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Deletional Analysis of the Rod Photoreceptor Cell Peripherin/RDS Carboxy-Terminal Region

Susan Muller-Weeks, **Kathleen Boesze-Battaglia**, and **Catherine Fitzgerald**

Department of Molecular Biology, University of Medicine and Dentistry of New Jersey–School of Osteopathic Medicine and UMDNJ-GSBS-Stratford Division, 2 Medical Center Drive, Stratford, NJ 08084, U.S.A.

Susan Muller-Weeks: muller@umdnj.edu

Abstract

The C-terminal region of peripherin/rds contains three predicted α-helical domains. One of these domains, corresponding to amino acids 311–322, form an amphiphilic α-helix previously shown to promote membrane fusion. The present studies were conducted to determine how the additional α-helical regions of the peripherin/rds C-terminus affect complex formation with rom-1, glycosylation, intracellular localization and membrane fusion properties. Bovine peripherin/rds and rom-1 were epitope tagged with an amino-terminal FLAG-tag or amino-terminal hemagglutinin (HA)-tag, respectively, and cloned into the pCI-neo expression vector for transient transfection into COS cells. Similarly, four C-terminal peripherin/rds truncation mutants (Δ1, Δ2, Δ3 and Δ4), corresponding to deletions of −19, −29, −39 and −59 amino acids were designed to disrupt the α-helical domains. Immunofluorescence microscopy and enzymatic digestions demonstrated that full-length peripherin/rds and the four C-terminal deletion mutants were localized to intracellular membranes and were all Endo-H sensitive. Western blotting and immunoprecipitation studies showed that the FLAG-tagged bovine peripherin/rds (full-length) was expressed as a 76 kDa dimer, which associates with HA-tagged rom-1 to form a higher order complex. The deletion mutants were also able to associate with rom-1. However, when analyzed using non-denaturing tricine electrophoresis, full-length peripherin/rds and the Δ1, Δ2 and Δ3 mutants formed homo-oligomeric complexes, while the Δ4 mutant appeared to form only homodimers suggesting a region upstream of amino acid 300 may be involved in C-terminal interactions. Membrane fusion was then evaluated using fluorescence resonance energy transfer (RET) techniques. Intracellular COS cell membranes containing full-length peripherin/rds fused with rod outer segment plasma membrane vesicles. This fusion was inhibited with the addition of a synthetic peptide (PP-5) corresponding to the fusion domain of peripherin/rds. In contrast, fusion was negligible with any of the C-terminal truncation mutants. Collectively, these results suggest that in addition to the fusion domain, other regions of the peripherin/rds C-terminus are required for fusion. Most interesting is the observation that the last 19 amino acids, a region downstream of the fusion peptide that is deleted in the Δ1 mutant, appear to be necessary for fusion. This region corresponds to the epitope for anti-peripherin/ rds monoclonal antibody 2B6, which is shown to partially inhibit peripherin/rds mediated membrane fusion.

Correspondence to: Susan Muller-Weeks, muller@umdnj.edu.

Keywords

rods; outer segments; membrane fusion; peripherin/rds; rom-1

1. Introduction

The retinal degeneration slow or rds mouse is a model for a group of inherited human diseases known collectively as retinitis pigmentosa (van Nie et al., 1978). Mice homozygous for the rds mutation do not develop photoreceptor outer segments and exhibit a slow progressive loss of rods and cones (Sanyal and Jansen, 1981). The underlying defect is an insertion of a 9·2 kbp repetitive sequence element into a gene known as peripherin/rds (Travis et al., 1989). Subsequent studies have shown that mutations in the human peripherin/rds gene also cause a variety of retinal dystrophies including autosomal dominant retinitis pigmentosa and macular dystrophy (Kohl et al., 1998).

Peripherin/rds is a 346 amino acid integral membrane glycoprotein expressed exclusively in the outer segments of rod and cone cells (Connell and Molday, 1990; Travis et al., 1991). Within the photoreceptor disk rim, peripherin/rds is oriented such that both the N-terminus and C-terminus are exposed to the cytoplasm (Arikawa et al., 1992). The protein has four predicted transmembrane domains and consequently two intradiskal loops (D1 and D2). The D1 loop is located between the first and second transmembrane domains while the D2 loop, the larger of the two, is located between the third and fourth transmembrane domains. Peripherin/rds is highly conserved with amino acid identities of greater than 90% between species (Kedzierski et al., 1996). It has two potential N-linked glycosylation sites although only asparagine 229 is glycosylated in vivo (Kedzierski et al., 1999).

In rod outer segment (ROS) disk membranes, peripherin/rds forms disulfide-linked homodimers, via cysteine residue 150 located within the D2 loop (Goldberg et al., 1998). A significant portion of these homodimers form disulfide-linked homotetrameric and octameric complexes (Loewen and Molday, 2000). Peripherin/rds homodimers also associate with homodimers of the related disk rim protein, rom-1 to form multisubunit complexes (Travis et al., 1991; Goldberg et al., 1995; Goldberg and Molday, 1996). Rom-1 is a 37 kDa, non-glycosylated structural homolog of peripherin/rds that is expressed in the rim region of rod disk membranes (Bascom et al., 1992; Moritz and Molday, 1996). Although the function(s) of peripherin/rds remains elusive, it is proposed to serve an important role in the structural maintenance of rod and cone outer segments (Travis et al., 1991; Bascom et al., 1992; Goldberg et al., 1995). This is directly supported by in vitro studies showing that expression of peripherin/rds confers a flattened morphology upon microsomal membranes (Wrigley et al., 2000). More recently, Poetsch et al. (2001) have shown that peripherin/rds interacts with glutamic acid rich proteins (GARP) associated with the β-subunit of the cGMP-gated channel located in ROS plasma membranes.

While the D-2 loop of peripherin/rds (the region containing the highest concentration of disease-linked mutations) has been well characterized, very little is known about this proteins' C-terminal domain. One postulated function for the peripherin/rds C-terminus is that of a membrane fusion protein. In ROS, membrane fusion events are necessary for both

disk morphogenesis and shedding (Steinberg et al., 1980; Matsumoto and Besharse, 1995). Purified, native peripherin/rds has been shown to promote fusion of recombinant disk lipid vesicles with ROS plasma membrane vesicles (Boesze-Battaglia et al., 1998). The putative fusion domain is an amphiphilic α-helical region located within the peripherin/rds C-terminus. Notably, a synthetic peptide (PP-5) corresponding to this 15 amino acid fusion domain, can mediate individual steps of membrane fusion including aggregation and membrane destabilization (Boesze-Bataglia et al., 1998, 2000).

In addition to the fusion domain, there are two other predicted α-helical domains located within the peripherin/rds C-terminus. This region may not only be involved in membrane fusion processes, but is proposed to contain a ROS specific localization signal (based on green fluorescent protein (GFP)-fusion constructs) (Tam et al., 2001). Its importance is also illustrated clinically by several chain termination and frame-shift mutations that result in proteins lacking various portions of the C-terminus. Individuals that have inherited these mutations present with heterogeneous disease phenotypes including pattern dystrophy and vitelliform macular dystrophy (Felbor et al., 1997; Kohl et al., 1997). Here we employ a well-characterized COS cell expression system to evaluate the contribution of the C-terminal α-helices to the established biochemical properties of peripherin/rds. A series of C-terminal deletion mutants were generated and shown to retain the capacity to interact with rom-1, although none are able to promote membrane fusion. Collectively, these results suggest that along with the fusion domain, other α-helical regions of the C-terminus contribute to the membrane fusion process. We propose that progressive deletion of the C-terminus abrogates the formation of a fusion competent peripherin/rds.

