The rod cGMP-gated channel is localized in the plasma membrane of rod photoreceptor outer segments, where it plays a central role in phototransduction. It consists of α- and β-subunits that assemble into a heterotetrameric protein. Each subunit contains structural features characteristic of nucleotide-gated channels, including a cGMP-binding domain, multiple membrane-spanning segments, and a pore region. In addition, the β-subunit contains a large glutamic acid- and proline-rich region called GARP that is also expressed as two soluble protein variants. Using monoclonal antibodies in conjunction with immunoprecipitation, cross-linking, and electrophoretic techniques, we show that the cGMP-gated channel associates with the Na/Ca-K exchanger in the rod outer segment plasma membrane. This complex and soluble GARP proteins also interact with peripherin-2 oligomers in the rim region of outer segment disc membranes. These results suggest that channel/peripherin protein interactions mediated by the GARP part of the channel β-subunit may play a role in connecting the rim region of discs to the plasma membrane and in anchoring the channel/exchanger complex in the rod outer segment plasma membrane.

The outer segment is a unique cellular compartment of vertebrate rod and cone photoreceptors where light is captured and converted to an electrical signal as the primary step in the visual process. The rod outer segment (ROS) consists of a highly ordered, axial array of hundreds of discs that is enclosed by a separate plasma membrane. Each disc consists of two, closely spaced, flattened membranes circumscribed by a specialized hairpin loop called the disc rim region. The perimeter of the disc is interrupted by one or more incisures that penetrate toward the center of the disc.

The protein composition of the ROS plasma membrane differs from that of the disc membranes. The plasma membrane contains the cGMP-gated channel (2), the Na/Ca-K exchanger (3), the GLUT-1 glucose transporter (4), and other membrane proteins, whereas disc membranes contain peripherin-2 (also known as peripherin/Rds) (5), Rom-1 (6), the retinal ATP-binding cassette transporter ABCR (also known as the rim protein) (7, 8), and guanylate cyclase (9). Peripherin-2 and Rom-1, homologous subunits that assemble as oligomeric complexes (10, 11), and ABCR are restricted to the rim and incisures of disc membranes (5, 7, 8, 12). Rhodopsin, the major membrane protein of rod outer segments, is present in both the flattened (lamellar) region of disc membranes and the plasma membrane. The molecular mechanisms responsible for the targeting of specific proteins to the disc and plasma membrane and protein/protein interactions involved in the formation and stabilization of the unique ROS structure are not known.

The cGMP-gated channel of photoreceptors plays a central role in phototransduction by controlling the flow of cations into the outer segment in response to light-mediated changes in intracellular cGMP. The rod channel consists of two homologous subunits termed α and β that assemble into a heterotetrameric complex (13–16). Each subunit has a cyclic nucleotide-binding domain near the C terminus, six putative transmembrane domains designated S1–S6, and a pore segment between the S5 and S6 membrane-spanning segments. In addition, the β-subunit contains a large cytoplasmic glutamic acid-rich region called GARP at its N terminus that is not present in the α-subunit (16, 17). GARP is also expressed in rod photoreceptors as two spliced variants known as GARP-1 or full-length GARP and GARP-2 or truncated GARP. GARP-1 is identical to the GARP part of the channel β-subunit except for a 19-amino acid C-terminal extension (16–18). GARP-2 is an abundantly expressed truncated variant lacking the C-terminal half of the protein, but containing an 8-amino acid C-terminal extension (17, 19). All three GARP variants have four proline-containing repeat regions within the common N-terminal half (20).

The distribution of GARP proteins in the retina has been examined by immunocytochemical techniques. Immunofluorescence microscopy has revealed that GARP-containing proteins are found in the outer segments of rod (but not cone) photoreceptors (17, 20, 21). Immunoelectron microscopy has more precisely localized GARP proteins to disc incisures and rims that are in close proximity to the plasma membrane (17, 20).

Recently, Korschen et al. (20) have investigated possible interactions of GARP proteins with various soluble and membrane proteins of ROS. In this study, synthetic peptides containing each of four proline-containing repeat regions were coupled to Sepharose beads and used as an affinity matrix to identify GARP-binding proteins. In this approach, the cGMP-
gated channel, guanylate cyclase, ABCR, phosphodiesterase, GARP-1, and GARP-2 all bound to these peptides. On the basis of these studies, it was concluded that GARP organizes cGMP signaling proteins and ABCR into a large multiprotein complex in ROS (20). Although peptide affinity chromatography has been used to investigate protein interactions, such an approach is prone to artifacts arising from nonspecific interactions.

