Calcium phosphate-mediated DNA transfection was performed using each of the plasmids containing the cDNA of interest (Wigler et al., 1978) and cells were harvested for experiments ~48 h post-transfection.

Plasmids and vectors

Myc-tagged bovine Grb14, Grb10 and Grb7 constructs have been described previously (Gupta et al., 2011). The RA (residues 105–195) domain of Grb14 was cloned into Myc-tagged pCDNA3 vector as described previously (Gupta et al., 2010). GST-Rab5 and GST-Rab5 (Q79L) mutant constructs were a kind gift from Dr. Guangpu Li (University of Oklahoma).

In vitro coupled transcription and translation

Myc-tagged Grb14, Grb10 or Grb7 were expressed individually *in vitro* using TNT-coupled transcription and translation system. The reaction mixture containing 1 μg of plasmid DNA, 1 mmol/L methionine and 40 μL of TNT-T7 quick master mix in a total volume of 50 μL, followed by incubation at 30°C for 90 min. The *in vitro* translation products were incubated with either GST or GST-RLD-CNGA1 followed by GST pull-down assays. The bound proteins were washed and subjected to immunoblot analysis with anti-Myc and reprobed with anti-GST antibodies.

Expression of GST-fusion proteins

An overnight culture of *E. coli* BL21 (DE3) (pGEX-RLD-CNGA1, pGEX-Rab5 and pGEX-Rab5 (Q79L) was diluted 1:10 with LB containing 100 μg/mL ampicillin grown for 1 h at 37°C, and induced for another hour by addition of IPTG to 1 mmol/L. Bacteria were sonicated three times for 20 s each time in lysis buffer containing 50 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Lysates were clarified by centrifugation, and the supernatants were incubated with 50 μL of 50% gluthathione-coupled beads (Amersham Pharmacia) for 30 min at 4°C. The GST-fusion proteins were washed in PBS [137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mmol/L KH_2PO_4] and subjected to pull-down assays.

Preparation of rat rod outer segments

ROSs were prepared from rat retinas using a discontinuous sucrose gradient as previously described (Rajala et al., 2002). Retinas were

