Localization of HRG4, a Photoreceptor Protein Homologous to Unc-119, in Ribbon Synapse

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PURPOSE. To characterize further HRG4, a novel photoreceptor protein recently identified by subtractive cDNA cloning, by sequence analysis and immunolocalization.

METHODS. The rat homolog of HRG4, RRG4, was expressed and used to prepare an antibody. The antibody was used in Western blot analysis, and immunofluorescent localization at the light and electron microscopic levels of HRG4-RRG4 protein. The HRG4-RRG4 sequence was also analyzed for homologies.

RESULTS. HRG4-RRG4 showed 57% homology with unc-119, a Caenorhabditis elegans neuroprotein causing defects in locomotion, feeding, and chemosensation when mutated. By Western blot analysis, the HRG4-RRG4 protein was demonstrable only in retina and was soluble in nature. Immunofluorescence microscopic study of human and rat retinas, using the HRG4-RRG4 antibody, and other rod and cone photoreceptor-specific antibodies showed that the HRG4-RRG4 protein is localized in the outer plexiform layer of the retina in the synaptic termini of rod and cone photoreceptors. Electron microscopic immunolocalization showed the protein in the cytoplasm and on the presynaptic membranes of the photoreceptor synapses.

CONCLUSIONS. The homology to unc-119 and localization to the photoreceptor synapse are suggestive of a function for HRG4-RRG4 in photoreceptor neurotransmission. HRG4 is the first photoreceptor-enriched synaptic protein to be reported, suggesting that its function may be unique to the specialized ribbon synapses formed between photoreceptors and the horizontal and bipolar cells of the retina. (Invest Ophthalmol Vis Sci. 1998;39:690-698)

Recently, it has been determined that HRG4 is related to a gene discovered in Caenorhabditis elegans. The C. elegans gene, unc-119, was isolated in a mutant nematode with defects in locomotion, feeding behavior, and chemosensation. The defect in locomotion was shown to be based in the nervous system, most likely involving sensory and motor neurons. Expression of unc-119 was seen in many neurons, consistent with the abnormal phenotype of the worm. Because unc-119 appears to be involved in nematode neuronal function, including chemosensation, the homology of HRG4 with unc-119 is suggestive that the novel photoreceptor protein might also be involved in neuronal signal induction or transmission in the photoreceptors. In this report, the production of an antibody to HRG4-RRG4 and its localization to the synaptic termini of rod and cone photoreceptors are described. Of the many known synaptic proteins expressed in photoreceptors, so far only HRG4 has been found to be mainly in photoreceptors, suggestive of a specialized function in visual neurotransmission.

MATERIALS AND METHODS

Production of a Recombinant RRG4 Protein

The recently identified HRG4 is a novel human photoreceptor cDNA encoding a 240 amino acid protein. The HRG4 cDNA was one of the clones isolated by a subtractive cDNA cloning strategy to obtain novel human retina-specific cDNAs. The expression of HRG4, determined by Northern blot analysis, appears only in the retina among the 14 different human protein or gene sequences.

Supported by the National Institutes of Health, Bethesda, Maryland; by Research to Prevent Blindness, New York, New York; and on the presynaptic membranes of the photoreceptor synapses.
The polymerase chain reaction product was digested with BamHI and ligated into a bacterial expression vector, pGEX4T-2 (Pharmacia Biotech; Uppsala, Sweden). Transformants with the correct orientation were selected according to the pattern of restriction fragments. Possible point mutations introduced in the coding region by Taq polymerase were excluded by DNA sequencing. Six hundred milliliters of bacterial culture with an OD600 of 1 was incubated at 30°C for 5 hours with 0.17 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce the expression of a glutathione-S-transferase-RRG4 fusion protein. The bacterial pellet was suspended in 20 ml phosphate-buffered saline (PBS; 150 mM NaCl, 16 mM Na2HPO4, and 4 mM NaH2PO4 [pH 7.3]) containing 2 mM ethylenediaminetetraacetic acid, 0.1% β-mercaptoethanol and 0.2 mM phenylmethylsulfonyl fluoride and lysed on ice by mild sonication. After solubilization with 1% Triton X-100, the fusion protein was adsorbed to 2 ml glutathione-Sepharose beads (Pharmacia Biotech). The beads were washed with PBS and were incubated with 8 NIH units of thrombin (Sigma, St. Louis, MO) at room temperature for 30 minutes, to release the recombinant RRG4 protein from the fusion partner binding to the glutathione-Sepharose (Pharmacia Biotech). The resultant protein contains two extra amino acids (Gly and Ser) at the aminoterminus of the RRG4 protein.

