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Photoreceptor synaptic protein HRG4 (UNC119) interacts with ARL2 via a putative conserved domain

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Abstract Human retinal gene 4 (HRG4) (UNC119) is a photoreceptor synaptic protein of unknown function, shown when mutated to cause retinal degeneration in a patient and in a confirmatory transgenic model. ADP-ribosylation factor-like protein 2 (ARL2) was identified as an interactor of HRG4 by the yeast two-hybrid strategy. The presence of ARL2 in the retina and co-localization with HRG4 was confirmed by Western blot and double immunofluorescence analysis, respectively. The interaction of ARL2 with HRG4 was further confirmed by co-immunoprecipitation and direct binding analysis. Phosphodiesterase δ (PDEδ) is an ARL2-binding protein homologous to HRG4. Amino acid residues of PDEδ involved in binding ARL2 and forming a hydrophobic pocket were shown to be highly conserved in HRG4, suggesting similarity in binding mechanism and function.

Key words: Human retinal gene 4; UNC119; Phosphodiesterase δ; ADP-ribosylation factor-like protein 2; Interactor; Yeast two-hybrid

1. Introduction

Human retinal gene 4 (HRG4) is a photoreceptor synaptic protein, originally isolated in our laboratory by a subtractive cDNA cloning strategy designed to isolate cDNAs specific to or enriched in the retina [1,2]. HRG4 is the first synaptic protein to be described that is highly enriched in the photoreceptor synapse, both of rods and cones. HRG4 appeared to be predominantly associated with synaptic vesicles in the photoreceptor synapses [2]. It is orthologous to UNC119 in *Caenorhabditis elegans* which was isolated in mutant nematodes with defects in coordination, feeding, and chemosensation [3]. A truncation mutation in HRG4 was discovered in a patient with late-onset cone–rod dystrophy, and a transgenic model expressing the same mutation was shown to develop late-onset retinal degeneration as did the human patient, accompanied by severe synaptic and trans-synaptic degeneration [4]. Specific changes in retinal synaptic proteins were demonstrated in the transgenic model before the onset of obvious pathology, including a decrease in peripheral membrane proteins of the synaptic vesicle [5]. Judging from its localization to the synaptic vesicles and the effect of the mutation on the synapse/synaptic vesicle proteins, the function of HRG4 appeared to involve the normal homeostasis of photoreceptor synaptic vesicles and their associated proteins.

The yeast two-hybrid technology has been a powerful tool in identifying the targets of many novel proteins, thereby elucidating or providing valuable clues as to the function of the protein [6,7]. In order to understand the function of HRG4, we determined the identity of its interacting protein(s) by the yeast two-hybrid strategy. A retinal cDNA prey library was constructed and used to identify the correct tissue-specific interactor for HRG4. As a result, ADP-ribosylation factor (ARF)-like protein 2, ARL2, was identified. The interaction between HRG4 and ARL2 was confirmed in in vitro experiments and shown to be similar to that between a homologous protein, phosphodiesterase δ (PDEδ), and ARL2. Co-localization of HRG4 and ARL2 in the retina was also demonstrated.

2. Materials and methods

2.1. Yeast two-hybrid system

Hybrid Hunter™ Two-Hybrid System (Invitrogen, Carlsbad, CA, USA) was used in this study. The yeast strain used was L40 (MATα his3Δ200trp1-901 leu2-3112 ade2 LYS2::4lexAop-His3 URA3::8lexAop-lacZ GAL4).

2.2. Construction of interactive trap (prey) cDNA library

40 μg of phage DNA was extracted from human retinal cDNA clones [8] using the Lambda Maxi Kit (Qiagen, Valencia, CA, USA), and the inserts were cut out. Inserts between the sizes of 500 bp and 5 kb were cut out from the gel after electrophoresis, and isolated using the QIAquick Gel Extraction Kit (Qiagen). 100 ng of inserts was ligated into 100 ng of digested, directional prey library plasmid (pYESTrp2). 10 μl out of 20 μl of the ligation mixture was used for transformation into *Escherichia coli* XL-10-Gold ultracompetent cells (Stratagene, La Jolla, CA, USA). 80 000 primary clones were obtained and used for the large transformation. Average size of the inserts was 1.7 kb (data not shown).

