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Heterologous Expression of WT and Mutant Photoreceptor Peripherin/rds in Madin Darby Canine Kidney Cells: an Assessment of Fusogenic Function

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Abstract

Peripherin/rds is proposed to function as a fusion protein within the rod outer segment and a fusion domain has been mapped to amino acids 311–325 within the C-terminus. To map regions within peripherin/rds required for membrane fusion a series of C-terminal mutants was analyzed. Madin Darby canine kidney cells were transiently transfected with an Xpress or FLAG epitope tagged peripherin/rds (wt) and three mutants of peripherin/rds. The mutants selected were a P296T mutant (replacement of the proline at position 296 with a threonine) and two C-terminal deletion mutants (one lacking the terminal 10 amino acids, Δ10 and one lacking the terminal 50 amino acids, Δ50). The wt protein, the P296T and Δ10 mutants were detected on SDS–PAGE as 84 kDa dimers, that resolved into 38–42 kDa monomers under reducing conditions. The Δ50 mutant showed a slightly increased mobility. The cellular localization of mutants differed from that of wt peripherin/rds. The wt Xpress-human and wt FLAG-bovine peripherin/rds were localized to both intracellular and plasma membranes. In contrast, the C-terminal deletion mutants were localized only to the intracellular membrane. The P296T mutant presented a still different pattern: initially the protein localized to intracellular membranes. Upon confluence, however, the localization appeared to become predominantly plasma membrane. To assess the fusion activity of the proteins, the cell membranes were fractionated using sucrose density gradient centrifugation and the various fractions identified based on immunoreactivity in Western blot analysis with Golgi (anti-rab 6) or plasma membrane (anti-ZO-3) specific marker proteins. All membrane fractions were assayed for fusion with ROS plasma membrane vesicles. The plasma membrane enriched fractions (isolated at densities of 1·08 and 1·125 g ml^{-1}) containing tagged peripherin/rds and the Δ10 mutant promoted membrane fusion with ROS plasma membrane vesicles. In contrast, fusion was not detected with plasma membrane vesicles from mock-transfected cells or the Δ50 peripherin/rds deletion mutant. Fusion was enhanced in a less dense fraction enriched in the P296T mutant (isolated from the 1·04/1·02 interface) relative to wt. Fusion was dependent on the presence of peripherin/rds in the membranes and could be inhibited with trypsinolysis and

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competition studies with the bovine fusion peptide, PP-5. Peptide competition suggests that the fusion domain of human peripherin/rds is most likely identical to that characterized in bovine and corresponds to amino acid residues 312–326. The C-terminal deletion mutants have allowed us to predict the minimal region of the C-terminus necessary for fusion to include residues starting at number 335. In addition a second region important in the formation of a fusion competent peripherin/rds has been mapped to a region upstream of the fusion peptide domain.

Keywords

peripherin/rds; rod outer segment; membrane fusion; MDCK cells; photoreceptor; FLAG-peripherin/rds; Xpress-peripherin/rds

1. Introduction

Photoreceptor rod cells are responsible for vision under dim light and mediate phototransduction within specialized compartments termed rod outer segments (ROSs). ROSs contain a densely packed stack of closed disk membranes (Roof and Heuser, 1982). These post-mitotic cells maintain their structure and thereby their physiological function through the co-ordinated processes of disk morphogenesis and disk shedding (Young, 1976). New disks are formed at the base of the ROS, and older disks form disk packets at the apical tip of the ROS, that are subsequently shed. The formation of new disks (Connell and Molday, 1990; Travis, Sutcliffe and Bok, 1991; Goldberg, Moritz and Molday, 1995), and the unusual flattened morphology of the disk rim require an integral membrane protein, peripherin/rds (Wrigley et al., 2000). Interestingly, peripherin/rds has been proposed to play a role in the membrane fusion processes that appear to be necessary in ROS renewal through morphogenesis and shedding (Boesze-Battaglia et al., 1997, 1998).

The first indication that peripherin, the product of the RDS gene (Connell et al., 1991), was essential for normal photoreceptor development came from the transgenic rescue of rds/rds mice with copies of normal peripherin/rds (Travis, Sutcliffe and Bok, 1991; Travis et al., 1992). In the rds mouse, a 10 kb insertion of exogenous DNA in the open reading frame of peripherin/rds (Ma et al., 1995) resulted in the deletion of the entire C-terminus (Travis et al., 1989). The disease process in rds/rds homozygotes and rds/+ heterozygotes is characterized by abnormal development of photoreceptors, with the subsequent degeneration of these cells leading to blindness (van Nie, Ivanyi and Demant, 1978; Jansen and Sanyal, 1984; Usukura and Bok, 1987). In heterozygotes, the ROSs are reduced in length, and irregularly arranged with abnormal disk shedding (Sanyal, De Ruiter and Hawkins, 1980; Cohen, 1983; Hawkins, Jansen and Sanyal, 1985). In the absence of the retinal pigment epithelium (RPE) in a xenopus culture system, Jablonski, Wohabrebbi and Ervin (1999) have observed the presence of whorl-like disorganized outer segments. In these disorganized retinas the expression of opsin appears to be normal however, peripherin/rds expression is up-regulated (Jablonski et al., 1999).

Peripherin/rds plays an essential role in the maintenance of the structure and stability of disk membranes (Goldberg et al., 1995; Goldberg and Molday, 1996a,b). This four pass transmembrane protein is localized to the rim region of photoreceptor cone and rod disk

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membranes (Arikawa et al., 1992), where it forms disulfide-linked homodimers (Goldberg et al., 1995; Goldberg and Molday, 1996b) through an essential cysteine at position 150 (Goldberg, Loewen and Molday, 1998). Peripherin/rds homodimers complex non-covalently (Loewen and Molday, 2000) with a non-glycosylated homologue, rom-1 (Bascom et al., 1992) to form a heterotetrameric complex (Moritz and Molday, 1996; Goldberg and Molday, 1996b). Detailed sedimentation velocity analysis of the oligomeric complexes formed showed that peripherin/rds forms both homotetramers and peripherin/rds-rom-1 heterotetramers (Loewen and Molday, 2000). These studies also indicated that peripherin/rds is present in a two-fold excess over rom-1 (Kedzierski, Weng and Travis, 1999a; Loewen and Molday, 2000). The formation of stable disulfide linked oligomers is necessary in disk morphogenesis, through the formation of a disk rim and contributes to disk membrane stability (Molday, 1994; Loewen and Molday, 2000). The inability to form such complexes, through defects in either protein folding or subunit assembly, has been demonstrated in disease-linked peripherin/rds mutants (Goldberg et al., 1995; Goldberg and Molday, 1996b) and in digenic peripherin/rds-rom-1 mutants (Goldberg and Molday, 1996a; Loewen, Moritz and Molday, 2001).

A novel function for peripherin/rds, namely that of a membrane fusion protein, has been identified. Purified peripherin/rds promotes membrane fusion between disk rims and ROS plasma membrane in a cell-free assay system (Boesze-Battaglia et al., 1997). Fusion is mediated through at least one region of the C-terminal domain from residues 311–325 (Boesze-Battaglia et al., 1998). A peptide analogue to this region, called PP-5, promotes two necessary steps in the fusion process, membrane adhesion (Boesze-Battaglia et al., 1998) and membrane destabilization (Boesze-Battaglia et al., 2000). This peptide is an amphiphilic α-helix based on Fourier transform infrared spectroscopy (FTIR) (Boesze-Battaglia et al., 1998), and this amphiphilic α-helical structure is necessary for the formation of a fusogenic PP-5 oligomer (Boesze-Battaglia et al., 2000).

Peripherin/rds shares structural and functional homology with a growing family of membrane fusion proteins (White, 1992; Pecheur et al., 1999). These proteins all contain a fusion peptide domain that causes destabilization of the target membrane in the formation of a fusion pore (for review, see Pecheur et al., 1999). Within the past decade many of these proteins have been shown to have domains in close proximity to the fusion peptide domain, that aid in providing for a fusion competent form of the protein (for review, see Mellman, 1995; Pecheur et al., 1999). In pH independent fusion processes, similar to those observed in the ROS, these augmenter regions are involved in a number of fusion steps including: (1) oligomerization of the fusion protein (Lawless et al., 1996); (2) binding of a fusion protein to the receptor (Rabenstein and Shin, 1995); and/or (3) formation of a ‘hairpin’, necessary to promote fusion pores (Weber et al., 1998; Wasserman, 1999). These processes lead to conformational changes bringing the fusion peptide region into close proximity to the target membrane. In vivo studies with peripherin/rds-rom-1 chimeric proteins support the hypothesis that regions in addition to the highly conserved D-2 loop are also necessary for normal ROS disk formation and proper peripherin/rds function (Kedzierski, Bok and Travis, 1999a). In vitro evidence also suggests that additional regions of the C-terminus of peripherin/rds are important for fusogenic function (Muller-Weeks, Boesze-Battaglia and Fitzgerald, 2001).

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The aim of the present study is to map regions within peripherin/rds required for membrane fusion through a series of C-terminal peripherin/rds mutants that have been transfected into Madin Darby canine kidney (MDCK) cells. The selection of MDCK cells rather than the more commonly used COS cells (Goldberg et al., 1995, 1998; Goldberg and Molday, 1996a,b; Loewen and Molday, 2000; Loewen et al., 2001) was made based on preliminary reports by Kim et al. (1998), who showed that peripherin/rds appeared to be localized to the plasma membranes of these cells. In contrast peripherin/rds in COS cell expression systems is where peripherin/rds is localized to the perinuclear membranes (Goldberg et al., 1995). The localization of a peripherin/rds to the plasma membrane aids in confirming the specificity of peripherin/rds mediated fusion events, independent of intracellular fusion proteins. Wt bovine or human peripherin/rds were each expressed with an N-terminal epitope tag, and thereby allowed us to detect and characterize a series of C-terminal mutants. The results presented herein characterize two C-terminal deletion mutants, corresponding to a 10 amino acid and a 50 amino acid deletion within the C-terminus. The development of an N-terminal epitope tagged heterologously expressed protein provides a valuable tool in mapping regions of the C-terminal domain involved in targeting peripherin/rds to the ROS (Tam et al., 2001) and in promoting membrane fusion processes within the ROS.