2. Materials and Methods

Generation of Peripherin/rds and Rom-1 Plasmid Constructs

The open reading frames of peripherin/rds and rom-1 were isolated from bovine retinal mRNA using RT–PCR (Ausubel, 1999). Peripherin/rds primers were designed using the sequence provided by Connell and Molday (1990, Genbank accession J02884). To enable protein detection, the upper 5′ primer was engineered with a 24-nucleotide sequence corresponding to an 8 amino acid FLAG epitope tag (5′-ATGGACTAGAAG-GACGACGACGACAAGGCGCTGCTCAAAGT-CAAATTT-3′)-The lower 3′ primer corresponded to the following sequence: 5′-CTTCAGTGTGCAGAGGG-GACT-3′. Similarly, rom-1 primers were designed using the sequence provided by Moritz and Molday (1996, Genbank accession U72027). The upper 5′ primer was engineered with a 36-nucleotide sequence corresponding to a 13 amino acid hemagglutinin (HA) epitope tag (5′-ATGTGCTACCCTTACGACGTGCCT-GACTACGCCAGCCTGGCGCCGGTGCTGCCCCTGGTC-3′)-The rom-1 lower 3′ primer was 5′-CTAGGCCT-CAGGCAGACACTCCTTGG-3′. All reactions were carried out for 35 cycles using an annealing temperature of 63°C. The resulting PCR products were purified, kinased and end-filled by standard procedures (Sambrook et al., 1989). The fragments were then ligated to Sma I-digested pCI-Neo vector (Promega, Madison, WI, U.S.A.) and verified by DNA sequencing.

To generate the peripherin/rds deletion mutants, four additional lower 3′ primers were designed. These primers correspond to progressive deletions of the C-terminus and are presented below:

Deletion number	3′ Lower PCR primer
Δ1	5′ - TCACAGCTTCTTCACACTCTCCAGAAA - 3′
Δ2	5′ - TCACTTCCAGGTCTCCGGCACGC - 3′
Δ3	5′ - TCAAAGCCAGCCCTCACTCTCGCAC - 3′
Δ4	5′ - TCAGTGCAGGTAGCGTAGCCCAA - 3′

Each 3′ primer was used in conjunction with the FLAG-tagged peripherin/rds 5′ primer to amplify the specific DNA fragments. All PCR reactions were performed for 35 cycles using an annealing temperature of 60°C and peripherin/rds/pCI-Neo construct as template DNA. Following PCR, the fragments were manipulated as described above and ligated to Sma I-digested pCI-Neo vector. All plasmid constructs were verified by sequence analysis and purified by density gradient centrifugation (Sambrook et al., 1989).

Cell Culture and Transfections

COS cells were grown in Dulbecco's Modified Essential Media (DMEM) supplemented with 10% fetal bovine serum. The cells were maintained in a humidified atmosphere of 5·5 % CO_2 at 37°C and split 1 : 3 every third or fourth day. Transfections were performed using Lipofectamine PLUS reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) as directed by the manufacturer. Cells were seeded 1 day prior to transfection as follows; 1×10^5 cells per well in a six well plate, 1×10^6 cells/10 cm dish, or 3×10^6 cells/15 cm dish. The amount of plasmid DNA was likewise based on culture vessel size; 1 μg per well, 4 μg/10 cm dish, or 12 μg/15 cm dish. All cells were harvested 48 hr post-transfection.

Immunolocalization and Immunoprecipitation Analysis

Immunolocalization studies were performed as described previously (Caradonna et al., 1996). The M5 anti-FLAG monoclonal antibody (Sigma, St. Louis, MO, U.S.A.) was used at a concentration of 10 μg ml^{-1} and the FITC-conjugated secondary antibody (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) was used at a 1 : 1000 dilution. The slides were viewed through a Ziess Axioscope fluorescence microscope equipped with an FITC-filter.

For immunoprecipitation analysis, cell extracts were prepared in 1 ml of cold NP40 lysis buffer (50 mM Tris–HCl, pH 7·5; 0·1% NP-40; 250 mM NaCl; 50 mM NaF; 5 mM EDTA). The extracts were sonicated and then centrifuged at 13 000 *g* for 10 min at 4°C to remove insoluble material. Protein concentrations were determined using the Bio-Rad protein assay system as directed. Immunoprecipitation reactions (0·5 mg ml^{-1}) were carried out as described previously (Muller-Weeks et al., 1998) with one modification; all washes were performed using NP40 lysis buffer. Again, the M5 anti-FLAG monoclonal antibody was used at a concentration of 10 μg ml^{-1} and the anti-HA monoclonal antibody (Covance) was

used at a 1 : 1000 dilution. Proteins recovered by immunoprecipitation were resolved by 10% SDS–PAGE and visualized by Western blotting.

Denaturing and Non-denaturing Gel Electrophoresis

SDS–PAGE was performed using the method of Laemmli (1970). Cell extracts were denatured by adding an equal volume of 2 × SDS–PAGE sample buffer (100 mM Tris–HCl, pH 6·8; 200 mM DTT; 4% SDS; 0·2 % bromophenol blue; 20 % glycerol) in the presence (reducing) or absence (non-reducing) of 5 % beta-mercaptoethanol. The samples were heated to 85°C for 10 min and loaded directly onto a 10% SDS–polyacrylamide gel. Non-denaturing tricine poly-acrylamide gel electrophoresis (10%) was performed by omitting SDS from the system described by Schagger and von Jagow (1987). In this case, the samples were prepared in the absence of BME and were not heated prior to loading. Gels were run at 30 mA for 2 hr.

Western Blotting and Enzymatic Deglycosylation Studies

Western blotting was performed according to the procedure of Towbin et al. (1979). Antibody binding and wash conditions were as previously described (Caradonna et al., 1996). Protein bands were visualized using Sigma Fast BCIP/NBT (5-Bromo-4-Chloro-3-Indolyl phosphate/ Nitro blue tetrazolium) substrate as directed.

Deglycosylation of peripherin/rds was performed using endoglycosidase H (Endo H) as outlined by New England Biolabs (Beverly, MA, U.S.A.). Initially, 2 μl of denaturing buffer (50 mM sodium citrate, pH 5·5) were added to the extracts in a 15 μl reaction. The extracts were denatured by heating to 100°C for 10 min. Subsequently, 1/10th volume of 10× G5 buffer (0·5 % SDS; 1 % β-mercaptoethanol) and 1 U of endoglycosidase H were added and the reactions were incubated at 37°C overnight. Control extracts were also prepared as described but lacked the enzyme.

Preparation and Labelling of ROS Membranes

Retinal ROS membranes were isolated from 50 frozen, dark-adapted bovine retinas (J.A. Lawson, Lincoln, NE, U.S.A.). Disk membranes and plasma membrane vesicles were isolated using ricin-agarose and differential sucrose gradient centrifugation as described (Boesze-Battaglia and Albert, 1989). The ROS plasma membranes were eluted from the ricin-agarose pellet with 1 M galactose in 0·1 M sodium borate, pH 8·5. Both ROS plasma membrane vesicle fractions and the isolated disk membranes were washed with 10 mM Hepes, 1·0 mM EGTA. The plasma membrane vesicles were resuspended in 500 μl of 10 mM Hepes, pH 7·4 and used within 24 hr of isolation. All manipulations were performed under dim red light.

The phospholipid concentration of both ROS disk and plasma membrane samples was determined as described by Bartlett (1959) and modified by Litman (1973). Disk membranes were labelled with octade-cylrhodamine B chloride (R_{18}, Molecular Probes, Junction City, OR, U.S.A.) at a concentration of 1 mol% relative to total phospholipid. Plasma membrane vesicles were similarly labelled with 5-(*N*-octadecanoyl) aminofluorescein (F_{18}, Molecular Probes, Junction City, OR, U.S.A.). Labelling was carried out in a minimum volume of

ethanol for 30 min at room temperature as described (Boesze-Battaglia and Yeagle, 1992). Labelled membranes were separated from unincorporated R_{18} or F_{18} by passing the samples through a Sephadex G50 column and eluting with 100 mM NaCl, 10 mM glycine pH 7·5.