We have used highly specific monoclonal antibodies in conjunction with immunoprecipitation, cross-linking, and electrophoretic techniques to identify ROS membrane proteins that interact with GARP proteins. Here, we report that the cGMP-gated channel and soluble GARP proteins specifically associate with peripherin-2-containing oligomeric complexes at the rim region of disc membranes, but not with ABCR or guanylate cyclase.

In addition, we show that the cGMP-gated channel interacts with the Na/Ca-K exchanger in the ROS plasma membrane, in agreement with earlier studies (21–23). These results suggest that GARP-mediated protein/protein interactions between the channel/exchanger complex in the ROS plasma membrane and peripherin-2 complexes in the rim region of disc membranes play a role in maintaining the spatial arrangement of the disc and plasma membrane in ROS and anchoring the channel/exchanger complex in the ROS plasma membrane.

EXPERIMENTAL PROCEDURES

Preparation of ROS and Separation of Membrane and Soluble Proteins—ROS were isolated from frozen bovine retinas by sucrose gradient centrifugation under dim red light as described previously (1). ROS membranes were separated from soluble proteins as follows. ROS (1 mg) were suspended in 0.2 ml of lysis buffer (2 mM HEPES and 2 mM EGTA, pH 7.4, with or without 10 mM dithiothreitol (DTT)) and incubated for 1 h on ice. The soluble fraction was separated from the membranes by centrifugation at 87,000 × g for 10 min. The membrane pellet was resuspended in 0.2 ml of lysis buffer, incubated for 30 min on ice, and washed by centrifugation. This procedure was repeated two more times. ROS membranes were finally suspended in 0.2 ml of lysis buffer.

Trypsin Digestion of ROS—ROS membranes were digested with trypsin as previously described (1). Briefly, bovine ROS membranes (2 mg/ml) in 0.1 M Tris-HCl, pH 7.5, 0.15 M KCl, and 0.2 mg/ml phenylmethylsulfonyl fluoride were digested with 7 μg of trypsin volume of trypsin (0.8 μg/ml) for 30 min at 4 °C. The reaction was stopped by the addition of excess soybean trypsin inhibitor, and the membranes were washed by centrifugation as described above.

Monoclonal Antibodies—Monoclonal antibodies were generated against glutathione S-transferase fusion proteins containing either the N-terminal 16 amino acids (Garp 8G8 and Garp 4B1) or the C-terminal 12 amino acids (Garp 15F10) of truncated GARP-2 (17). The 8G8 monoclonal antibody was purified from a hybridoma cell culture supernatant by affinity chromatography on a Protein G-Sepharose column. Monoclonal antibodies to peripherin-2 (Per 2B6), Rom-1 (Rom 1C6), ABCR (Rim 3F4), the rod cGMP-gated channel (PME 6E7 and PME 1D1 to the α-subunit and PMB 3C9 to the β-subunit), the Na/Ca-K exchanger (PME 2D9), and human RetGC-1 guanylate cyclase (GC 2H6) have been described (1, 5, 8, 12, 16, 24–26). For immunoprecipitation studies, purified antibodies were covalently coupled to CNBr-activated Sepharose 2B at 1–2 mg of protein/ml of packed beads as previously described (27).

Chemical Cross-linking—Cross-linking experiments were performed on either dark-adapted ROS under dim red light or light-adapted ROS under normal room light. ROS were washed with 20 mM HEPES/KOH, pH 7.5, 0.15 M KCl, and 20% (v/v) sucrose by centrifugation at 12,000 rpm (7800 × g) in a Beckman TL1A100 rotor for 8 min and resuspended at a protein concentration of 2.2 mg/ml. Cross-linking was carried out by adding a 10× stock solution of dithiobis(succinimidyl propionate) (DSP) in dimethyl sulfoxide to the samples on ice. After 2 h, the reaction was terminated by the addition of an equal volume of stop buffer (0.1 M Tris-HCl and 0.5 mM glycerine, pH 7.5). ROS glycerinated at 7800 × g for 8 min, resuspended in stop buffer at a protein concentration of 0.15 mg/ml, incubated for 30 min on ice, and centrifuged again.

