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Abstract

Peripherin-2 (also known as peripherin/rds), a photoreceptor specific tetraspanin protein, is required to maintain normal cell structure through its role in renewal processes requiring membrane fusion. It is the first tetraspanin fusogen and has been shown to directly mediate fusion between disk membranes and opposing membranes to maintain the highly ordered structure of rod outer segments. Localized to the C terminus of human, bovine, and murine peripherin-2 is an amphiphilic fusion peptide domain (residues 312-326) and a highly conserved region upstream of this domain that we hypothesize is essential for fusogenic function. Our previous studies indicated that substitution of a threonine for a proline at position 296 within this highly conserved region enhanced fusion activity. In this study we wanted to determine whether this proline is essential with the introduction of three additional substitutions of proline with alanine, leucine, and glutamic acid. Wild type, P296T, P296A, P296L, and P296E mutants of peripherin-2 were expressed as His_6-tagged full-length proteins in Madin-Darby canine kidney (MDCK) cells. All of the proteins were localized to intracellular membranes and detected as 42-kDa monomers by Western blot analysis. The wild type, P296A, and P296L assembled into core tetramers; in contrast the P296T and P296E formed higher order oligomers. Fusogenic activity of full-length protein expressed in MDCK membranes and purified protein reconstituted in model membrane liposomes was determined using fluorescence quenching techniques. Fusion activity was decreased in the P296L, P296A, and P296E mutants both in endogenous MDCK membranes and in model liposomes. Collectively, these results suggest that the proline at position 296 is necessary for optimal function. © 2005 by The American Society for Biochemistry and Molecular Biology, Inc.

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A Novel Tetraspanin Fusion Protein, Peripherin-2, Requires a Region Upstream of the Fusion Domain for Activity*

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Peripherin-2 (also known as peripherin/*rds*), a photoreceptor specific tetraspanin protein, is required to maintain normal cell structure through its role in renewal processes requiring membrane fusion. It is the first tetraspanin fusogen and has been shown to directly mediate fusion between disk membranes and opposing membranes to maintain the highly ordered structure of rod outer segments. Localized to the C terminus of human, bovine, and murine peripherin-2 is an amphiphilic fusion peptide domain (residues 312–326) and a highly conserved region upstream of this domain that we hypothesize is essential for fusogenic function. Our previous studies indicated that substitution of a threonine for a proline at position 296 within this highly conserved region enhanced fusion activity. In this study we wanted to determine whether this proline is essential with the introduction of three additional substitutions of proline with alanine, leucine, and glutamic acid. Wild type, P296T, P296A, P296L, and P296E mutants of peripherin-2 were expressed as His_6-tagged full-length proteins in Madin-Darby canine kidney (MDCK) cells. All of the proteins were localized to intracellular membranes and detected as 42-kDa monomers by Western blot analysis. The wild type, P296A, and P296L assembled into core tetramers; in contrast the P296T and P296E formed higher order oligomers. Fusogenic activity of full-length protein expressed in MDCK membranes and purified protein reconstituted in model membrane liposomes was determined using fluorescence quenching techniques. Fusion activity was decreased in the P296L, P296A, and P296E mutants both in endogenous MDCK membranes and in model liposomes. Collectively, these results suggest that the proline at position 296 is necessary for optimal function.

Tetraspanins belong to a growing family of transmembrane proteins, with over 40 family members identified in mammals, *Drosophila*, and *Caenorhabditis elegans* (1). Similar to other transmembrane 4 superfamily proteins, tetraspanins contain four transmembrane domains. Mutations of tetraspanin genes are associated with various degenerative diseases. For example, the human tetraspanin, TMS4SF/A15, is associated with mental retardation (2); furthermore over 60 mutations of the photoreceptor-specific tetraspanin, peripherin-2, result in a variety of retinal degenerative disorders (3). The unique structural and functional domains of this protein family includes four to six highly conserved cysteine residues linked into two to three disulfide bonds in a highly conserved EC-2 domain. Mutations within this domain result in retinal degeneration and mental retardation. Tetraspanins also contain polar residues in the first, third, and fourth transmembrane domains postulated to be involved in protein-protein interactions necessary to form signaling complexes (4–8). Thus tetraspanins form homo- and hetero-oligomers resulting in large multiple protein complexes or tetraspanin webs that are involved in the regulation of cell motility, fertilization, cell signaling, and fusion processes.

Work in our laboratory has focused on elucidating the role of photoreceptor peripherin-2 as a membrane fusion protein. Although peripherin-2 is first the tetraspanin protein that is directly involved in promoting fusion, several other tetraspanins have important, although poorly defined, functional roles in membrane fusion. CD-9-deficient mice showed impaired egg-sperm fusion (9, 10). Interestingly, injection of CD81 mRNA into CD9 null mouse oocytes rescued membrane fusion, suggesting that in some instances tetraspanins may functionally substitute for each other. CD81 has also been implicated in human leukemia virus 1 fusion but not HIV[1] fusion.

More recently, both CD9 and CD81 have been shown to promote muscle cell fusion (11). The mechanism by which these proteins participate in fusion is not clear; however, the notion that these tetraspanins may have intrinsic fusogenic function has not been ruled out.

Photoreceptor cells contain a unique set of tetraspanin proteins known as peripherin-2 and its nonglycosylated homologue rom-1 (12–16). Purified peripherin-2 promotes membrane fusion with ROS plasma membrane in a cell-free assay system (17, 18). This system serves as a model for two key fusion-dependent events in rod cell renewal: disk morphogenesis and disk shedding (19). Fusion is mediated through a fusion peptide domain within the C-terminal domain of peripherin-2 corresponding to residues 312–326. The amphiphilic, α-helical structure of the fusion peptide is necessary for the formation of a fusogenic oligomer of the peptide to promote the requisite steps in membrane fusion (20, 21). Homology among human, murine, bovine, *Xenopus*, and avian forms of the protein in a region upstream of the fusion peptide, residues 294–314, suggests functional importance within this region of the C

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[1] The abbreviations used are: HIV, human immunodeficiency virus; MDCK, Madin-Darby canine kidney; wt, wild type; PBS, phosphate-buffered saline; ROS, rod outer segment; PM, plasma membrane; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors.

