Purification and Light-Dependent Phosphorylation of a Candidate Fusion Protein, the Photoreceptor Cell Peripherin/rds[†]

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ABSTRACT: The proteins peripherin/rds and rom-1 form a protein complex in the rims of photoreceptor outer segment disk membranes. Peripherin/rds plays an essential role in the morphogenesis and maintenance of disk membrane structure, with peripherin/rds gene mutations resulting in photoreceptor cell degeneration. We report two different chromatographic procedures for the purification of native peripherin/rds from bovine photoreceptor cell outer segments and show that the protein is a phosphoprotein that promotes membrane fusion *in vitro.* During one procedure, peripherin/rds was copurified in association with rom-1 by hyroxylapatite and Mono Q FPLC. During the other, it was purified free from rom-1 by concanavalin-A affinity chromatography and chromatofocusing. Analysis of homogeneous peripherin/rds from the second procedure showed that exposure of photoreceptor outer segments to light resulted in the incorporation of nearly 2 mol of phosphate per mole of peripherin/rds and a concomitant shift in the isoelectric point of the protein. In addition, we found that recombination of purified peripherin/rds into lipid vesicles increased membrane fusion, with more rapid fusion detected with phosphorylated peripherin/rds. In conclusion, studies with purified peripherin/rds reveal that the protein undergoes light-dependent phosphorylation and that it may function in membrane fusion.

Vertebrate photoreceptor rod cells mediate phototransduction within specialized compartments termed outer segments. Rod photoreceptor cell outer segments (ROSs)[1] contain a densely packed stack of closed disk membranes. The unique morphology of the disk membrane is maintained through a sorting of protein constituents within the membrane bilayer. Peripherin/rds is located solely in the rim of the disk, where it forms a noncovalent association with rom-1, a 37 kDa, nonglycosylated homologue, while the lamellar region of the disk contains rhodopsin (Molday et al., 1987; Arikawa et al., 1992; Bascom et al., 1992; Moritz & Molday, 1996).

The cDNA for peripherin/rds has been cloned and codes for a 39 kDa integral membrane glycoprotein with four putative transmembrane domains (Travis et al., 1989; Connell & Molday, 1990). In an animal model of retinal degeneration, the rds mouse, the peripherin/rds gene is defective (hence, part of the name peripherin/rds) (Connell et al., 1991). rds mouse homozygotes exhibit abnormal disk membrane morphogenesis; ROSs do not develop, and the photoreceptor cells eventually die (Jansen & Sanyal, 1984; Usukura & Bok, 1987). The insertion of a normal copy of the rds gene into rds mice results in the formation of normal ROSs and no morphological indications of photoreceptor degeneration (Travis et al., 1991). The molecular mechanisms of photoreceptor degeneration are not known. Moreover, the normal function(s) of peripherin/rds remains obscure.

Experiments confirming peripherin/rds as a structural protein have been facilitated by the development of a heterologous COS-1 cell expression system. Goldberg et al. (1995) have shown that a stable and specific peripherin/rds−rom-1 complex is formed when these cells are cotransfected with cDNAs for peripherin/rds and rom-1, although this same complex does not form if the proteins are expressed individually and the cell extracts subsequently recombined (Goldberg & Molday, 1996a). A limitation of peripherin/rds from cDNA expression systems is that it is unlikely to possess the same post-translational modifications as peripherin/rds *in vivo.* For example, peripherin/rds from COS-1 cells is glycosylated abnormally (Goldberg et al., 1995). Yet glycosylation of ROS membrane proteins is essential for normal disk membrane morphogenesis; disk morphogenesis is affected by changes in protein sialic acid content (Stiemke & Hollyfield, 1994) and is drastically perturbed by tunicamycin treatment, which selectively inhibits N-linked glycosylation and leads to cell death (Fliesler et al., 1985, 1986).

To define the biochemical function of peripherin/rds rigorously, sufficient quantities of purified native protein are required. The major difficulty in purifying native peripherin/rds has been separating it from rhodopsin, especially for

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[1] Abbreviations: ROS, rod outer segment; IEF, isoelectric focusing; BSA, bovine serum albumin; OG, octyl glucopyranoside; Con-A, concanavalin-A; CMC, critical micelle concentration; RT, room temperature.

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studying peripherin/rds phosphorylation; rhodopsin makes up over 95% of the disk membrane protein (Krebs & Kühn, 1977), is similar in size to peripherin/rds, and is polyphosphorylated (Wilden & Kühn, 1982). In the present study, we report the biochemical copurification of bovine peripherin/rds and rom-1 using hydroxylapatite and Mono Q FPLC and the homogeneous purification of a peripherin/rds homodimer using concanavalin-A (Con-A) Sepharose affinity chromatography and chromatofocusing. The development of these procedures has allowed us to begin the biochemical characterization of native peripherin/rds. Previous results have suggested that peripherin/rds might be phosphorylated in response to light in photoreceptor rod cells (Boesze-Battaglia et al., 1996). Here, we have used purified peripherin/rds to document the phosphorylation of this protein.

We have previously shown that ROS disk membranes fuse with R_{18}-labeled plasma membrane vesicles in a calcium-dependent manner (Boesze-Battaglia & Yeagle, 1992). Given the location of peripherin/rds in the rim of the disk membranes, the region that must interact with the plasma membrane upon membrane fusion, we postulated that peripherin/rds may mediate *in vitro* disk−plasma membrane fusion. In the last part of the present study, we demonstrated the ability of reconstituted peripherin/rds to promote membrane fusion. Furthermore, we studied the effect of phosphorylation of peripherin/rds on this newly documented property of the protein.

MATERIALS AND METHODS

Materials

Fresh dark- or light-adapted bovine retinas were obtained from a local slaughterhouse. Alternatively, frozen, dark-adapted bovine retinas were purchased from J. Lawson Inc. (Lincoln, NE). Monoclonal antibodies, 2B6 and 3B6, against peripherin/rds and 1D5, against rom-1 were given to us as hybridoma culture media from R. Molday (Moritz & Molday, 1996). A polyclonal antibody against bovine rhodopsin was raised in rabbits by standard procedures.

Hydroxylapatite was purchased from Bio-Rad, and HR 15/30 and HR 10/30 columns (Pharmacia) were packed according to the Bio-Rad instruction manual. A prepacked Mono Q HR 5/5 column was purchased from Pharmacia. These columns were used on a Pharmacia FPLC instrument. PBE-94 and Polybuffer were also purchased from Pharmacia. Con-A Sepharose and octyl glucopyranoside (OG) were purchased from Calbiochem (La Jolla, CA). All chemicals were purchased from Sigma (St. Louis, MO), except for *N*,*N*-dimethyldodecylamine *N*-oxide from Fluka. Peptides to the C-terminal and N-terminal region of peripherin/rds (PP-1 and PP-2, respectively) were synthesized using solid phase methods by Research Genetics Inc.

Methods

Rod Outer Segment Membrane Preparations. For hydroxylapatite chromatography, ROSs were prepared from fresh retinas, using sucrose density gradients as in Azarian et al. (1995). Light-adapted ROSs were prepared from previously light-adapted eyes under room light. For dark-adapted ROSs, eyes were placed in the dark at the slaughterhouse and ROSs were prepared under dim red light. ROSs were washed three times in low-salt buffer [10 mM imidazole (pH 7.2) and 1 mM $MgCl_2$] plus 0.1 mM GTP to remove transducin and cytosolic proteins. In earlier procedures, the ROS membranes were also stripped by washing once in 10 mM imidazole (pH 7.2) plus 5 M urea.

For concanavalin-A chromatography, ROS disk membranes were prepared from frozen, dark-adapted retinas (J. Lawson Inc.) using Ficoll flotation (Smith et al., 1975). The isolated ROS disk membranes were washed in hypotonic buffer (5 mM Hepes and 1 mM EDTA at pH 7.8) prior to Con-A chromatography. Leupeptin (10 μM) and aprotinin (1 μM) (both from Sigma) were included in all of the isolation and phosphorylation buffers. All manipulations of ROS membranes were performed under dim red light, and buffers were purged with argon to reduce lipid oxidation.