Preparation and Labelling of COS Intracellular Membranes

COS cell intracellular membranes were isolated essentially as described by Oprian (1993). Briefly, cell pellets were resuspended in 600 μl Tris–$MgCl_2$ buffer (10 mM Tris–HCl pH 7·2, 2 mM $MgCl_2$, and 10 mM glucose) and lysed by passage through a 26-gauge needle two times. The resulting lysate was layered on a 3·8 ml cushion of 37% W/V sucrose in Tris–$MgCl_2$ buffer and centrifuged at 18 000 rpm for 20 min at 10°C (XL70 Ultra Centrifuge, SW 60 rotor, Beckmann Instrument, Papo Gilo, CA, U.S.A.). The intracellular membranes were collected in a 500 μl volume and an equal volume of Tris–$MgCl_2$ buffer was added to dilute the sucrose. Phosphate assays were performed to determine the total phospholipid content of the intracellular membranes. The membranes were then labelled with F_{18} as described above. A total volume of 3 ml of labelled membrane was collected from the Sephadex G50 column.

Resonance Energy Transfer Fusion Assays

The fusion between R_{18}-labelled ROS plasma membrane vesicles and F_{18}-labelled membranes (ROS disk or intracellular COS) was measured using a well-characterized resonance energy transfer (RET) assay (Struck et al., 1981; Partearroyo et al., 1994). The fusion assays were performed at room temperature on a Perkin-Elmer LS 50B spectrofluorometer (Gaithers-burg, MD, U.S.A.) equipped with a 96 well plate reader. Fusion was initiated with addition of R_{18}-labelled plasma membranes (final phospholipid concentration = 0·20 nM) to F_{18}-labelled membranes (final phospholipid concentration = 0·40 nM) already present in the well. Fluorescence intensity was measured at λ_{ex} = 460 nm (F_{18} excitation) and at λ_{em} =524 nm (F_{18} emission) and λ_{em} = 592 nm (R_{18} emission) over a 2 min period. There was no additional change in R_{18} fluorescence for 30 min. The extent of fusion ($\Delta R \times 100$) was calculated as a percent change in R_{18} intensity over time as described previously (Partearroyo et al., 1994). The change in R_{18} intensity at a given time (ΔR) was computed as follows:

$$\Delta R = (I_{592}/I_{524}+I_{592})_{T} - (I_{592}/I_{524}+I_{592})_{I},$$

where I_{524} and I_{592} are the fluorescence intensities at 524 and 592 nm, respectively. The subscripts T and I represent a given time point and the initial time point of each sample, respectively. The extent of fusion (i.e., percent change in R_{18}) was determined by multiplying each ΔR value by 100 and this value was normalized to the calculated extent of fusion observed between F_{18}-labelled disks and R_{18}-labelled plasma membrane. This latter value was set as 100 % and all extents of fusion ($\Delta R \times 100$) were normalized to this standard. The $\Delta R \times 100$ value obtained with mock-transfected cells was routinely subtracted from $\Delta R \times 100$ of the samples. This value was variable, accounting for 25–40 % of ΔR measured for peripherin/rds expressing cells. The final Ca^{2+} concentration in all assays was

100 nM. Fusion could be inhibited with the addition of EDTA at a final concentration of 10 mM.

For specificity studies, F_{18}-labelled COS intracellular membranes were preincubated with PP-5 (72·5 nM) at 37°C for 20 min prior to the addition of R_{18}-labelled ROS plasma membrane. The sequence of the PP-5 peptide is H_2N-V-P-E-T-W-K-A-F-L-E-S-V-K-K-L-COOH.

3. Results

Construction and Expression of Peripherin/rds Deletion Mutants

The peripherin/rds C-terminus is a 64 amino acid extradiskal domain that spans amino acid residues 282–345. This region is highly conserved (78% amino acid identity) between human, bovine, canine, feline and rodent species suggesting a functional importance. Chou-Fasman and Robson-Garnier analyses of the peripherin/rds C-terminus predict three α-helical regions that are labelled 1, 2 and 3 in Fig. 1. To evaluate the functional significance of these α-helical domains, a series of C-terminal truncation mutants were constructed. The deletion endpoints were selected using the predicted secondary structural analysis (Fig. 1). The Δ1 mutant, removes residues 326–345, corresponding to the third predicted α-helical region. The Δ2 mutant, deletes residues 317–345, truncating the second α-helical region or fusion peptide domain (Boesze-Battaglia et al., 1997, 1998). The Δ3 mutant removes residues 307–345, thus eliminating the entire fusion domain. Finally, the Δ4 mutant truncates the entire C-terminus (residues 288–345) encompassing all three α-helical regions. Of note, both the Δ2 and Δ4 mutants approximate disease-linked mutations shown to co-segregate with retinal degenerative diseases (Felbor et al., 1997; Kohl et al., 1997).

The full-length peripherin/rds and all mutants were engineered with an amino-terminal FLAG-epitope tag to facilitate detection. Following transient transfections into COS cells, protein expression was confirmed by immunohistochemical localization using monoclonal antibody against the FLAG-tag (M5, Sigma, St. Louis, MO, U.S.A.) and FITC-conjugated secondary antibody. The full-length, FLAG-tagged peripherin/rds is localized to the internal membranes of COS cells (Fig. 2(A), panel 1). Importantly, control transfections using pCI-neo vector alone are negative for specific peripherin/rds immunoreactivity (Fig. 2(A), compare panels 1 and 2). These results are consistent with those previously reported by Goldberg et al. (1995) for heterologously expressed peripherin/rds. Immuno-fluorescent localization of mutants Δ2 and Δ4 shows that the staining pattern is unchanged, remaining confined to the intracellular membranes (Fig. 2(A), panels 3 and 4). While it appears that the Δ4 mutant may be expressed at higher levels (as compared with wild-type and Δ2), Western blot analysis described below, demonstrates that it is not the case. Identical results were obtained for mutants Δ1 and Δ3 (data not shown).

Peripherin/rds expression was also evaluated by Western blot analysis using equivalent amounts of protein extract (25 μg) derived from each transfected sample. As shown in Fig. 2(B), the full-length peripherin/rds and deletion mutants are expressed to approximately equal levels and can be readily detected using the FLAG-epitope tag. Full-length peripherin/rds migrates as a 38 kDa protein. This is slightly larger than the native

peripherin/rds purified from ROS owing to the presence of the 1 kDa FLAG-tag. Molecular weights of the peripherin/rds mutants are reduced accordingly, ranging from 36 (Δ1) to 31·5 kDa (Δ4). It is also apparent that the peripherin/ rds proteins resolve as doublets or possibly, as triplets. This is most likely due to the presence of both glycosylated and non-glycosylated forms. Others have observed similar doublet patterns under both in vivo (Boesze-Battaglia et al., 1997) and in vitro conditions (Wrigley et al., 2000). Collectively, these results suggest that the epitope tag does not interfere with protein expression or localization. In addition, progressive deletions of the C-terminus do not significantly alter protein expression levels or cellular localization in the COS cell system.

Biochemical Characterization

The consequences of these C-terminal mutations on several well-established biochemical properties of peripherin/rds were then examined. These features include: (1) glycosylation, (2) association with rom-1 and (3) the ability to form higher order complexes. Peripherin/rds contains three possible N-linked glycosylation sites. In ROS, peripherin/rds is glycosylated at a single site, asparagine 229, located within the D2 intradiskal loop (Kedzierski et al, 1999). Goldberg et al. (1995) have shown that heterologously expressed peripherin/rds is incompletely glycosylated with both modified and unmodified forms present in extracts. To examine the effects of the C-terminal deletions on glycosylation, extracts prepared from transfected COS cells were treated with endoglycosidase H (Endo-H) as shown in Fig. 3. In untreated extracts (− lanes), peripherin/rds migrates as doublets (full-length, Δ1 and Δ2) or triplets (Δ3 and Δ4). Upon Endo-H treatment (+ lanes) however, all samples resolve as a single, lower molecular weight band. An HA-tagged Rom-1 was also included in this analysis and there was no apparent change in the presence or absence of Endo-H treatment indicating that it is not glycosylated in this system (data not shown). The glycosylation pattern associated with full-length peripherin/rds and mutants Δ1 and Δ2 is consistent with previous results (Goldberg et al., 1995) and indicates that removing the terminal 29 amino acids does not markedly interfere with glycosylation in this system. Interestingly, there is a third, higher molecular weight band identifiable in mutants Δ3 and Δ4, which suggests the use of alternate glycosylation sites.