Production and Affinity Purification of the Polyclonal Anti-RRG4 Antibody

All procedures using animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A New Zealand White rabbit was anesthetized and injected intradermally at multiple sites with 150 µg of the recombinant RRG4 protein, emulsified in Freund's complete adjuvant (Sigma). One hundred micrograms of the antigen, emulsified in Freund's incomplete adjuvant (Sigma), was given as a booster injection every 2 weeks thereafter. The activity of the antisera was tested on Western blots. Antisera with high activity were purified using an affinity column with the recombinant RRG4 protein attached to CNBr-activated Sepharose 4B (Pharmacia Biotech), according to the manufacturer's protocol. The antibodies were adsorbed to the antigen Sepharose gel and were eluted with 100 mM glycine (pH 3).

The concentration of the antibody was estimated from the OD280 (1 OD280 = 0.75 mg/ml) with a spectrophotometer (DU Series 60; Beckman Instruments; Fullerton, CA).

Protein Extracts from Tissues

Adult rat (Long Evans) tissues (retina, brain, lung, heart, muscle, liver, and kidney) were homogenized in a 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 were centrifuged at 10,000g for 10 minutes at 4°C. Protein concentration of the supernatant was measured by the modified Lowry method using a kit (Micro Protein Determination; Sigma). Ten micrograms of protein per lane was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

Protein Fractionation of the Rat Retina

Adult rat retinas were homogenized in a buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 0.02% sodium azide, 100 µg/ml phenylmethylsulfonyl fluoride, and 1 µg/ml aprotinin and was centrifuged at 1,000g for 10 minutes at 4°C to remove cellular debris. The supernatant was centrifuged at 105,000g for 1 hour at 4°C to separate the cytosolic fraction from the membrane pellet. Ten micrograms of protein from the supernatant fraction was used for Western blot analysis. The membrane fraction was suspended in the same volume of sample buffer (50 mM Tris–HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) for SDS–PAGE as the supernatant fraction, and an amount equivalent to that used for the supernatant fraction was subjected to Western blot analysis.

Triton X-114 phase separation was also performed to separate the hydrophilic proteins from the amphiphilic membrane proteins, as described by Bordier. Briefly, adult rat retina was homogenized in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 100 µg/ml phenylmethylsulfonyl fluoride and centrifuged at 1,000g for 10 minutes at 4°C to remove tissue debris. Proteins in the supernatant were solubilized with 1% Triton X-114 (Sigma) on ice and overlaid on a cushion of 6% (wt/vol) sucrose, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.06% Triton X-114, and incubated at 30°C for 5 minutes to induce phase separation. After centrifugation at 300g for 3 minutes, the upper aqueous phase and the detergent phase were supplemented with Triton X-114 and buffer, respectively, to obtain equal concentrations of salt and detergent. Ten micrograms of protein from the aqueous phase and an equivalent amount of the detergent phase were used for Western blot analysis.

Western Blot Analysis

Protein samples were suspended in the sample buffer and fractionated by SDS–PAGE (12.5% acrylamide). Equal amounts of each sample were loaded in two gels, except for the recombinant RRG4 protein (approximately 200 ng for Coomassie blue staining, 10 ng for Western blot analysis). One gel was stained with Coomassie blue (Bio-Rad; Hercules, CA) to check the quantity of the protein in each sample, and the duplicate was electroblotted onto a membrane (Immobilon-P; Millipore, Bedford, MA). The blot was incubated at 4°C overnight in a blocking buffer containing 5% (wt/vol) nonfat dried milk in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20. After the blocking of nonspecific binding sites, the blot was incubated, first with 1 µg/ml affinity-purified anti-RRG4 antibody in the blocking buffer for 2 hours at room temperature, then with the complex of biotinylated goat antirabbit IgG (immunoglobulin G; Life Technologies, Gaithersburg, MD) and streptavidin–alkaline phosphatase conjugate (Life Technologies) in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20 for 1 hour at room temperature. The antibody-binding sites on the blot were visualized by incubation with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt. For the control experiment, preimmune serum (1:1000 dilution or 2 µg/ml immunoglobulin, affinity-purified with a protein A-Sepharose CL-4B column [Pharmacia Biotech]), and the flowthrough fraction from the affinity purification of anti-RRG4 antibody (1:5000 dilution) were used for the substitution for the primary antibody.