Hydroxylapatite Chromatography. ROS membranes from 60 retinas were resuspended in 50 mL of 10 mM imidazole (pH 7.2) plus either 0.5% dimethyldodecylamine *N*-oxide or 50 mM OG (buffer A). After centrifugation at 360 000 *g* for 40 min, the supernatant was removed and loaded onto the first hydroxylapatite column (HR 15/30) at 0.35 mL/min. The column was washed at the same rate with 30 mL of buffer A, followed by 40 mL of buffer A plus 150 mM NaH_2PO_4, 30 mL of buffer A plus 210 mM NaH_2PO_4, and 30 mL of buffer A plus 300 mM NaH_2PO_4. Buffer containing NaH_2PO_4 was kept at room temperature to avoid crystallization of the sodium phosphate. Two-millilter fractions were collected, and the elution of protein was monitored by absorbance at 280 nm. The two fractions containing protein eluted by the 210 mM NaH_2PO_4 were diluted with 4−5 volumes of buffer A and loaded onto the second hydroxylapatite column (HR 10/30), which was developed as before. The 210 mM NaH_2PO_4 eluate was then diluted with 8−10 volumes of buffer A and loaded onto a Mono Q column. After the sample was loaded, an additional 10 mL of buffer A was run through the column. Proteins were then eluted with a linear gradient of 90 mL of buffer A plus 0 to 1 M NaCl applied at 0.3 mL/min. One-milliliter fractions were collected.

Concanavalin-A Affinity Chromatography. Con-A chromatography was carried out as described (Litman, 1983). The hypotonically washed ROS disk membranes were washed and resuspended in Con-A standard buffer and solubilized in OG. Fractions were monitored at 280 nm, and peak fractions corresponding to the flow-through (unbound) peak were pooled. The rhodopsin-rich bound fractions (eluted with 0.5 M α-methyl mannoside) were also pooled. Both unbound and bound fractions were concentrated by Amicon ultrafiltration (model 8050) using a YM-30 filter, to $^1/_{10}$ the original volume. The concentrated samples were dialyzed overnight versus either 0.025 M imidazole hydrochloride, (pH 7.4) or 0.025 M piperazine hydrochloride, (pH 5.5), depending on the subsequent chromatofocusing procedure. The dialysis reduced the OG concentration in the samples from 167 mM (bound) and 146 mM (unbound) to 32 mM (bound) and 40 mM (unbound), respectively.

Chromatofocusing. The chromatofocusing column was poured and run essentially as described by Pharmacia-LKB (Uppsula, Sweden) in their technical bulletin (52-1586-00). All buffers were degassed prior to use. Briefly, PBE-94 column material was equilibrated with either 0.025 M imidazole hydrochloride and 10 mM OG (pH 7.4) or 0.025

M piperazine hydrochloride and 10 mM OG (pH 5.5) until a stable pH was established. Prior to the loading of the column, 1 mL of start buffer was added so that the sample proteins were not exposed to extremes of pH. After the loading of the sample, the various proteins were eluted with the appropriate dilution of Polybuffer-74 and 10 mM OG as described below. Eluate absorbance was monitored at 280 nm. Routinely, 0.750−1.2 mL fractions were collected. The pH of every second fraction was recorded immediately. Chromatofocusing of bound fractions was done under dim red light. For pH range 7−4, PBE-94 was used in a 5−7 mL bed volume. Start buffer was 0.025 M imidazole hydrochloride and 10 mM OG at pH 7.4; eluate was a 1:8 dilution of polybuffer-74 hydrochloride at pH 4.0 and 10 mM OG. For pH range 5−4, PBE-94 gel was used in a 1−2 mL bed volume. Start buffer was 0.025 M piperazine hydrochloride and 10 mM OG at pH 5.5; eluate was a 1:10 dilution of polybuffer-74 hydrochloride at pH 4.0 and 10 mM OG. The chromatofocused fractions were assayed for protein (Bio-Rad) and the fractions pooled (as described in the figures) and concentrated to 0.5 mL using Centricon-30 concentrators (Amicon) prior to IEF and SDS−PAGE analysis.

Light-Stimulated Protein Phosphorylation in Intact ROS. In phosphorylation experiments, ROS membranes were isolated as a float on 40% w/w sucrose (Smith et al., 1975). Phosphorylation of ROS proteins was then carried out essentially as described (Miller et al., 1986). Briefly, purified intact leaky ROS membranes were washed twice and then resuspended in phosphorylation buffer (100 mM NaH_2PO_4, 1 mM $MgCl_2$, and 5 mM DTT at (pH 7.4) to a final total protein concentration of 0.7 mg/mL. Routinely, between 0.5 and 1.2 mg of peripherin/rds was present in the incubation mixtures, with 1 mCi [γ-^{32}P]ATP (Amersham, Arlington Heights, VA) and 0.1 mM ATP. Dark control samples were run parallel to the light-stimulated samples. The samples were sonicated in a bath sonicator for 90 s and incubated at 35 °C for 15 min. The sample was exposed to light for 90−120 s and then incubated in the dark at for RT 30 min. This resulted in a bleaching of 80−85% of the rhodopsin based on a change in absorbance at 500 nm. The phosphorylation reaction was quenched with ice cold EDTA at a final concentration of 20 mM. The light-stimulated and dark control ROS membranes were lysed and Ficoll floated (Smith et al., 1975). The phosphorylated disk membranes were regenerated with a 5-fold molar excess of 11-*cis*-retinal, and rhodopsin was separated from peripherin/rds using Con-A chromatography and chromatofocusing as described above. The 11-*cis*-retinal was provided by the National Eye Institute of the National Institutes of Health. Samples were analyzed for peripherin/rds content using either SDS−PAGE or isoelectric focusing and silver-stained. The protein in each lane was quantified by densitometric analysis. Dried gels were analyzed by autoradiography; peripherin/rds bands were excised, and radioactivity was counted by liquid scintillation counting.

Preparation and R_{18} Labeling of ROS Plasma Membranes. ROS disk membrane and plasma membrane vesicles were isolated using ricin−agarose and differential sucrose density gradient centrifugation as described (Boesze-Battaglia & Albert, 1989). The ROS plasma membrane was eluted from the ricin−agarose using 1 M galactose in 0.1 M sodium borate at pH 8.0. The isolated plasma membrane was washed free of calcium and resuspended in calcium-chelating buffer (Robertson & Potter, 1984). The ROS plasma membranes were labeled with octadecylrhodamine B chloride (R_{18}, Molecular Probes, Inc., Junction City, OR) for 30 min at room temperature in the dark as described (Boesze-Battaglia et al., 1992b). R_{18} was incorporated at self-quenching levels; typically, it was added at 3−5 mol % relative to the phospholipid. Upon membrane fusion, the R_{18} probe is diluted throughout the target membrane by lateral diffusion, which leads to a dequenching and an increase in observed fluorescence intensity that is proportional to the membrane fusion (Hoekstra et al., 1984; Boesze-Battaglia & Yeagle, 1992; Boesze-Battaglia et al., 1992a,b).

Peripherin/rds recombinants were prepared from purified peripherin/rds isolated from the chromatofocusing procedure. The purified protein was recombined with Con-A disk lipid vesicles (from extracted disk lipid membranes) using detergent dialysis essentially as described by Wilson et al. (1993). During the final dialysis step, the recombinant vesicles were dialyzed against SM-2 BioBeads (Bio-Rad). Using this method, less than 0.05 mol % of the OG remained associated with the vesicles. Rhodopsin recombinants were prepared as described by Jackson and Litman (1985). The peripherin/rds and rhodopsin recombinants were subjected to five freeze−thaw cycles so they would form large unilamellar vesicles (Boesze-Battaglia & Yeagle, 1992). The disk lipid vesicles containing no protein were prepared as large unilamellar vesicles as described (Boesze-Battaglia et al., 1992a). In the preparation of the disk lipids used in all of the recombinants, the retinal Schiff base linkage was reduced using sodium cyanoborohydride, thereby eliminating any retinal-induced membrane destabilization and membrane fusion due to a lipid-mediated mechanism (Boesze-Battaglia et al., 1992a). The peripherin/rds vesicle recombinants were treated with trypsin as described (Molday et al., 1987). The trypsin-treated large unilammelar vesicles were washed in 10 mM Hepes at pH 7.2 and the recombinants pelleted at 60 000 rpm for 40 min. The resulting pellet was resuspended in 10 mM Hepes at pH 7.2 for the fusion assays.

R_{18} Lipid Mixing Assay. Fusion assays characterizing R_{18}-labeled plasma membrane−disk membrane fusions were performed exactly as described previously (Boesze-Battaglia et al., 1992b). In some experiments, the disk membranes were pretreated with either anti-peripherin monoclonal antibody 2B6, anti-opsin antibody, or various peptides for 10 min in the dark at 37 °C. Fusion was initiated with the addition of R_{18}-labeled plasma membrane vesicles to these suspensions as described below. The synthetic peripherin/rds peptides PP-1 and PP-2 correspond to the C-terminal region and the N-terminal region of peripherin/rds, respectively. These peptides have the following sequence:

PP-1 H_2N-V-P-E-T-W-K-A-F-L-E-S-V-K-K-L-COOH

PP-2 H_2N-R-K-R-S-D-V-M-N-N-S-D-S-H-F-V-COOH

Bradykinin (Sigma) was used as a nonspecific control peptide in these studies. All peptides were added at a concentration equivalent to 10 mol of peptide per mole peripherin/rds.