2. Materials and Methods

Preparation of Human Peripherin/rds cDNA Expression Constructs and P296T Mutant

Human poly A+ retina RNA (Clontech, Palo Alto, CA, U.S.A.) was reverse transcribed into cDNA using an oligo-dT primer (Stratascript, Stratagene, La Jolla, CA, U.S.A.). The human peripherin/rds was amplified by the polymerase chain reaction (PCR) using cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). Oligonucleotide PCR primers were designed to introduce Not1 restriction sites immediately upstream of the initiator methionine codon and downstream of the stop codon of the human sequence (Travis et al., 1991). The sense primer utilized was 5′-TAGCGGCCGCATGGCGCTACTGAAAGTCAAG and the anti-sense primer used was 5′-TAGCGGCCGCTCAGCCAGCCTCTGGGGCCT. PCR was performed as described (Stratagene, La Jolla, CA, U.S.A.). Following denaturation at 94°C, amplification consisted of 30 cycles of 1 min at 94°C + 1 min at 54°C + 1 min at 72°C. The resultant peripherin/rds cDNA was blunt end ligated into the Bluescript II SK(+0) (Stratagene, La Jolla, CA, U.S.A.) vector using T4 ligase and subsequently cloned into XL-1Blue cells. Both strands were sequenced to exclude any PCR induced mutations. A Not1 restriction fragment was then subcloned into the vector pCDNA3.1/His B (Invitrogen, Carlsbad, CA, U.S.A.) to introduce epitope tag sequences to the N-terminus of the cDNA and places it under the control of a constitutive cytomegalovirus promoter. A similar construct, pCDNA3.1/His/lacZ, expressing B-galactosidase with an N-terminal Xpress-epitope tag was used as a control (Invitrogen, Carlsbad, CA, U.S.A.).

The human peripherin/rds Not1 restriction fragment was subcloned into pGEMEX-2 (Promega) for subsequent mutagenesis using the GeneEditor in vitro Site Directed Mutagenesis System (Promega) and transformed in the *Escherichia coli* strain JM109 (Promega). Orientation of the Not1 peripherin/rds cDNA fragment was determined by restriction mapping. Mutations were oligonucleotide directed using custom primers obtained

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from Integrated DNA Technologies, Inc. The mutagenic primer with the sequence 5′-GAGATTCCTCGGTGTTGGACACC-3′ was used to generate a substitution of proline at position 296 to threonine. The mutation was verified by sequence analysis and subcloned by Not1 restriction into the mammalian expressionvector pcDNA 3.1 His B.

Xpress-tagged peripherin/rds was purified using a Ni 2+ column. Briefly, 5×10^7 cells expressing either wt or the P296T mutant were harvested in PBS, pelleted and resuspended in native binding buffer consisting of 20 mM Na phosphate, 500 mM NaCl, and 30 mM octylglucoside, pH 7·4. The cell lysates were sonicated 3× for 10 sec each and Poly-His-tagged protein purified using the Xpress purification system under native conditions (protocol as provided by Invitrogen, Carlsbad, CA, U.S.A.). The protein was eluted from the column with increasing concentrations of imidazole (50, 200, 350 and 500 mM). The eluted fractions were concentrated using Centricon 30 concentrators to 1 ml. Typically the yield of Xpress-peripherin/rds from 5×10^7 cells was between 0·4 and 1·0 mg. The purity of the protein was confirmed by SDS–PAGE and Western blot analysis using the anti-Xpress antibody.

Preparation of Bovine Peripherin/rds cDNA Expression Constructs and C-terminal Deletions of Peripherin/rds

Isolation and cloning of wild type and FLAG tagged peripherin/rds were obtained by PCR using wild type bovine peripherin/rds as the template. Peripherin/rds PCR primers were generated using the sequence published by Connell and Molday (1990). The PCR reactions were performed as per the GIBCO/BRL Hot Start PCR protocol (Gibco/BRL, Rockville, MD, U.S.A.). The optimum annealing temperature, 63°C for both reactions, was determined using Oligo 6.3 software. The resulting PCR products, 1.·2 kb FLAG-peripherin/rds, were purified, kinased and end filled by standard procedures (Sambrook, Fisher and Anderson, 1989). The FLAG-peripherin/rds cDNA were ligated into the SmaI cut mammalian expression vector pCI-Neo (Promega, Madison, WI, U.S.A.) and verified by DNA sequencing. To generate the C-terminal deletions, two 3′ peripherin/rds synthetic primers were generated that correspond to progressive deletions of the C terminus. Each primer was used in conjunction with the FLAG tagged peripherin/rds 5′ primer to amplify the specific DNA fragments. The optimum annealing temperature for each reaction was 60°C. Each FLAG-peripherin/rds deletion cDNA was purified and blunt end ligated into the pCI-Neo mammalian expression vector (Promega, Madison, WI, U.S.A.) by standard methods (Sambrook, et al., 1989). After transformation into DH5α *E. coli*, individual colonies were selected and grown for DNA isolation. One wt FLAG-peripherin/rds, one Δ10 mutant and one Δ50 mutant clone were chosen and confirmed by sequence analysis.

Cell Culture and Transfections

MDCK strain II cells (Weimbs et al., 1997) were maintained in Eagle's minimal essential growth medium (MEM) with Eagle's salts and L-glutamine (Mediatech, Herndon, VA, U.S.A.), 10 % fetal bovine serum (FBS, Hyclone, Logan, UT, U.S.A.), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C in 5 % CO_2 and 95 % air. The MDCK cells were transfected using calcium phosphate precipitation (Breitfeld et al., 1989). Briefly, 10 cm plates with MDCK cells in log-phase growth were trypsinized and transfected in

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suspension with 20 μg of $CaCl_2$ precipitated DNA. Cells were subsequently grown in growth media for 3 days and passed into the selection medium, consisting of growth medium in which G418 (Gibco BRL, Grand Island, NY, U.S.A.) 450 μg ml^{-1} was substituted for penicillin and streptomycin. Ten to 14 days following selection, clones were isolated using clone rings. Aliquots of stably transfected positive clones were banked and thawed as needed for the experiments described below. The cell lines were passed weekly with MEM containing 10 % FBS and 10 % penicillin/streptomycin solution and selected using G418 (Wong, Low and Hong, 1992). Transient transfections were performed using Lipofectamine PLUS reagent (Gibco BRL, Grand Island, NY, U.S.A.). The day before transfection, cells were seeded according to the size of the culture vessel used, 1×10^6 cells well in a six well plate or 3×10^6 cells per 15 cm dish. The amount of plasmid DNA was likewise based on culture vessel size, 1 μg per well, or 2 μg per 15 cm dish. The transfections were incubated for 3 hr at 37°C. Subsequently, the transfection media were replaced with complete culture media and cells harvested within 48–72 hr.

Immunofluorescence and Laser Scanning Confocal Microscopy

For immunohistochemistry. MDCK cells were grown for 4–5 days on sterilized glass coverslips in 100 × 200 mm dishes, supplemented with MEM with 10 % FBS and fixed in 1:1 methanol/acetone solution for 2 min. Alternatively, the cells were washed in PBS containing 2 mM MgCl and 0·2 mM CaCl (PBS-C/M) and fixed in 2 % paraformaldehyde for 30 min at 4°C. The paraformaldehyde fixed cells were rinsed in PBS-C/M, quenched with 50 mM glycine for 10 min at 4°C and rinsed in PBS-C/M. Sequential incubations of primary and secondary antibodies were performed for 1 hr each. The primary antibodies included monoclonal anti-Xpress (Invitrogen, Calsbad, CA, U.S.A.), used at 1:2500 dilution, monoclonal anti-FLAG-M5 antibody (10 μg ml^{-1}) (Sigma, St. Louis, MO, U.S.A.). The secondary antibodies utilized included goat anti-rabbit IgG FITC (Gibco BRL, Grand Island, NY, U.S.A.), goat anti-mouse IgG FITC 594 (Gibco-BRL, Grand Island, NY, U.S.A.), goat anti-mouse IgG Alex Fluor 594 (Molecular Probes, Eugene, OR, U.S.A.) and goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR, U.S.A.). For surface immunofluorescence staining, coverslips were washed in PBS-C/M and incubated with primary antibody for 1 hr at 4°C, prior to fixation with 2 % paraformaldehyde and incubation with the seconday antibodies. Samples were analyzed on a Zeiss AXIOSKOP fluorescent microscope capturing images at the magnifications indicated in the figures. Scanning laser confocal microscopy was performed on a Zeiss AXIOVERT 100TV LSM.

Subcellular Fractionation of Membranes from Peripherin/rds Transfected and Control MDCK Cells

Transfected MDCK cells were harvested upon confluency. The cells were scraped in homogenizing buffer (0·1 M sodium phosphate, 1 mM DTT, 1 μg ml^{-1} leupeptin and 2 μg ml^{-1} aprotinin) and collected by centrifugation (1000 *g* for 5 min). The resulting pellet was resuspended in homogenizing buffer and the cells lysed by passage through a 26 gauge needle (2×) and sonication (twice for 10 sec each). The membranes were fractionated on a discontinuous sucrose density gradient as described (Oprian, 1993). The gradient was prepared in a total volume of 8 ml, with four layers of each 2·0 ml of 5, 10, 20 and 30 % w/w sucrose in homogenizing buffer. The gradients were centrifuged at 21·1 K for 25 min in

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a SW-41 rotor at 4°C. Fractions corresponding to the 1·04, 1·08 and 1·125 g ml^{-1} interfaces and the pellet were collected, washed in homogenization buffer and collected by centrifugation in a Ti-70 rotor, at 60 K for 25 min at 4°C. The resulting pellets were resuspended in 50 mM Hepes, pH 7·4. The total protein content (Bio-Rad) and phosphate content (Bartlett, 1959) of each individual fraction were determined.