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Abbreviations: HRG4, human retinal gene 4; PDEδ, phosphodiesterase δ; ARL2, ADP-ribosylation factor-like protein 2; TE, Tris–ethylenediamine tetraacetic acid (EDTA)

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2.3. Construction of the bait yeast strain

The bait plasmid pHybLex/Zeo-HRG4 was constructed by ligating a polymerase chain reaction (PCR)-derived fragment (PCR primers; pHyb-1, 5′-CCGGAATTCATGAAGGTGAAGAAGGGCGGCG-3′, pHyb-2, 5′-ATGGGCTTGACTGGGGACACCA-3′, 901 bp product) encoding the complete amino acid sequence of HRG4 (5′ end beginning at the initiation codon ATG (position 55 in the cDNA sequence) with a 9 bp built-in *Eco*RI restriction site and the 3′ end at position 946 in the cDNA sequence just after a *Kpn*I site) into pHybLex/Zeo that had been digested with *Eco*RI and *Kpn*I. The ligation reaction was performed with 80 ng of pHybLex/Zeo and 78 ng of inserts in a total reaction volume of 20 μl. 1 μl of the ligation mixture was used for transformation into XL-1 blue (Stratagene) competent bacteria. Construction of the bait plasmid (pHybLex/Zeo-HRG4) was confirmed by sequencing, and 4 μg of the bait plasmid was transformed into L40 yeast strain using the S.c. EasyComp Kit (Invitrogen). Expression of the HRG4 protein in the yeast was confirmed by enhanced chemiluminescence (ECL)™ Western blotting (Amersham, Piscataway, NJ, USA). In brief, zeocin-resistant transformants (putative bait strain) and untransformed L40 were cultured overnight, centrifuged, and the aqueous phase was decanted. The cell pellets were placed in a −80°C freezer for 10 min, thawed in 100 μl of pre-warmed (−60°C) cracking buffer, and transferred to a 1.5 ml microcentrifuge tube containing 100 μl of glass beads. The solution was incubated at −70°C for 10 min, vortexed for 1 min, and centrifuged at 14 000 rpm for 5 min at room temperature. 50 μl of the supernatant was used for immunoblot analysis. Antibody used to assay the level of fusion protein expression was the polyclonal anti-LexA antibody (Invitrogen) and a polyclonal anti-rat RRG4 antibody [2]. Further confirmation was performed to determine that the bait clone does not show prototrophy for histidine (to confirm the absence of contamination by other yeast strains or leakage of expression of histidine-related genes, L40 being auxotrophic for histidine), and it does not show β-galactosidase activity (to confirm the absence of non-specific transactivation of the reporter construct in the L40 strain) (data not shown).

2.4. Large-scale transformation and screening

500 μg of interactive trap cDNA clones (prey library) was large-scale transformed into the bait strain according to the manufacturer's protocol. In brief, the L40 strain containing the bait was cultured overnight at 30°C and centrifuged. The pellets were washed with Tris–ethylenediamine tetraacetic acid (TE) and mixed with lithium acetate/0.55×TE. 500 μg of prey cDNA was mixed with 1 ml of salmon sperm DNA, added to the cell suspension, and incubated with lithium acetate/40% polyethylene glycol (PEG)/TE at 30°C for 30 min. After addition of 17.6 ml dimethyl sulfoxide (DMSO), the mixture was heat shocked at 42°C for 6 min. After several washes with the yeast extract peptone dextrose medium, the cells were suspended in 10 ml of medium. 100 μl of the suspension was plated out in 40 plates and incubated at 30°C for 2–4 days. Clones were picked and arranged in a grid on new plates for easy manipulation of the β-galactosidase filter lifting assay. Positive colonies were detected by selecting on plates lacking the auxotrophic marker (histidine), followed by a second screen for β-galactosidase activity by filter lifting assay in addition to the zeocin selection. Plasmids encoding the putative interactors were retrieved according to the manufacturer's protocol. Briefly, an overnight culture of a positive yeast clone was mixed with the lysis buffer, vortexed with glass beads, extracted with phenol/chloroform, centrifuged, and the aqueous phase was ethanol precipitated to obtain the DNA. The plasmid DNA was resuspended in 25 μl of TE, and transformed into *E. coli* (XL-1 blue) with ampicillin selection in order to obtain only the pYEST plasmid, not the bait plasmid. All double-selected colonies were identified by DNA sequencing. To eliminate the possibility of false positives, the L40 yeast, transformed with each putative positive interactor clone, was tested for expression of β-galactosidase by the filter lifting assay as is, or after additional transformation with the bait vector, bait vector containing HRG4, and bait vector containing lamin. A true positive clone showed the blue color only when the putative interactor clone and the HRG4-containing bait vector were present together.

2.5. Antibody production

All procedures using animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Polyclonal antibody against ARL2 was produced using recombinantly expressed human ARL2 as previously described for rat HRG4 (RRG4) antibody [2]. Briefly, glutathione-S-transferase (GST)-ARL2 hybrid protein was expressed from a pGEX clone containing the PCR-amplified human ARL2 sequence, and ARL2 was separated from the GST protein by thrombin digestion. 500 μg of ARL2 was injected into rabbit with complete and incomplete Freunds adjuvant in three different doses over 4 weeks, and serum was collected over a total of 9 weeks. The activity of the antiserum was confirmed by Western blotting of expressed ARL2. The antiserum was affinity-purified by chromatography through ARL2-bound Sepharose 4B. The specificity of the antibody for ARL2 was tested by inactivation of the antibody activity by mixing with excess ARL2 antigen and use in Western blot analysis of expressed ARL2.