Immunofluorescent Staining

Adult rat retinas and human donor retinas were fixed in 4% paraformaldehyde in PBS (10 mM phosphate, 130 mM NaCl [pH
After washing, sections were again blocked with goat serum (Technologies).

red-green cone opsin in the human retina, sections were first stained for HRG4, using rhodamine-conjugated goat antirabbit IgG polyclonal antibody (1:50 dilution; Life Technologies) in PBS with 1% BSA at room temperature for 30 minutes. Slides were then washed in PBS and mounted in glycerol–PBS. Pre-immune serum (1:100 dilution) and the flowthrough fraction from the affinity purification of anti-RRG4 antibody (1:100 dilution) were also used to check the background staining and the specificity of the anti-RRG4 antibody.

For the double immunofluorescent staining of HRG4 and Santigent in the human retina, sections were first stained for HRG4 as described earlier, except rhodamine-conjugated goat antirabbit IgG polyclonal antibody (1:50 dilution; Boehringer Mannheim, Indianapolis, IN) was used as a secondary antibody. After washing, sections were again blocked with goat serum and reacted with 1:100 antiovine Santigent monoclonal antibody, A9-C6 (a generous gift from L. Donoso) followed by FITC-conjugated antimouse IgG antibody (1:50 dilution; Life Technologies).

For the double immunofluorescent staining of HRG4 and red-green cone opsins in the human retina, sections were first stained for HRG4, using rhodamine-conjugated goat antirabbit IgG polyclonal antibody, followed by postfixation in cold methanol to mask the epitopes of the anti-RRG4 antibody that are recognizable by the second polyclonal antirabbit IgG antibody. The sections were washed, blocked with goat serum, and incubated with the polyclonal antihuman red-green cone opsin antibody (generous gift from J. Saari) and FITC-conjugated antirabbit IgG antibody. All slides were examined using a Zeiss photomicroscope III (Carl Zeiss, Oberkochen, Germany) by phase contrast and fluorescence microscopy, using filter sets selective for fluorescein and rhodamine.

Electron Microscopic Immunocytochemistry

Adult rat retina was fixed as described earlier and frozen-sectioned at 16 μm thickness. The sections were postfixed in 4% paraformaldehyde, washed in PBS, immersed in 0.3% hydrogen peroxide, and blocked with 10% normal goat serum in PBS and 0.1% Triton X-100 for 30 minutes, followed by incubation with the affinity-purified rabbit anti-RRG4 antibody (2 μg/ml) in PBS with 1% bovine serum albumin (BSA) at room temperature for 1.5 hours. After washing in PBS, the sections were treated with fluorescein isothiocyanate–conjugated (FITC) goat antirabbit IgG polyclonal antibody (1:50 dilution; Life Technologies) in PBS with 1% BSA at room temperature for 30 minutes. Slides were then washed in PBS and mounted in glycerol–PBS. Pre-immune serum (1:100 dilution) and the flowthrough fraction from the affinity purification of anti-RRG4 antibody (1:100 dilution) were also used to check the background staining and the specificity of the anti-RRG4 antibody.

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RESULTS

Figure 1 shows the alignment of unc-119 with the sequences of the human (HRG4) and rat (RRG4) photoreceptor proteins. The overall homology of unc-119 with the photoreceptor proteins was 57%. As previously demonstrated, the homology between HRG4 and RRG4 reflects the presence of a two-domain structure in this protein, in that the region of the first domain (proximal one fourth) is moderately homologous, whereas the region of the second domain (distal three fourths) is 100% conserved between the two sequences. It is interesting to note that unc-119 showed the same pattern of homology with HRG4–RRG4: The homology was at most 29%, with huge gaps in the proximal one fourth, whereas it was 59% in the distal three fourths.