Peripherin/rds has been shown to complex non-covalently with a non-glycosylated homolog, rom-1 (Bascom et al., 1992). The contribution of the peripherin/rds C-terminus to this complex formation was determined by co-immunoprecipitation analysis using extracts from cells co-transfected with each peripherin/rds construct (Δ1, Δ2, Δ3, and Δ4) and rom-1. For detection purposes, rom-1 was engineered with a HA-tag and cloned into pCI-Neo. Extracts prepared from COS cells co-transfected with the individual peripherin/rds constructs and rom-1 were immunoprecipitated using anti-FLAG antibody (detects peripherin/rds). The products were separated by 10 % SDS–PAGE and analysed by Western blotting with the anti-HA antibody (detects rom-1). Fig. 4(A) shows that full-length peripherin/rds and each of the mutants are able to co-precipitate the 39 kDa HA-tagged rom-1 protein. Control reactions are presented in Fig. 4(B). Rom-1 is not detected in FLAG-immuno-precipitates from mock-transfected cells or cells transfected with peripherin/rds or rom-1 individually (Fig. 4(B), lanes 1–3). In a reciprocal experiment, all of the anti-HA immunoprecipitates were shown to contain peripherin/rds (data not shown). Together these results show that

removal of the peripherin/rds C-terminus does not eliminate its ability to interact with rom-1.

Native peripherin/rds also forms covalently linked homodimers, tetrameric and higher order oligomeric complexes (Goldberg et al., 1995; Loewen and Molday, 2000). Of particular significance are recent studies by Boesze-Battaglia et al. (2000) showing that a synthetic peptide corresponding to the peripherin/rds fusion domain (PP-5) can assume a homo-tetrameric state mediated through non-covalent interactions between the α-helices. This suggested that the C-terminus of peripherin/rds may participate in the formation of homotetramers (oligomers) through non-covalent interaction. We assessed the ability of full-length peripherin/rds and each deletion mutant to assume dimeric and oligomeric forms using non-denaturing tricine gel electrophoresis. The proteins were visualized by Western blotting with FLAG antibody and the results are presented in Fig. 5. Full-length peripherin/rds, as well as peripherin/rds co-transfected with rom-1 predominantly form dimeric (approximately 80 kDa) and higher order oligomeric (≥160 kDa) complexes. The same is evident for mutants Δ1 and Δ2. Interestingly, while mutant Δ3 is likewise able to form dimeric and higher order complexes, a significant monomeric band is also visible. The Δ4 mutant is present exclusively in monomeric and dimeric forms and is apparently unable to efficiently form higher order complexes.

Analysis of Peripherin/rds Dependent Fusion

Full-length bovine peripherin/rds has been shown to promote membrane fusion. The fusion domain has been localized to residues 311–325 within the C-terminus of the protein. To investigate how the predicted secondary structural domains within the C-terminus contribute to membrane fusion, the peripherin/rds deletion mutants were studied for their ability to promote membrane fusion. Using a RET method (Struck et al., 1981; Partearroyo et al., 1994), the fusion between intracellular COS cell membranes containing the expressed peripherin/rds proteins and bovine ROS plasma membrane vesicles was measured. In this assay, each membrane species is labelled with a distinct fluorescent probe (F_{18} or R_{18} as described in the ‘Materials and Methods’). As the membranes fuse, the lateral diffusion of the fluorescent probes within the lipid bilayer allows them to come in close proximity. This causes energy to be transferred from F_{18} (excited at 460 nm) to R_{18}, thereby indirectly exciting R_{18}. As shown in Fig. 6(A), when R_{18}-labelled ROS plasma membrane vesicles are mixed with F_{18}-labelled COS cell membranes isolated from cells expressing the full-length peripherin/rds, an increase in R_{18} (solid line) and a decrease in F_{18} (dashed line) fluorescence intensity is observed. Notably, the increase in fluorescence was specific for only peripherin/rds containing COS cell membranes; when COS cells were transfected with rom-1 alone, no fusion was detected (data not shown). The increase in R_{18} fluorescence intensity was quantitated as described in the ‘Materials and Methods’ and normalized to maximal fusion. Maximal fusion is designated as that between Relabelled plasma membrane vesicles and F_{18}-labelled disk membranes (containing native peripherin/rds) (Fig. 6(B), bar 1). Fusion mediated by COS cell membranes containing full-length peripherin/rds is presented in Fig. 6(B), bar 2. All assays were performed using equivalent amounts of phospholipid (0·40 n_M) and peripherin/rds expression was verified by Western blot analysis as shown in the inset. To confirm the specificity of the fusion as that due to peripherin/rds,

the F_{18}-labelled COS cell intracellular membranes were treated with PP-5, a synthetic peptide, previously shown to inhibit disk-plasma membrane and peripherin/rds LUV-plasma membrane fusion. The addition of PP-5 resulted in a decrease in the percent change in R_{18} fluorescence, suggesting inhibition of membrane fusion (Fig. 6(C)). Importantly, the addition of bradykinin or PP-1 (a synthetic peptide to the N-terminal domain of peripherin/rds (Boesze-Battaglia et al., 1997)) had no effect on fusion. The concentration of PP-5 added is equal to the amount that inhibits fusion in model membrane studies.

A comparison of the percent maximal fusion between ROS plasma membranes and COS cell membranes expressing either the full-length or the four peripherin/rds deletion mutants can be seen in Fig. 7. In contrast to the full-length peripherin/rds, very little to no fusion was detectable with any of the four C-terminal deletion mutants. These values are in fact equivalent or below those obtained with mock-transfected cells. Routinely, the fluorescence observed with the mock-transfected samples corresponded to 2–3 % of maximal fusion and importantly no increase in fluorescence intensity was observed over the time course of the experiments with these cells. This inability to promote fusion is not due to the level of protein expression, since all membranes contained similar levels of expressed protein as shown in the inset of Fig. 7. Together these results suggest that none of deletion mutants contained the minimal structural requirements necessary to promote fusion at a level consistent with that of the full-length protein.

4. Discussion

The results presented herein confirm and expand upon those reported by Goldberg et al. (1995). As described previously, peripherin/rds expressed in COS cells localizes to intracellular membranes, is glycosylated, forms heterotetramers with rom-1, and can form homodimers and homotetramers (Goldberg et al., 1995; Loewen and Molday, 2000). Our results extend these findings by showing that peripherin/rds expressed in COS cells can function as a fusion protein by promoting fusion between COS cell membranes containing peripherin/rds and vesicles prepared from ROS plasma membranes. The characteristics of fusion between COS cell membranes and ROS plasma membrane vesicles are identical to those observed with the native disk membranes and purified peripherin/rds. Specifically, it is dependent upon nanomolar levels of calcium and can be inhibited by addition of PP-5, the synthetic peptide mimicking the proposed fusion domain of peripherin/rds. Moreover, it is specific for ROS plasma membrane as the target (data not shown), observations which suggest that the fusion event is indeed dependent upon the presence of peripherin/rds in the membranes and is not mediated by any of the other putative fusion proteins likely to also be present in COS intracellular membranes.

The present experiments were conducted to evaluate the role of the C-terminal a-helices of peripherin/ rds. To facilitate protein detection, an N-terminal FLAG epitope tagged peripherin/rds construct was generated for expression in COS cells. Importantly, our data show that the presence of the N-terminal epitope tag does not alter the expression, or the localization of peripherin/rds to intracellular membranes. As expected, the FLAG-tagged peripherin/rds formed either homo-oligomers or hetero-oligomers with rom-1. The presence of the N-terminal epitope tag also did not alter the fusogenic function of peripherin/ rds.