2.6. Preparation of retinal protein extracts

Rat and mouse retinas were homogenized in buffer containing 50 mM Tris–HCl (pH 8),150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100 and centrifuged at 10 000×*g* for 10 min at 4°C. Protein concentration of the supernatant was measured by the modified Lowry method (Micro Protein Determination; Sigma, St. Louis, MO, USA).

2.7. Western blotting

10–30 μg of protein samples was suspended in the same volume of sample buffer (50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 10% glycerol), incubated at 95°C for 5 min, and electrophoresed in Tris–tricine SDS–polyacrylamide gel (SDS–PAGE). The proteins in the gel were transferred to polyvinylidene difluoride (PVDF) filters (Millipore, Bedford, MA, USA) by electroblotting. After the transfer, the polyacrylamide gels were stained with Coomassie blue to visualize the remaining proteins and size markers. The filters were blocked in 4 ml of PBST buffer (phosphate-buffered saline (PBS) with 0.1% Tween 20) supplemented with 0.1% non-fat dry milk for 1 h with gentle shaking at room temperature, rinsed twice, and washed three times with 150 ml each of fresh PBST. The blots were incubated with the ARL2 (1:200) or RRG4 (1:100) antibody for 1 h, washed, reacted with horseradish peroxidase-conjugated goat anti-rabbit antibody, washed, and processed for detection by enhanced chemiluminescence (ECL; Amersham).

2.8. Preparation of frozen sections

5 months old rats were killed, and eyes were enucleated. Incisions were made around the limbus, and the eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 2 h, followed by soaking in 20% sucrose in 0.1 M phosphate buffer overnight. Before sectioning, the eyes were embedded in OCT (Miles, Elkhart, IN, USA) and frozen in liquid nitrogen. 6 μm sections were cut on a cryostat and kept dry at −20°C until used.

2.9. Immunofluorescence

6 μm frozen sections of the rat retinas were analyzed by double immunofluorescence with polyclonal antibodies to human ARL2 and rat HRG4 (RRG4) [2] as described before [9]. Briefly, the frozen sections were dried at room temperature for 20 min, blocked overnight with blocking buffer (10% goat serum, 1% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS, pH 7.4), and incubated with the first primary antibody, ARL2, in PBS at a dilution of 1:50 for 90 min. After three washes in PBS, the sections were incubated with the secondary antibody (fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG; Sigma) at a dilution of 1:50 for 30 min, followed by three washes in PBS. Then the sections were post-fixed in cold methanol for 10 min, washed, and treated with the second primary antibody, RRG4, at 1:100 for 1 h. The sections were washed, treated with the secondary antibody (rhodamine-labeled goat anti-rabbit IgG; Sigma), and washed. Control sections were treated with the two secondary antibodies in a similar fashion. The sections were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc, Birmingham, AL, USA) and digitally photographed with a Zeiss photomicroscope III (Carl Zeiss, Oberkochen, Germany).

2.10. Co-immunoprecipitation of HRG4 and ARL2

The rat retinal extract was first pre-cleaned for use in immunoprecipitation. 50 μl of pre-immune rabbit serum was added to 2.5 mg of rat retinal extract, and the mixture was incubated for 1 h at 4°C.

18 mg of Protein A-Sepharose (Sigma) was added, and the mixture was incubated for additional 30 min at 4°C. The Protein A-Sepharose complex was removed by centrifugation at 12 000×*g* for 5 min at 4°C, and the supernatant was used for immunoprecipitation. The supernatant was divided into two 250 μl portions, 5 μl of HRG4 antiserum [2] was added to one portion, 5 μl of pre-immune serum was added to the other portion, both in NET gel buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0, 0.25% (w/v) gelatin, 0.02% (v/v) sodium azide), and the mixtures were incubated for 1 h at 4°C. 15 mg of Protein A-Sepharose was added to both, and the mixtures were incubated for additional 1 h at 4°C. The samples were centrifuged at 12 000×*g* for 20 s to collect the immunoprecipitates which were then washed three times with 1 ml each of PBS. The final samples were subjected to Western blot analysis as described above with the ARL2 and HRG4 antibodies.