Detergent Solubilization and Immunoprecipitation—Untreated or cross-linked ROS pellets were resuspended at a protein concentration of 2 mg/ml in phosphate-buffered saline (PBS; 0.01 M sodium phosphate and 0.15 M NaCl, pH 7.4) or HEPES/KCl (10 mM HEPES and 0.15 M KCl, pH 7.4). ROS were solubilized by the dropwise addition of an equal volume of 2% Triton X-100 in PBS or 30 mM CHAPS in 10 mM HEPES, 0.15 M KCl, and 0.2 mg/ml phenylmethylsulfonyl fluoride. The solution was stirred for 30 min at 4 °C and centrifuged at 87,000 × g for 15 min to remove any residual unsolubilized material.

An antibody-Sepharose matrix was washed four times with 0.3 ml of PBS containing 0.2% Triton X-100 or 15 mM CHAPS by low speed centrifugation in a Millipore Ultrafree MC 0.45-μm filter unit. The detergent-solubilized ROS solution (0.75 mg of protein) was incubated with 100 μl of Sepharose matrix with gentle agitation for 40 min at 4 °C. Thebound protein was recovered by low speed centrifugation, and the matrix was washed four times with 0.3 ml of 0.2% Triton-X-100 or 15 mM CHAPS in PBS containing 0.2% Triton-X-100. Finally, the matrix was incubated at room temperature for 20–30 min with 30–50 μl of 0.2% Triton-X-100 or 10 mM CHAPS in PBS containing the competing peptide or 2% SDS. This procedure was repeated, and the two eluted fractions were combined to produce the bound fraction. Competing peptides were as follows: 0.1 mg/ml DQGGAPAPAG for the Per 2B6 antibody, 1 mg/ml MGHWVRQVLQPPOTIQ for the Garp 8G8-Sepharose, 0.4 mg/ml FROG antibody, and 1 mg/ml SNKQEQKEKKKKKK for the PMe 6E7 antibody.

Immunoprecipitation of Proteins Solubilized in SDS—Untreated or cross-linked ROS membranes were resuspended in PBS at a concentration of 6 mg/ml of protein. An equal volume of 1% (w/v) SDS, 0.1 M N-ethylmaleimide, and 0.2 mg/ml phenylmethylsulfonyl fluoride was added, and the mixture was incubated for 30 min at 37 °C on a shaker. After centrifugation at 100,000 × g for 30 min at 15 °C, the supernatant was diluted 10-fold in PBS containing 0.1% Triton-X-100 and incubated with the antibody-Sepharose matrix for 3 h at 4 °C on a rocker. The antibody-Sepharose matrix was then washed with the same buffer to collect the unbound protein. The bound protein was eluted with competing peptides as described above or with 4% SDS.

Quantification of the Interacting Protein Fraction—ROS were solubilized in Triton-X-100 and immunoprecipitated as described above. To quantify the fraction of Rom-1 and peripherin-2 that was bound to GARP, solubilized ROS were added to Garp 8G8-Sepharose, and the unbound fraction was then added to Per 2B6-Sepharose. To quantify the fraction of GARP in complex with peripherin-2 and Rom-1, solubilized ROS were incubated with Per 2B6-Sepharose to quantitatively bind peripherin-2, and the unbound fraction was then added to Garp 8G8-Sepharose to bind GARP. The bound protein was eluted from the Sepharose matrix with 4% SDS for 15 min at room temperature. Fractions were subjected to SDS-PAGE and Western blotting for ECL. The exposed film was scanned with a laser densitometer to determine the ratio of coprecipitated and free proteins for GARP, peripherin-2, and Rom-1.

SDS-PAGE and Western Blotting—Samples were denatured with an equal volume of loading buffer (4% SDS, 0.02 M Tris-HCl, pH 6.8, 40% glycerine, 0.1% β-mercaptoethanol, and 0.2 mg/ml phenylmethylsulfonyl fluoride) and applied to 10% polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) in a semidy transfer apparatus using the buffer system of Towbin et al. (29). The membranes were labeled with hydride cyclase protein supranant containing the primary monoclonal antibody and peroxidase-conjugated sheep anti-mouse immunoglobulin for detection by ECL (Amersham Biosciences, Inc., Baie d’Urfé, Quebec, Canada).