Fusion assays using disk lipid vesicle recombinants and plasma membrane were carried out and the initial rates of fusion calculated as described (Hoekstra et al., 1984; Boesze-

Battaglia et al., 1992b). Fluorescence and light scattering were measured using a Perkin-Elmer LS-50B spectrofluorometer. Fluorescence was monitored at an excitation wavelength of 560 nm and an emission wavelength of 586 nm. Fusion was initiated with the addition of R_{18}-labeled plasma membrane into a suspension of disk lipid vesicles. The increase in R_{18} fluorescence due to the dilution of the probe in the target membrane was monitored continuously and increased linearly with probe dilution. The fluorescence intensity obtained without the addition of plasma membrane was taken as a baseline. The fluorescence at infinite probe dilution (100% fluorescence) was determined with the addition of 100 μL of 10% Triton X-100 to the membrane mixture. Initial rates of fusion were determined from the rate of increase in fluorescence intensity as a function of time as described previously (Hoekstra et al., 1984). In these experiments, the increase in fluorescence was recorded for 10 min. Under these conditions, the increase in fluorescence reached a plateau and the change in fluorescence over the first 5 min was used to calculate the initial rate of fusion. This assay has been shown to be sensitive (without artifacts) to the fusion of disk membranes with large unilamellar vesicles of phosphatidylethanolamine and disk lipid vesicles as well as fusion between plasma membrane and disk membranes (Boesze-Battaglia, 1997; Boesze-Battaglia & Yeagle, 1992; Boesze-Battaglia et al., 1992a,b). Fusion assays in the presence of peptides were carried out as described above in the R_{18}-labeled plasma membrane−disk membrane fusion studies.

Fourier Transform Infrared Spectroscopy (FTIR) of Purified Peripherin/rds. Infrared data were acquired with a Nicolet (model Magna IR-550) FTIR spectrometer equipped with a TGS detector essentially as described (Lamba et al., 1993, 1994). Sample was layered on a 13 mm diameter AgCl infrared window which was sandwiched by another window spaced by a 0.015 mm Teflon spacer. Approximately 200 interferograms were recorded, coadded, and apodized with a Happ-Genzel function prior to fourier transform, yielding an effective resolution of 1.0 cm^{-1}. Heat-denatured spectra were recorded at ambient temperature after heating the sample at 100 °C for 15 min.

Isoelectric Focusing and SDS−PAGE. Prior to either isoelectric focusing or electrophoresis, samples containing OG were dialyzed overnight against 0.5 M NaCl, 10 mM Hepes, and 5.0 mM OG at pH 7.4 to decrease the OG concentration below the CMC. Isoelectric focusing (IEF) was performed essentially as described (Aton et al., 1984). The pH gradient was determined by running a blank lane and measuring pH using a surface electrode (Sigma) at the focusing temperature, 5 °C. All IEF gels were run under dim red light to prevent any residual rhodopsin (if present) from aggregating. Focusing time for a 0.2 mm mini-slab gel was 1.25 h at 7 W constant power at 5 °C. Routinely, the pH gradient was established approximately 0.5 cm into the gel from pH 6.5 to 4.0 using the following recipe: 1.5 mL of 30% acrylamide, 2.1 mL of D_2O, 1.25 mL of 5% w/v OG, 20 μL of Ampholines (pH 3−10, LKB), 130 μL of Ampholines 4−6.5 (LKB), 15 μL TEMED, and 20 μL of ammonium persulfate (100 mg/mL). The cathode and anode buffers (0.01 M NAOH and 0.01 M H_3PO_4, respectively) were cooled prior to focusing. Cytochrome *c* (pI = 9.0) and phycocyanine (pI = 4.3) were used as standards. Samples were diluted 1:1 in sample buffer as described (Aton et al., 1984). The pI of peripherin/rds was calculated using standards obtained from Pharmacia. The pH of the IEF gels changed linearly over the regions indicated in the figures.

SDS−polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970) and modified (Smith et al., 1975). Routinely, 7.5 or 10% acrylamide gels were run. Sample buffer containing 200 mM DTT was used when reducing conditions were desired.

Western Blotting. For Western blotting, proteins were electrophoretically transferred from either SDS gels or IEF gels to either Nitrocellulose or Immobolin-P (Millipore). Prior to protein transfer, the gels were soaked in transfer buffer for 30−60 min in the cold.

Electrophoretic transfer of SDS gels was carried out in transfer buffer containing 25 mM Tris base, 190 mM glycine, 20% v/v methanol, and 0.1% v/v SDS and transfer of IEF gels in 0.7% acetic acid.

The Western blots were blocked with 5% w/v nonfat dry milk and 3% BSA in TBS (Tris-buffered saline), incubated with anti-peripherin/rds antibody (mAb 2B6 or mAb 3B6), anti-rom-1 antibody (1D5), anti-opsin antibody, or anti-phosphoserine (Sigma) washed extensively, subsequently incubated with goat anti-mouse IgG conjugated to HRP (Amersham, Arlington Heights, IL) or alkaline phosphatase, and washed again. When opsin (polyclonal) antibody was used, the blots were incubated with goat anti-rabbit IgG-linked antibody (Gibco-BRL). The opsin (polyclonal) antibody appears to recognize both extracellular and intracellular domains of opsin (preliminary unpublished observation). HRP was visualized using the ECL detection system (Amersham). The molecular weights of the immunoreactive species were determined using R_f measurements of molecular weight markers. When low-pH samples from the chromatofocusing procedure were immunolabeled, the sample pH was adjusted to approximately pH 7.0 with a minimal volume of 0.l M imidazole at pH 8.0.

Additional Assays. Phosphate was determined as described (Bartlett, 1959) and modified (Litman, 1973). Total protein was determined using a Bio-Rad microassay procedure (Sigma). OG concentration was determined as described (Seifter et al., 1950). Gels were silver-stained as described (Merril et al., 1982). Rhodopsin concentration was determined by measuring the difference in absorbance at 500 nm before and after illumination in the presence of 50 mM neutralized hydroxylamine using an extinction coefficient of 40 000.

RESULTS

Purification of Peripherin/rds−rom-1 by Hydroxylapatite Chromatography. The major hurdle in the purification of peripherin/rds, especially for phosphorylation studies, is its separation from rhodopsin, which makes up over 95% of the ROS disk membrane protein (Krebs & Kühn, 1977) and is polyphosphorylated (Wilden & Kühn, 1982). In our first attempt at this separation, we exploited the observation of Dean and Akhtar (1993) that phosphoopsin is only sparingly soluble in 2% ammonyx-LO or 0.5% dimethyldodecylamine *N*-oxide, and thus removed most of the opsin from light-adapted retinas, prior to hydroxylapatite chromatography. However, as we observed that peripherin/rds required 210 mM sodium phosphate to be eluted from a hydroxylapatite column, whereas rhodopsin requires only 130 mM sodium

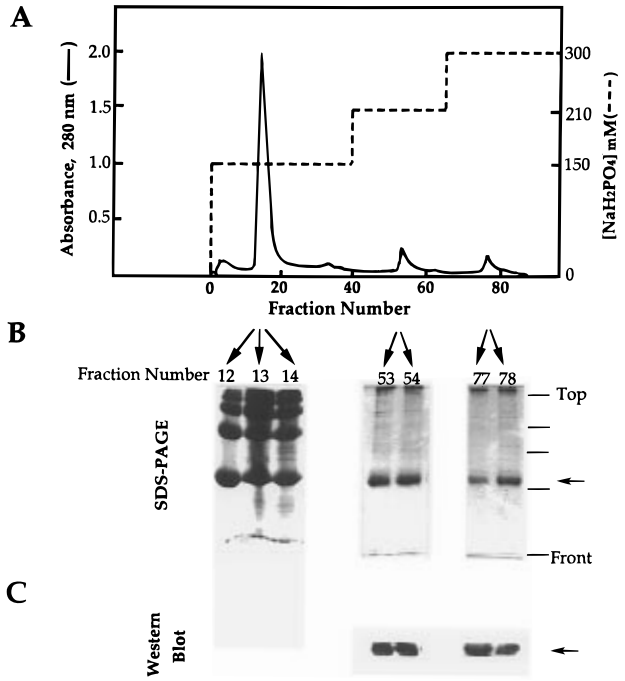

FIGURE 1: Separation of peripherin/rds and rhodopsin by hydroxylapatite chromatography. (A) Protein elution as a result of washing with stepwise increases of sodium phosphate. (B) SDS−PAGE of the fractions corresponding to peaks of protein elution. Positions of molecular mass markers are indicated on the right. The three lines correspond to 29, 45, and 66 kDa; the arrow corresponds to the position of peripherin/rds. (C) Western blot of the same fractions, labeled with antiperipherin/rds (mAb 3B6). The region of the Western blot, containing polypeptides with apparent molecular masses of 30−35 kDa (arrow), was cut out routinely for labeling in order to conserve antibody (specificity of this antibody for peripherin/rds is illustrated in lane 2 of Figure 2B). Rhodopsin (which oligomerizes in SDS−PAGE) was eluted by the first step (fractions 12−14). Peripherin/rds was eluted only by the subsequent steps (fractions 53 and 54 and fractions 77 and 78).

phosphate (Dean & Akhtar, 1993), it seemed that hydroxylapatite chromatography could be used with different detergents and dark-adapted retinas. We thus developed the following procedure.