2.11. Pull-down of ARL2 with HRG4

RRG4-GST hybrid protein and GST protein were prepared from 600 ml of bacterial cultures as previously described [2], and purified by absorption to 300 μl of glutathione-Sepharose. ^{35}S-labeled ARL2-B42 hybrid protein was prepared by using 1 μg of the ARL2-containing pYESTrp2 yeast two-hybrid clone in an in vitro transcription–translation reaction in 40 μl of TNT T7 Quick Master Mix (TNT Quick Coupled Transcription/Translation Systems, Promega, Madison, WI, USA) and 2 μl (30 μCi) of [^{35}S]methionine (Amersham) at 30°C for 90 min. To 15 μl each of the transcription–translation product containing the ^{35}S-labeled ARL2-B42 hybrid protein were added 35 μg of RRG4-GST-Sepharose or 35 μg of GST-Sepharose, and the mixtures were incubated at 4°C overnight. The mixtures were centrifuged at 2000×*g* for 3 min, washed with PBS five times, resuspended in 50 μl of PBS containing 1 μg/μl aprotinin and 100 μg/ml phenylmethylsulfonylfluoride, and analyzed by SDS–PAGE and autoradiography.

3. Results

Clones from our human retinal cDNA library [8] were used to construct the interactive trap cDNA library (prey library) for the yeast two-hybrid strategy. A number of important retina-specific and -enriched clones has been isolated from this library, indicating the suitability of this library for construction of the prey library [1,8,10–12]. 80 000 primary clones were obtained in the prey library with an average insert size of 1.7 kb. For the construction of the HRG4 bait strain, the coding sequence of HRG4 was PCR-amplified, inserted into the vector, confirmed by sequencing, and used for transformation of the L40 yeast strain. Expression of the LexA DNA-binding domain-HRG4 hybrid protein by the transformant

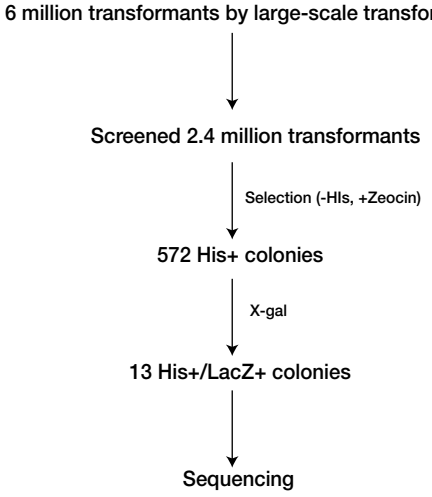

Fig. 1. Summary of the isolation of HRG4-interacting proteins by the yeast two-hybrid strategy.

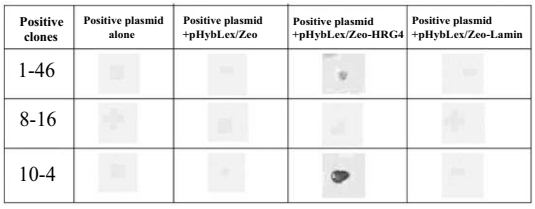

Fig. 2. Test of putative positive clones for false positivity. The 13 putative positive clones obtained by the yeast two-hybrid strategy (representative clones 1-46, 8-16, and 10-4 shown) were tested for true positivity by transformation into L40 strain and the filter lift assay for β-galactosidase as is or after transformation with the bait vector alone (pHybLex/Zeo), bait vector containing HRG4 (pHybLex/Zeo-HRG4), or bait vector containing lamin (pHybLex/Zeo-lamin). The strong positive reaction for clone 10-4 is shown only in the presence of the HRG4-containing bait.

was confirmed by Western blot analysis, and the histidine auxotrophy and the absence of β-galactosidase activity were also confirmed (data not shown).

A large-scale transformation of the L40 bait strain with 500 μg of the prey library clones resulted in approximately 6 million initial transformants (Fig. 1). 2.4 million transformants were subjected to selection, first for histidine prototrophy and zeocin resistance which yielded 572 colonies. A second selection for β-galactosidase activity and zeocin resistance resulted in 13 colonies. The cDNA clones were extracted from the positive colonies and sequenced for identification. In order to rule out false positive clones, the L40 strain was transformed with the putative positive clones, and tested for β-galactosidase expression after additional transformation with various bait constructs (Fig. 2). Nine out of the 13 clones showed strong β-galactosidase activity only after transformation with the correct HRG4 bait construct, indicating true positivity. All nine clones contained the cDNA for ARL2.