Equal amounts of total protein from the fractionated membranes were separated using SDS–PAGE (either 7·5 or 10 % acrylamide, as described in the figure legends), transferred to nitrocellulose and Western blotting performed using anti-Xpress (Invitrogen, Carlsbad, CA, U.S.A.), or anti-Rab-6 (CalBiochem; La Jolla, CA, U.S.A.); or anti-ZO-3 (Chemicon, Temecula, CA, U.S.A.). In the case of anti-ZO-3 and anti-Rab 6 the secondary antibody used was goat anti-rabbit IgG HRP (Life-Technologies, Grand Island, NY, U.S.A.).

Preparation of Xpress-Peripherin/rds Large Unilamellar Vesicle (LUV) Recombinants

Vesicles composed of phosphatidylcholine:phosphatidylserine:cholesterol (4:4:1 mole ratio) and 1 mol % F_{18} were prepared as described previously (Boesze-Battaglia et al., 2000). Briefly, the lipids were co-solubilized in chloroform, dried under N_2, lyophilized and resuspended in 10 mM Hepes, pH 7·4. The lipid suspension was sonicated 3 × for 3 min each using a probe sonicator to form small unilamellar vesicles. The vesicles were combined with purified Xpress-peripherin/rds using detergent dialysis as described previously for the preparation of native peripherin/rds recombinants (Boesze-Battaglia et al., 1997). The recombined membranes were dialyzed for 72 hr against 10 mM Hepes, 100 mM NaCl to remove all traces of detergent. The recombined membranes were separated from unincorporated proteins and pure lipid vesicles on a 5–40 % w/w sucrose density gradient. The Xpress-peripherin/rds LUV recombinants were isolated at 32–35 % w/w sucrose. These recombinants were spun down at 60 000 K for 20 min and resuspended in 10 mM Hepes, pH 7·4, prior to fusion assays.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation studies were performed as described (Springer, 1996). Briefly, cell extracts prepared in NP-40 extraction buffer (50 mM Tris–HCl, pH 7·4, 250 mM NaCl, 0.1 % NP-40, 5 mM EDTA, 50 mM NaF) were incubated on ice for 20 min, sonicated (twice for 10 sec each) and centrifuged at 14 000 rpm for 10 min. Anti-Xpress monoclonal antibody was added to each of the cell extracts and incubated overnight while rotating at 4°C. Following immunoprecipitation with 150 μl of Protein A sepharose the complexes were washed five times in NP-40 buffer and resuspended in 2× SDS–PAGE sample buffer containing β-mercaptoethanol (β-ME). After heating at 85°C for 10 min the complexes were centrifuged at 14 000 rpm for 30 sec and the immunoprecipitated complexes separated on SDS–PAGE and transferred to nitrocellulose for Western blot analysis (Boesze-Battaglia et al., 1997) or silver stained (Merrill, Goldman and Van Keuran, 1982). In some experiments, the immunoprecipitated products were subsequently treated with Endoglycosidase H (ENDO-H, Connell and Molday, 1990) prior to electrophoresis. The immunoreactive bands were visualized using the ECL detection system (Pierce) and molecular weights calculated using R_f measurements of molecular weight makers.

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R_{18} Lipid Mixing Fusion Assay

Fusion between the R_{18} labeled ROS plasma membranes (R_{18}-ROSPM) and the MDCK cell lysates and membranes fractioned as described above was measured essentially as described for disk membranes and peripherin/rds recombinants (Boesze-Battaglia, 2000). Bovine ROS plasma membrane vesicles were isolated (Boesze-Battaglia and Albert, 1989) and labeled with octadecylrhodamine B-chloride (R_{18}) (Boesze-Battaglia, Albert and Yeagle, 1992). The purity of the plasma membrane fraction was determined by the cholesterol to phospholipid ratio (0·35) and confirmed by Western blot analysis of disk and plasma membrane fractions probed with anti-63 kDa cGMP channel monoclonal antibody, PMc 1D1 (a gift from Dr Om Lamba) and mAb 2B6 to bovine peripherin/rds (a generous gift from Dr R. Molday). The ROS plasma membrane was found to be immunoreactive with the channel antibody PMc 1D1, and not immunoreactive with monoclonal antibody 2B6 (data not shown). The labeled vesicles were stored on ice for 1–2 hr or used immediately.

Upon fusion, the R_{18} probe is diluted throughout the target membrane by lateral diffusion leading to a dequenching and an increase in the observed fluorescence intensity that is proportional to membrane fusion (Hoekstra, et al., 1984). Fusion was initiated with the addition of R_{18} labeled plasma membrane (R_{18}-ROSPM) to target membranes at 37°C. All target membranes were 1 m_M total phospholipid based on phosphate analysis (Bartlett, 1959). In some cases the target membranes were pretreated with various peptides for 30 min in the dark or treated with trypsin as described (Boesze-Battaglia et al., 1998). Fluorescence was monitored with λ_{ex} = 560 nm and λ_{em} = 586 nm using a Perkin-Elmer LS 50B spectro-fluorometer. The fluorescence intensity in the absence of R_{18}-ROSPM was taken as baseline. The fluorescence at infinite probe dilution (100 % fluorescence) was determined with the addition of 100 μl of 10 % Triton X-100. Initial rates of fusion were determined from the increase in fluorescence intensity as a function of time.

Resonance Energy Transfer (RET) Fusion Assay

The fusion between R_{18} labeled plasma membranes and F_{18} labeled membranes was measured using a fluorescence resonance energy transfer (RET) assay (Partearroyo et al., 1994) at room temperature on a Perkin-Elmer LS 50B spectrofluorometer (Gaithersburg, MD, U.S.A.) equipped with a 96 well plate reader. Fusion was initiated with addition of R_{18} labeled plasma membranes to F_{18} labeled membranes 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. The extent of fusion was calculated as the change in R_{18} intensity over time as described (Partearroyo et al., 1994). The change in R_{18} intensity (ΔR) at a given time was computed as follows:

$$\Delta R=(I_{592}/I_{524}+I_{592})_T-(I_{592}/I_{524}+I_{592})_I$$

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 percentage change in R_{18}, indicated in the graphs as the percent fusion, was determined my multiplying each ΔR value by 100. Background percentage change in R_{18} was calculated

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for the mock-transfected cells and subtracted from the peripherin/rds MDCK cell membrane results, the background values accounted for less than 2 % of the total change in R_{18} fluorescence. In some studies the target membranes were pretreated with peptides for 30 min at 37°C as indicated in the legends to the figures. The amino acid sequences of the peptides used were; NP-1; H_2N–ALLKVKFDQKKRVKLAQ–COOH and PP-5; H_2N–VPETWKAFLESVKKL–COOH. In the resonance energy transfer assays, 10 μg of PP-5 was added to the F_{18} labeled target membrane samples prior to the addition of R_{18} labeled ROS plasma membrane.

3. Results

Heterologous Expression of Xpress-human Peripherin/rds and FLAG-bovine Peripherin/rds wt and Mutants in MDCK Cells

To map regions within the C-terminus of peripherin/rds that contribute to membrane fusion, two C-terminal deletion mutants and a single amino acid substitution at position 296 (position based on human sequence, Dryja et al., 1997) were studied. To facilitate detection of the wt and mutant proteins, the bovine peripherin/rds was engineered with an N-terminal FLAG epitope tag and the human peripherin/rds with an N-terminal Xpress tag. The C terminus of peripherin/rds is a 64 amino acid extradiskal domain that spans amino acid residues 282–345. A Chou–Fasman helix analysis of this region predicts three α-helical regions that are labeled 1, 2 and 3 in Fig. 1. The Δ10 mutant removes residues 345–335, thereby truncating the first α-helical domain. The Δ50 mutant removes residues 345–295, removing the fusion peptide domain and essentially the entire C terminus. The single amino acid substitution replaces proline at position 296 with threonine (P296T) in a highly conserved region of the C terminus (Kedzierski et al., 1996) postulated to be involved in fusion due to its homology with other membrane fusion proteins (Mellman, 1995; Gerst, 1999; Pecheur et al., 1999).

Wild type human Xpress-peripherin/rds, the P296T mutant, the full-length bovine FLAG-peripherin/rds and C-terminal deletion constructs were each individually transiently transfected into MDCK cells. Protein expression was confirmed by Western blot analysis of cell lysates. In addition to the transient transfections, an N-terminal Xpress-epitope tagged human peripherin/rds was stably transfected into MDCK cells in three distinct cell lines designated 13, 22 and 38. The level of Xpress-peripherin/rds expression was highest in cell line 22 and the characterization of Xpressperipherin/rds in this cell line is discussed. The other two cell lines showed qualitatively similar results (data not shown). WT Xpress-peripherin/rds expression in MDCK line 22 [Fig. 2(B), lanes 7 and 8] and in transiently transfected MDCK cells [Fig. 2(B), lanes 1 and 2] was compared to mock-transfected MDCK cells [Fig. 2(B), lanes 3 and 4] transiently transfected COS cells [Fig. 2(B), lanes 5 and 6; Goldberg, et al., 1995], and native bovine peripherin/rds in ROS disk membranes [Fig. 2(A), lane 1] by Western blot analysis. The native disk membrane peripherin/rds was detected with monoclonal antibody 2B6 and shown to be a 34–38 kDa monomer under reducing conditions [Fig. 2(A), lane 1]. The heterologously expressed human protein was detected using anti-Xpress (monoclonal) antibody [Fig. 2, lanes 1 and 2, transiently transfected; lanes 7 and 8 stably transfected]. Both the Xpress-tagged human peripherin/rds

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and the FLAG tagged bovine peripherin/rds (data not shown) banded at 82 kDa indicative of a dimer that was resolved into a single 42 kDa band in the presence of BME, consistent with in vivo disulfide homodimer formation. The slightly higher molecular weights of the Xpress and FLAG tagged proteins are due to the presence of the epitope tag on the peripherin/rds in the MDCK cell transfections. The Xpress-P296T peripherin/rds mutant was also detected as a dimer that resolved into a 38 kDa monomer in the presence of BME [Fig. 2(C), lanes 1 and 2]. The wt and P296T Xpress-peripherin/rds proteins were subsequently purified using from a Ni^{2+} column and fractions eluted with 200 m_M imidizole found to contain the Xpressperipherin/rds. These proteins were detected as 42 kDa monomers under reducing conditions [Fig. 2(D), lanes 1 and 2].