Progressive deletion of the peripherin/rds C-terminus did not appear to alter significantly protein expression or localization in the COS cell system. However, the utility of COS cells is limited in that they are not polarized neuronal cells and certainly lack specific machinery required for transport/sorting. Our results therefore, do not preclude the possibility that the peripherin/rds C-terminus is important for directing localization of this protein in ROS. This question is currently being addressed using peripherin/rds-GFP fusion constructs to create transgenic *Xenopus laevis* (Lee et al., 2001; Tam et al., 2001). Initial results are conflicting however, with one group reporting that the C-terminus is required for proper targeting to ROS (Tam et al., 2001) and another reporting that the C-terminus is not required (Lee et al., 2001).

The C-terminal mutants also behave normally with respect to glycosylation and complex formation with rom-1. Interestingly, when the entire C-terminal domain was removed in the Δ4 mutant, additional slower migrating bands were seen (Fig. 3). It is possible that the Δ4 mutant is somewhat misfolded, thereby exposing a previously inaccessible glycosylation site, which resulted in the additional bands. Alternatively, conformational changes resulting from the loss of the entire C-terminus may have caused the changes in migration. In either case, all protein forms were reduced to a single non-glycosylated form in the presence of Endo-H. Native ROS peripherin/rds forms heterotetramers and higher order complexes with rom-1 (Goldberg and Molday, 1996; Loewen and Molday, 2000). Loewen and Molday (2000) have presented evidence suggesting that the sites of non-covalent interactions between peripherin/rds and rom-1 are within the D2 loop of peripherin/rds. Our results showing complex formation between rom-1 and each of the four mutated forms of peripherin/rds suggest that the C-terminus is most likely not the site of interaction between peripherin/rds and rom-1 and are consistent with previous results.

Prior work has identified a region within the C-terminus of peripherin/rds proposed to be the fusion domain of this protein (Boesze-Battaglia et al., 1997, 1998). This region corresponding to amino acids 311–325 is an amphiphilic α-helix (Boesze-Battaglia et al., 1998), a secondary structure common to other fusion proteins (White, 1990). Deletion mutants within the C-terminus of peripherin/rds that either disrupt (Δ2 mutant) or omit (Δ3 and Δ4 mutants) this fusion domain, do not promote membrane fusion. These observations provide strong support for our conclusions that the fusogenic region of peripherin/ rds resides in the C-terminus and that the fusogenic domain within the C-terminus lies between amino acids 311–325. In addition, the Δ2 mutant approximates the stop mutation that is associated with a retinal degenerative disease state described by Felbor et al. (1997). Similarly, Kohl et al. (1997) have described a retinal degenerative disease that is associated with a second stop mutation in peripherin/rds corresponding to the Δ4 mutant. Our findings represent the first biochemical abnormalities associated with these mutated forms of peripherin/rds found in either degenerative disease.

Interestingly, the truncation mutant (Δ1) which lacks the last 20 amino acids of the C-terminus, but which retains an intact fusion domain (i.e., amino acids 311–325) did not promote fusion. This observation was not altogether unexpected since the anti-peripherin/rds monoclonal antibody 2B6, partially inhibits disk-plasma membrane and peripherin/rds LUV-plasma membrane fusion. The epitope for this monoclonal antibody resides within the

terminal 30 amino acids of the protein (Connell and Molday, 1990). Most likely, the 20 amino acid region missing in the Δ1 mutant is itself not a fusion domain because in vitro studies using a synthetic peptide mimicking this region did not by itself promote fusion between ROS plasma membrane vesicles and lipid vesicles (Boesze-Battaglia et al., 1997). Of note, Stefano et al. (2002) have recently generated a peripherin/rds mutant lacking the C-terminal 10 amino acids and expressed this protein in MDCK cells. This mutation does not disrupt the third α-helical domain and is capable of mediating membrane fusion. Similar results were also seen in COS cells (Boesze-Battaglia, unpubl. res).

Our previous studies have suggested that the fusion competent form of the fusion peptide (a.k.a. PP-5) and by inference, peripherin/rds, requires the formation of a homotetrameric complex. It is unlikely, however, that the failure of the Δ1 mutant to promote fusion can be ascribed to an inability of this mutant to form tetramers because evidence of homo-oligomer formation was detected with this mutant (Fig. 5). The finding that the Δ1 mutant forms higher order complexes but is not fusion competent indicates that formation of a tetrameric state is not solely responsible for conferring fusion competency. It can also be concluded that domains within the C-terminus, other than the fusion domain (amino acids 311–322), are required for the formation of a fusion competent form of peripherin/rds. Thus, the region corresponding to the terminal 20 amino acids most likely aids in promoting the fusion competency of this protein. Such domains have been described for viral membrane fusion proteins and intracellular trafficking fusion proteins as contributing to the formation of hairpins (a.k.a., SNAREpins), necessary to form a fusion pore (Weber et al., 1998). How these regions interact and their structural identity remains to be determined.

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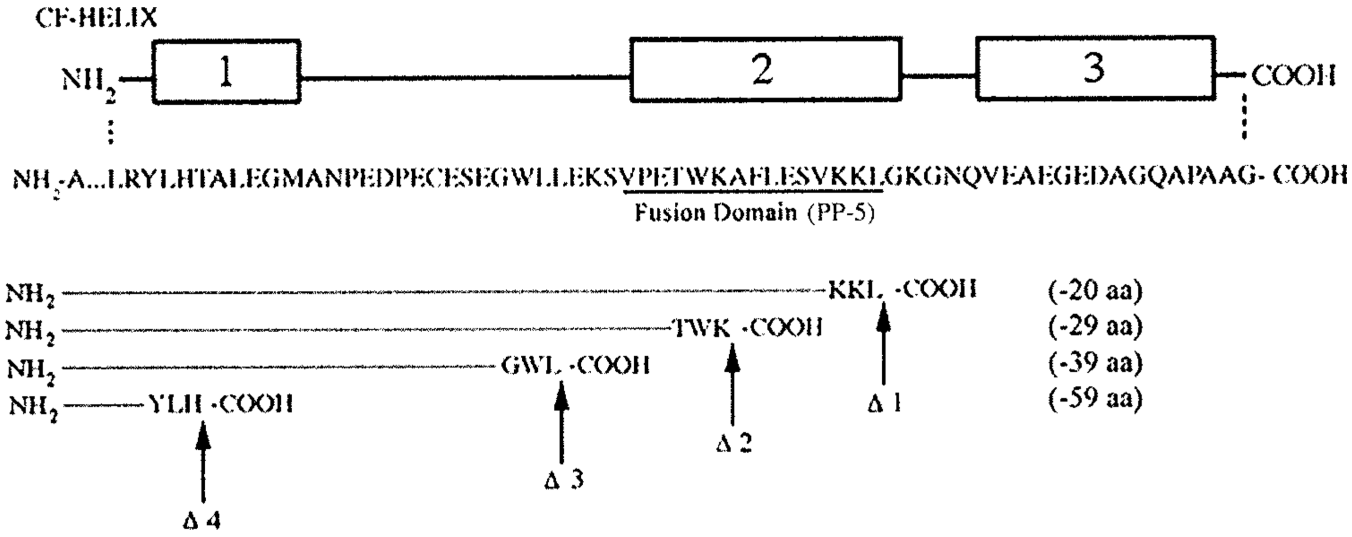

Fig. 1.

Schematic representation of the peripherin/rds C-terminus and deletion mutants. Amino acid sequence of the peripherin/rds C-terminus is presented (residues 282–345). Chou-Fasman and Robison-Garnier analyses of the region reveals three, α-helical regions that are denoted by ▭ and numbered 1, 2 and 3. Helix 2 is the previously characterized fusion domain and the corresponding amino acid sequence is underlined. This sequence also corresponds to the peptide referred to as PP-5. Four C-terminal deletion mutants were constructed to disrupt the α-helical structures and are denoted Δ1–Δ4. Deletion endpoints are marked with an arrow and the length of each deletion is indicated on the right.