A prokaryotic expression clone of RRG4 was constructed using the glutathione-S-transferase gene fusion system, and recombinant RRG4 was produced and purified by glutathione affinity chromatography and thrombin cleavage. The recombinant RRG4 was used to produce a polyclonal rabbit antiserum, which was subsequently affinity-purified. In Western blot analysis of human and rat retinal proteins, the affinity-purified antibody demonstrated a specific reaction with a 35- and 33-kDa species for HRG4 and RRG4, respectively, along with a strong reaction with the 33-kDa recombinant RRG4 (Fig. 2). The antibody to the rat protein showed a strong cross-reactivity with the human antigen. The preimmune serum gave no reaction with the retinal proteins or the recombinant RRG4. Significantly, the sizes of the immunoreactive native HRG4 and RRG4 were identical with those of the in vitro–expressed products, confirming that the larger-than-calculated sizes are real characteristics of these proteins. The size of the recombinant RRG4 expressed through the glutathione-S-transferase gene fusion system also matched that expressed by in vitro transcription and translation.

To determine the subcellular distribution of RRG4 protein, retinal extract was fractionated by high-speed centrifugation, and the membrane fraction and supernatant were examined by Western blot analysis. The result demonstrated the immunoreactivity to be in the supernatant (Fig. 2, lanes 4, 5). Fractionation of the retinal proteins into membrane and non-membrane portions by phase separation, using Triton X-114, was also performed. Western blot analysis of the resultant aqueous and detergent phases also indicated RRG4 to be in the soluble, nonmembranous fraction (Fig. 2, lanes 6, 7).

The expression of HRG4–RRG4 was previously shown to be only in the retina by Northern analysis of RNAs from 14 different tissues, including the retina, iris, cornea, and retinal pigment epithelium from the eye. Tissue expression at the protein level was examined by Western blot analysis, using the antibody. Analysis of proteins from seven different rat tissues demonstrated the presence of 33-kDa RRG4 protein only in the retina (Fig. 3), consistent with the results of the mRNA expression studies.
HRG4 alone. The positive staining of cone outer segments for diffusible HRG4 component may be because of the shortness and the more open access of the cone outer segments for diffusible HRG4 component compared with that of rod outer segments. The presence of HRG4 in the rod and cone inner/outer segments could also be appreciated in sections stained with the HRG4 antibody alone (Fig. 5A). This result thus indicates that HRG4 might be present in the rod inner segments indicating the presence of HRG4 and S-antigen, and red immunofluorescence of the rod inner segments indicating the expression of HRG4 and S-antigen in the rod axons, in the termini, or in both, with some pockets of red staining, representing sites of localization of HRG4 alone in the cone terminals (Fig. 5C). The same pattern was also seen in the outer portion of the photoreceptors, with yellow immunofluorescence of the rod outer segments indicating the presence of HRG4 and S-antigen, and red immunofluorescence of the cone inner and outer segments indicating the presence of HRG4 alone. The positive staining of cone outer segments for HRG4 may be because of the shortness and the more open access of the cone outer segments for diffusible HRG4 component compared with that of rod outer segments. The presence of HRG4 in the rod and cone inner/outer segments could also be appreciated in sections stained with the HRG4 antibody alone (Fig. 5A). This result thus indicates that HRG4 might be present in the outer portion of the photoreceptors, with yellow immunofluorescence of the rod outer segments indicating the presence of HRG4 and S-antigen, and red immunofluorescence of the cone inner and outer segments indicating the presence of HRG4 alone.

As previously shown, human retinal sections immunostained with an antibody against red-green cone opsin display intense staining of the cone outer segments, as well as faint staining of the cone axons and termini (Fig. 5F); thus, the distribution of cone termini within the OPL can be visualized with this antibody. To take advantage of this, a comparison was made of the immunoreactivity observed in the HL-OPL of human retinas double stained with antibodies to HRG4-RRG4 (Fig. 5E) and cone opsin (Fig. 5F). The result of this combina-
The precise localization of HRG4 within the photoreceptor synaptic termini was investigated by electron microscopic immunocytochemistry. A preembedding method of electron microscopic immunocytochemistry\textsuperscript{11} was used, because RRG4 antigenicity was lost after fixation with glutaraldehyde. Analysis of ultrathin sections from immunoreacted rat retinal samples showed electron-dense particles in the presynaptic space of rod photoreceptor spherules in the outer plexiform layer, whereas the horizontal and bipolar cell processes were devoid of reaction (Fig. 6A). Within the spherules, the particles were homogeneously distributed in the presynaptic space and were localized to the presynaptic membranes of the horizontal and bipolar cell processes. In the inner plexiform layer, the termini were devoid of reaction (data not shown). Control sections reacted with preimmune serum were devoid of immunoreactivity (Fig. 6B). Because of the paucity of cones in rat retina\textsuperscript{12} these tissues were not suitable for electron microscopic immunocytochemical study of this protein in cone termini.