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terminus. A P296T substitution in this region was previously shown to enhance fusion, although the contribution of subunit assembly to the fusogenicity of that mutant was not addressed (22).

The role of peripherin-2 as a membrane fusion protein is evident in the phenotypes observed in a series of animal models of retinal degeneration. Initially, the *rds* (retinal degeneration slow) mouse model confirmed that disruption of the *RDS* tetraspanin gene caused retinal degeneration. *rds* homozygotes fail to develop photoreceptor outer segments and have limited phototransduction capability, and consequently, the photoreceptors undergo a slow, progressive apoptosis (23, 24). Mice heterozygous for the *rds* defect develop shortened outer segments, which are abnormally phagocytosed. *In vivo* evidence in support of a peripherin-2 C-terminally mediated function comes from three mouse models of *RDS* action. Data from a chimeric mouse line expressing the D-2 loop of peripherin-2 in the context of rom-1 suggests that functional efficacy is not restricted to the D-2 loop (26). The dominant negative phenotype of the codon 307-del mouse model of RP, in which the C-terminal domain is altered, shows a more rapid retinopathy than the *rds* −/−, suggesting that the C terminus of peripherin-2 contains a unique functional domain that contributes to the degenerative process (25). Lastly, the haplo-insufficiency mouse model argues that two functional alleles are necessary to assemble the ROS. Collectively, although the phenotypes are complex, these *in vivo* studies coupled with *in vitro* work (18–21) provide support for the classification of peripherin-2 as the first tetraspanin fusion protein. In this study we have advanced our hypothesis that the C-terminal domain of peripheirn-2 is a unique functional domain of this protein and possibly other tetraspanins. In this regard we have shown that a single amino acid substitution at position 296 from a proline to an alanine, leucine, or glutamic acid inhibits membrane fusion, whereas substitution to threonine enhances fusion but abrogates normal subunit assembly. Collectively, these studies suggest that this highly conserved region of peripherin-2 may play a role in regulating fusion activity.

MATERIALS AND METHODS

Preparation of Human Peripherin-2 cDNA Mutants and Expression Constructs—Human peripherin-2 cDNA flanked by NotI restriction sites was subcloned into the multiple cloning site of pGEMEX-2 bacterial expression vector (Promega). Single amino acid substitutions were made using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). wt template DNA was prepared through transformation of JM109 competent *Escherichia coli* cells (Promega) with 10 ng of the peripherin-2 pGemex-2 construct and subsequent plasmid DNA purification using a Qiaprep spin miniprep kit (Qiagen). Custom primers were obtained from Integrated DNA Technologies, Inc. as 100-nmol oligonucleotides using standard desalting. Mutagenic oligonucleotides were designed corresponding to the coding strand of peripherin-2 to contain ~50% GC content and a T_m of <75 °C. The mutagenic primers used to change the proline at position 296 to alanine and leucine are indicated by the sequences 5′-GTGTCCAACGCCGAGGAATC-3′ and 5′-GTCCAACCTCGAGGAATC-3′. Generation of the P296E mutant required a 27-base primer: 5′GGTGTGTCCAACGAGGAGGAATCTGAG-3′. The mutagenic primers were 5′-phosphorylated and used in combination with the bottom strand selection oligonucleotide provided in the GeneEditor kit. The mutations were verified by sequence analysis (Davis Sequencing). The 1.1-kb wt and mutant peripherin-2 cDNA NotI fragments were subcloned into the NotI site of the multiple cloning site of the pcDNA 3.1 His B mammalian expression vector (Invitrogen). The correct orientation of the peripherin-2 NotI fragments in the vector was confirmed through restriction enzyme analysis by digestion with SstI (New England Biolabs).

Cell Culture and DNA Transfections—MDCK strain II cells (ATCC) were maintained in Eagle's minimum essential media containing Eagle's salts and L-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 1% nonessential amino acids at 37 °C in 5% CO_2 (all reagents from Sigma). The cells were seeded 2 days before transient transfections in 150-cm^2 tissue culture flasks, allowing the cells to reach 70–80% confluency at the time of transfection. Transfections were performed using Optimem I media with no serum or antibiotics. Peripherin-2 used for fusion analyses was obtained from cells transfected with Lipofectamine (Invitrogen). For each transfection, 10 μg of DNA and 23 μl of Lipofectamine 2000 (Invitrogen) was used. The cells were incubated for 3 h at 37 °C with the transfection complexes, and the medium was replaced with the complete culture medium. Proteins for sedimentation velocity assay were isolated from cells transfected with FuGENE (Roche Applied Science). 12 μg of DNA and 108 μl of FuGENE were added followed by incubation for 5 h. Then the Optimem I medium with transfection complexes was replaced with the complete culture medium. In both cases, the cells were harvested by scraping in PBS 48 h post-transfection.

Purification of Peripherin-2—Five confluent 150-cm^2 flasks of MDCK cells expressing either wt or the proline 296 mutants of peripherin-2 were harvested in PBS, pelleted, and resuspended in native binding buffer consisting of 20 mM sodium phosphate, 500 mM NaCl, and 30 mM octylglucoside, pH 7.4. The cell lysates were sonicated three times for 10 s each time, and His-tagged peripherin-2 His_6-peripherin/rds was purified using the Xpress purification system under native conditions (protocol as provided by Invitrogen). 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-2 from five confluent 150-cm^2 flasks cells was between 0.4 and 1.0 mg. The purity of the protein was confirmed by SDS-PAGE and Western blot analysis using anti-Xpress antibody.