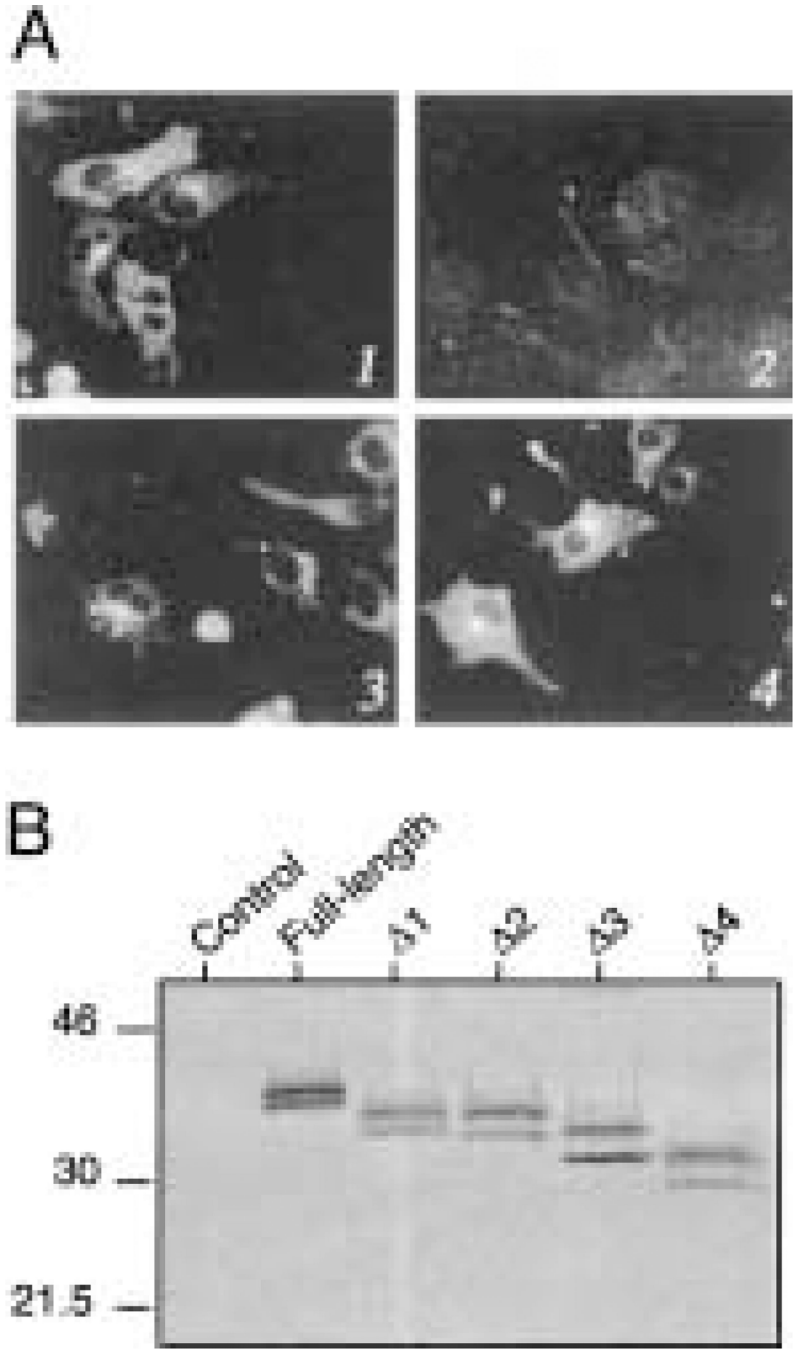

Fig. 2.

Expression of the peripherin/rds constructs in COS cells. (A) Immunofluorescent localization using M5 anti-FLAG primary antibody and FITC-labelled secondary antibody. Cells were transiently transfected with the full-length peripherin/rds (panel 1), Δ2 (panel 3) and Δ4 (panel 4) constructs. Panel 2 shows that no immunofluorescence is detected in control cells transfected with pCI-Neo vector alone. (Note: The exposure time for panel 2 was increased in order to visualize cell structure since no specific fluorescence is detectable.) (B) Western blot analysis of full-length peripherin/rds and each deletion mutant

(Δ1–Δ4). Extracts (25 μg) from transiently transfected COS cells were fractionated by 10% SDS–PAGE under reducing conditions. The proteins were analysed by Western blotting using M5 anti-FLAG antibody. Molecular weight standards in kilodaltons are indicated on the left.

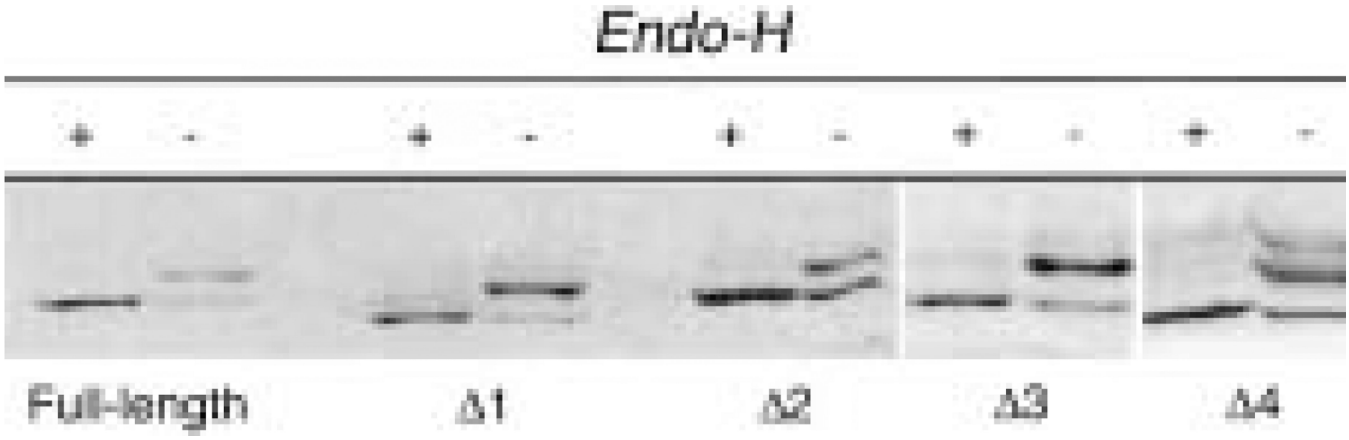

Fig. 3.

Enzymatic deglycosylation studies. Extracts (25 μg) prepared from transiently transfected COS cells were treated with Endo H (+) or left untreated (−) as indicated. Proteins were fractionated by 10% SDS–PAGE under reducing conditions and transferred to nitrocellulose for Western blot analysis using the anti-FLAG M5 antibody. Each construct name is indicated below the lanes.