FIGURE 2. Western blot analysis of HRG4 protein. (A) Protein samples were fractionated in a 12.5% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto a polyvinylidene-fluoride membrane. The blot was reacted with the affinity-purified anti-RRG4 antibody (1 \( \mu \)g/ml), and color development was performed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt. Lanes: 1, protein extract of adult human retina (10 \( \mu \)g); 2, recombinant RRG4 protein (10 ng); 3, protein extract of adult rat retina (10 \( \mu \)g); 4, supernatant of the homogenate of adult rat retina centrifuged at 105,000g for 1 hour at 4°C (10 \( \mu \)g); 5, pellet of the homogenate of adult rat retina (membrane fraction) centrifuged at 105,000g for 1 hour at 4°C and resuspended in the same volume of the sample buffer as the supernatant; 6, aqueous phase of Triton X-114 phase separation of the homogenate of adult rat retina (10 \( \mu \)g); and 7, detergent phase of Triton X-114 phase separation of the homogenate of adult rat retina diluted back to the same volume as the aqueous phase. (B) Coomassie blue staining of the duplicate gel of (A). A larger amount of recombinant RRG4 (200 ng) was loaded in lane 2 for the visualization of the protein. For other lanes, the same amount of protein was loaded as in (A). (C) Protein samples were electrophoresed and blotted as described. The blot was reacted with the preimmune serum (2 \( \mu \)g/ml immunoglobulin affinity-purified with a protein A-Sepharose [Pharmacia Biotech] CL-4B column). Lanes: 1, protein extract of adult rat retina (10 \( \mu \)g); and 2, recombinant RRG4 (10 ng). (D) Coomassie blue staining of the duplicate gel of (C). A larger amount of recombinant RRG4 (200 ng) was loaded in lane 2 for the visualization of the protein.

FIGURE 3. RRG4 protein expression in various tissues of the adult rat. (A) Protein extracts (10 \( \mu \)g) from adult rat tissues (lanes: 1, retina; 2, brain; 3, lung; 4, heart; 5, muscle; 6, liver; and 7, kidney) were separated in a 12.5% sodium dodecyl sulfate-polyacrylamide gel and electroblotted. The blot was reacted with the affinity-purified anti-RRG4 antibody (1 \( \mu \)g/ml), and color development was performed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt. Arrowhead indicates the position of the band (approximately 33 kDa) seen only in the retina lane. (B) Coomassie blue staining of the duplicate gel of (A). A larger amount of protein was loaded as in (A). (C) Protein samples were electrophoresed and blotted as described. The blot was reacted with the preimmune serum (2 \( \mu \)g/ml immunoglobulin affinity-purified with a protein A-Sepharose [Pharmacia Biotech] CL-4B column). Lanes: 1, protein extract of adult rat retina (10 \( \mu \)g); and 2, recombinant RRG4 (10 ng). (D) Coomassie blue staining of the duplicate gel of (C). A larger amount of recombinant RRG4 (200 ng) was loaded in lane 2 for the visualization of the protein.
A Photoreceptor HRG4 Localizes to Ribbon Synapse 695

FIGURE 4. Immunofluorescent staining of HRG4-RRG4 protein in rat and human retina. Adult rat (A, B, C) and human (D, E, F) retina was fixed in 4% paraformaldehyde and frozen-sectioned. The sections were incubated with 2 μg/ml affinity-purified anti-RRG4 antibody (A, D) or the preimmune serum diluted at 1:100 (C, F) followed by reaction with FITC-conjugated secondary antibody. Phase contrast images of rat (B) and human (E) retina are shown for the identification of retinal layers. The paracentral region of the fundus is shown. The immunoreactivity is mainly localized to the OPL in both species, whereas inner segments of rods and cones (human) show weak staining (A, D), not seen in control specimens (C, F). In the paracentral human retina, the outer plexiform layer is thickened by the presence of elongated photoreceptor axons forming Henle's fiber layer. The human retina shows autofluorescence in the retinal pigment epithelium (D, F). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; HL-OPL, Henle's fiber layer-outer plexiform layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium. Scale, 50 μm.