Peripherin/rds isolated from bovine ROSs, and as a heterologously expressed protein in COS cells (Goldberg et al., 1995) is glycosylated and glycosidase treatment produces a core polypeptide (Travis et al., 1991; Goldberg et al., 1995). The post nuclear lysates from the transfected MDCK cells were analyzed for Endogylcosidase H (Endo-H) sensitivity. As seen in Fig. 3, there is an increase in the mobility of Endo-H treated peripherin/rds suggesting a high mannose or hybrid carbohydrate modification of the protein (Travis et al., 1991). The molecular weight of the core polypeptide was 34 kDa, in close agreement with that observed in vivo and in transgenic animals with a S231A mutation (Kedzierski et al., 1999b). A portion of the FLAG tagged peripherin/rds and the two C-terminal deletion mutants were sensitive to Endo-H as shown in Fig. 3(A). The Xpress-P296T mutant was also found to be Endo-H sensitive as shown in Fig. 3(B). Residual amounts of Endo-H resistant proteins were found to be variable with transfections, however they were higher with the FLAG tagged peripherin/rds than the Xpress-tagged proteins. Collectively, these results suggest that the expressed peripherin/rds forms disulfide linked dimers, and is glycosylated suggesting that it is not grossly misfolded. The properties shown here are similar to those observed in vivo (Connell and Molday, 1990; Travis et al., 1991) and in other cell expression systems (Goldberg et al., 1995).

Immunolocalization of Xpress-human Peripherin/rds and FLAG-bovine Peripherin/rds wt and Mutants in MDCK Cells

Protein expression of human peripherin/rds was confirmed in MDCK cells and localization was accessed microscopically using a fluorescently labeled secondary antibody to the monoclonal anti-Xpress antibody [Fig. 4(A) and (B)], and to a polyclonal antibody to peripherin/rds [Fig. 4(C)]. This polyclonal antibody was generated against a peptide to the C-terminal domain of peripherin/rds (Covance Inc.). In transfected MDCK cells, labeling appears to be localized to both the intracellular membranes and to the plasma membrane surface based on labeling with either anti-Xpress or the polyclonal antibody [Fig. 4(A) and (C)]. When these same cells were fixed in paraformaldehyde, and labeled with anti-Xpress, labeling also appeared to be plasma membrane associated [Fig. 4(B)]. In a complementary experiment, pretreatment of the transfected MDCK cells with trypsin, prior to fixation, decreased plasma membrane associated immunofluorescence [inset, Fig. 4(C)] as detected by a peripherin/rds polyclonal antibody. Qualitatively similar results were observed with anti-Xpress (data not shown). In contrast, mock-transfected MDCK cells do not show appreciable immunoreactivity with either the polyclonal antibody [Fig. 4(D)] or anti-Xpress

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[Fig. 6(D)] or anti-FLAG [Fig. 5(A), inset]. Collectively, the paraformaldehyde fixation and pretreatment of cells with trypsin suggest that a portion of the peripherin/rds is localized to the plasma membrane, a conclusion suggested first by Kim et al., (1998). Immunofluorescent localization of the Δ10 and Δ50 mutant shows a different pattern, with very little to no immunoreactivity at the plasma membrane when cells were fixed with MeOH/acetone or 2 % paraformalde-hyde [Fig. 5(B), (C) and (D)]. These deletion mutants appear to be localized almost exclusively intracellularly.

The immunofluorescent localization of Xpress wt peripherin/rds and the P296T mutant was accessed in more detail using laser confocal microscopy. The P296T mutant exhibited a very interesting localization pattern [Fig. 6(A) and (B)]. In single non-confluent cells, the labeling appeared to be localized exclusively to intracellular vesicles [Fig. 6(A), inset]. Upon confluence the label was further distributed to the plasma membrane, specifically to areas that appear to be tight junctions [Fig. 6(A), see arrow]. A similar localization pattern was also observed when the P296T expressing cells were fixed in 2 % paraformaldehyde [Fig. 6(B)]. As shown in Fig. 6(C), using a fluorescently labeled secondary antibody to the monoclonal anti-Xpress antibody, wt protein is expressed in the intracellular membranes with some signal seen in the plasma membrane.

Fractionation of MDCK Cell Membranes Expressing Peripherin/rds

The immunofluorescent localization studies presented in Figs 4 and 6 suggest that a portion of both wt Xpress-human peripherin/rds localizes to the plasma membrane. To corroborate the localization of the expressed proteins biochemically, membranes isolated from the transfected MDCK cells were fractionated using sucrose density gradient centrifugation. Four fractions were isolated at the 1·02/1·04 g ml^{-1} interface, 1·04/1·08 g ml^{-1} interface, 1·08/1·125 g ml^{-1} interface and are designated fraction 1, 2, 3 and pellet, respectively. Using an antibody to a plasma membrane specific protein, ZO-3, and an antibody to a Golgi specific protein, rab-6, the predominant membrane species in each of the four fractions were identified based on Western blot analysis. As shown in Fig. 7, all four fractions were immunoreactive to anti-ZO-3 antibody. Only two of these fractions, fraction 1 and the pellet contained detectable levels of intracellular membrane based on immunoreactivity with rab-6. In contrast, fractions 2 and 3 contained no detectable levels of Golgi membranes, based on the absence of immunoreactivity with anti-rab 6 antibody, suggesting that these two fractions were the most highly enriched in plasma membrane relative to intracellular membrane fragments. All four membrane fractions contained wt Xpress-peripherin/rds detected by Western blot analysis (Fig. 7). Similar results were obtained with the wt FLAG-bovine peripherin/rds (data not shown). The detection of Xpress-peripherin/rds in fractions enriched in plasma membrane (fractions 2 and 3) is consistent with the immunofluorescence microscopy (Figs 4 and 6), which suggests that a portion of the Xpress-tagged peripherin/rds has localized to the MDCK cell plasma membrane.

Membrane Fusion Studies

To establish the fusion competency of the expressed wt peripherin/rds, fusion between the wt peripherin/ rds containing membrane fractions and bovine ROS plasma membrane was determined using fluorescence dequenching. This assay was chosen to allow a direct

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comparison of the fusion kinetics and parameters of the expressed peripherin/rds to those characterized previously in native disk membranes and purified peripherin/rds LUVs (Boesze-Battaglia, et al., 1992, 1997, 1998). The mock-transfected and wt Xpress-peripherin/rds MDCK cell membrane fractions, 1, 2, 3 and pellet, described in Fig. 8 were isolated and used immediately for fusion assays. Upon the addition of R_{18} labeled ROS plasma membrane vesicles, heretofore designated as R_{18}-ROS PM, to MDCK cell plasma membrane enriched fraction 2, an increase in fluorescence intensity at 586 nm indicative of fusion was observed (data not shown). From this increase in fluorescence intensity the initial rate of fusion (IRF) was calculated as described (Boesze-Battaglia et al., 1992) and is shown in Table I. Fraction 3 showed qualitatively similar results, although the initial rate of fusion in those experiments was somewhat lower (Table I). In contrast to fractions 2 and 3, the pellet and fraction 1 from the transfected cells showed no detectable fusion with R_{18}-ROS PM as shown in Table I. Little detectable fusion was observed between mock-transfected MDCK cell membrane fractions (1–3) and R_{18}-ROS PM (Table I). Quantitatively similar results were obtained with the FLAG-peripherin/rds (data not shown).

Peripherin/rds promoted fusion is mediated by a 14 amino acid long C-terminal region of the protein that is 100 % homologous to a corresponding region in human peripherin/rds (amino acids 312–326). Trypsinolysis of peripherin/rds cleaves the entire C terminus, including the fusion domain thereby resulting in a decrease in bovine peripherin/rds-dependent fusion with R_{18}-ROS PM (Boesze-Battaglia et al., 1998). The specificity of the fusion between MDCK cell plasma membrane enriched fractions containing Xpress-peripherin/rds (fractions 2 and 3) and R_{18}-ROS PM was addressed by trypsinolysis. When R_{18}-ROS PM was added to trypsin treated Xpress-peripherin/rds membrane fractions 2 and 3, very little change in R_{18} fluorescence intensity was observed (Table I). When the trypsin supernatant of these cell fractions was assayed it was found to be immunoreactive with anti-peripherin/rds monoclonal antibody 2B6, suggesting that the C-terminal domain of the expressed peripherin/rds had been cleaved (data not shown). These results suggest that trypsinolysis inhibits fusion most likely in a manner similar to that characterized in bovine, by cleaving the fusion promoting region of the Xpress-peripherin/rds.