Blue Native Gel Electrophoresis—Untreated or DTT-treated ROS were solubilized in Triton-X-100, incubated with Per 2B6-Sepharose or Garp 8G8-Sepharose, and eluted from the matrix with a competing peptide as described above. After the addition of glycerol (final concentration of ~20%), the samples were applied to a 4% polyacrylamide stacking gel and a linear 5–15% acrylamide separation gel (dimensions 12.5 × 12.5 cm, 0.1 cm). Blue native gel electrophoresis was performed as described (30). Electrophoresis was started with a cathode buffer containing 0.02% Coomassie Blue G-250. After one-third of the run, the buffer was changed to a cathode buffer containing 0.002% Cooilmassie Blue G-250. Immunoblotting of the native gels on two sheets of Immobilon-P membranes and background destaining were carried out as described (31).

Treatment of ROS—ROS were washed three times with 0.25 ml of PBS/mg of protein by centrifugation and resuspended at 2 mg/ml in PBS with or without 10 mM DTT. After 90 min at room temperature, an equal volume of ice-cold solubilization buffer (2% Triton-X-100, 0.2 mg/ml phenylmethylsulfonyl fluoride, and 80 mM N-ethylmaleimide in PBS) was added. The samples were stirred for 30 min at 4 °C, centrifuged at 87,000 × g for 30 min, solubilized in Triton-X-100, and immunoprecipitated as described above.
RESULTS

Co-immunoprecipitation of ROS Proteins with GARP Proteins Using a GARP Immunoadfinity Matrix—An immunoadfinity matrix consisting of a monoclonal antibody (Garp 8G8) to the N terminus of GARP coupled to Sepharose 2B was used to identify ROS proteins that coprecipitate with GARP proteins. In these experiments, detergent-solubilized ROS were mixed with the Garp 8G8 immunoadfinity matrix and the unbound and bound (peptide-eluted) fractions were analyzed on SDS gels and Western blots. A, Coomassie Blue (CB)-stained gel (left panel) and GARP N terminus-specific Garp 4B1-labeled Western blot (right panel) showing the quantitative immunoprecipitation of the three GARP-containing proteins. B, Western blots of untreated ROS labeled with monoclonal antibodies to the β-subunit of the cGMP-gated channel (PMb 3C9), GARP-2 (Garp 1H5), the α-subunit of the cGMP-gated channel (PMc 1D1), the Na/Ca-K exchanger (PMe 2D9), peripherin-2 (Per 2B6), Rom-1 (Rom 1C6), ABCR (Rim 3F4), and RetGC-1 guanylate cyclase (GC 2H6). C, Western blots of DSP-cross-linked ROS labeled as described for B.

Fig. 1. Immunoprecipitation of ROS proteins on a GARP immunoadfinity matrix. Untreated or DSP-cross-linked, light-adapted ROS membranes were solubilized in Triton X-100 and immunoprecipitated on an immunoadfinity matrix consisting of the GARP N terminus-specific Garp 8G8 monoclonal antibody coupled to Sepharose 2B. The solubilized membranes (Extract) and unbound (Unbound) and bound (Bound) fractions were analyzed on SDS gels and Western blots. A, Coomassie Blue-stained gel (left panel) and GARP N terminus-specific Garp 4B1-labeled Western blot (right panel) showing the quantitative immunoprecipitation of the three GARP-containing proteins. B, Western blots of untreated ROS labeled with monoclonal antibodies to the β-subunit of the cGMP-gated channel (PMb 3C9), GARP-2 (Garp 1H5), the α-subunit of the cGMP-gated channel (PMc 1D1), the Na/Ca-K exchanger (PMe 2D9), peripherin-2 (Per 2B6), Rom-1 (Rom 1C6), ABCR (Rim 3F4), and RetGC-1 guanylate cyclase (GC 2H6). C, Western blots of DSP-cross-linked ROS labeled as described for B.