DISCUSSION

The results indicate that HRG4-RRG4 is a very abundant synaptic protein in photoreceptors. As such, it would be the first of its kind because, although a number of proteins have been identified in the OPL or in the photoreceptor ribbon synapse, none qualifies as a protein that is predominantly, if not exclusively, in the photoreceptor synapse. B16 is an 88-kDa antigen present in the synaptic ribbon of photoreceptor terminals, but it is also present in the brain. Dystrophin has also been localized to the OPL presynaptic region in which it is thought to control the calcium channel and neurotransmission, but it is also present in other tissues, including brain. RET52 is a 52-kDa protein, detected in the inner segments of photoreceptors and the OPL and is known to be related to the actin-bundling erythrocyte membrane component, dematin; its localization in the photoreceptor synapse, however, has not been demonstrated.

What might be the function of HRG4? Results of the electron microscopic immunocytochemical study showed that HRG4 is localized in the cytoplasm and on the presynaptic membrane of the horizontal and bipolar processes within the photoreceptor synapse (rod spherule). The images produced by this technique are very similar to those observed in retinal synapses immunostained with antibodies against the synaptic vesicle proteins synaptotagmin, synapsin, and SV2. Thus, HRG4 certainly could be associated with synaptic vesicles, which are present in the cytoplasm and on the presynaptic membrane during docking and exocytosis. HRG4 may play a role in the mechanism of photoreceptor neurotransmitter release through the synaptic ves-
Double immunofluorescent staining of HRG4 with S-antigen and red-green cone opsin.

(A, B) Frozen sections of adult human retina (paracentral region) were stained with anti-RRG4 antibody and rhodamine-conjugated secondary antibody, followed by anti-S-antigen antibody and FITC-conjugated secondary antibody. Color photographs were taken with appropriate sets of filters for HRG4 (A) and S-antigen (B). (C) Double exposure showing superimposition of images seen in (A, B). (D) Phase contrast image of (A, B, C). (A) HRG4 immunoreactivity (red) is prominent in Henle’s fiber layer-outer plexiform layer and is discernible in the inner segments of rods and cones, and the outer segments of cones, but not of rods. Fluorescence in the retinal pigment epithelial layer (A, B, C) is unrelated to the immunostaining and is caused by autofluorescence of lipofuschin in these cells. (B) Prominent S-antigen immunoreactivity is seen in the rod outer segments and in the Henle’s fiber layer-outer plexiform layer. (C) In the double exposure, areas of overlap of HRG4 and S-antigen fluorescent signals in rod photoreceptors are indicated by yellow immunofluorescence, seen in a punctate pattern in the Henle’s fiber layer-outer plexiform layer and in the rod inner segments. A double-labeled (yellow) rod inner segment is indicated (R). The inner segments of most cones (C), by contrast, label only with HRG4 antibody, and appear red. (E, F) Double immunofluorescent staining of HRG4 and red-green cone opsin. Adjacent sections from the human retina in (A, B, C, D) were stained with anti-RRG4 antibody and rhodamine-conjugated secondary antibody, followed by anti-red-green cone opsin antibody and FITC-conjugated secondary antibody. Photographs were taken with appropriate filters for HRG4 (E), and red-green cone opsin (F), and with both filter sets (G). (H) Phase contrast of (E, F, G). (F) Red and green sensitive cones show immunoreactivity for red-green cone opsin predominantly in their outer segments. (B) indicates an unlabeled blue cone also marked in (E, G). Red-green cones also exhibit weak opsin staining throughout the cell, enabling visualization of their synaptic terminals in the Henle’s fiber layer-outer plexiform layer (F). In the double exposure (G), areas of colocalization of the signal for HRG4 and red-green cone opsin appear as yellow-orange immunofluorescence, brightest in the Henle’s fiber layer-outer plexiform layer, and weaker in the inner segments of red and green cones. Inner segments of blue cones display red fluorescence due to labeling of HRG4 alone. In the double exposures, superimposition of the two images is not perfect, because of slight misalignment of the two filter sets. INL, inner nuclear layer; HL-OPL, Henle’s fiber layer-outer plexiform layer; ONL, outer nuclear layer; IS, rod and cone inner segments; OS, rod and cone outer segments; RPE, retinal pigment epithelium; CIS, cone inner segments; COS, cone outer segments; ROS, rod outer segments. Scale, 24 μm.
HRG4 is a novel synaptic protein present in ribbon synapses of rod and cone photoreceptors. It is likely to be involved in some aspect of the synaptic vesicle cycle by interaction with other synaptic proteins in the rod and cone synapse. The homology of HRG4 with unc-119 is consistent with the possibility that HRG4 may play an important role in the synaptic vesicle cycle. Although localization within the synapse or the function of unc-119 in C. elegans has not yet been demonstrated, the existence of a defect in this gene leading to significant problems in movement, feeding, and chemosensation is consistent with a defect in neurotransmission.