In order to confirm the interaction of HRG4 with ARL2 in the retina, we first demonstrated the presence of ARL2 in the mammalian retina. Antibody against ARL2 was obtained from two sources, one being a generous gift from Emory University (Daniel Sharer) and the other being our own polyclonal antibody produced as described with recombinantly expressed human ARL2. The specificity of our antibody activity against ARL2 was confirmed by its inactivation with excess antigen (data not shown). The two antibodies were used in Western blot analysis of rat retinal protein extract. Results demonstrated the presence of the 21 kDa ARL2 in the retina (Fig. 3). Next, the specific localization of ARL2 in the retina in relation to HRG4 was determined by double immunofluorescence. The ARL2 antibody showed reaction in various layers of the rat retina, namely the ganglion cell layer, inner nuclear layer, outer plexiform layer (OPL), and the photoreceptor inner segments (IS) (Fig. 4B). There was also a low level of reaction present in the inner plexiform layer. The HRG4 antibody showed reaction in the OPL as previously reported [2] and in the IS (Fig. 4C). Thus, the co-localization of these two proteins in the OPL and IS was clearly demonstrated, consistent with their interaction as revealed by the yeast two-hybrid strategy. Controls using secondary antibodies alone were negative (Fig. 4D, E).

Two approaches were used to directly demonstrate and confirm the interaction between ARL2 and HRG4, i.e. co-immunoprecipitation and direct binding of recombinantly ex-

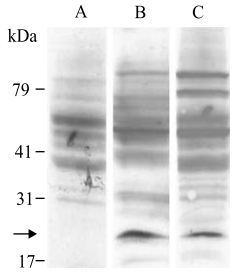

Fig. 3. Western blot analysis of rat retinal proteins with ARL2 antibodies. 30 μg of rat retinal proteins were Western blotted with two different polyclonal antibodies against human ARL2. The blots were reacted with A, no antibody; B, affinity-purified polyclonal ARL2 antibody from Emory University at 1:200; C, our affinity-purified polyclonal ARL2 antibody at 1:200. The 21 kDa ARL2 band (arrow) was visualized with both antibodies. The source of the faint upper background bands is identified as non-specific reaction of the secondary antibody in lane A. Molecular sizes in kDa are shown.

pressed proteins. HRG4 was immunoprecipitated from rat retinal protein extract using anti-HRG4 serum and Protein A, and co-precipitated proteins were analyzed by Western blotting with the HRG4 and ARL2 antibody. ARL2 was clearly demonstrated as a co-precipitated protein when anti-HRG4 serum was used, but not when pre-immune serum was used for the immunoprecipitation (Fig. 5). Finally in a direct binding experiment, ^{35}S-labeled ARL2-B42 activating domain hybrid protein was expressed from the pYESTrp2 clone containing the ARL2 cDNA. The RRG4-GST-glutathione-Sepharose complex was prepared by bacterial expression, and the two were mixed and tested for binding. The ^{35}S-labeled ARL2 hybrid protein was observed to bind the RRG4-GST hybrid protein but not the GST protein alone (Fig. 6), again confirming the interaction of ARL2 and HRG4.

PDEδ is a protein that was isolated from the soluble form of PDE6, the rod-specific cGMP phosphodiesterase complex [13], and it shows homology to HRG4, especially in the COOH terminal region (30% identity) [14]. Like HRG4, PDEδ also interacts with ARL2 [15]. Recently, the crystal structure of the complex between PDEδ and ARL2-GTP was elucidated, and the amino acid residues in PDEδ that are important for its interaction with ARL2 were delineated [16]. Alignment and comparison of the homologous regions of the PDEδ and HRG4 sequences demonstrated a high degree of conservation of these residues in HRG4 (10 of 13 residues (77%) identical or similar) (Fig. 7). A hydrophobic pocket has been shown to be present in PDEδ, and the residues that are important for this pocket were also delineated [16]. Again,