Velocity Sedimentation Assay—Sedimentation coefficient ($S_{20,w}$) estimates were made in a Beckman Optima LE-80K ultracentrifuge using a SW 50.1 rotor essentially as described by Goldberg and Molday (26), with the following modifications. The purified Xpress tagged wild type peripherin-2 and P296T, P296A, P296L, and P296E mutant samples were loaded onto a 5–20% (w/w) sucrose gradient and centrifuged for 20 h at 40,000 rpm. Sucrose gradient fractions (~250 μl) were collected from the bottom of the gradient by peristaltic elution through the capillary micropipette. Sedimentation profiles of fractionated sucrose gradients were determined by chemiluminescent Western blotting using anti-X-press antibody (Invitrogen). Digital analysis of Western blots was performed using Kodak Image Station 440CF.

The following equations (27) were used to calculate the sedimentation coefficient,

$$S_{T_m} = (dr/dt)/\omega^2 r \qquad \text{(Eq. 1)}$$

in which S_{Tm} is the sedimentation coefficient S at a given temperature T in a solvent, dr/dt is the distance traveled by the sample over time in mm/s, ω is the angular velocity of the rotor in radians/s, and r is the distance from the axis of rotation in mm.

$$S_{20,w} = S_{T_m}\frac{\eta_{T_m}(1/\omega - \rho_{20,\mathrm{w}})}{\eta_{20,w}(1/\omega - \rho_{T_m})} \qquad \text{(Eq. 2)}$$

where $S_{20,w}$ is the sedimentation coefficient $S_{\mathrm{T_m}}$ corrected to the "standard state" of water at 20 °C, $\eta_{\mathrm{T_m}}$ and $\rho_{\mathrm{T_m}}$ are the viscosity and density, respectively, of the medium m at the temperature T of the experiment, and $\eta_{20,\mathrm{w}}$ and $\rho_{20,\mathrm{w}}$ are the viscosity and density of water at 20 °C. Partial specific volume (υ) of the analyzed proteins were assumed to be 0.83 ml/g (28).

Preparation of F_{18}-labeled Recombinant Liposomes—Vesicles consisting of phosphatidylcholine:phosphatidylethanolamine:phosphatidylserine:cholesterol in a 4:4:1:1 mole ratio and 1 mol % F_{18} were prepared as described (29–31). The lipids were cosolubilized in chloroform, dried under N_2, lyophilized, and resuspended in 10 mM Hepes, pH 7.4. The lipid suspension was sonicated three times for 3 min each using a probe sonicator to form small unilamellar vesicles. A final volume of 1 ml of vesicles was added to purified Xpress peripherin-2, which was detergent solubilized in 20 mM octylglucoside while vortexing (32). A 1:100 nmol ratio of purified protein:vesicle phospholipid was used for all recombinants. The recombined membranes were dialyzed for 72 h against 10 mM Hepes, 100 mM NaCl and for 10 h in the presence of Bio-Beads (Sigma) 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 spun at 25,000 rpm overnight at 4 °C. The Xpress peripherin-2-LUV recombinants were isolated from the sucrose gradients. 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. All of the recombinants were assayed for total phosphate (33) and protein (Bio-Rad).

Preparation of F_{18}-labeled Total Cell Membranes from Peripherin-2-transfected MDCK Cells—Five confluent 150-cm^2 flasks of MDCK cells were transfected with wt and mutant peripherin-2 as described above. The cells were harvested by scraping in homogenizing buffer (100 mM monobasic sodium phosphate, 1 mM dithiothreitol, 1 μg/ml of leupeptin, and 2 μg/ml of aprotinin, pH 7.4), pelleted (1,000 rpm for 5 min), and resuspended in 4 ml of homogenizing buffer. The cells were then lysed by passage through a 26-gauge needle twice and sonicated three times for 10 s. Homogenized cell lysates were layered onto a single layer 30% sucrose gradient (30% sucrose (w/w) in homogenizing buffer) and spun at 25,000 rpm for 20 min at 4 °C in a SW28 Beckman ultracentrifuge rotor. The plasma membrane fraction was collected from the first interface and pelleted by centrifugation (50,000 rpm for 30 min at 4 °C), and the membranes were resuspended in 1 ml of 10 mM Hepes, pH 7.4. The membranes were labeled with 1 mol % F_{18} (1 mg/ml in ethanol) while vortexing and incubated at 37 °C for 30 min in subdued light. Unincorporated label was removed with the addition of 500 μl of 2% bovine serum albumin incubating for 10 min at 37 °C and recovering labeled membranes by spinning at 50,000 rpm for 30 min at 4 °C. The pellets were resuspended in 500 μl of 10 mM Hepes, pH 7.4.

Preparation R_{18}-labeled of Rod Outer Segment Plasma Membranes (ROS-PMs)—Bovine ROS plasma membrane vesicles were isolated in the dark from frozen bovine retinas (Lawson, Lincoln, NE) as described previously (34). Purified plasma membrane was resuspended in 2 ml of 10 mM Hepes, 0.5 M NaCl and labeled with 50 μl of R_{18} (2 mg/ml solution in ethanol) while vortexing and then incubated at 37 °C for 1 h to incorporate the fluorescent label. Unincorporated R_{18} label was separated from labeled PM on a Sephadex G-50 size exclusion column as described (31).

Resonance Energy Transfer Fusion Assay—Fusion between R_{18}-labeled plasma membrane and F_{18}-labeled cell membranes or F_{18}-labeled recombinant vesicles was measured using a fluorescence resonance energy transfer assay (35) at room temperature on a PerkinElmer Life Sciences 50B spectrofluorometer (Gaithersburg, MD). Fusion was initiated with the 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 (36). The change in R_{18} intensity (ΔR) at a given time was computed as follows.

$$\Delta R = (I_{592}/I_{524} + I_{592})_T - (I_{592}/I_{524} + I_{592})_I \quad \text{(Eq. 3)}$$

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 of change in R_{18}, indicated in the graphs as the percentage of fusion, was determined by multiplying each ΔR value by 100. The fusion values were normalized for peripherin-2 content. Total cell membrane fusion values represent total fusion/mg of peripherin-2 as determined by densitometry of anti-Xpress Western blots of isolated MDCK cell membranes. In the figures, the percentage of fusion values are normalized to the total percentage of fusion seen with wild type, which is set at 100% of wild type fusion.