Dark-adapted ROS membranes were washed in low-salt buffer, containing GTP, solubilized in buffer containing 50 mM OG, and loaded onto a hydroxylapatite column. Stepwise elution with 150, 210, and, finally, 300 mM sodium phosphate resulted in the collection of most of the rhodopsin after the first step, and most of the peripherin/rds after the second step, although the third step yielded additional peripherin/rds (Figure 1). That eluted by 210 mM sodium phosphate (fractions 53 and 54, Figure 1) was purified further by a second hydroxylapatite column, followed by application of the 210 mM sodium phosphate eluate from this second column to a Mono Q column.

Using a linear NaCl gradient, peripherin/rds was eluted from the Mono Q column with 150 to 200 mM NaCl (fractions 26−30 were pooled, Figure 2A). No opsin was detected in these fractions by Western blot analysis (lane 4, Figure 2B). By silver staining after SDS−PAGE, two bands of protein were evident (lane 8, Figure 2B). Western blot analysis showed that the upper band was peripherin/rds and the lower band was rom-1 (lanes 5−7, Figure 2B). On Coomassie blue-stained gels (not shown), there appeared to be equal amounts of peripherin/rds and rom-1. (On the silver-stained gel shown in lane 8 of Figure 2B, rom-1

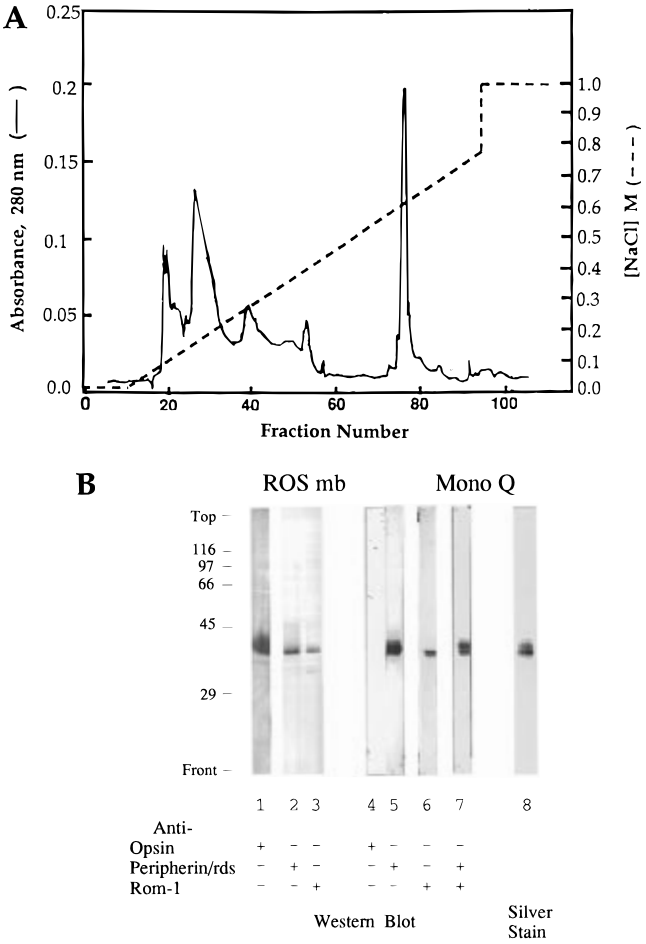

FIGURE 2: (A) Profile of protein elution from a Mono Q column in response to a linear NaCl gradient. The Mono Q column was used to purify peripherin/rds following hydroxylapatite chromatography. One millilter fractions were collected. (B) Western blot and SDS−PAGE of ROS membranes (ROS mb) and purified peripherin/rds (and rom-1) after Mono Q chromatography. The ROS membranes are labeled with antiopsin (lane 1), peripherin/rds 3B6 antibody (lane 2), and rom-1 1 D5 antibody (lane 3). Fractions from the Mono Q column were labeled with the same antibodies (lanes 4−7) or silver-stained (lane 8). Lanes 4 and 5 are derived from a single wide lane that was cut in half lengthwise and labeled with antiopsin and antiperipherin/rds, respectively. Lane 6 is the same as lane 4 but was reprobed with rom-1 antibody. To obtain lane 7, the same lane was probed again, but this time with peripherin/rds antibody. Consequently, lane 6 shows labeled rom-1, and lane 7 shows both labeled rom-1 (below) and peripherin/rds (above). Lane 8 shows the same sample as in lanes 4−7, except that it is from the gel that was silver-stained instead of transblotted. In this lane, the peripherin/rds band is just above the somewhat sharper rom-1 band (relative positions indicated from labeled Western blots).

actually appears to be stained more intensely than peripherin/rds, but the peripherin/rds band is more diffuse.) Consequently, the presence of rom-1 is unlikely to have resulted from contamination of the peripherin/rds fractions; instead, copurification of rom-1 is most likely because of an interaction with peripherin/rds. Peripherin/rds and rom-1 are structurally related subunits of the same protein complex; in the native ROS membrane, these proteins are found as heterotetramers (Moritz & Molday, 1996). Therefore, this purification procedure provides a means of purifying the peripherin/rds−rom-1 complex. Note that, when the two proteins are expressed individually and the cell extracts are subsequently combined, they fail to associate and form this complex (Goldberg & Molday, 1996a).

The yield of peripherin/rds from 60 bovine retinas was about 400 μg. Given that peripherin/rds accounts for 5−6% of ROS membrane protein (Connell & Molday, 1990), our yield is 23−25% from 1.75 mg of peripherin/rds in our starting material. The yield of rom-1 is similar.

Purification of Homogeneous Peripherin/rds by Chromatofocusing. To investigate the phosphorylation of the peripherin/rds subunit, we needed to obtain a preparation free of rom-1 (and any other proteins with similar mobility in SDS−PAGE). This was achieved by a procedure which includes chromatofocusing.

ROS disk membranes were washed with hypotonic buffer to remove membrane-associated proteins. The washed membranes were solubilized in OG and chromatographed on a Con-A Sepahrose affinity column. Although Con-A chromatography has traditionally been used to purify rhodopsin (Litman, 1982), the rationale for the use of this chromatographic step in the isolation of peripherin/rds was to aid in the removal of rhodopsin. Thus, fractions were collected immediately after loading the solubilized ROS membrane preparation onto the column. A large peak, containing the membrane phospholipids and various proteins, eluted within 10 min. This eluate was designated the unbound fraction. The Con-A bound proteins, mostly rhodopsin, were subsequently eluted with α-methyl mannoside and designated the bound fraction. Transblotted samples from both unbound and bound fractions were labeled with the peripherin/rds monoclonal antibody, 2B6, using slot blot analysis. The unbound fraction was found to be enriched in peripherin/rds relative to the bound fraction (data not shown). On the basis of these results, it was expected that peripherin/rds may be purified readily from the rhodopsin-poor, unbound fraction.

The abundance of negatively charged amino acid residues at the C terminus of peripherin/rds makes this protein relatively acidic (Connell & Molday, 1990). Its theoretical p*I* is 5.3 (not accounting for glycosylation), which is considerably lower than that of native unphosphorylated rhodopsin (p*I* = 6.0; Aton et al., 1984) and rom-1 (calculated p*I* = 5.98). This property pointed to chromatofocusing as a viable means of separating peripherin/rds from rhodopsin and rom-1. Because the actual isoelectric point of peripherin/rds was unknown, both the unbound and bound fractions were chromatofocused on PBE-74 exchange resin over a broad pH range (pH 7−4). Chromatofocusing of the Con-A unbound fraction showed enrichment of peripherin/rds in fractions corresponding to a pH of 4.89−4.35. The Con-A bound fraction was also chromatofocused. Opsin was immunodetected in fractions corresponding to a pH of 7.6−6.24. Lower levels of opsin, possibly phosphoopsin (Aton et al., 1984; Mc Dowell et al., 1993), were also detected in fractions of pH 6.0 and 5.76. Aliquots from the same fractions were probed with mAb 2B6 to detect peripherin/rds. The majority of the detectable peripherin/rds eluted with a small fraction of the opsin at pH 5.76; very little peripherin/rds was seen in subsequent fractions. The amount of peripherin/rds eluted in this fraction corresponded to less than 5% of the total purified peripherin/rds.