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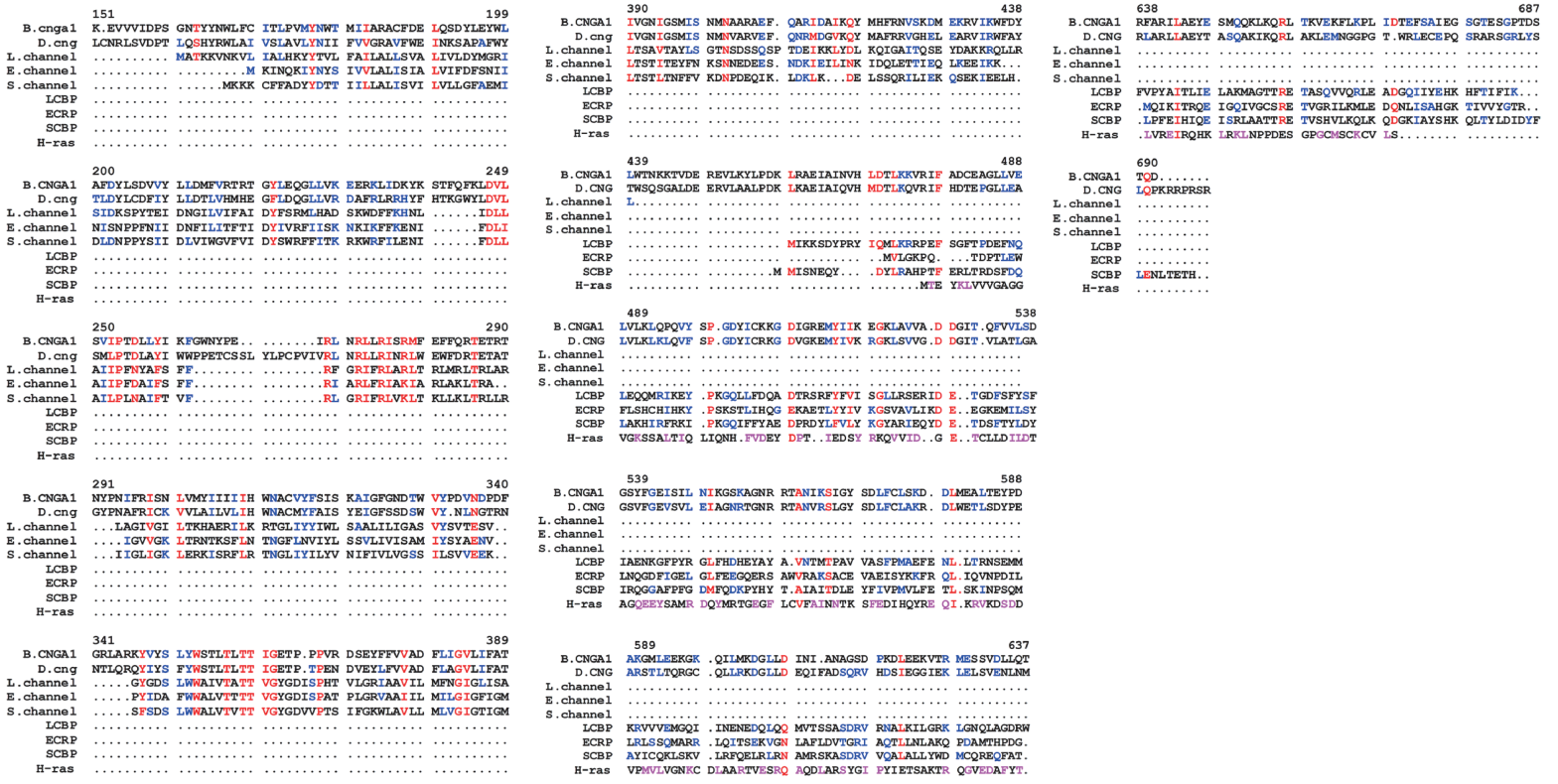

Figure 7. Analysis of predicted primary structures of orthologous proteins. The multiple sequence alignment was generated using the CLUSTAL PC/GENE program. The evolutionary conservation between eukaryotic CNG channels and prokaryotic potassium channel proteins is represented by the following colors: primary (red, 100%), and secondary (blue, 80%). The evolutionary conservation between prokaryotic cyclic nucleotide binding proteins (CNBPs) and H-Ras is represented as pink (80%). The primary sequences of B. CNGA1 (Bovine CNGA1; Uniprot ID: Q00194), D. CNG (Drosophilla cyclic nucleotide gated cation channel; Uniprot ID: Q24278), L. Channel (Potassium/ion channel *Lactobacillus casei*; Uniprot ID: B3WCR6), E. Channel (Potassium/ion channel *Enterococcus faecalis*; Uniprot ID: C7VGQ7), S. Channel (Potassium/ion channel *Streptococcus pneumoniae*; Uniprot ID: C1CKW2), LCBP (cAMP-binding protein-catabolite gene activator *Lactobacillus brevis*; Uniprot ID: Q03NZ3), ECRP (cAMP-regulatory protein *Enterobacter* sp. 638; NCBI Accession No. YP_001178491), SCBP (cAMP-binding protein-catabolite gene activator *Streptococcus suis*; Uniprot ID: A4W091) and H-Ras: (Human-Ras protein; Uniprot ID: P01112). Numbers indicate amino acid positions. Percent similarity: 74/190 = 35% Prokaryotic catabolite gene activator proteins with H-Ras (Pink); 97/234 ≈ 40% Eukaryotic CNG channel (C-terminus) with prokaryotic catabolite gene activator proteins and 108/281 ≈ 35% Eukaryotic CNG channel with prokaryotic potassium channel proteins. However the eukaryotic CNG channels are highly conserved amongst themselves (65%–75%).