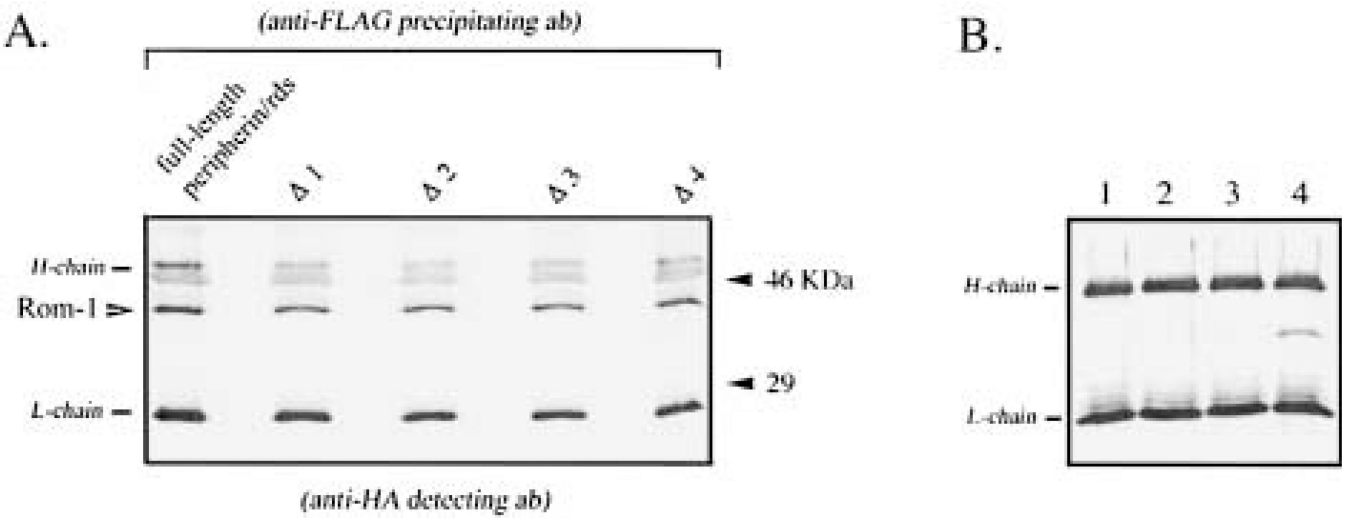

Fig. 4.

Immunoprecipitation of peripherin/rds and rom-1 complexes. (A) Extracts (0·5 mg ml^{-1}) were prepared from COS cells transiently co-transfected with rom-1 plus the indicated constructs and immunoprecipitated using the M5 anti-FLAG antibody (detects peripherin/rds). The immunoprecipitates were fractionated by 10% SDS–PAGE and then analyzed by Western blotting using the anti-HA monoclonal antibody (detects rom-1). Molecular weight markers are indicated on the right and the location of antibody heavy and light chains are indicated on the left. (B) Control reactions showing the absence of rom-1 staining in immunoprecipitates from mock-transfected cells and cells transfected individually with rom-1 and peripherin/rds. Immunoprecipitation reactions and Western blotting were performed as described in (A), however, fractionation was by 12·6% SDS–PAGE. Lanes: (1) mock-transfected COS cells; (2) cells transfected with HA-rom-1; (3) cells transfected with FLAG-peripherin/rds; (4) cells co-transfected with HA-rom-1 and FLAG-peripherin/rds.

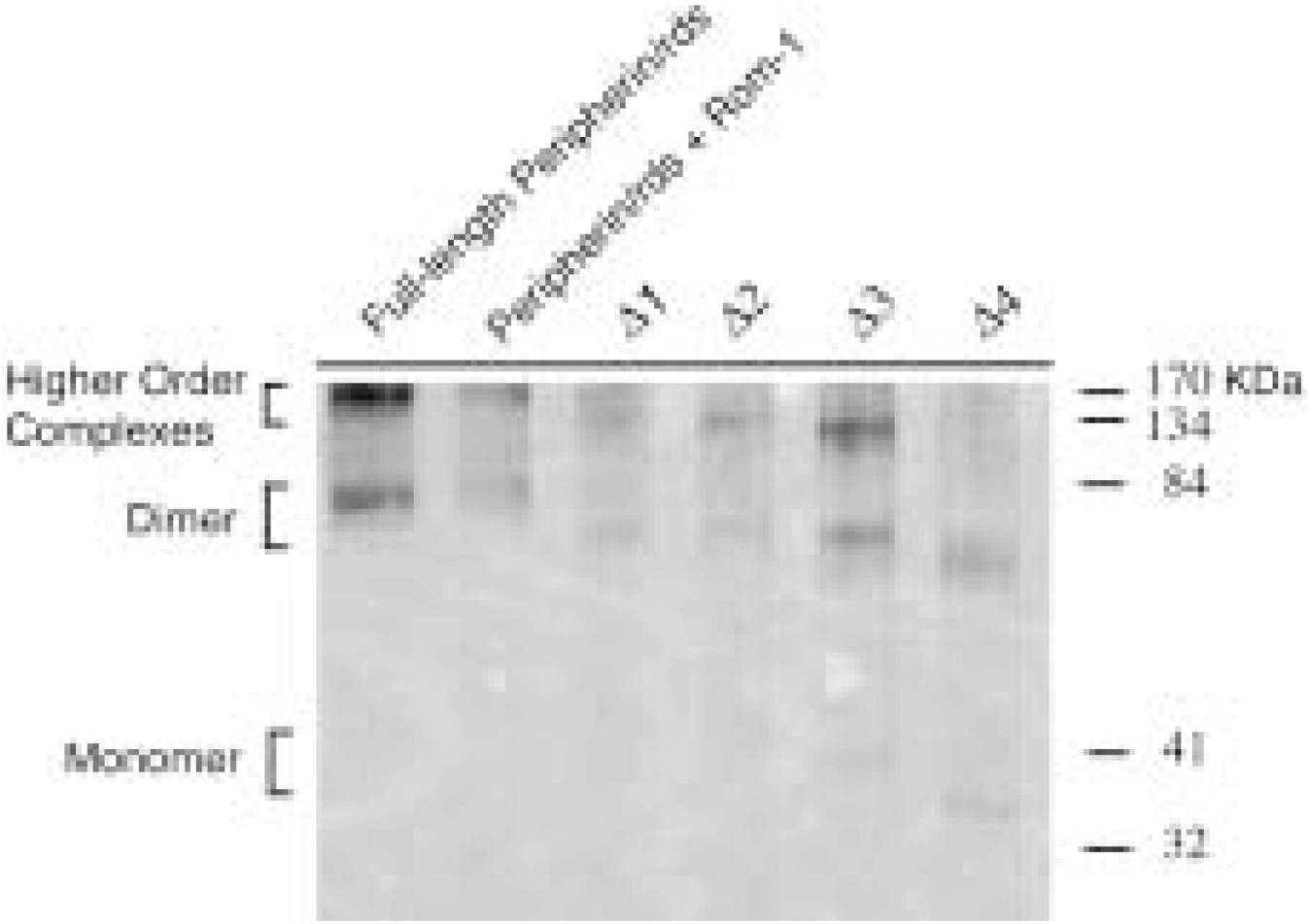

Fig. 5.

Non-denaturing electrophoresis of full-length peripherin/rds and the deletion mutants. Extracts (30 μg) from cells transiently transfected with the indicated constructs were fractionated by 10 % non-denaturing tricine gel electrophoresis. The proteins were transferred to nitrocellulose for Western blotting using anti-FLAG M5 monoclonal antibody. Monomeric, dimeric and higher order complex protein forms are indicated by brackets on the left. Molecular weight markers in kilodaltons are indicated on the right.