Once it was determined that the isoelectric point of peripherin/rds was in the range of pH 5−4, the Con-A unbound fraction was chromatofocused over a narrower range, using a PBE-94 gel with a 0.025 M piperazine hydrochloride (pH 5.5) start buffer. For comparison, the Con-A bound fraction was also chromatofocused in the same type of gel. Elution with a pH 5 to 4 gradient gave profiles for the Con-A unbound and bound fractions as shown in panels A and C of Figure 3. Pooled fractions are indicated by brackets and are designated as Con-A unbound peak 1 (U1), Con-A unbound peak 2 (U2), Con-A bound peak 1 (Bl), and Con-A bound peak 2 (B2). The pH of U1 and B1 was 5.4−4.8, and that of U2 and B2 was 4.5−4.4.

A number of different proteins were detected in U1 and B1 fractions by SDS−PAGE (Figure 3B,D). The B1 fraction contained mostly rhodopsin (lane 2, Figure 3D), while the U1 fraction contained several proteins, with the two main ones having apparent molecular masses of 38 and 35 kDa (lane 2, Figure 3B). A single polypeptide was detected in the U2 fraction with an apparent molecular mass of approximately 35 kDa (lane 4, Figure 3B). No detectable protein was seen by silver staining in the corresponding fraction, B2, from Con-A bound proteins (lane 4, Figure 3D). Western blot analysis using mAb 2B6 of fractions U1 and U2 confirmed that peripherin/rds was present in both of these samples (lanes 3 and 5, Figure 3B). In the absence of reducing agent, the apparent molecular mass of peripherin/rds in SDS−PAGE was about 68 kDa (data not shown), indicating that the protein was present as a homodimer [cf. Goldberg and Molday (1996a)]. Immunoblot analysis of the B1 and B2 fractions, using mAb 2B6, showed no detectable peripherin/rds in B2 (lane 5, Figure 3D), but a small amount of peripherin/rds which cofractionated with rhodopsin in B1 (lane 3, Figure 3D).

Both U2 and B2 fractions were subsequently subjected to isoelectric focusing to confirm the homogeneity of peripherin/rds isolated using chromatofocusing techniques and to determine the isoelectric point. Figure 4 shows a single isoelectric species with an isoelectric point at 4.7 in lane 1, which contained the U2 fraction, and no detectable protein in lane 2, which contains the B2 fraction. Western blot analysis using mAb 2B6 confirmed that the protein in lane 1 was peripherin/rds (lane 3, Figure 4). Chromatofocusing results yielded an isoelectric point of approximately 4.5, which was in reasonable agreement, given that the IEF gel was run at 5 °C, while the chromatography was routinely performed at 20 °C [cf. Vesterberg (1971)].

The U2 fraction appeared to contain only peripherin/rds and not rom-1. First, only a single protein band was detected by SDS−PAGE (lane 4 of Figure 3B; contrast with the doublet evident in lane 8 of Figure 2). Second, this protein was detected by peripherin/rds antibodies. Third, the isoelectric point (4.7) of the protein in the U2 fraction was close to the theoretical p*I* of peripherin/rds, which is 5.3 (not accounting for glycosylation), but not to the theoretical p*I* of rom-1, which is 5.98. Fourth, protein could not be detected using rom-1 mAb, 1D5, to probe Western blots of U2 (lane 6 of Figure 3B; although rom-1 is present in the Con-A unbound fraction, as shown in lane 7 of Figure 3B). Goldberg and Molday (1996a) showed that in the absence of reducing agents the peripherin/rds−rom-1 complex dissociates only in the presence of strong chaotropic agents. This observation suggests that these proteins are noncovalently linked, through a significant hydrophobic component. In this second purification procedure, the ability to isolate peripherin/rds free from rom-1 using a pH gradient is consistent with a noncovalent interaction between these two proteins.

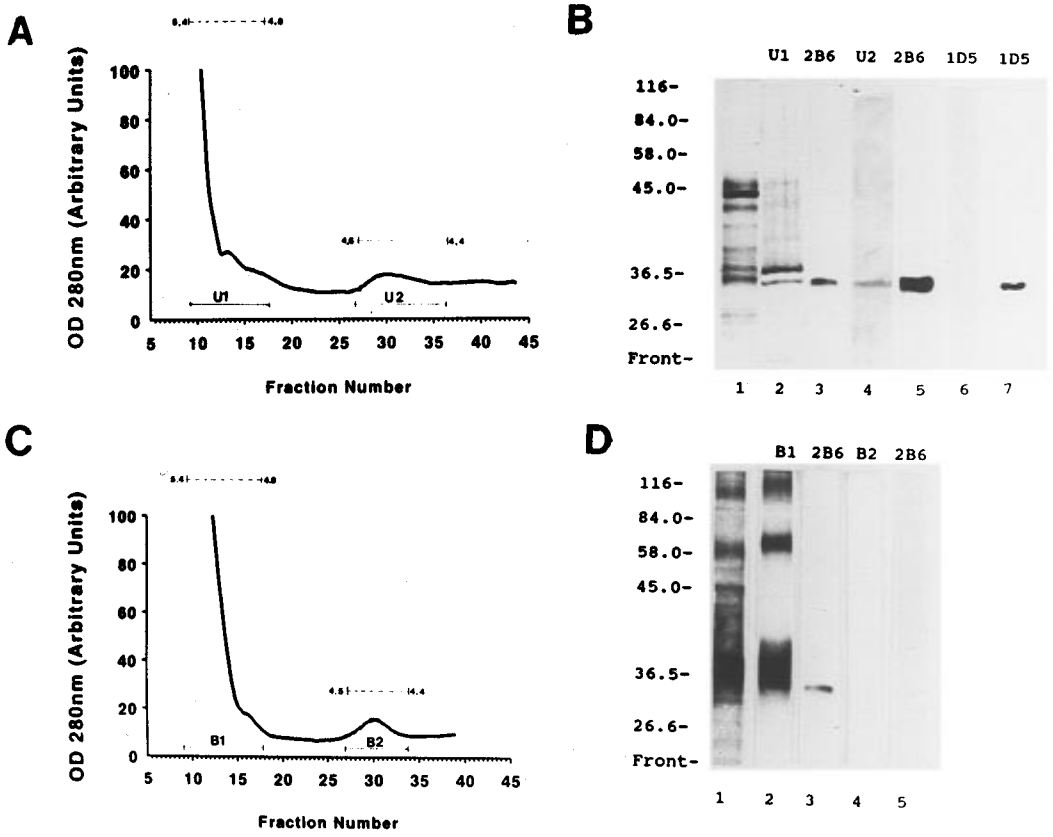

FIGURE 3: Purification of peripherin/rds by chromatofocusing. (A) Unbound fraction. The unbound fraction from a Con-A column was applied to a PBE-94 column and eluted with a pH 5 to 4 gradient. Fractions (1.2 mL) were collected, and the absorbance of the fractions at 280 nm is shown. Fractions 9−18 were pooled and are designated U1; fractions 26−36 were pooled and are designated U2. The pH of the isolated fractions is shown above the pooled peaks. (B) Western blot and SDS−PAGE of unbound fraction and purified peripherin/rds: lane 1, silver-stained pre-chromatofocused unbound sample; lane 2, U1 (silver-stained); lane 3, U1 transferred and probed with mAb 2B6; lane 4, U2 (silver-stained); lane 5, U2 transferred and immunobloted with mAb 2B6; lane 6, U2 transferred and immunobloted with mAb 1D5; and lane 7, Con-A unbound fraction, transferred and immunobloted with mAb 1 D5. (C) Purification of peripherin/rds by chromatofocusing of the bound fraction. The bound fraction from a Con-A column was applied to a PBE-94 column and eluted with a pH 5 to 4 gradient as described in Materials and Methods. Fractions (1.2 mL) were collected, and the absorbence of the fractions at 280 nm is shown. Fractions 9−18 were pooled and are designated Bl; fractions 26−33 were pooled and are designated B2. The pH of the isolated fractions is shown above the pooled peaks. (D) Western blot and SDS−PAGE of chromatofocused bound fractions: lane 1, pre-chromatofocused protein sample (silver-stained); lane 2, B1 (silver-stained); lane 3, B1 transferred and probed with mAb 2B6; lane 4, B2, (silver-stained); and lane 5, B2 immunobloted with mAb 2B6.