To provide additional evidence for the specificity of fusion detected as that due to Xpress-peripherin/rds, the authors exploited an observation made previously when studying fusion between peripherin/rds LUVs and R_{18}-ROS PM (Boesze-Battaglia et al., 1997, 1998). In this system, addition of PP-5, a synthetic peptide analogue to the fusion domain, inhibited peripherin/rds-dependent fusion. The addition of PP-5 to the Xpress-peripherin/rds MDCK membrane fractions 2 and 3 prior to fusion with R_{18}-ROS PM resulted in a decrease in the initial rate of fusion (Table I). These results suggest that the fusogenic species in the MDCK cell membrane fractions was peripherin/rds. In contrast, pretreatment of fractions 2 and 3 with a peptide to the N-terminal domain of peripherin/rds, called NP-1, had no effect on fusion. Additionally when the membrane fractions from both peripherin/rds and mock-transfected MDCK cells, fractions 1, 2, 3 and pellet were incubated with R_{18} labeled disk membranes or R_{18} labeled phosphatidylcholine vesicles, no increase in fluorescence intensity was observed (data not shown), indicating that the fusion detected is specific to a ROS plasma membrane, peripherin/rds mediated process. These results also confirm that no

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fusion independent transfer of R_{18} is contributing to the increase in fluorescence intensity observed.

To access the fusogenic function of the various peripherin/rds mutants an alternative fluorescence based fusion assay was utilized. In these studies [Fig. 8(A)–(C)] fusion was detected using fluorescence RET methods. This assay is advantageous with the mutants because it requires equal amounts of target and donor membrane vesicles, in contrast to the R_{18} lipid-mixing assay, which requires a 10-fold excess of target membrane. The RET assay is preferred since the yield of MDCK cell membrane material is limited in the transiently transfected cells. In the RET assays, upon fusion of an F_{18} labeled target membrane with R_{18}-ROS PM vesicles an increase in R_{18} fluorescence (λ_{em} = 592 nm) is observed due to a transfer of energy from the F_{18} (λ_{ex} = 470 nm) when it is in close proximity to the R_{18} (i.e. upon fusion of the two membranes). Upon the addition of R_{18}-ROS PM to F_{18} labeled unfractionated MDCK cell membranes an increase in R_{18} fluorescence was observed with a concomitant decrease in F_{18} fluorescence (data not shown). The time course of fusion between ROS PM and cell membranes isolated from wt Xpress-human p/rds, Δ10, Δ50 and the P296T mutant is shown in Fig. 8(A). Relative to the wt, a slight increase in fusion was observed between the Δ10 mutant and R_{18}-ROS PM at all time points. A much larger enhancement of fusion was observed when fusion between the P296T mutant and R_{18}-ROS PM was compared to wt. The observed increase in fusion could be due to increased levels of protein expression in the Δ10 and the P296T mutant. However, when levels of expressed protein were compared, less protein was detected in the P296T mutant, suggesting that the higher fusion rate was not attributable to the presence of more fusion protein [Fig. 8(B), inset]. The fusion between R_{18}-ROS PM and wt, P296T or the Δ10 mutants was inhibited with the addition of PP-5 (data not shown), further suggesting that the fusion event measured with the RET assay was specific for peripherin/rds.

The fusion between R_{18}-ROS PM and subcellular fractions isolated from the transiently transfected MDCK cell lines was compared. The transiently transfected MDCK cells were lysed and the membrane subpopulations separated on sucrose density gradients as described above (Fig. 7). The percent fusion between R_{18}-ROS PM and the various membrane fractions is shown in Fig. 8(B) and the level of protein expression in each fraction is shown in the inset. The wt and the Δ10 mutants both show maximal fusion activity in membranes isolated in fraction 2, enriched in Z0-3, a plasma membrane specific marker. In contrast, the P296T mutant has highest fusion activity in less dense membrane species, isolated at the 1·04/1·08 g ml^{-1} interface. This fraction is enriched in rab-6 suggesting that it is a peripherin/rds bearing post-Golgi vesicle. The percentage fusion in this figure was normalized to protein content. Since the argument can be made that there is substantially more lipid surface area in the lighter fraction the phospholipid was compared to the protein ratio of the various fractions. The phospholipid/protein ratios are similar, corresponding to 3·0, 2·04, 1·72 and 2·10 (mg/mg) for fractions 1–3 and pellet, respectively, further suggesting that the enhanced fusion seen with the P296T mutant is most likely due to a change in the C-terminal domain of this protein. In all of the fractionated membranes fusion was completely inhibited upon trypsinolysis of the membranes and fusion between P296T and R_{18}-ROS PM and wt p/rds and R_{18}-ROS PM could also be inhibited with the addition of PP-5 (data not shown). To further support the conclusion that the P296T mutation results in

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an enhancement of peripherin/rds-dependent fusion, lipid recombinants containing either the WT Xpressperipherin/rds or the P296T mutant were prepared and assayed for fusion. The result of the RET fusion measurements between F_{18} labeled recombinants and R_{18}-ROS PM are shown in Table II. When compared to WT fusion is enhanced with the P296T mutant. This enhancement is not due to variations in protein incorporation since both recombinants had phospholipid protein ratios on the order of 90 mol phospholipid per mol protein. Collectively, these results have allowed us to map additional regions within the C-terminus specifically a region of as yet undefined length upstream of the fusion peptide domain (residues 311–325), which contains proline 296.

4. Discussion

The results herein report the development of a heterologous expression system (MDCK cells) for the study of photoreceptor peripherin/rds. Both FLAG N-terminal epitope tagged bovine peripherin/rds and Xpress-N-terminal epitope tagged human peripherin/rds were expressed as disulfide linked oligomeric glycoproteins (Fig. 2), that were able to promote membrane fusion with ROS PM vesicles (Table I). The biochemical properties of the expressed proteins are similar to the native protein in ROS. The expressed wt and mutant proteins all form dimers that are reduced to monomers in the presence of reducing agents as observed in vivo (Connell and Molday, 1990; Travis et al., 1991) and in other heterologous expression systems (Goldberg et al., 1995; Goldberg and Molday, 1996a,b). The expressed proteins appear to be processed through the Golgi and are glycosylated as evidenced by sensitivity to Endo-H. The level of Endo-H resistant protein appears to be higher in cells expressing the FLAG tagged bovine construct when compared to the Xpress-tagged constructs (Fig. 3). These resistant species may be due to incompletely processed or misfolded proteins; incompletely processed species were also observed when bovine peripherin/rds was expressed in COS cells (Goldberg et al., 1995). As observed previously, glycosylation is not required for subunit assembly in vivo (Kedzierski et al., 1999b) and does not alter the flattened shape of microsomal membranes in an in vitro cell free system (Wrigley et al., 2000).

Immunofluorescence microscopy data show localization of wt peripherin/rds to both intracellular membranes and plasma membrane of MDCK cells. Peripherin/rds appears to be properly folded in the plasma membrane fraction based on analysis of fusogenic function of subcellular fractions. The localization of peripherin/rds in the MDCK cells is in contrast to COS cells, in which peripherin/rds is localized exclusively to the perinuclear membrane (Goldberg et al., 1995). In MDCK cells human peripherin/rds also sorts to the plasma membrane. Moreover, this sorting does not require rom-1, the non-glycosylated homolog of peripherin/rds in vivo. Rhodopsin has been expressed in MDCK cells and this protein localizes to the apical membrane (Chuang and Sung, 1998). Kim et al. (1998) reported that the Xpress-human peripherin/rds was localized to the MDCK cell basolateral membrane. Collectively, the work presented herein and the characterization of rhodopsin expression in MDCK cells suggests that the MDCK cell expression system may provide a tool used to identify localization signals that direct these proteins to different membranes in rod cells.

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The localization pattern of the Xpress-P296T peripherin/rds mutant protein from intracellular membranes to the plasma membrane upon confluence is somewhat unusual. The confocal images shown in Fig. 6, suggest that the P296T mutant may become associated with the tight junctions, suggesting that peripherin/rds may co-localize with other proteins associated with this region. This possibility is presently under investigation. Interestingly, glycoprotein B of cytomegalovirus, a functional homolog (i.e. fusion protein) of peripherin/rds (Navaro et al., 1993), is endocytosed from the plasma membrane of MDCK cells (Tugizov et al., 1999). The signal for endocytosis is a cluster of acidic amino acids within the C-terminal domain. Similar staining patterns are observed as the glycoprotein B is sorted within the MDCK cells. Whether peripherin/rds sorts by a similar mechanism remains to be determined.

Functionality of the expressed proteins was defined as the ability to promote fusion with ROS PM vesicles. The Xpress-tagged human peripherin/rds and the FLAG tagged bovine peripherin/rds were able to promote membrane fusion between ROS plasma membrane vesicles and MDCK cell membrane fractions in a manner similar to native purified bovine peripherin/rds. In both cases, fusion required nano-molar levels of calcium (Table I), was inhibited upon trypsinolysis (Table I) and with the addition of PP-5 (Table I), a synthetic peptide analogue to the fusion domain of bovine peripherin/rds. The specificity of the fusion was inferred from competition studies using PP-5, which corresponds to amino acids 312–326 of human peripherin/rds (Dryja et al., 1997). Within this region there is complete conservation of amino acid between human and bovine peripherin/rds lending further support for a common functional domain. The ability to promote fusion is most likely not due to an artifact of misfolding since it has been previously shown that misfolded protein, as determined by heat denturation, is unable to promote fusion (Boesze-Battaglia et al., 1997).

Peripherin/rds shares structural and functional homology with an extensive and well-characterized group of membrane fusion proteins. In addition to a fusion peptide domain, these membrane fusion proteins contain regions that are required not only for the formation of a fusion pore but also to confer fusion competency to the protein and specificity to the fusion process (Mellman, 1995; Skehel and Wiley, 1998). Recently, the fusion protein fertilin has been shown to contain regions upstream of the fusion peptide domain, which contribute to targeting and fusion pore formation (Pecheur et al., 1997, 1999). The HIV fusion protein, gp41, also contains conserved amino acid residues essential for the formation of an ectodomain in an area upstream of the fusion peptide and adjacent to the transmembrane domain (Liu et al., 2001).