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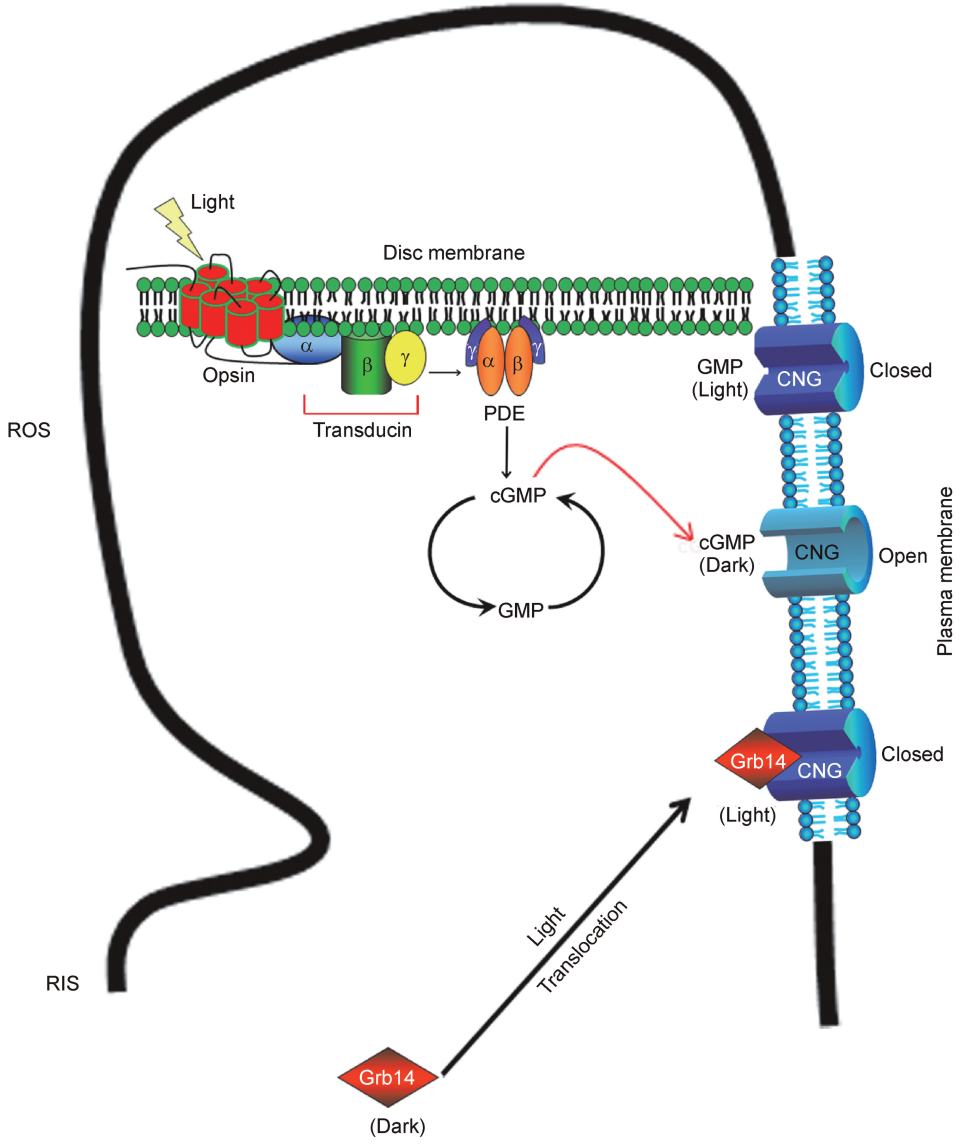

Figure 8. Light-activated rhodopsin signaling resulting in the hydrolysis of cGMP. Light induced activation of rhodopsin activates G-protein, transducin which leads to the further activation of cGMP phosphodiesterase (PDE). PDE hydrolyses cGMP to GMP. Interaction of Grb14 with CNGA1 in light-adapted conditions. cGMP, cyclic guanosine monophosphate; CNG, cyclic nucleotide gated channel; ROS, rod outer segment; RIS, rod inner segment; Grb14, growth factor receptor-bound protein 14.