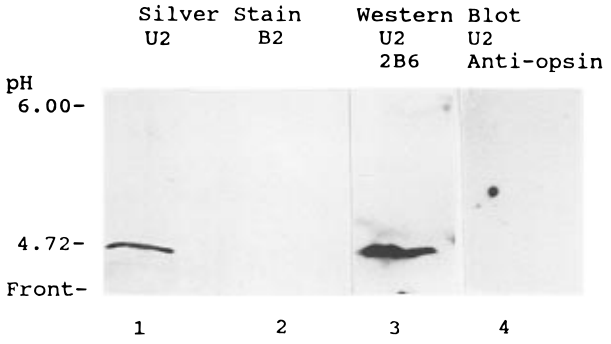

FIGURE 4: Isoelectric focusing and Western blot analysis of purified peripherin/rds. Fractions U2 and B2 were run on isoelectric focusing gels: lane 1, silver-stained U2 fraction; and lane 2, silver-stained B2 fraction. After transfer to Immobilon, the U2 fraction was labeled with mAb 2B6 (lane 3) or anti-opsin antibody (lane 4).

As peripherin/rds accounts for 5−6% of ROS membrane protein (Connell & Molday, 1990), we would expect to have 1.0−1.25 mg of peripherin/rds prior to the Con-A chromatography step (from 50 retinas). From the U2 fraction (after chromatography with Con-A and PBE-94), we were able to recover 400−800 μg of purified peripherin/rds (based on Bio Rad total protein assay and densitometric scans of 10% SDS gels).

Phosphorylation of Peripherin/rds. Studies using magic angle spinning ^{31}P-NMR have suggested that a protein with the same SDS−PAGE mobility as peripherin/rds might be phosphorylated in disk membranes in response to light (Boesze-Battaglia et al., 1996). However, the extensive light-dependent phosphorylation of rhodopsin, which tends to form oligomers with a variety of mobilities in SDS−PAGE, has made it difficult to detect the phosphorylation of other ROS transmembrane proteins by autoradiography. As native peripherin/rds has now been separated from rhodopsin (as well as rom-1 and other proteins), we are able to begin to characterize peripherin/rds phosphorylation.

The phosphorylation of ROS membrane proteins was initiated by light in the presence of [γ-^{32}P]ATP. Samples were regenerated with 11-*cis*-retinal and subjected to Con-A chromatography. The Con-A unbound fraction was isoelectric focused. In some cases, peripherin/rds was purified further by chromatofocusing prior to isoelectric focusing. The IEF gel was cut in half. One half was used for Western blot analysis; the other half was dried for autoradiography.

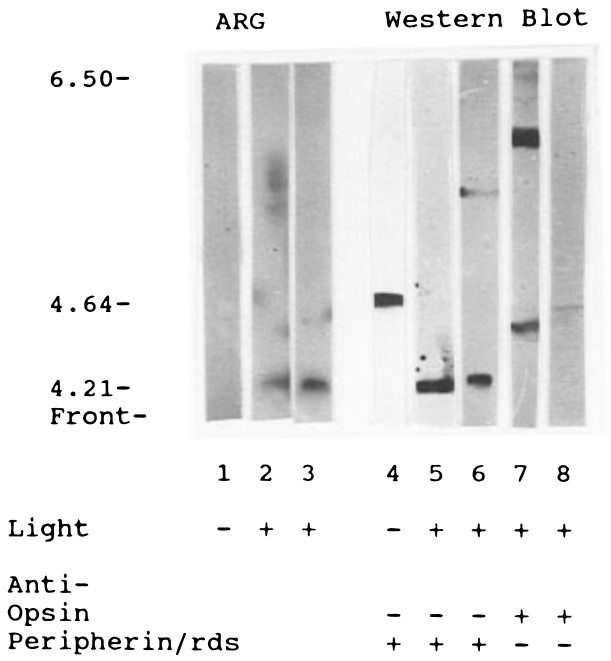

FIGURE 5: Light-dependent phosphorylation of peripherin/rds. Autoradiogram and western blot analysis of an IEF gel of a Con-A unbound fraction and purified peripherin/rds upon light-stimulated phosphorylation. Autoradiograms of the dark control Con-A unbound fraction (lane 1), light-stimulated Con-A fraction (lane 2), and purified peripherin/rds (lane 3). Western blot analysis of an IEF gel of a Con-A unbound fraction of a dark control ROS sample (lane 4), the light-stimulated Con-A unbound fraction (lane 5), and purified peripherin/rds isolated from light-stimulated ROSs (lane 6) probed with anti-peripherin/rds mAb 2B6. The Con-A unbound sample (lane 7) and purified peripherin/rds (lane 8) isolated from light-stimulated ROSs probed with antiopsin.

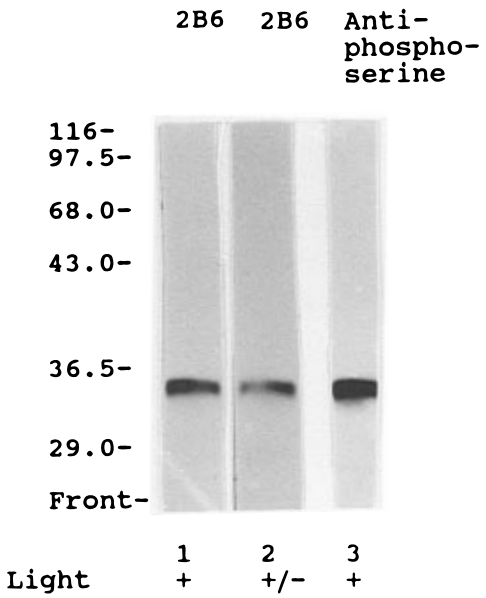

FIGURE 6: Western blot of Con-A unbound and bound fractions. Western blot of a dark Con-A unbound fraction (lane 1) probed with mAb 2B6. Western blot of a dark Con-A unbound fraction and a light-stimulated Con-A unbound fraction 50:50 mix (lane 2) probed with mAb 2B6. Western blot of the U2 fraction of phosphoperipherin/rds probed with anti-phosphoserine antibody (lane 3).

In the absence of light, no phosphorylation of peripherin/rds (or any other polypeptide) was detected in the Con-A unbound fraction (lane 1, Figure 5). As described above, the isoelectric point of peripherin/rds in these samples was about 4.7 (actually 4.64 in lane 4, Figure 5). Exposure of the ROSs to light caused the isoelectric point of peripherin/rds to shift to 4.2 (lane 5, Figure 5). Autoradiography showed that a protein in the Con-A unbound fraction with the same isoelectric point was phosphorylated (lane 2, Figure 5). Parallel treatment with homogeneous peripherin/rds (the U2 fraction obtained from chromatofocusing) confirmed that this protein was peripherin/rds (lane 3, Figure 5). Western blots of both the Con-A unbound fraction and the U2 fraction were probed with antiopsin. As expected, no opsin was detected in the U2 fraction (lane 8, Figure 5). Some phosphoopsin was detected in the Con-A unbound fraction, but even in light-exposed samples, its isoelectric point was about 0.2−0.3 pH unit less acidic than peripherin/rds in the same sample (lane 7, Figure 5). This result is consistent with previously published work showing that the isoelectric point of phosphorhodopsin species ranges from 5.5 to 4.46 (Aton et al., 1986; Mc Dowell et al., 1993).

Peripherin/rds from the U2 fraction was excised from IEF gels and the ^{32}P counted. It was determined that 1.8−2.0 mol of phosphate was associated with each mole of peripherin/rds monomer, suggesting at least two potential phosphorylation sites. These results are based on two independent preparations, each excised from two IEF gels, for an *n* of 4. Phosphorylated peripherin/rds was also probed with antibodies against phosphoserine, phosphothreonine, and phosphotyrosine. Only the phosphoserine antibodies reacted with the peripherin/rds, indicating that all the phosphates are on serine residues (lane 3, Figure 6). Finally, we investigated the relative mobilities of phosphorylated and unphosphorylated peripherin/rds in SDS−PAGE. A 50:50 mixture of dark and light-stimulated Con-A unbound fractions was Western blotted after SDS−PAGE. Only a single immunoreactive band was evident (lane 2, Figure 6), indicating that phosphorylation does not significantly alter the mobility of peripherin/rds in SDS−PAGE. Upon finding two bands in SDS−PAGE after our first purification procedure (hydroxylapatite and Mono Q chromatography), we had initially wondered whether the two bands might result from a mixture of phosphorylated and unphosphorylated peripherin/rds. The result here is consistent with the different conclusion made above, namely, that the two bands correspond to two different polypeptides (peripherin/rds and rom-1).