Western Blot Analysis—MDCK cell lysates, purified peripherin-2, and total cell membranes isolated from MDCK cells were mixed in a 1:1 ratio with 2× SDS sample buffer and separated on 10–15% SDS-PAGE and transferred to nitrocellulose for Western blot analysis. For trypsin treatment of total cell membrane fractions, total protein content of the membranes was determined, and a 3× excess μg amount of trypsin was added, followed by incubation at 37 °C for 30 min. Western blots were probed with a 1:2500 dilution of anti-Xpress primary antibody and a 1:1000 dilution of goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Western blots were visualized by enhanced chemiluminescence (Supersignal ECL; Pierce).

Immunohistochemistry of Peripherin-2-transfected MDCK Cells—For immunohistochemistry, MDCK cells were seeded on sterilized glass coverslips in 6-well plates and grown for 24 h. The cells were transiently transfected with wt or mutant peripherin-2 using Lipofectamine 2000 as described above. Twenty-four hours post-transfection, the cells were washed in PBS containing 2 mM $MgCl_2$ and 0.2 mM $CaCl_2$ (PBS-C/M). For surface staining, the intact cells were incubated with a 1:500 dilution of anti-Xpress antibody for 1 h. The cells were then fixed in 2% paraformaldehyde for 30 min at 4 °C, rinsed in PBS-C/M, quenched with 50 mM glycine for 10 min at 4 °C, and rinsed in PBS-C/M. The cells were subsequently incubated for 1 h with goat anti-mouse IgG Alexa Fluor 488 secondary antibody at a concentration of 10 μg/ml (Molecular Probes). For permeabilized cell staining, the cells were fixed in a 1:1 methanol/acetone solution for 2 min prior to the 1-h incubation with anti-Xpress primary antibody. The cells were rinsed with PBS-C/M and then incubated with secondary antibody as described above. The samples were analyzed on a Zeiss AXIOSKOP fluorescent microscope, capturing images at the magnifications indicated in the figures.

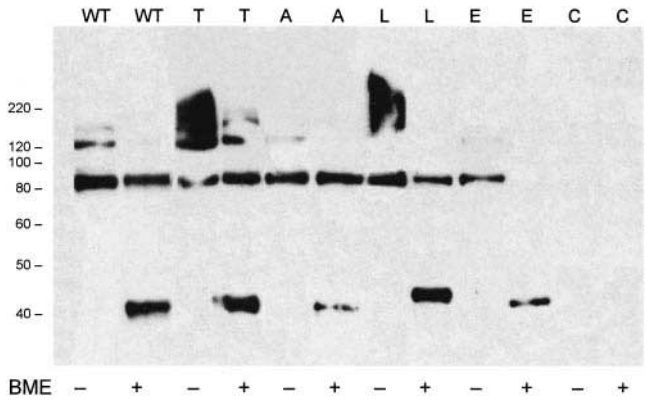

FIG. 1. **Expression and dimerization of wt and mutant peripherin-2.** Purified wt and mutated peripherin-2 (~5 μg of protein/lane) were subjected to either nonreducing β-mercaptoethanol (*BME* −) or reducing β-mercaptoethanol (*BME* +) electrophoresis, immunoblotted and probed with anti-Xpress antibody. Under nonreducing conditions the proteins were detected as 84-kDa dimers; P296T (*T*) and P296L (*L*) mutants also formed substantial amount of higher order aggregates. In the presence of BME, 42-kDa monomers were observed.

Statistical Analysis—Analysis of variance was first performed to test whether there is any difference among groups. When a significant difference was found from analysis of variance, pairwise, post-hoc *t* testing (Dunnett's *t* test) of each mutant with wild type peripherin-2 was performed while controlling for the overall type I error. All of these statistical analyses were perform using SAS 9.1 (SAS Institute, Cary, NC).

RESULTS

Peripherin-2 and Pro^{296} Mutants Are Heterologously Expressed in and Localized to Intracellular Membranes of MDCK Cells—To determine whether regions upstream of the fusion peptide are necessary for fusogenic function, we generated four human peripherin-2 mutants bearing single amino acid substitutions of proline at position 296. These substitutions include P296T, P296A, P296L, and P296E and were chosen to alter the polarity, size, and charge of this region. The mutants contained an N-terminal Xpress epitope tag and a poly-His_6 tag at the N terminus to allow for detection and purification of the proteins. Each of the four Pro^{296} mutant expression plasmids was transiently transfected individually into MDCK cells. The His_6-tagged proteins were purified by nickel column chromatography, and protein expression was assessed by Western blot analysis using anti-Xpress antibody. As shown in Fig. 1, wt peripherin-2 migrated as an 84–86-kDa dimer under nonreducing conditions, with the majority reduced to a 41–43-kDa monomer in the presence of β-mercaptoethanol. Similarly, the four mutants typically migrated as 84–86-kDa dimers under nonreducing denaturing conditions. A fraction of the dimers was reduced to 41–43-kDa monomers in all cases, with all of the expressed P296E protein reduced to the monomeric form. Two mutants, the P296T and the P296L proteins, showed slower migrating proteins consistent with higher order aggregates in the range of 120–220 kDa. Similar pattern of higher order complex formation is not unexpected and was observed in case of insertional mutants: IM5, IM7, and IM11 expressed in COS-1 cells (37). Thus peripherin-2 was successfully expressed in MDCK cells, and peripherin-2 mutants form disulfide-linked dimers. These observations are consistent with data from other