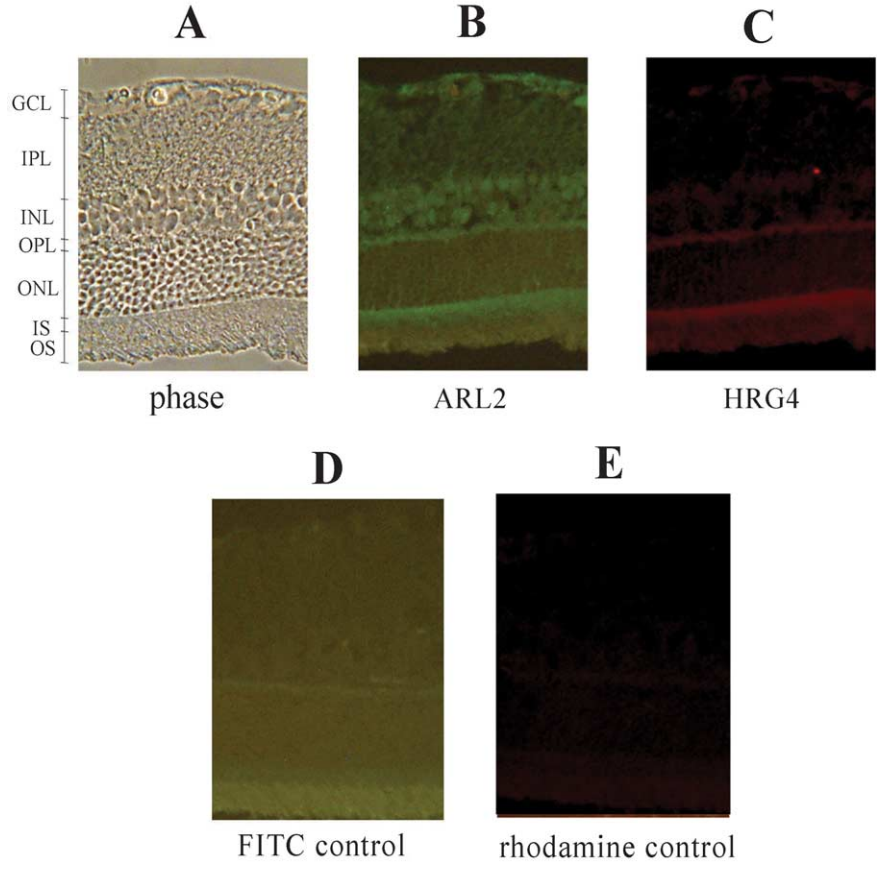

Fig. 4. Double immunofluorescence of rat retina with ARL2 and HRG4 antibodies. 5 months old rat retina was reacted with the human ARL2 antibody at 1:50 and rat HRG4 (RRG4) antibody at 1:100 and visualized with FITC-conjugated secondary antibody and rhodamine-conjugated secondary antibody, respectively. The micrographs of the phase (A), ARL2-reacted FITC (B), HRG4-reacted rhodamine (C), and the controls for the FITC and rhodamine secondary antibodies (D, E) are shown. The ARL2 reaction is present predominantly in the ganglion cell, inner nuclear, outer plexiform, and inner segment layers with low activity in the inner plexiform layer. HRG4 is present in the outer plexiform and inner segment layers. The co-localization of ARL2 and HRG4 in the outer plexiform and inner segment layers is demonstrated. The FITC and rhodamine controls do not show any significant reaction in any of the layers. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment.

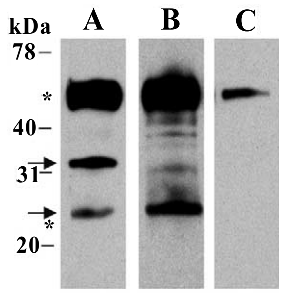

Fig. 5. Co-immunoprecipitation of ARL2 with HRG4 in rat retinal extract. Rat retinal extract was treated with antiserum against HRG4 or pre-immune serum and Protein A-Sepharose, and the precipitated complex was Western blotted with HRG4 and ARL2 antibodies. A, precipitated with HRG4 antiserum and Western blotted with HRG4 antibody. B, precipitated with HRG4 antiserum and Western blotted with ARL2 antibody. C, precipitated with pre-immune serum and Western blotted with HRG4 and ARL2 antibody (no reaction seen with either). Immunoprecipitation of HRG4 (upper arrow, A) and co-precipitation of the bound ARL2 (lower arrow, B) are shown. Superimposition of the ARL2 and antibody light chain signals was confirmed by densitometry. Upper asterisk, antibody heavy chain; lower asterisk, antibody light chain. Molecular sizes in kDa are shown.

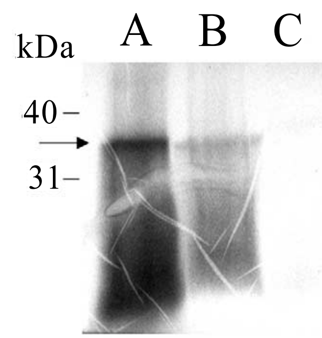

Fig. 6. Pull-down of ^{35}S-labeled ARL2-B42 hybrid protein with HRG4-GST-glutathione-Sepharose. ^{35}S-labeled ARL2-B42 hybrid protein expressed from the positive pYESTrp2 clone was mixed with HRG4-GST-glutathione-Sepharose or GST-glutathione-Sepharose, and the interaction between the two was examined after centrifugation by electrophoresis and autoradiography. A, original ^{35}S-labeled ARL2-B42 hybrid protein. B, mixed with HRG4-GST-glutathione-Sepharose and analyzed. C, mixed with GST-glutathione-Sepharose and analyzed. Binding of the ARL2-B42 hybrid protein (arrow) to the HRG4-GST complex (B) but not to the GST complex (C) is shown. Molecular sizes in kDa are shown.

there was significant conservation of these residues in HRG4 (11 of 17 residues (65%) identical or similar).