To define broadly the regions of the C-terminus that contribute to the fusion process, a series of six deletion mutants was constructed. Two of these deletions, a Δ10 and a Δ50 mutant are described here and the other four deletion mutants (Δ20, Δ29, Δ39 and Δ59) are described elsewhere (Muller-Weeks et al., unpublished res.). In these previous studies, all four deletion mutants lost fusogenic function. The Δ20 mutant in those studies still retained the fusion domain but removed the first predicted α-helix (based on Chou-Fasman analysis). It was proposed that the inactivity of the Δ20 mutant was due to the inability of the fusion peptide domain to maintain proper helical conformation. Herein it was found that the Δ10 mutant, the shortest deletion still retained fusogenic function. It has been previously shown that

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addition of monoclonal antibody 2B6 (with epitope corresponding to amino acids 330–345) to either disk membranes or peripherin/rds LUVs results in a partial inhibition of fusion. Deletion of the entire 2B6 epitope (Δ20 mutant) resulted in no detectable fusion between peripherin/rds and ROS PM vesicles (Muller-Weeks et al., unpublished res.). Collectively these results suggest that a region from amino acids 325–335 may aid in promoting fusion. While the experiments as designed did not address the lag-time associated with fusion, slightly longer 'lag-time' was observed with the Δ10 mutant when compared with wt (data not shown). Taken together these results suggest that the terminal 10 amino acids may be necessary to render peripherin/rds fusion competent, as evidenced by a slight increase in lag-time associated with this mutant. As expected deletion of 50 amino acids resulted in no detectable fusion.

Phylogenetic homology has been observed in the peripherin/rds protein between residues 294–314, a region containing the fusion peptide domain (Kedzierski, et al., 1996). Using well-characterized membrane fusion processes (i.e. fertilin, synaptobrevin and intracellular fusion) as a paradigm, it was hypothesized that the region corresponding to amino acids 294–312 in bovine and human peripherin/rds may be necessary for membrane fusion. To determine if this region of peripherin/rds is involved in the fusion process a threonine residue was substituted for the proline residue at position 296. A higher initial rate of fusion between membranes containing the P296T mutant and ROS PM was observed when compared to wt Xpress-peripherin/rds (Fig. 8 and Table II). The P296T mutant is a non-conservative substitution, resulting in a change in this region of the protein from aliphatic to a more polar, hydrophilic region. If peripherin/rds functions in a manner analogous to other fusion proteins, it is likely that the fusion permissive form of this protein requires a coiled-coil structure (Bentz, 2000), which would require that a 'hairpin' form upstream of the fusion peptide. In this case an increase in hydrophilicity as would occur with a substitution of proline to threonine could alter the properties of the fusion protein. A similar mutation in gp-41 has been shown to favor the hairpin structure of the protein and enhance fusion (Liu et al., 2001).

Collectively the results herein allow us to refine further our working hypothesis of peripherin/rds mediated fusion. It is proposed that a region upstream of the fusion peptide domain of peripherin/rds aids in rendering the protein fusion competent most likely by aiding in a required change in conformation, possibly through the formation of a required 'hairpin'. Disease-linked C-terminal mutations of peripherin/rds are assembled into two small but distinct clusters, a group within the homologous region and a smaller group within the fusion peptide domain (residues 311–325) (Kohl et al., 1998). The identification of three genetic polymorphisms in this region may make it more difficult to screen for defects in this area using conventional genetic approaches (Kohl et al., 1998). Such a cluster of mutations is consistent with the high functional efficacy of these regions.

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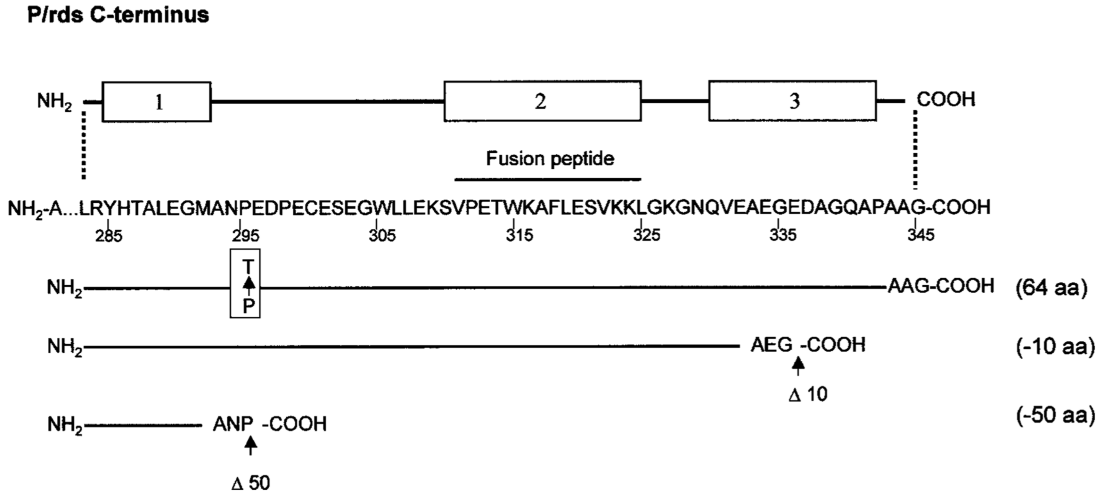

Fig. 1.

Schematic representation of the peripherin/rds C terminus and mutants. The amino acid sequence of bovine peripherin/rds is presented (residues 282–345). A Chou-Fasman analysis of the C-terminus reveals three alpha-helical regions that are denoted as □ and numbered 1, 2 and 3. The fusion peptide domain is underlined and corresponds to helix 2. Two C-terminal deletion mutants were constructed as designated Δ10 (removal of residues 335–345) and Δ50 (removal of residues 295–345). An arrow indicates deletion endpoints. A third single amino acid substitution from proline to threonine at position 296 is indicated by an arrow and is boxed.

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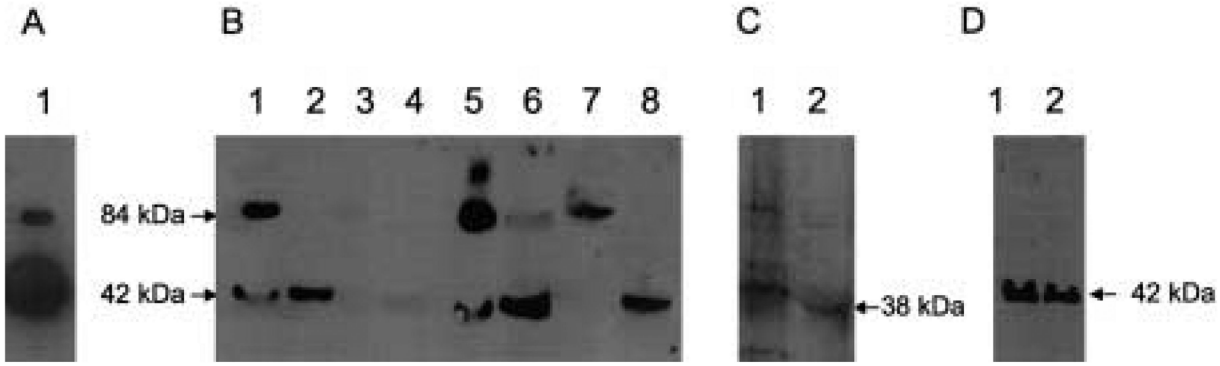

Fig. 2.

Western blot analysis of Xpress-human peripherin/rds in MDCK and COS-1 cells. (A) Bovine ROS disk membrane proteins were separated by electrophoresis under reducing (+β-ME) denaturing conditions and then Western blotted with anti-peripherin/rds monoclonal antibody 2B6. (B) Detergent extracts of MDCK cells transiently transfected with Xpress-human peripherin/rds (lanes 1 and 2), mock transfected (vector alone, lanes 3 and 4), or stably transfected (lanes 7 and 8) and detergent extract of COS-1 cells transiently transfected with Xpress-peripherin/rds (lanes 5 and 6) were electrophoresed under reducing or non-reducing (±β-ME) denaturing conditions and then Western blotted with a monoclonal antibody to the Xpressepitope tag on the human peripherin/rds. (C) Cell extracts of MDCK cells transfected with the Xpress-P296T mutant human peripherin/rds was electrophoresed under reducing or non-reducing (±β-ME) denaturing conditions and then Western blotted with a monoclonal antibody to the Xpress-epitope tag on the human peripherin/rds. (D) Fractions isolated from Ni^{2+} column with 200 m_M imidazole corresponding to wt Xpress-peripherin/rds (lane 1) and P296T Xpress-peripherin/rds (lane 2) were electrophoresed and then Western blotted with a monoclonal antibody to the Xpress-epitope tag on the human peripherin/rds.

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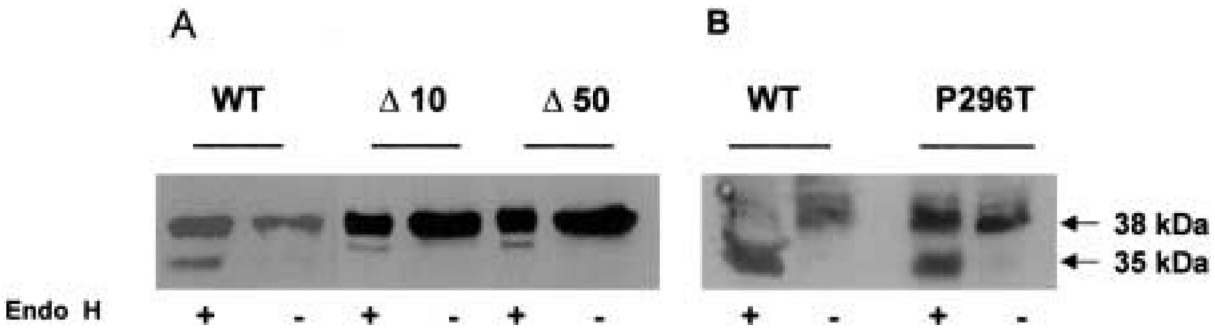

Fig. 3.