Western blots labeled with the Garp 4B1 antibody showed that the three GARP proteins, the β-subunit of the channel (240 kDa), GARP-1 (140 kDa), and GARP-2 (62 kDa), quantitatively bound to the GARP immunoadfinity matrix and were eluted with the competing Garp 8G8 peptide. Western blots of the fractions from the Garp 8G8 affinity matrix were also labeled with antibodies to various ROS proteins to identify proteins that coprecipitate with GARP variants. The PMb 3C9 antibody against the C terminus of the channel β-subunit and the Garp 1H5 antibody against the unique C terminus of GARP-2 confirmed that the β-subunit of the channel and the GARP-2 protein quantitatively bound to the affinity matrix (Fig. 1B). The 63-kDa α-subunit of the cGMP-gated channel also quantitatively bound to the Garp 8G8 matrix, a result consistent with earlier studies showing that the α-subunit of the cGMP-gated channel interacts strongly with the β-subunit to form a heteromeric complex (15, 16, 32). The Na/Ca-K exchanger was detected in both the unbound and eluted fractions of the immunoadfinity matrix, indicating that a significant fraction of the exchanger is associated with the channel in detergent solution, in agreement with several earlier studies (21–23).
Interestingly, a significant fraction of peripherin-2 and Rom-1, two homologous tetra-spanning membrane proteins that assemble into multimeric complexes in the rim region and incisures of disc membranes, coprecipitated with the GARP proteins (Fig. 1B). Monomeric bovine peripherin-2 typically migrated on SDS gels as a major 35-kDa and a minor 37-kDa species, presumably due to differences in the extent of N-linked glycosylation, whereas Rom-1, which is not glycosylated, migrated as a single species. In addition, a small amount of SDS-resistant peripherin-2 dimer was observed when large amounts of untreated or cross-linked sample were applied to the gel (Fig. 1, B and C).

The possible interaction of two other disc membrane proteins, ABCR and guanylate cyclase (RetGC-1), with GARP proteins was also investigated. As shown in Fig. 1B, ABCR and RetGC-1 were detected only in the unbound fraction from the GARP immunoaffinity column, indicating that these proteins do not form stable associations with GARP proteins in detergent solution.

Chemical cross-linking was carried out to further evaluate these protein/protein interactions. In these studies, ROS were treated with the disulfide-cleavable cross-linker DSP prior to detergent solubilization and immunoprecipitation on a Garp 8G8-Sepharose matrix. The samples were subsequently subjected to SDS gel electrophoresis under reducing conditions. As indicated in Fig. 1C, the coprecipitation profiles obtained from DSP-cross-linked ROS were generally similar to those of untreated ROS. However, cross-linking had an effect of increasing the amount of peripherin-2 and, to a lesser extent, the Na/Ca-K exchanger that coprecipitated with GARP proteins. Cross-linking also resulted in the appearance of additional higher molecular mass proteins in some instances (β-subunit, GARP-2, and peripherin-2) presumably due to incomplete dissociation of the cross-linked products.

**Co-immunoprecipitation of ROS Proteins with the cGMP-gated Channel on a Channel Immunoaffinity Matrix**—An immunoaffinity matrix consisting of monoclonal antibody PMc 6E7 to the cGMP-gated channel α-subunit coupled to Sepharose was used to identify ROS proteins that coprecipitate with the channel. As shown in Fig. 2, both the α- and β-subunits of the cGMP-gated channel quantitatively bound to the matrix. Peripherin-2 and the Na/Ca-K exchanger were detected in both the unbound and bound fractions, indicating that a significant fraction of these proteins is associated with the channel complex in detergent solution. In contrast, truncated GARP-2, ABCR, and guanylate cyclase (RetGC-1) were detected only in the unbound fraction, indicating that these proteins do not associate with the channel.

**Coprecipitation of GARP Proteins with Peripherin-2 on a Peripherin-2 Immunoaffinity Matrix**—The interaction of GARP proteins and peripherin-2 was further examined using a peripherin-2 immunoaffinity matrix. Untreated and DSP-cross-linked ROS were solubilized in detergent and added to a Per 2B6-Sepharose matrix, and the unbound and bound (Per 2B6 peptide-eluted) fractions were analyzed on Western blots labeled with anti-GARP and anti-peripherin-2 antibodies. As shown in Fig. 3A, a significant fraction of both GARP-2 and the cGMP-gated channel β-subunit coeluted with peripherin-2 with or without prior DSP cross-linking.

To further confirm the existence of covalent cross-links between the GARP proteins and peripherin-2, we developed a method to immunoprecipitate cross-linked proteins that had been solubilized in SDS. In this procedure, DSP-cross-linked ROS membranes were solubilized in SDS to disrupt noncovalent protein interactions, diluted in Triton X-100-containing buffer to reduce the SDS concentration, and immunoprecipitated on a peripherin-2 immunoaffinity matrix. As shown in Fig. 3B, GARP proteins coprecipitated with peripherin-2 only when ROS were cross-linked with DSP prior to SDS solubilization.