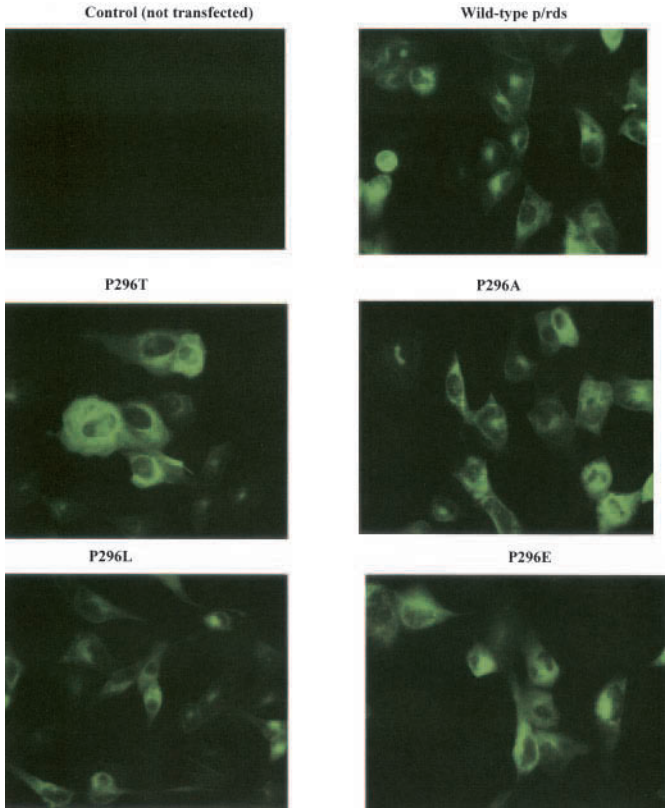

FIG. 2. **Wild type and mutant Xpress peripherin-2 is localized to intracellular MDCK cell membranes.** MDCK cells grown on glass coverslips were transiently transfected with the indicated plasmids. The cells were fixed in methanol/acetone and incubated with monoclonal anti-Xpress antibody and subsequently anti-mouse IgG Alexa Fluor 488 (fluorescein-conjugated) secondary antibody. The immunofluorescence is compared with the labeling of mock transfected (control) MDCK cells labeled with anti-Xpress antibody. The cells were analyzed on a Zeiss AXIOSKOP fluorescent microscope with images captured at 40× magnification.

investigations of the protein *in vivo* and in other cell expression systems (38, 39).

We next focused our attention on the localization pattern of wt and Pro^{296} mutants in MDCK cells. As shown in Fig. 2, MDCK cells transfected with wt and Xpress peripherin-2 Pro^{296} mutants all show a distinct subcellular localization pattern. Peripherin-2, the P296A, P296L, and P296E mutants all exhibited a stereotypical Golgi localization pattern, forming a small cap over the nuclei of expressing MDCK cells. In contrast the P296T mutant showed a more diffuse pattern consistent with protein aggregation. We previously noted that the distribution of the P296T mutant was perinuclear in subconfluent cells but appeared to be plasma membrane-associated upon confluence (22). Similar results were noted in these studies (data not shown).

Analysis of Pro^{296} Mutant Subunit Assembly by Velocity Sedimentation—wt peripherin-2 assembles into a tetramer in both retinal photoreceptor and in COS cell expression systems. The formation of core tetramers appear to be essential for function because the disruption of subunit assembly results in peripherin-2 mislocalization and is linked to dysmorphic outer segment in mice and to some forms of human retinal degenerative diseases (40–42). To determine whether single point mutations at position 296 in the highly conserved region of the C termini affect subunit assembly, the purified poly-His_6-tagged peripherin-2 mutants were analyzed using sedimentation velocity assays. wt and Pro^{296} mutants were centrifuged through continuous sucrose density gradients, and gradient fractions were analyzed for peripherin-2 by Western blotting (as shown in Fig. 3). Estimated sedimentation coefficients for the analyzed proteins are given in Table I, and sedimentation profiles are shown in Fig. 3. The sedimentation velocity profiles for the wt peripherin-2 as well as P296A and P296L mutants indicate that they form homotetrameric complexes (27, 43). Additionally, the P296L mutant formed larger complexes with sedimentation coefficients of 7.1 S. In contrast, the increased mobility of P296T and P296E mutants suggests that these proteins do not assemble into normal homotetramers; the sedimentation coefficient (7.1S) is consistent with the formation of octamers (40) or unstable protein. These observations suggest that the replacement of the conserved proline with alanine or leucine has no effect on subunit assembly, whereas substitution of proline with threonine or glutamate affects subunit assembly and possibly protein folding.

Analysis of Fusion between MDCK Cell Membranes and ROS-PM—The fusogenic function of wt and mutant peripherin-2 was assessed in both MDCK cell membrane preparations and model membranes containing pure protein. Intracellular MDCK cell membranes, shown to contain significant amounts of peripherin-2 (Fig. 2) were isolated from transfected MDCK cells as described in our previous studies (44). The fusion activity of wild type and peripherin-2 mutants were compared using a fluorescence resonance energy transfer assay. This assay relies on fluorescence resonance energy transfer between two lipophilic probes R_{18} and F_{18} (45), which readily incorporate into the membrane bilayer of ROS-PM and the target membranes; (intracellular MDCK cells membranes or model membrane liposomes), respectively. Fusion is detected when the F_{18} molecule excited at 460 nm transfers energy to an R_{18} molecule that is in close proximity. The close contact between the two probes and the resulting transfer of energy occurs through lateral diffusion of the fluorescent lipids in the membrane of a newly formed fused species of the F_{18}-labeled membranes and the R_{18} ROS-PM. Fusion between ROS-PM vesicles and MDCK cell intracellular membrane fractions was measured at 37 °C. When R_{18} ROS-PM was added to F_{18} MDCK cell membranes, an increase in R_{18} fluorescence was observed, with a concomitant decrease in F_{18} intensity. This increase in fluorescence was followed over time, and the extent of fusion after 15 min (time point at which no additional change in fluorescence was detectable) was calculated and compared with the percentage of fusion observed with wild type. Because the transfection efficiency, although similar, was not identical with all of the mutants, the fusion assays were normalized to the amount of peripherin-2 expressed. As shown in Fig. 4, substitution of the proline with a leucine, glutamic acid, or alanine decreased fusion by 67, 72, and 41%, respectively, when compared with wt peripherin-2, indicating an inhibition of fusogenic activity. In fact these mutations abolished fusion activity almost completely when compared with control (nonperipherin/*rds*-expressing) MDCK cells. In contrast, the P296T mutant showed a 2-fold increase in fusion when compared with wt peripherin-2. Collectively, these results suggest that the proline at position 296 lies in a critical position for the fusogenic properties of the recombinant peripherin-2. Further, the specific biochemical or structural characteristics of the amino acid residues at position 296 likely dictate the fusion capacity of the fusion peptide.