WT peripherin/rds and mutant proteins are deglycosylated by Endo-H. (A) Anti-FLAG-M5 monoclonal antibody immunoprecipitated complexes of MDCK cell extracts transfected with FLAG-bovine peripherin/rds, designated WT, Δ10 mutant and Δ50 mutant treated with ENDO-H (+) or untreated (−) were separated on 10 % SDS–polyacrylamide gels in the presence of β-ME, transferred to nitrocellulose and probed with anti-FLAG antibody, M-5. (B) MDCK cell extracts transfected with Xpress-human wt or P296T mutant peripherin/rds treated with ENDO-H (+) or untreated (−) were separated on 10 % SDS–polyacrylamide gels in the presence of β-ME, transferred to nitrocellulose and probed with anti-Xpress antibody.

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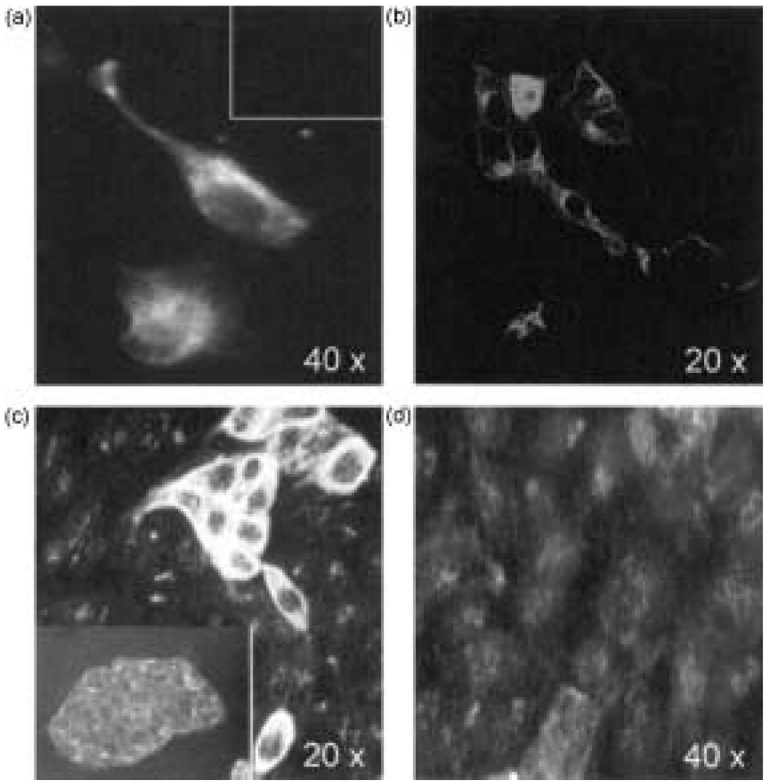

Fig. 4.

Immunolocalization of human Xpress-peripherin/rds in transfected MDCK cells. MDCK cells expressing wt Xpress-human peripherin/rds were grown on glass coverslips, fixed in MeOH/acetone (A) or 2 % paraformaldehyde (B) and incubated with anti-Xpress antibody. MDCK cells expressing wt Xpress-human peripherin/rds were grown on glass coverslips, fixed in MeOH/acetone and incubated with polyclonal antibody to peripherin/rds (C) followed by incubation with Alexa Fluor 594 secondary antibody. The immunofluorescence is compared to labeling of mock-transfected (vector alone) MDCK cells labelled with anti-Xpress (Inset, A) or a polyclonal antibody to peripherin/rds (D).

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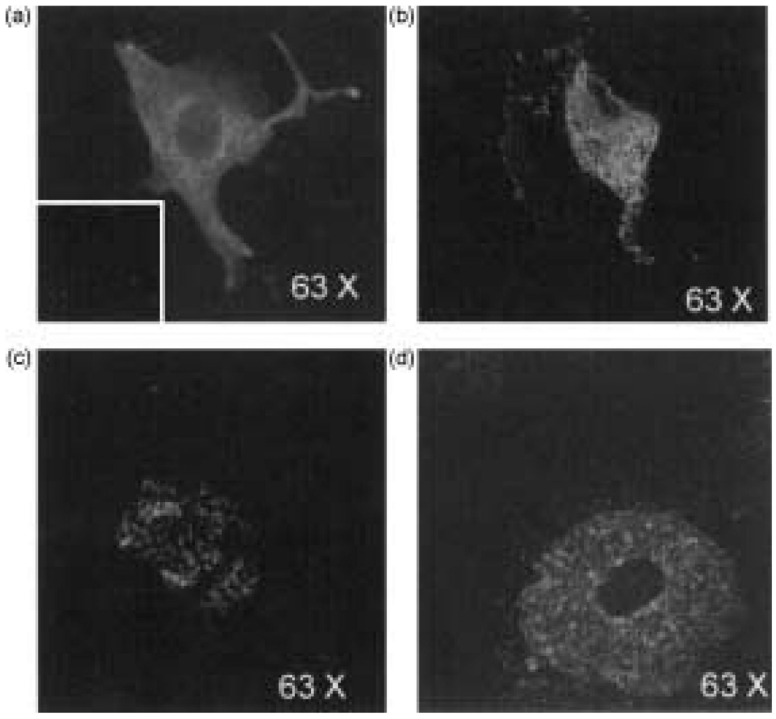

Fig. 5.

Immunofluorescent localization of FLAG tagged wt and C-terminal deletion mutant of peripherin/rds in transiently transfected MDCK cells. MDCK cells expressing wt FLAG-peripherin/rds were grown on glass coverslips, fixed in MeOH/acetone (A, inset mock-transfected), Δ10 FLAG-peripherin/rds mutant (C), Δ50 FLAG-peripherin/rds mutant (D) were incubated with M5 mAb (anti-FLAG antibody) followed by incubation with Alexa Fluor 594 secondary antibody. (B) MDCK cells expressing Δ10 FLAG-peripherin/rds mutant were grown on glass coverslips, fixed in 2 % paraformaldehyde and incubated with M5 mAb (anti-FLAG antibody) followed by incubation with Alexa Fluor 594 secondary antibody. Immunofluorescent staining was analysed by laser scanning confocal microscopy. Optical sections horizontal (X–Y) to the monolayer are shown. Magnification is indicated in panels.

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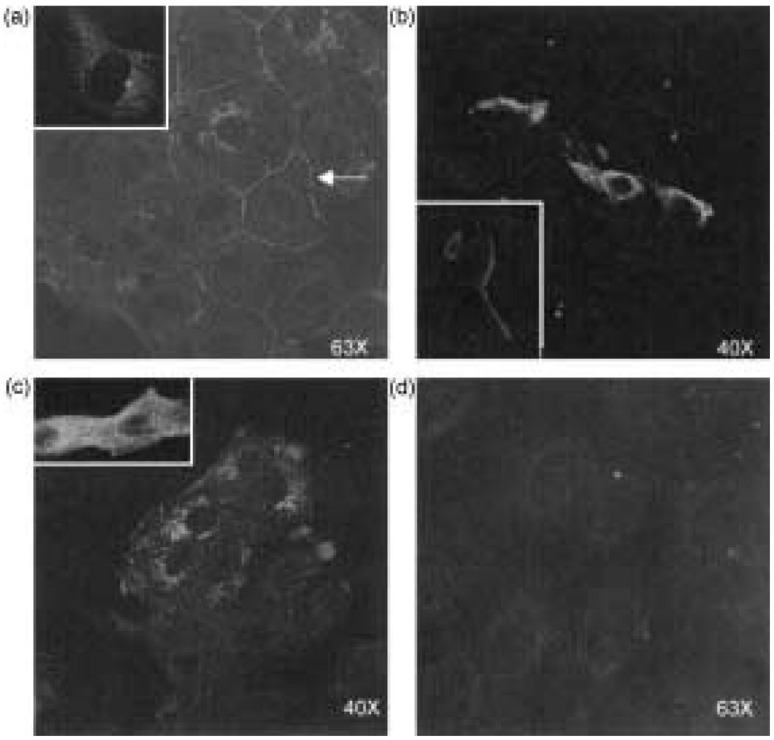

Fig. 6.

Immunolocalization of wt and P296T Xpress-peripherin/rds in transiently transfected MDCK cells. Cells grown on coverslips were transfected with Xpress-peripherin/rds P296T mutant, in (A) cells were fixed in MeOH/acetone, incubated with peripherin/rds polyclonal antibody followed by incubation with Alexa Fluor 594 secondary antibody. (B) Xpress-peripherin/rds P296T mutant cells were fixed in 2 % paraformaldehyde, incubated with anti-Xpress monoclonal antibody followed by incubation with Alexa Fluor 594 secondary antibody. Inset to (B) Xpress-peripherin/rds P296T mutant expressing cells were incubated with anti-Xpress antibody prior to fixation with 2 % paraformaldehyde. (C) MDCK cells transfected with wt Xpressperipherin/rds or (D) mock-transfected cells were fixed in MeOH/acetone incubated with anti-Xpress monoclonal antibody followed by incubation with Alexa Fluor 594 secondary antibody. (A) and (C) Insets represent individual cells of each group. Arrow indicates the location of cell junction. Immunofluorescent staining was analysed by laser scanning confocal microscopy. Optical sections horizontal (X-Y) to the monolayer are shown. Magnification is at 63×.