4. Discussion

This study demonstrates that HRG4, highly expressed in retinal photoreceptor synapses, interacts with ARL2. The precise function of ARL2 is not yet known. Nevertheless, an analysis of structurally similar proteins and other proteins found to interact with ARL2 may provide insight into the function of the HRG4–ARL2 interaction.

ADP-ribosylation factors (ARFs) are ubiquitous, highly conserved, Ras-like GTP-binding proteins [17,18]. The function of ARFs, consisting of at least six different genes/proteins, was originally demonstrated to be a cofactor activity of ADP-ribosylation of $G_{\alpha S}$ by bacteria toxins [19], but it has since expanded to include vesicular traffic [20–23], complementation of an arf1–arf1 double deletion in *Saccharomyces cerevisiae* [24,25], and activation of phospholipase D [26,27]. ARF-like proteins (ARLs) are more structurally divergent proteins that are 40–60% homologous to ARFs or to each other. At least 10 distinct ARLs have been identified [28–32]. The ARLs lack the specific functions that have been described for the ARFs, with exception of ADP-ribosylation factor-like protein 1 (ARL1) showing similarity to ARFs in its effect on the Golgi and some of its interacting proteins [15,33].

ARL2 is 45% homologous to ARF1 and unique among the

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                               +   +*        +                        +
                    10        20          30             40
human PDEδ  MSAKDERAREILRGFKLNWM--NLRDAETGKILWQG-----TEDLSV------PGVEHEA
                          :| :::  ::|| ::| :|::      :| | :     |:: : :
human HRG4  RITGDYLCSPEENIYKIDFVRFKIRDMDSGTVLFEIKKPPVSERLPINRRDLDPNAGRFV
                     80        90       100       110       120       130

             +     ++    + +*  *               +           * * * *   ***
               50        60        70         80        90       100
human PDEδ  RV--PKKILKCKAVSRELNFSSTEQ-MEKFRLEQKVYFKGQCLEEWFFEFGFVIPNSTNT
            |      :|: : |:  ::|:  :: :::||: :: ||::| |: : |:||| ||:| ||
human HRG4  RYQFTPAFLRLRQVGATVEFTVGDKPVNNFRMIERHYFRNQLLKSFDFHFGFCIPSSKNT
                    140       150       160       170       180       190

            ** *         +    +   + +                 + + +
               110       120       130         140       150
human PDEδ  WQSLIEAAPESQMMPASVLTGNVIIETK--FFDDDLLVSTSRVRLFYV
             : : :  | |: : : ::      ::   :| || ||  :::   |
human HRG4  CEHIYDFPPLSEELISEMIRHPYETQSDSFYFVDDRLVMHNKADYSYSGTP
                    200       210       220       230       240
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Fig. 7. Homology between human PDEδ and HRG4 and conservation of residues important for the interaction with ARL2 and the hydrophobic pocket. The alignment of the human PDEδ and HRG4 amino acid sequences in the homologous region as determined by the FASTA program (GCG, Madison, WI, USA) is shown. This region shows an identity of 23% in 153 amino acid residues between the two sequences. Residues identical between the two sequences in the alignment are designated by bars, and residues that are similar (conservative changes) are designated by colons. Amino acid residues that have been determined to be at the interface of interaction between PDEδ and ARL2 are designated by asterisks above them, and residues shown to be important for the formation of a hydrophobic pocket in PDEδ are designated by plus symbols above them [16]. A significant number of both types of residues are conserved in HRG4; of the 13 residues important for interaction with ARL2 (*), eight are identical and two are similar, and for 17 residues important for the hydrophobic pocket (+), three are identical and eight are similar.

ARF/ARLs in its high affinity for GTP and GDP and its lack of *N*-myristoylation which is present in all the other members of the ARF family [29,34]. It is expressed ubiquitously like the ARFs, but is most abundant in neural tissue. The precise function of ARL2 and the other ARLs is not yet known, but there have been some interesting recent reports. Alp41, a yeast ortholog of ARL2, has been shown to play an important role in the assembly of tubulin to form microtubules [35]. Specifically, Alp41 was shown to act upstream of cofactor D in the α-tubulin pathway, and to be essential for the viability of fission yeast. ARL2 was actually shown to interact with cofactor D and prevent the destruction of tubulin and microtubules mediated by cofactor D [36]. *TITAN5*, a gene in Arabidopsis important for cytokinesis and proposed to regulate microtubule function in seed development, was also found to be the plant ortholog of ARL2, consistent with its involvement in microtubule formation [37,38]. A 19 kDa protein named binder of ARL2 (BART) was found to interact with ARL2 by a GTPase overlay strategy and was determined to be the first identified effector for ARL2 [39]. More recently, the BART-ARL2-GTP complex was shown to enter the mitochondria and bind the adenine nucleotide transporter 1 (ANT1) [34]. The ANT proteins are integral inner membrane proteins of mitochondria that form channels used in exchange of ATP and ADP, thus controlling the ATP level in the cytoplasm [40]. Besides BART, additional proteins have been shown to interact with ARL2 by the yeast two-hybrid strategy [15]. Eight overlapping different binding partners of ARL1, ARL2, and ARL3 were identified, including HRG4, BART, and PDEδ for ARL2. Thus, the identification of HRG4 as an ARL2-binding protein was consistent with our result.