Role of Peripherin/rds in Rod Outer Segment Membrane Fusion. In the following series of studies, we investigated the role peripherin/rds may play in membrane fusion between disks and plasma membrane. The fusion between R_{18}-labeled plasma membrane vesicles and a variety of target liposomes can be measured using a cell free lipid mixing assay described previously (Boesze-Battaglia & Yeagle, 1990; Boesze-Battaglia, et al., 1992a,b). As the R_{18}-labeled plasma membrane vesicles are introduced into a sample of target liposomes, the increase in R_{18} fluorescence due to the dilution of the probe into the target membrane is monitored continuously and recorded as the initial rate of fusion. Using this assay, we compared the fusogenic properties of native peripherin/rds in disk membranes to those of purified peripherin/rds in disk lipid vesicle recombinants. In an initial series of experiments, we looked at the contribution native peripherin/rds makes to disk−plasma membrane fusion by determining the effect of various inhibitors on R_{18}-labeled plasma membrane−disk membrane fusion. The effect of

Table 1: Fusion between R_{18}-Labeled Plasma Membrane and Disk Membranes (A) and between R_{18}-Labeled Plasma Membrane and Disk Lipid Vesicle Recombinants (B)[a]

target membrane	additions	lag time (s)	initial rate of fusion (%Δ fluorescence/min)
(A)			
disks	10 μM Ca^{2+}	20−30	0.29 ± 0.04
disks	10 μM Ca^{2+} and mAb 2B6	80−100	0.22 ± 0.01
disks	10 μM Ca^{2+} PP-1 (1.8 μM)	100−200	0.23 ± 0.02
phosphorylated disks	10 μM Ca^{2+}	20−30	1.54 ± 0.05
trypsin-treated disks	10 μM Ca^{2+}	90−200	0.18 ± 0.04
(B) LUVs			
(−)-peripherin/rds	1 μM Ca^{2+}	20−25	0.051 ± 0.003
(−)-peripherin/rds	1 μM Ca^{2+} and 0.5% all-trans retinal	20−30	0.120 ± 0.005
(+)-peripherin/rds	1 μM Ca^{2+}	20−30	0.300 ± 0.030
(+)-peripherin/rds	1 μM Ca^{2+} and PP-1 (6 nM)	100−120	0.350 ± 0.004
(+)-phosphorylated peripherin/rds	1 μM Ca^{2+}	20−30	0.450 ± 0.050
trypsin-treated peripherin/rds	1 μM Ca^{2+}	120−130	0.150 ± 0.005

[a] Membrane fusion was measured as an increase in R_{18} fluorescence intensity upon the addition of labeled plasma membrane to the unlabeled recombinant membranes (at a final phospholipid concentration of 1 μM). All fusion assays were performed at 37 °C. The various recombinants were prepared as described in Methods. The initial rates of fusion are calculated as the percent change in maximal fluorescence intensity per minute. Each value is reported as the mean initial rate of fusion and standard deviation of at least three independent assays.

peripherin/rds monoclonal antibody 2B6 and a synthetic peptide corresponding to a region of the C terminus of peripherin/rds (called PP-1) on R_{18}-labeled plasma membrane−disk membrane fusion was investigated. As shown in Table 1A, the initial rate of disk−plasma membrane fusion is slightly decreased with the addition of PP-1 and mAb 2B6. Perhaps more striking is the increase in "lag time" (time it takes prior to a change in fluorescence, the initiation of fusion) with both PP-1 and mAb 2B6. These results suggest that both mAb 2B6 and PP-1 may competitively inhibit a plasma membrane−disk membrane interaction necessary for membrane fusion. It is likely that such an interaction may be involved in membrane adhesion, a step necessary for the progress of the fusion process. Peptides corresponding to the N terminus of peripherin/rds or bradykinin (a nine-amino acid nonspecific peptide) and rhodopsin antibody had no effect on disk−plasma membrane fusion (data not shown). Light-induced phosphorylation of disk membranes resulted in a 5-fold increase in the initial rates of fusion (Table 1A). However, given that both peripherin/rds and rhodopsin are phosphorylated, it is not possible to determine the specificity of this phosphorylation effect in this fusion system.

To determine the effect of the individual protein components (specifically peripherin/rds) on this membrane fusion process, both peripherin/rds and rhodopsin recombinants were made and fusion was assessed. The effect of purified peripherin/rds on disk lipid large unilammellar vesicle fusion with R_{18}-labeled plasma membrane was determined. The disk lipid vesicles containing no protein were prepared as large unilamellar vesicles as described (Boesze-Battaglia et al., 1992a). In the preparation of the disk lipids used in all of the recombinants, the retinal Schiff base linkage was reduced, thereby eliminating any retinal-induced membrane destabilization and membrane fusion due to a lipid-mediated mechanism (Boesze-Battaglia et al., 1992a). As seen in Table 1B, when peripherin/rds was reconstituted into disk lipid large unilamellar vesicles, an increase in the initial rate of fusion was observed when compared with controls containing no protein. The phospholipid to protein ratio of the peripherin/rds recombinants ranged from 83 to 226 mol of phospholipid per mole of peripherin/rds monomer. The initial rates of fusion were essentially identical in the recombinants with ratios of 83 and 110 mol of phospholipid per mole of peripherin/rds monomer; they were 0.32 ± 0.01 and 0.39 ± 0.05, respectively. Those with a higher phospholipid:peripherin/rds ratio (226) fused to a lesser extent, with an initial rate of fusion of 0.10 ± 0.023. The enhancement of the fusion rate is specific for peripherin/rds. When rhodopsin recombinants were allowed to fuse with R_{18}-labeled plasma membrane, there was no increase in the initial rates of fusion when compared with controls containing no protein.

When the peripherin/rds recombinant was pretreated with PP-1, there was an increase in the lag time and essentially no change in the initial rate of fusion observed. These results are qualitatively similar to those obtained with the disk−plasma membrane fusion assays in Table 1A, suggesting that purified peripherin/rds was in fact in a native-like state.

Trypsin treatment of the peripherin/rds recombinant confirmed that the majority of the peripherin/rds was oriented as it would be in the native disk, with the C-terminal region on the outside (data not shown). In addition, trypsin treatment of peripherin/rds resulted in a decrease in the fusion seen between large unilammellar vesicles and plasma membrane. When phosphorylated peripherin/rds was incorporated into the recombinant large unilammellar vesicles, more rapid fusion between the large unilammellar vesicles and plasma membrane was observed than with unphosphorylated peripherin/rds. Collectively, these results suggest that peripherin/rds may play a role in membrane fusion events in photoreceptor cells, with phosphorylation of peripherin/rds affecting this role.

To test whether the extremes of pH used in the purification procedure for peripherin/rds (pH 4.7 and 4.3 for phosphorylated peripherin/rds) might have denatured the protein, we compared fusion results at pH 7.4 and 4.5 as shown in Figure 7. When the pH of the target membrane was lowered to 4.5, there was no change in the initial rates of fusion when compared to that of liposomes at pH 7.4. However, when peripherin/rds was intentionally denatured by boiling and subsequently incorporated into a liposome, there was a substantial decrease in the fusion rates observed (Figure 7). Essentially identical results were obtained if the peripherin/rds recombinant was boiled immediately prior to the fusion assay. Collectively, these results suggest that the fusion assays using peripherin/rds recombinants exhibit the same

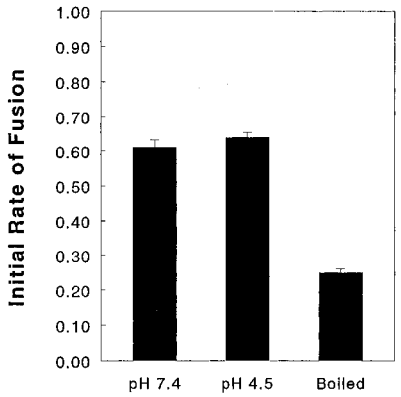

FIGURE 7: Membrane fusion between R_{18}-labeled plasma membrane and peripherin/rds recombinants. The initial rate of fusion between R_{18}-labeled plasma membrane and peripherin/rds recombinants under the indicated conditions (pH 7.4 and 4.5) and boiled protein is shown. Fusion was measured at 37 °C, as described in Materials and Methods. The data represent two independent peripherin/rds recombinant preparations, with fusion assays done in triplicate for an *n* of 6.