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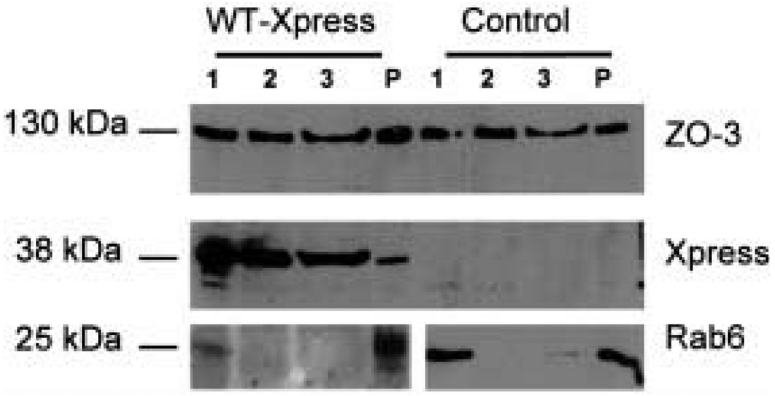

Fig. 7.

Western blot analysis of membrane fractions isolated from sucrose density gradients. Proteins from membrane fractions isolated at 1·02/1·04 g ml^{-1} interface, designated fraction 1 (1), 1·04/1·.08 g ml^{-1} interface, designated fraction 2(2), and 1·08/1·125 g ml^{-1} interface, designated fraction 3(3) and the pellet fraction (P) were separated by SDS–PAGE and analysed by Western blot analysis as described in Materials and Methods. The various fractions 1–3 and P (pellet) are indicated for both Xpress-peripherin/rds cell line 22, mock-transfected MDCK cells (control cells) and the P296T mutant. The immunoreactivity of the proteins within the fractions with anti-ZO-3 antibody, anti-Xpress antibody and anti-rab 6 antibody is indicated on the right and the respective molecular masses of the proteins identified in kDa are indicated on the left.

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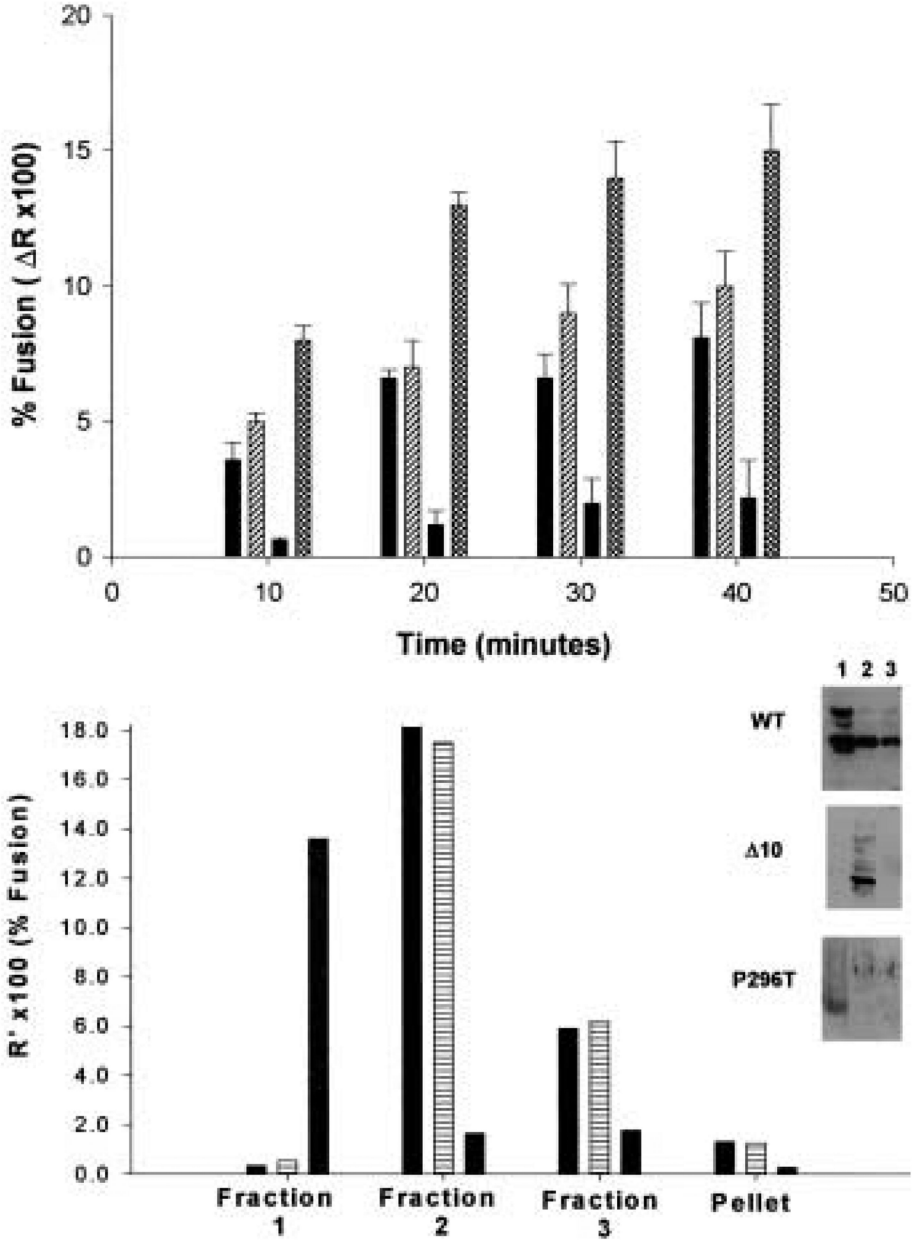

Fig. 8.

Fusion between R_{18}-ROS PM and F_{18} labelled MDCK cell membranes containing wt and mutant peripherin/rds. Resonance energy transfer from F_{18} labelled MDCK cell membranes to R_{18} labelled ROS PM vesicles upon fusion of the membranes was measured at room temperature. The results are presented as percentage fusion calculated as described in Materials and Methods. (A) Time course of fusion between R_{18} labelled ROS PM vesicles and F_{18} labelled wt Xpress-peripherin/rds containing membranes (■), or Δ10 FLAG-peripherin/rds deletion mutant (▨), or Δ50 FLAG- peripherin/rds deletion mutant (▧), or P296T peripherin/rds mutant (☒). (B) Fusion between R_{18}-ROS PM and F18 labelled membrane fractionated from wt Xpress-peripherin/rds expressing MDCK cells (■), FLAG-peripherin/rds Δ10 mutant (▤) and Xpress-peripherin/rds P296T mutant (▤). Fractions were

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isolated as described in Materials and Methods. Fractions 1–3 and pellet are indicated. Resonance energy transfer from F_{18} labelled MDCK cell membranes to R_{18} labelled ROS PM vesicles upon fusion of the membranes was measured at room temperature. The results are presented as percentage fusion calculated as described in Materials and Methods. Inset. The relative amount of expressed protein in fractions 1, 2 and 3 was determined using Western blot analysis. The Xpress wt and P296T mutant were detected with anti-Xpress and the 10 FLAG-peripherin/rds mutant with anti-FLAG M5 antibody.

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Table I

Fusion between R_{18}-ROS PM and MDCK cell membrane fractions

	X-p/rds MDCK cells		Mock-transfected MDCK cells	
Target membrane	**Additions**	**IRF**	**Additions**	**IRF**
Fraction 1	1 μM Ca^{2+}	ND	1 μM Ca^{2+}	ND
Fraction 2	1 μM Ca^{2+}	0·88 ± 0·02	1 μM Ca^{2+}	0·20 ± 0·01
Fraction 2	1 μM Ca^{2+} 10 nM PP-5	0·41 ± 0·03	1 μM Ca^{2+} 10 nM PP-5	0·23 ± 0·005
Fraction 2	1 μM Ca^{2+} 10 nM NP-1	0·82 ± 0·03	1 μM Ca^{2+}	0·22 ± 0·009
Fraction 3	1 μM Ca^{2+}	0·65 ± 0·08	1 μM Ca^{2+}	0·11 ± 0·02
Fraction 3	1 μM Ca^{2+} 10 nM PP-5	0·44 ± 0·04	1 μM Ca^{2+}	ND
Fraction 3	1 μM Ca^{2+} 10 nM NP-1	0·69 ± 0·01	1 μM Ca^{2+}	ND
Pellet	1 μM Ca^{2+}	ND	1 μM Ca^{2+}	ND
Trypsin treated fraction 2	1 μM Ca^{2+}	0·40 ± 0·08	1 μM Ca^{2+}	ND
Trypsin treated fraction 3	1 μM Ca^{2+}	0·28 ± 0·04	1 μM Ca^{2+}	ND
			1 uM Ca^{2+}	ND

IRF were normalized to total membrane phospholipid in the target membrane. The data shown are the change in fluorescence intensity per min per umol phospholipid. All assays were carried out at 37°C under dim red light. The target membrane was incubated with either the PP-5 peptide or the NP-1 peptide for 30 min prior to the initiation of fusion. X-p/rds refers to Xpress-tagged human peripherin/rds. ND, Not detected.

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Table II

Fusion between R_{18}-ROS PM and Xpress-peripherin/rds recombinants

	Extent of fusion (ΔR × 100)		
Target membrane	**5 min**	**10 min**	**20 min**
WT Xp/rds recombinant	15·1 %	23·5 %	26 %
P296T mutant Xp/rds recombinant	40 %	60 %	70 %

The extent of fusion between R_{18}-ROS PM and F_{18} labeled recombinants was measured as described in Materials and Methods. All assays were carried out at 37°C under dim red light. Xp/rds refers to Xpress-tagged peripherin/rds isolated from transfected MDCK cells.