Because the precise function of ARL2 is not yet known, the functional significance of the interaction of HRG4 with ARL2 remains to be determined. Since HRG4 is found in the photoreceptor synapse and would be expected to be present in the photoreceptor axon as well, the involvement of ARL2 in tubulin assembly and microtubule formation suggests that HRG4 may be involved in microtubule formation in the photoreceptor axon and synapse via ARL2. Microtubules are important for vesicular transport of proteins in axons and synapses. Thus, HRG4 may have a role in regulating vesicle trafficking of proteins. Interestingly, another protein related to retinal degeneration, the retinitis pigmentosa protein 2 (RP2), is similar to the tubulin-specific chaperone cofactor C and has been shown to interact with ADP-ribosylation factor-like protein 3, ARL3 [41]. RP2 was shown to be a tubulin GTPase activator, thus possibly playing a role in the regulation of microtubule dynamics which may be a common theme in retinal degeneration. As for a possible relationship to mitochondria, as in the case of BART interacting with ARL2, immunocytochemical analysis of HRG4 did not reveal any obvious association with the mitochondria in synapses, but further investigation is necessary to answer this question fully [2].

The recently demonstrated interaction of PDEδ with ARL2 provides an interesting possibility for the function of HRG4. PDEδ, in addition to being a binding partner of ARL2 like HRG4, is homologous to HRG4. The two proteins are 23% identical overall, and 30% identical in the COOH-terminal 1/3 [14]. PDEδ is expressed ubiquitously, whereas HRG4 is predominantly expressed in the photoreceptors [1,42]. PDEδ was originally isolated from the soluble form of PDE6, the rod-specific cGMP phosphodiesterase, but was found not to affect the catalytic activity of the enzyme [13,42]. Rather, it was found to bind the α and β subunits of PDE6 by their prenylated COOH ends and to solubilize them from the photoreceptor membrane [42]. PDEδ has also been shown to interact with and solubilize Rab 13, a small GTPase, involved in membrane trafficking in fibroblasts [43]. PDEδ also interacts with the retinitis pigmentosa GTPase regulator (RPGR), the pathogenic gene for X-linked retinitis pigmentosa, RP3 [44]. The amino-terminal half of RPGR is homologous to regulator of chromosome condensation (RCC1), which is essential for nucleo-cytoplasmic transport [45,46]. Cumulatively, these results point to a pattern of involvement of PDEδ in intracellular protein transport and localization.

The crystal structure of the complex between PDEδ and ARL2-GTP was recently elucidated, and the amino acid residues in PDEδ that are involved in its interaction with ARL2 were delineated [16]. Importantly, there is a high degree of conservation of these residues in HRG4 (10 of 13 residues (77%) identical or similar), suggesting that the molecular nature of the interaction between HRG4 and ARL2 may be very similar to that between PDEδ and ARL2 (Fig. 7). A hydrophobic pocket has been shown to be important in PDEδ for the binding of prenylated proteins, such as the subunits of PDE6, and the residues that are important for this pocket were also delineated [16]. Again, there is significant conservation of these residues in HRG4 (11 of 17 residues (65%) identical or similar), suggesting that HRG4 may also have such a hydrophobic pocket that may play a role in binding prenylated proteins (Fig. 7). Based on the findings, PDEδ was suggested to be a soluble transport factor for certain prenylated proteins which is mediated by interaction with ARL2-GTP [16]. HRG4 was shown not to be able to extract PDE6 from the photoreceptor outer segment membrane [47], but it may play a role in the insertion/extraction of some other membrane protein. Because of its association with synaptic vesicles, it is tempting to speculate that HRG4 may be involved in the membrane trafficking of certain synaptic vesicle proteins. Interestingly, we demonstrated a specific decrease in the levels of several peripheral membrane proteins of photoreceptor synaptic vesicles in a transgenic model expressing the identical truncation mutation of HRG4 as found in a human patient with late-onset cone–rod dystrophy [5]. This phenomenon may be consistent with HRG4 playing a role in membrane trafficking of photoreceptor synaptic vesicle proteins. Whether HRG4 has such a function will be determined through further investigation of HRG4 in vitro.

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