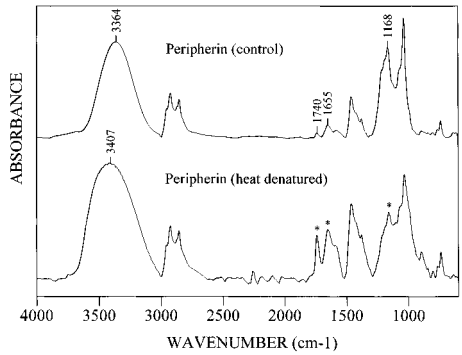

FIGURE 8: FTIR spectra of the thin films of chromatofocused purified peripherin/rds stabilized in octylglucoside. The sample was dispersed in 5 mM Hepes at pH 4.7 (control). The heat-denatured form of the detergent-stabilized peripherin/rds was recorded after cooling the sample for 1 h at room temperature. Asterisks (*) indicate the heat-sensitive spectral bands. Irreversible increased intensity changes in these bands indicate a decrease in the structural stability of this protein due to a loss of peripherin/rds−lipid contacts.

characteristics (i.e., increased lag time with PP-1, with little change in the initial rate of fusion) as fusion using intact disk membranes. Fusion in disks may require peripherin/rds given that rhodopsin recombinants fused only minimally with R_{18}-labeled plasma membrane, suggesting a role for peripherin/rds in this membrane fusion process.

In addition to the functional assays described above, which indicate indirectly that purified peripherin/rds is in a native-like state, the purified protein was analyzed by Fourier transform infrared spectroscopy (FTIR). As seen in Figure 8, purified peripherin/rds (isolated at its isoelectric point, pH 4.7) is in a native-like conformation relative to heat-denatured purified peripherin/rds. Upon heating, peripherin/rds seems to lose most of its contacts with the lipid environment as indicated by a significant increase in intensity of the prominent lipid−protein bands indicated by asterisks in Figure 8. These intensity changes are irreversible, suggesting that the protein is structurally unstable in the absence of the lipid matrix. When compared with the heat-denatured protein, purified peripherin/rds shows strong hydrogen bonding between the purified protein and the lipid environment. The hydrogen bonding is subsequently broken, and there is a 30% increase in the half-width of the 3407 cm^{-1} band largely due to protein and water OH groups and protein NH group vibrations upon denaturation. This band also shifts to higher frequency and is indicative of weakening of the water−protein contacts with the membrane matrix. A weakening of these interactions results in the irreversible loss of certain C−C backbone skeleton bands of ca. 1168 cm^{-1}. Heat denaturation of the peripherin/rds is irreversible as indicated by the FTIR data recorded after cooling of the sample (Figure 8). These results confirm that the chromatofocused purified peripherin/rds is in a native-like conformation under the experimental conditions described.

DISCUSSION

We have purified peripherin/rds from bovine ROSs by two procedures, each giving a different product. Hydroxylapatite and Mono Q FPLC of ROS membrane proteins yielded peripherin/rds in association with rom-1, the other subunit of the same protein complex (Moritz & Molday, 1996). This first procedure should be useful for studies of the peripherin/rds−rom-1 complex, which cannot be formed by simply adding the two proteins together using a transfection system (Goldberg & Molday, 1996a). In the second purification procedure, Con-A and chromatofocusing produced peripherin/rds as a homogeneous dimer. The latter part of the present study was concerned with the phosphorylation of peripherin/rds and the role of peripherin/rds in membrane fusion. Therefore, we used peripherin/rds purified by the second procedure to obviate the possibility of any contaminating signal from rom-1.

In the Con-A chromatofocusing purification procedure, peripherin/rds was isolated from the unbound fraction of ROS membrane proteins. This was unexpected given that peripherin/rds is sensitive to Endo-H (Connell & Molday, 1990). The most likely explanation for these results is that, while OG is a sufficiently strong detergent to solubilize rhodopsin, it results in incomplete solubilization of peripherin/rds. As all the postulated N-linked glycosylation sites are located within the intradiskal region of peripherin/rds (Connell & Molday, 1990), incomplete solubilization of peripherin/rds would mask the Con-A binding mannose residues within a disk membrane lipid−detergent micelle, thus not allowing peripherin/rds to bind to Con-A. The tight association between the Con-A unbound fraction and peripherin/rds also suggests that peripherin/rds may be associated with cytoskeletal proteins. Indeed, a significant proportion of peripherin/rds tends to remain with the Triton X-100 insoluble fraction of ROSs (unpublished observations). Experimentally, it allows us to reconstitute peripherin/rds into native disk membrane liposomes to look at the functional properties of this protein and to characterize post-translational modifications, such as phosphorylation.

Protein phosphorylation plays an important role in the regulation of metabolism and function within rod photoreceptors. In this study, we document the light-dependent phosphorylation of peripherin/rds. Phosphorylation lowers the p*I* of the protein from around 4.7 to 4.2, thereby allowing the two species to be isolated independently using chromatofocusing or electrophoresis techniques for further functional studies. Under the experimental conditions described, an average stoichiometry of nearly 2 mol of phosphate per mole of peripherin/rds monomer was measured, and yet all the peripherin/rds had the same p*I* (4.2). The most likely

explanation is that all the peripherin/rds molecules were phosphorylated to the same extent, each with two phosphates.

The possibility that other ROS phosphoproteins contributed to the phosphorylation signal is unlikely given that we purified peripherin/rds to apparent homogeneity. However, in considering what known ROS proteins have mobilities in SDS−PAGE and isoelectric focusing that are similar to those of peripherin/rds, and thus could provide a contaminating signal if they were present, the only candidate is phosducin (molecular mass of 33 kDa, p*I* of 4.5) (Lee et al., 1990a,b). But the phosphorylation of phosducin requires cyclic AMP and occurs in the dark (Lee et al., 1990a,b).

We present evidence suggesting that peripherin/rds can promote the *in vitro* fusion between disk lipid vesicles and ROS plasma membranes. The incorporation of peripherin/rds into disk lipid vesicles rendered these vesicles more fusogenic. When phosphorylated peripherin/rds was used, a slightly higher rate of this fusion was detected. As trypsin preferentially cleaves the C terminus of peripherin/rds (Molday et al., 1987), the lower rate of fusion observed with trypsin-treated peripherin/rds suggests that the ability of this protein to promote fusion depends upon its C terminus. Interestingly, the C terminus is also the most likely site of peripherin/rds phosphorylation. According to the current topographical model of the protein (Connell & Molday, 1990), it contains all of the nine exposed serine and threonine residues and one of the four exposed tyrosine residues. In these studies, we detected only phosphoserine on Western blots of phosphoperipherin/rds.

In a series of studies, Molday and his colleagues (Goldberg et al., 1995; Goldberg & Molday, 1996a; Moritz & Molday, 1996) have presented evidence that peripherin/rds and rom-1 interact noncovalently and form a tetrameric structure in the rim of the disk membrane. On the basis of these studies, they have suggested that the proteins function in a multimeric structural unit and not as individual proteins. Additional evidence supporting this hypothesis has been obtained from a characterization of mutated peripherin/rds and a null rom-1 mutation found in a digenic form of retinitis pigmentosa (Goldberg & Molday, 1996b). The present data suggest a membrane fusion role for peripherin/rds that can be performed without rom-1 in an *in vitro* fusion system. This role appears to be modulated by phosphorylation of peripherin/rds.

The necessity of transmembrane fusion proteins, with properties similar to those of peripherin/rds, is well documented; these proteins regulate such diverse processes as viral infection, endocytosis, exocytosis, and egg−sperm fusion [for review, see White (1992)]. The changes in photoreceptor outer segment membrane organization during disk membrane morphogenesis and shedding (Laties et al., 1976; Young, 1976; Steinberg et al., 1980; Corless & Costello, 1981; Tsukamoto & Yamada, 1982; Matsumoto & Besharse, 1985) seem to require membrane fusion. Fusion between disk and plasma membranes appears to occur during disk shedding; see the electron micrographs in Young (1976). Our results indicate that peripherin/rds would be a likely candidate to mediate this event. Alternatively, since the rds mouse, which has a null mutation for peripherin/rds, shows defective disk membrane morphogenesis (i.e., no disks appear to form), peripherin/rds may be involved in a critical fusion event during this process. Membrane fusion occurs as the two outgrowing rims meet and fuse, thus completing the formation of a mature discrete disk; see part 4 of Figure 9 in Steinberg et al. (1980). Peripherin/rds is present in these outgrowing rims (Arikawa et al., 1992) and therefore could mediate this fusion event. However, this fusion occurs between membranes containing peripherin/rds molecules with their extracellular domains exposed, and tests with peripherin/rds in this orientation have yet to be performed.

Insight into the role played by peripherin/rds and rom-1 has been gained from COS cell cDNA expression (Goldberg et al., 1995). The present purification procedures should help extend our understanding of peripherin/rds by permitting biochemical studies on the native protein. Here, we have demonstrated light-dependent phosphorylation of peripherin/rds and have shown that homogeneous peripherin/rds and, in particular, phosphoperipherin/rds may function in membrane fusion.

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