To confirm that the changes in fusogenic function observed were due specially to peripherin-2 and not endogenous MDCK cell fusion effectors, individual lipid recombinants containing each of the purified proteins were prepared and assayed for fusion activity with ROS-PM as described for purified bovine ROS peripherin-2 (46). This method enables us to study the

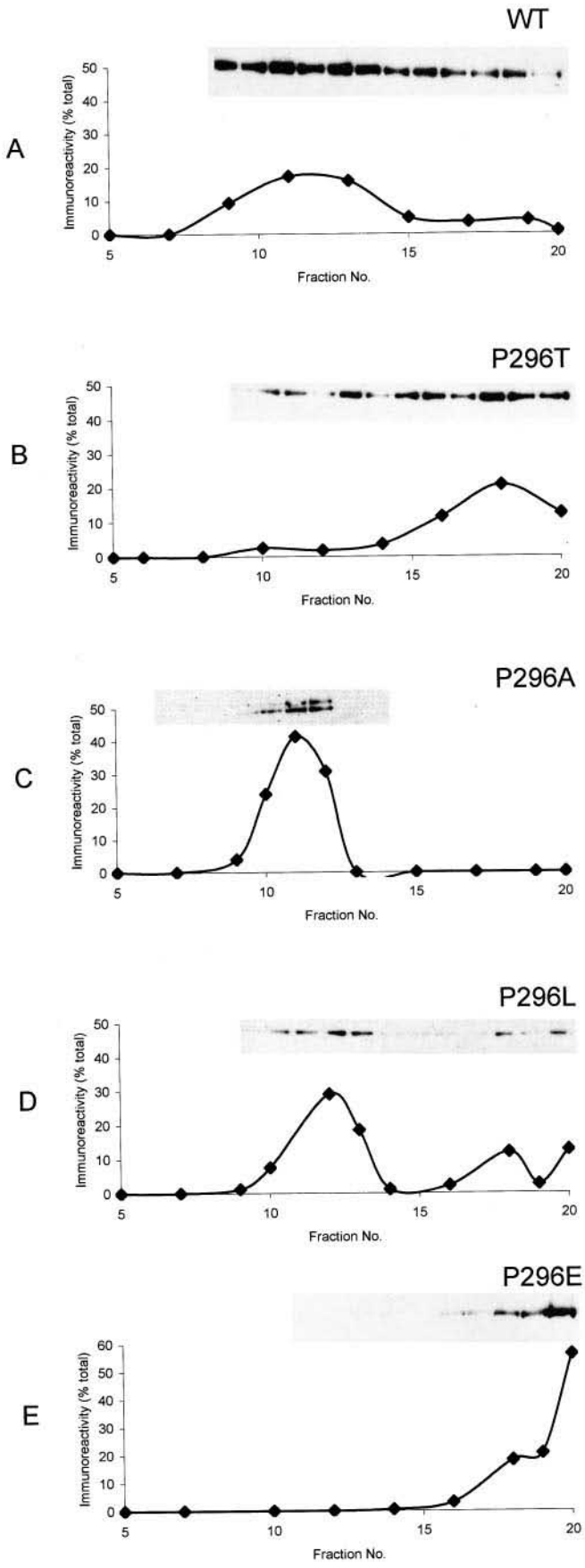

FIG. 3. **Subunit assembly of peripherin-2 Pro^{296} mutants by velocity sedimentation.** *A*, MDCK cells were transfected with wt or mutant peripherin-2 Pro^{296} mutant plasmids as indicated. The expressed poly-His$_6$-tagged peripherin-2 mutants were purified on a nickel column, and peripherin-2-containing complexes were sedimented through a continuous 5–20% (w/w) sucrose gradient. Fractionated gradients were assayed for peripherin-2 and Pro^{296} mutant expression by Western blot analysis with anti-Xpress antibody. Representative chemiluminescent blots and corresponding plots generated by image analysis are shown for wt peripherin-2 and the Pro^{296} mutants as indicated. Immunoreactivity in the individual gradient fractions was calculated as a percentage of the total. wt peripherin-2 and the P296A (*C*) mutant sediment as a single major pick characteristic of tetrameric complex. *D*, P296L mutant forms not only tetramers but assemble also into complexes of higher sedimentation velocity. In contrast, P296T (*B*) and P296E (*E*) mutant were recovered in higher density fractions.

TABLE I
Sedimentation coefficients ($S_{20,w}$) of wild type and mutated peripherin-2

Variant	$S_{20,w}$[a]
wt	4.6 (5.1)[b]
P296T	7.1
P296A	4.6
P296L	5.1/7.1[c]
P296E	7.5

[a] Each value is an average of two independent sedimentation trials.
[b] Sedimentation coefficient of individually expressed peripherin/rds in COS-1 cells provided by Goldberg *et al.* (35).
[c] Two peaks of intensity were observed.

fusogenic activity of peripherin-2 and its mutants while eliminating other proteins present in the MDCK cell membranes. Briefly, detergent-solubilized Xpress peripherin-2 wt and mutants were recombined into F_{18}-labeled lipid vesicles as the target membrane. These recombinant model membranes had phospholipid to protein (mol:mol) ratios of 88–101, 92–108, 90–101, 81–99, and 85–102 for the P296T, P296A, P296L, and P296E mutants, respectively. Because the C terminus of peripherin-2 contains the fusion peptide domain, to assay functionality in the membrane preparations we needed to confirm that this domain was exposed to the opposing membrane to mediate fusion. To determine the orientation of peripherin-2, the isolated membranes were treated with trypsin as described for ROS disk membranes and pure peripherin-2 LUVs (17, 46). Trypsin specifically cleaves the N and C termini of peripherin-2 (20); these regions would only be accessible to cleavage if oriented on the outside of membrane vesicles. As shown in Fig. 4 (*inset*), the intact protein containing the N-terminal Xpress tag is present in untreated cell membrane samples; in contrast the Xpress tag is cleaved in the presence of trypsin under nonsolubilizing conditions. In addition, when the nitrocellulose membrane were stained with colloidal gold two major bands are detected, with molecular masses equivalent to 12.5 and ~20 kDa, corresponding to the cleaved C terminus and to the major membrane-associated domain, respectively. These results suggest that the majority of the peripherin-2 is oriented with the N- and C-terminal regions exposed and support the rationale for using these membrane vesicles to study the fusion properties of these mutants.