**Quantification of the Immunoprecipitated Peripherin-2, Rom-1, and GARP-2 Proteins**—The percentage of total ROS peripherin-2 and Rom-1 that coprecipitates with GARP-2 was determined. This was carried out by comparing the amount of peripherin-2 and Rom-1 in the bound and unbound fractions from a Garp 8G8 immunoaffinity matrix. Since rhodopsin interferes with the transfer of peripherin-2 and, to a lesser extent, Rom-1, the amount of these proteins in the unbound fraction was determined after adsorption to a peripherin-2 (Per 2B6) affinity matrix. Using this approach, 8.8 ± 3.1% of peripherin-2 and 8.9 ± 2.7% of Rom-1 from Triton X-100-solubilized ROS coprecipitated with GARP-2 on a Garp 8G8 immunoaffinity matrix, and 9.7 ± 7.5% of GARP-2 coprecipitated with peripherin-2 on a Per 2B6 affinity matrix.

**Effect of DTT on GARP/Peripherin-2 Interactions**—Previous studies have shown that exposure of ROS to hypotonic buffer containing DTT results in the complete removal of soluble GARP proteins from ROS membranes and their recovery in the soluble fraction after centrifugation (20). We have examined the effect of DTT on the association of the soluble GARP proteins with ROS membranes. Fig. 4A shows that in the presence of DTT, essentially all of GARP-2 and GARP-1 were recovered in the soluble fraction, whereas the β-subunit of the cGMP-gated channel remained in the membrane pellet, a result that is consistent with an earlier study (20).
however, a significant fraction of GARP-2 was still associated with the membrane fraction.

The effect of DTT on the interaction of peripherin-2 and GARP proteins was further examined by immunoprecipitation. In these studies, ROS were treated with DTT under conditions known to reduce disulfide-linked peripherin-2 oligomers (11). After quenching the DTT with excess N-ethylmaleimide, ROS were solubilized in detergent and immunoprecipitated on a Garp 8G8-Sepharose matrix. As shown in Fig. 4B, prior treatment of ROS with DTT eliminated the coprecipitation of peripherin-2 with GARP. Glutathione, which does not reduce intermolecular disulfide bonds responsible for peripherin-2 oligomerization, did not prevent coprecipitation of GARP and peripherin-2 (data not shown), suggesting that the DTT effect is specific for peripherin-2 and is not due to secondary oxidation.

Blue Native Gel Electrophoresis—Blue native polyacrylamide gel electrophoresis is a technique used to analyze membrane proteins under nondenaturing conditions (30). We have used this technique to further analyze peripherin-2 and GARP complexes under reducing and nonreducing conditions. Untreated and DTT-treated ROS solubilized in Triton X-100 were immunoprecipitated on either a Garp 4B1 monoclonal antibody or a Per 2B6-Sepharose affinity matrix. The bound proteins were resolved using blue native electrophoresis and analyzed by Western blotting. As shown in Fig. 5 (lane a), GARP-2 isolated from DTT-reduced ROS migrated as a single species (band 1) just ahead of the monomeric bovine serum albumin standard. In contrast, GARP-2 isolated from nonreduced ROS migrated as a ladder of slower migrating species (bands 3–5) in addition to the faster migrating species (band 1) observed under reducing conditions (lane b). When this blot was reprobed with the anti-peripherin-2 Per 2B6 antibody, bands 3–5 were labeled, whereas band 1 was not (lane c). Peripherin-2 immunoprecipitated from nonreduced ROS (lane d) showed a similar pattern, except for an additional species (band 2), which most likely represents peripherin-2 not bound to GARP-2.

ABCR Does Not Coprecipitate with GARP Proteins—We further investigated the possible association of ABCR with GARP proteins using an ABCR immunoaffinity matrix. Fig. 6 shows
The migration positions of the bovine serum albumin (BSA) antibody and for peripherin-2 with the Per 2B6 antibody as indicated. Western blots were labeled for GARP-2 with the Garp 1H5 antibody or Per 2B6 peptide and resolved by blue native gel electrophoresis. Untreated (lanes a–c) or DTT-treated (lanes d) immunoprecipitation on a Garp 8G8 (lanes a–c) or Per 2B6 (lane d) immunoprecipitation matrix. The bound complex was eluted with the Garp 8G8 or Per 2B6 peptide and resolved by blue native gel electrophoresis. Western blots were labeled for GARP-2 with the Garp 1H5 antibody and for peripherin-2 with the Per 2B6 antibody as indicated. The migration positions of the bovine serum albumin (BSA) monomer, dimer, and trimer markers are indicated for references. Band 1, GARP-2; band 2, peripherin-2 protein; bands 3–5, higher order peripherin-2-GARP-2 complexes.