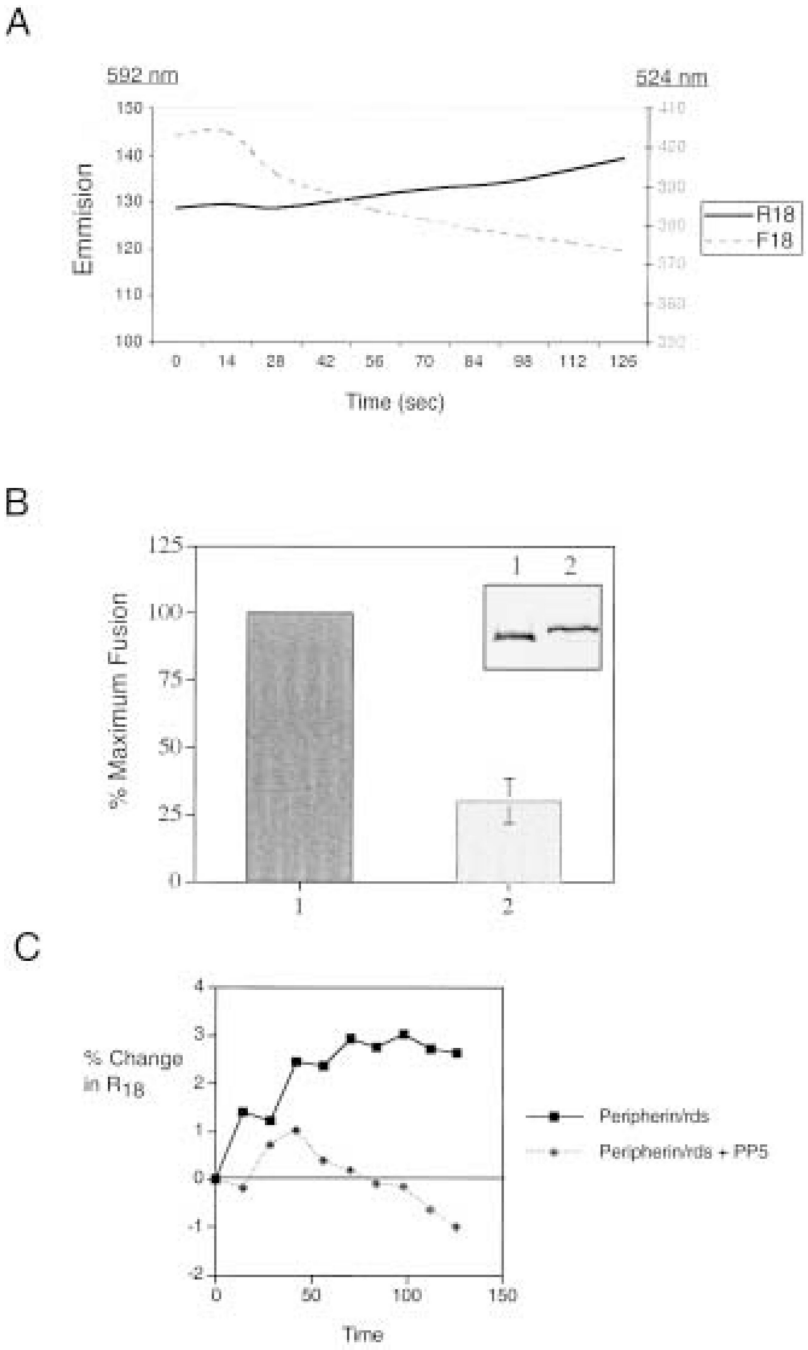

Fig. 6.

Resonance energy transfer from F_{18} to R_{18}. (A) Presented above is a representative scan of the increase in R_{18} fluorescence emission at 592 nm (solid line) and the concomitant decrease in F_{18} fluorescence emission at 524 nm (dashed line) when F_{18}-labelled COS cell membranes containing peripherin/rds fuse with Relabelled ROS plasma membrane. The excitation wavelength is 460 nm. (B) Maximal fusion between Relabelled ROS plasma membrane and F_{18}-labelled COS cell membranes containing peripherin/rds or F_{18}-labelled disk membranes. The RET assay was performed as described in the 'Materials and Methods'

and maximal percent fusion was calculated. Western blot analysis was also performed to confirm peripherin/rds expression in both samples (shown in inset). Inset, lane 1 disk membrane peripherin/rds; Western blot probed with monoclonal antibody 2B6 and lane 2, FLAG-tagged peripherin/rds in COS cell membranes; Western probed with anti-FLAG antibody. (C) The percent change in R_{18} fluorescence over time (in sec) upon energy transfer when Relabelled ROS plasma membranes are mixed with F_{18}-labelled COS cell membranes, in the presence [—◆—] and absence [—■—] of PP-5.

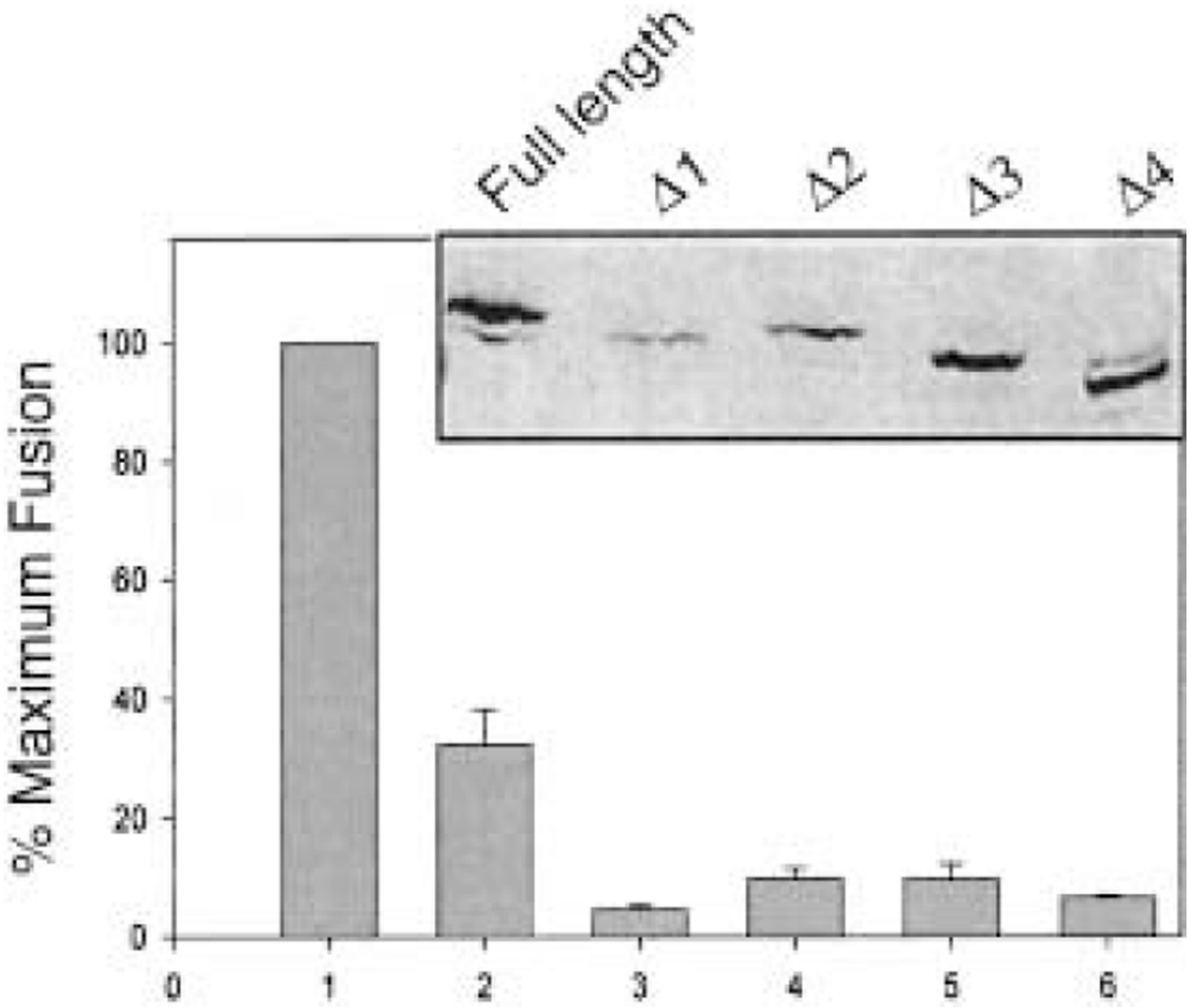

Fig. 7.

RET fusion assay between R_{18}-labelled plasma membrane vesicles and various target membranes. The percent maximal fusion is shown for mixtures of Relabelled plasma membrane vesicles and F_{18}-labelled, disks (bar 1), or COS cell membranes from cells transfected with full-length FLAG-peripherin/rds (bar 2), Δ1 mutant (bar 3), Δ2 mutant (bar 4), Δ3 mutant (bar 5) or Δ4 mutant (bar 6). Inset, Western blot probed with anti-FLAG antibody showing the amount of expressed protein in each sample.