When R_{18}-labeled ROS-PM was mixed with F_{18}-labeled recombinant vesicles containing each of the peripherin-2 proteins, an increase in R_{18} fluorescence was observed (data not shown). All of the fusion assays were normalized to peripherin-2 incorporated into the recombinant liposomes based on protein analysis. As shown in Fig. 4, fusion activity of the peripherin-2 recombinants was similar to the fusion activity observed when intracellular MDCK cell membranes were utilized, and the overall range of percentage of fusion (wt and Pro^{296} mutants) was also comparable, with an observed range of 4.2–13.9% fusion in the recombinant samples (in a typical experiment) and a range of 1.8–10.6% fusion when the MDCK cell membranes were used. The P2965A, P296L, and P296E mutants were less fusogenic than the wt peripherin-2 with

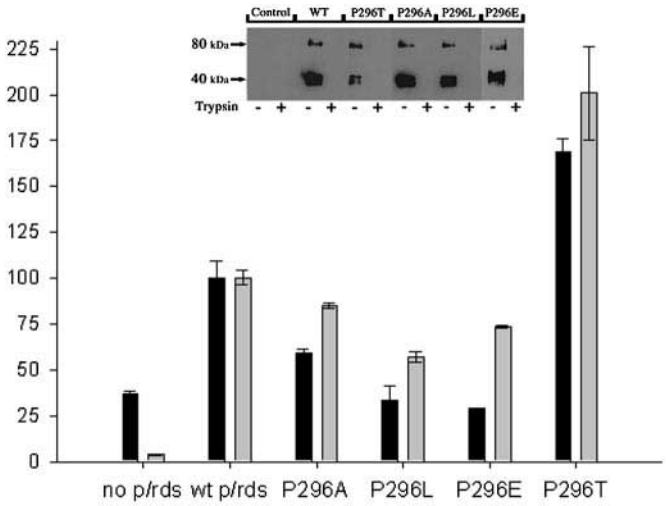

FIG. 4. **Peripherin-2 containing MDCK cell intracellular membranes fuse with ROS plasma membrane vesicles.** Intracellular MDCK cell membranes from cells transfected with wt or mutant peripherin-2 Pro^{296} mutant plasmids were isolated by sucrose gradient centrifugation as indicated. Fusion between F_{18}-labeled intracellular MDCK membranes and R_{18}-labeled ROS plasma membrane vesicles (*solid bars*) was measured using fluorescence resonance energy transfer at 37 °C as described under "Materials and Methods. " The average total fusion values from resonance energy transfer fusion assays are presented. Three separate preparations of membranes isolated from MDCK cells transfected with no DNA (control), wild type, or proline 296 mutants of peripherin-2 were used for the fusion assays. Total fusion was calculated based on fluorescence emission and normalized to peripherin-2 concentrations as described under "Materials and Methods." All of the values are expressed as percentages of fusion observed with wild type peripherin. The data are presented as percentages of this value. The *shaded bars* represent fusion between peripherin-2 recombinant vesicles and ROS plasma membrane vesicles. Purified poly-His_6-tagged wild type or Pro^{296} mutant periphern-2 was reconstituted into model membranes as described under "Materials and Methods." Fusion between F_{18}-labeled model membranes containing purified peripherin-2 (wt and mutants) and R_{18}-labeled ROS plasma membrane vesicles was measured using fluorescence resonance energy transfer at 37 °C as described under "Materials and Methods." The total extent of fusion was calculated based on fluorescence values and normalized to peripherin-2 concentrations as described under "Materials and Methods." All of the values are normalized to the extent of fusion observed with wild type. The data are presented as percentages of this value. *, $p < 0.05$ from Dunnett's t tests, which controls type 1 error for the pairwise comparisons of each mutant groups against wt p/*rds* was observed for all of the mutants. *Inset*, expression and proteolysis of wt and Pro^{296} peripherin-2 mutants reconstituted into liposomes. The peripherin-2 containing model membranes were untreated (−) or treated with trypsin (+). The samples were then separated on a 10% SDS gel under denaturing and reducing conditions. Western blots were probed with monoclonal anti-Xpress antibody. Monomer (42 kDa) and dimer (84 kDa) bands are present in untreated (*Trypsin* −) liposomes. There are no detectable anti-Xpress bands in the trypsin-treated liposomes.

activity corresponding to 85, 56, and 73% of wild type, respectively. Finally, the P296T mutant displayed a fusion capacity that was roughly double that of the wild type protein as reported previously (21).