homogenized in 4.0 mL of ice-cold 47% sucrose solution containing 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L $NaVO_3$, 1 mmol/L PMSF, and 10 mmol/L Tris-HCl (pH 7.4) (buffer A). Retinal homogenates were transferred to 15-mL centrifuge tubes and sequentially overlaid with 3.0 mL of 42%, 3.0 mL of 37%, and 4.0 mL of 32% sucrose dissolved in buffer A. The gradients were spun at 82,000 *g* for 1 h at 4°C. The 32/37% interfacial sucrose band containing ROS membranes was harvested and diluted with 10 mmol/L Tris-HCl (pH 7.4) containing 100 mmol/L NaCl and 1 mmol/L EDTA, and centrifuged at 27,000 *g* for 30 min. The ROS pellets were resuspended in 10 mmol/L Tris-HCl (pH 7.4) containing 100 mmol/L NaCl and 1 mmol/L EDTA, and stored at −20°C. All protein concentrations were determined by the BCA reagent following the manufacturer's instructions.

Preparation of osmotically intact ROS disks

Osmotically intact ROS disks were prepared by Ficoll flotation (Smith and Litman, 1982). The ROSs were prepared from frozen bovine retinas according to the method described (Papermaster, 1982).The ROS pellet was resuspended in 30 mL Ficoll in distilled water and the suspension was kept at 4% under nitrogen for at least 2 h to allow the ROS plasma membrane to burst. This suspension of ROS was divided between two small SW-28 centrifuge tubes and layered with

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cold water. After 2 h of centrifugation in a SW-20 rotor at 25,000 rpm, the intact disks were collected from 5% Ficoll water interface. The bottom pellet contained the ROS plasma membrane-enriched fraction.

Molecular modeling studies

The protein tertiary structural modeling was done using the program MODELLER (Eswar et al., 2008). Ribbon model of CNGA1 (residues 489–653) was constructed based on template [PDB id. 1Q3E]. VMD (Humphrey et al., 1996), Arguslab (Thompson, Planaria software), and DaliLite v3 (Holm et al., 2008) were used for structural superimpositions and graphical manipulations. The extensive energy minimization was carried out using Deep View (Guex and Peitsch, 1997). The energy calculations were performed using the universal force field method (UFF) of molecular mechanics. A few knotty side-chain conformations were acknowledged and rectified. Structural parameters and prediction quality of the modeled structure were evaluated based on bond angle stereo-chemistry using WHATIF (Vriend, 1990) and PROCHECK (Morris et al., 1992).

Immunoprecipitation

Immunoprecipitation was carried out according to the method described earlier (Li et al., 2007). HEK-293T cells were harvested and solubilized for 30 min at 4°C in a lysis buffer containing 1% Triton X-100, 137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 10% glycerol, 1 mmol/L EGTA, 1 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L Na_3VO_4, 10 μg/mL leupeptin, and 1 μg/mL aprotinin. Insoluble material was removed by centrifugation at 17,000 *g* for 20 min at 4°C, and the solubilized proteins were pre-cleared by incubation with 40 μL of protein A Sepharose for 1 h at 4°C with mixing. The supernatant was incubated with primary antibodies or normal IgG (control) overnight at 4°C and subsequently with 40 μLof protein A-Sepharose for 2 h at 4°C. Following centrifugation at 17,000 *g* for 1 min at 4°C, immune complexes were washed three times with ice-cold wash buffer [50 mmol/L HEPES (pH 7.4), 118 mmol/L NaCl, 100 mmol/L NaF, 2 mmol/L $NaVO_3$, 0.1% (*w/v*) SDS and 1% (*v/v*) Triton X-100]. The immunoprecipitates were subjected to immunoblot analysis with indicated antibodies in the respective figures.

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ABBREVIATIONS

Grb14, growth factor receptor-bound protein 14; CNBPs, cyclic nucleotide binding proteins; CNG, cyclic nucleotide gated channel; ROS, rod outer segment; RA, Ras-associating domain; RLD, Ras-like domain

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