FIG. 5. Analysis of GARP-2/peripherin-2 association by blue native gel electrophoresis. Untreated (−DTT) or DTT-treated (+DTT), light-adapted ROS were solubilized in Triton X-100 and subjected to immunoprecipitation on a Garp 8G8 (lanes a–c) or Per 2B6 (lane d) immunoprecipitation matrix. The bound complex was eluted with the Garp 8G8 or Per 2B6 peptide and resolved by blue native gel electrophoresis. Western blots were labeled for GARP-2 with the Garp 1H5 antibody and for peripherin-2 with the Per 2B6 antibody as indicated. The migration positions of the bovine serum albumin (BSA) monomer, dimer, and trimer markers are indicated for references. Band 1, GARP-2; band 2, peripherin-2 protein; bands 3–5, higher order peripherin-2-GARP-2 complexes.

In this study, we have focused on protein/protein interactions involving GARP-containing proteins of ROS. GARP domains are characterized by their relatively high content of glutamic acid residues. However, the N-terminal 291-amino acid region common to the three GARP proteins (β-subunit of the cGMP-gated channel, GARP-1, and GARP-2) possesses an even higher content of proline residues (17% proline compared with 13% glutamic acid for bovine GARP proteins). Since proline residues are commonly involved in protein/protein interactions (33), we reasoned that GARP proteins might also function in this capacity in ROS.

Our studies indicate that the GARP proteins specifically associate with peripherin-2 complexes at the rim and incisures of ROS discs. Evidence for this interaction is based on a series of experiments employing highly specific monoclonal antibodies with immunoprecipitation, cross-linking, and electrophoretic techniques. First, a GARP immunoaffinity matrix that quantitatively binds all GARP proteins coprecipitated peripherin-2/Rom-1 oligomeric complexes, but not two other disc membrane proteins, ABCR and guanylate cyclase (RetGC-1), even when ROS were chemically cross-linked prior to immunoprecipitation (Fig. 1). Second, a channel α-subunit immunoaffinity matrix that quantitatively binds the channel α-subunit immunoaffinity matrix that quantitatively binds the cGMP-gated channel, GARP-1, and GARP-2) possesses an even higher content of proline residues (17% proline compared with 13% glutamic acid for bovine GARP proteins). Since proline residues are commonly involved in protein/protein interactions (33), we reasoned that GARP proteins might also function in this capacity in ROS.

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2-Rom-1 complexes is consistent with the localization of these proteins in ROS. Electron microscopic studies employing both pre-embedding and post-embedding immunogold labeling techniques have confined peripherin-2 and Rom-1 to the rim and incisures of ROS disc membranes (5, 6, 12). More recently, GARP proteins have been localized to disc rims and incisures by post-embedding immunogold labeling studies for electron microscopy (20). Sequestration of soluble GARP proteins to the disc rim and incisures is consistent with their interaction with peripherin-2 complexes.

GARP proteins appear to bind to peripherin-2 complexes with variable affinities. Under nonreducing conditions, a considerable fraction of the soluble GARP proteins is weakly bound and released from the disc membranes upon hypotonic lysis of ROS. A significant fraction (~10%), however, is more tightly bound and remains associated with the peripherin-2 complexes even after hypotonic lysis and detergent solubilization. The tightly bound fraction is lost when ROS are pretreated with DTT under conditions that result in the reduction of disulfide-linked peripherin-2 oligomers to core tetramers (11). This behavior suggests that GARP proteins bind tightly to disulfide-linked peripherin-2 oligomers. Since all GARP proteins colocalize with peripherin-2 to the rim and incisures of discs, soluble GARP proteins most likely also bind to reduced core peripherin-2 homotetramers and peripherin-2-Rom-1 heterotetramers in ROS, although with reduced affinity, such that this interaction is disrupted by low ionic strength and detergent extraction.