What type of synaptic protein could HRG4 be? HRG4 is a 240-amino acid, 35-kDa, acidic (pI 5.96), hydrophilic protein with three different types of putative phosphorylation sites (five casein kinase II, two protein kinase C, and two tyrosine kinase) and a proline- and glycine-rich amino-terminal region of approximately 60 amino acids. Proline-rich regions in proteins are important for protein-protein interactions, many of which also involve phosphorylation. The proline-rich amino terminal region of HRG4, which has several casein kinase II phosphorylation sites, may be important in such interactions. The absence of a hydrophobic membrane-insertion sequence tends to argue against HRG4 as an integral synaptic membrane protein, such as synaptotagmin, synaptobrevin, synaptophysin, or SNAP-25. It could, however, be a peripheral membrane-associated protein, and the association with membrane could be through an N-myristoyl group that may be attached to HRG4 at a consensus site, if the amino-terminal 5 to 8 residues, which are conserved between human and rat, are cleaved off to expose the glycine residue. The electron microscopic picture is consistent with association with synaptic vesicles and the presynaptic plasma membrane, as mentioned earlier.

HRG4 shares several molecular features in common with a number of known synaptic proteins involved in exocytosis and endocytosis. Among these, synapsin 1 is a peripheral membrane phosphoprotein that binds to synaptic vesicles and actin, and is thereby postulated to control their interaction and release, leading to exocytosis. This control is thought to be mediated by phosphorylation: A carboxyl-terminal, proline-rich region with phosphorylation sites is important in binding to, and possibly in controlling the exocytosis of, synaptic vesicles. Synapsin is notable in that it is absent in photoreceptor ribbon synapses, whereas HRG4 appears to be present abundantly in this location. Considering the importance of the postulated functions of synapsin, however, it seems likely that a synapsin homolog would exist in ribbon synapses. It is possible that HRG4 could be such an entity. Similarly, an essential synaptic protein, syntaxin 1, is absent in the retinal ribbon synapse; however, a homolog, syntaxin 3, is present instead.

The phenotype of unc-119 C. elegans indicates that the effects of this gene are multisystem, involving neurons in various locations in the worm. On the basis of levels of HRG4 mRNA and protein expression detectable by Northern and Western blot analyses of multiple tissues, it is interesting that the current data indicated that HRG4, the putative mammalian homolog of unc-119, was expressed predominantly in the photoreceptors. There are at least three possible explanations for this: After divergence, the gene may have evolved to have a more limited expression in mammals; HRG4 may be a distant homolog of unc-119, and there may be a closer homolog of unc-119 with a wider range of neuronal expression; and HRG4 may be expressed at very low levels in other neurons, undetectable by the methods used in this study.

In summary, HRG4 appears to be a novel synaptic protein present in ribbon synapses of rod and cone photoreceptors. It will be of interest to elucidate its precise function in...
the mammalian visual system, through biochemical, physiological, and molecular genetic analyses, including the production of transgenic and knockout mice.

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