The interaction of the cGMP-gated channel with peripherin-2 complexes is of particular interest. The cGMP-gated channel is exclusively localized in the ROS plasma membrane (2), whereas peripherin-2 complexes are present along the rim region of disc membranes (5). The interaction of these membrane protein complexes must therefore occur within the narrow cytoplasmic space between the disc and plasma membrane. The exact distance between the rim region of discs and the plasma membrane has not been accurately determined due to the difficulties inherent in sample preparations. However, one report utilizing freeze-fracture techniques indicates that this gap is <10 nm (35), a distance over which the GARP part of the channel and the C-terminal domain of peripherin-2 could interact. Previous pre-embedding immunogold labeling studies are consistent with this interaction. In hypotonically lysed and washed ROS, GARP labeling is most pronounced in regions where the discs come in close contact with the plasma membrane (17). Protein/protein interactions are known to mediate the association of discs with the plasma membrane since mild trypsin treatment of hypotonically lysed ROS results in complete dissociation of discs from the ROS plasma membrane (1, 5). In this study, we have shown that these same conditions result in cleavage of GARP from the channel β-subunit (Fig. 7). These results suggest that channel/peripherin-2 interactions contribute to the connections between the disc and plasma membrane that are important in the formation and stabilization of the ROS structure. Fibrous elements have been observed by electron microscopy to link the rim region of disc membranes to the ROS plasma membrane (35, 36). It remains to be determined if such structures represent channel-peripherin-2 complexes or if they correspond to other trypsin-sensitive proteins that help to maintain the ROS structure. The interaction of peripherin-2 and the channel may also serve to anchor the channel-exchanger complex within the plasma membrane, preventing it from freely diffusing in the plane of the membrane (37). Such an interaction may explain the highly variable channel density observed in excised patches from salamander retinal rod outer segments (38).

These studies lead to a working model for protein/protein interactions that occur between ROS disc and plasma membrane proteins (Fig. 8). In this model, the channel associates with the exchanger in the ROS plasma membrane to form a large multiprotein complex. This complex interacts with disulfide-linked peripherin-2-containing oligomers and possibly core peripherin-2 tetramers across the gap between the disc and plasma membrane. Soluble GARP proteins also interact strongly with disulfide-linked peripherin-2-Rom-1 oligomeric complexes and more weakly with core tetramers. This interaction confines soluble GARP proteins to the rim and incisures of disc membranes. On the other hand, ABCR and guanylate cyclase do not appear to interact with the peripherin-2-GARP complexes.

Our results differ from those of Korschchen et al. (20). These investigators have reported that a number of proteins, including GARP-1, GARP-2, the channel α- and β-subunits, ABCR, guanylate cyclase, and phosphodiesterase, associate with GARP proteins on the basis of the binding of these proteins to short, immobilized, synthetic, proline-rich GARP peptides. Using similar buffer and detergent conditions, however, we have been unable to demonstrate any association of ABCR or guanylate cyclase with GARP-containing proteins including the channel, and we have not observed any interaction of soluble GARP proteins with the channel with or without covalent cross-linking. The reason for these differences is unclear, but it may arise from nonspecific interactions associated with the use of small peptide affinity matrices in the former study.

The role of soluble GARP proteins in rod photoreceptors remains to be firmly established. Soluble GARP proteins may serve to cap peripherin-2 complexes at the rim region, preventing the C terminus of peripherin-2 from initiating fusion of the discs with the plasma membrane (39). Alternatively, these GARP proteins may play a role in inhibiting light-activated phosphodiesterase in the vicinity of the channel, as suggested by the in vitro studies of Korschchen et al. (20). In a preliminary study, we investigated the possible interaction of phosphodiesterase with soluble GARP using immunoprecipitation techniques; we observed that a minor fraction of phosphodiesterase coprecipitated with soluble GARP proteins on a GARP immu-
no affinity matrix. Further studies are being carried out to evaluate the possible role of GARP proteins in modulating phosphodiesterase activity.

In summary, our studies indicate that soluble GARP proteins and the GARP part of the channel \( \beta \)-subunit associate with the peripherin-2 complexes at the rim region of ROS disc membranes. The interaction of the channel/exchanger complex with peripherin-2 complexes via the GARP part of the channel provides the first direct evidence for specific protein/protein interactions across the space between ROS disc and plasma membranes.

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