DISCUSSION

Peripherin-2 shares structural and functional similarities with a well defined group of membrane fusion proteins (37). It is unique, however, in that it is the only tetraspanin protein shown to mediate fusion in the absence of additional cofactors. Other tetraspanins involved but not directly mediating fusion include CD9 and CD81. In contrast to the well characterized EC-2 domains of peripherin-2 and the other tetraspanins, it is the C terminus of peripherin-2 that mediates fusion required during photoreceptor renewal. As shown in Fig. 5, peripherin-2 contains an amphiphilic α-helix that promotes membrane fusion as a functional tetramer *in vitro* (17, 18). To understand

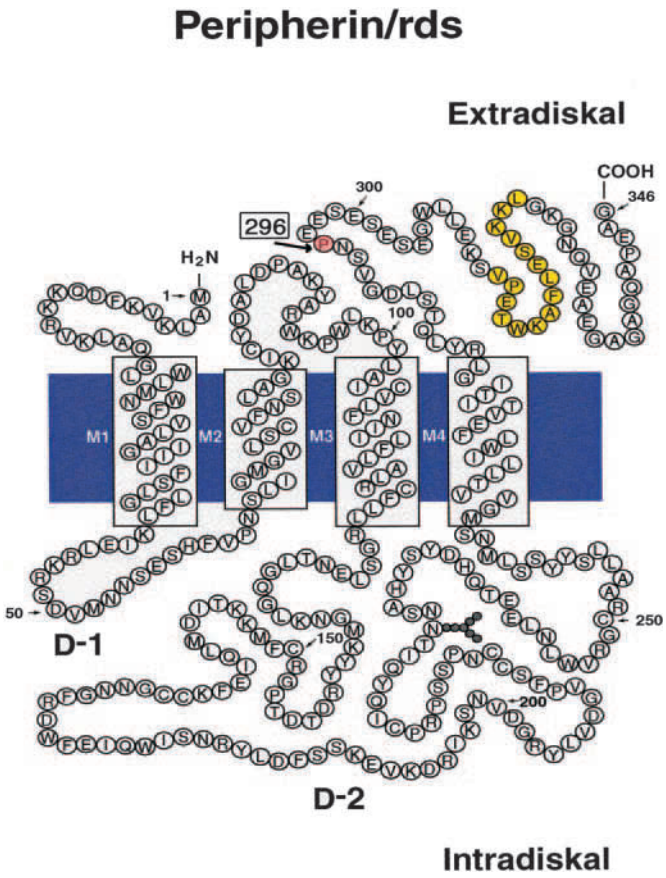

FIG. 5. **Schematic representation of peripherin-2.** Peripherin-2 has four predicted membrane spanning regions, shown as *M1–M4*. There are two intradiskal loops, indicated as *D1* and *D2*. The N and C termini are oriented extradiskally, in the space between the disk membrane and the rod outer segment plasma membrane. The fusion peptide region from residues 312–326 is indicated in *yellow*. Proline 296, indicated in *pink*, lies just upstream of the fusion peptide region within the C terminus. Figured modified from Ref. 19.

the contribution of other C-terminal regions to protein biosynthesis and fusogenic function, we have chosen to focus on a highly conserved region upstream of the fusion domain, corresponding to residues 294–315. A series of four mutants was generated in which proline at position 296 was replaced with threonine, alanine, leucine, and glutamic acid to alter the size, charge, and polarity of this residue.

The biochemical properties of the wt and Pro^{296} mutant proteins are consistent with the native peripherin-2 in photoreceptor rod outer segments. When expressed in MDCK cells they form 84-kDa dimers that are reduced to 42-kDa monomers in the presence of reducing agents as observed *in vivo* and in other heterologous expression systems (16, 21, 38, 39). Two of the mutants, P296T and P296E, also form larger complexes in a molecular mass range of 120–220 kDa. The localization pattern of the wt peripherin-2 to intracellular membranes was similar to that of the Pro^{296} mutants. The P296T and P296E mutants exhibited a more diffuse staining pattern, but localization did not appear to be clustered around the nucleus as described for unstable protein (34). The localization pattern and migration pattern in reducing denaturing gels of the P296T and P296E mutants suggest that these mutants form homo-oligomers. *In vivo*, wt peripherin-2 forms disulfide linked homo-dimers that associate noncovalently with rom-1 disulfide-linked homo-dimers to form a hetero-tetramer. Peripherin-2 self-associates into functional tetramers forming the requisite complex for delivery to the outer segment (35, 36). In these studies we demonstrated that alteration of a proline in a

highly conserved region of the C terminus results in altered subunit assembly. When alanine or leucine were substituted for proline, subunit assembly was normal. In contrast, the P296T and P296E mutants sedimented more rapidly and were recovered in fractions corresponding to sedimentation coefficients on the order of 7 S (Fig. 3), presumably because of higher order aggregation or misfolding of the proteins. These results are in contrast to other studies in which insertional mutations in the C terminus and mis-sense mutations in the C-terminal helical domains did not alter peripherin-2 biosynthesis, including homotetramer formation (26, 28, 29). It should be noted that in those studies, a number of mutants showed heterogeneous subunit assembly with some large oligomeric complexes. We anticipate that the difference in subunit assembly in our studies is due to expression in a polarized MDCK cell system (*versus* COS cells in work by Goldberg *et al.* (35) and Ritter *et al.* (39)) and to the fact that the mutated amino acid was a proline, a residue known to alter loop-turn structures.

A common paradigm in membrane fusion protein function is the formation of a metastable fusion inactive form of the protein that requires an activating event. Activation may result from a conformational change that exposes the fusion peptide to the target membrane to mediate fusion (37). Regions in close proximity to the fusion peptide of these proteins are involved in promoting a fusion competent form of the protein (40). For example, some fusion proteins contain a "fusion trigger" region (41–43) and/or a hairpin-like domain (44). In synaptic vesicle fusion, the fusion protein functions in active complexes with other soluble or integral membrane proteins (45). The HIV fusion protein contains distinct regions upstream and/or downstream of the fusion peptide domain that stabilize a fusion-active conformation (also known as fusion competent form) (46, 47). Some fusion processes, for example SNARE fusion, contain elements of both (48–50). Structural changes within fusion proteins involving these domains are required to allow a protein to go from a prefusion to a fusion competent conformation (51–54). Moreover, proline residues in or near the fusion peptide domain are essential for the formation of the metastable active structure as well as for membrane interactions leading to fusion (55–57).

We predicted that the highly conserved region upstream of the fusion peptide from residues 294–315 also plays an important role in the fusion properties of peripherin-2 and that proline at position 296 is involved in this function. Two separate fusion assays were used to confirm the ability of the expressed proteins to promote fusion. First, intracellular membranes containing peripherin-2 isolated from transiently transfected MDCK cells were shown to promote fusion activity with plasma membrane vesicles isolated from rod outer segments. This membrane fraction was previously shown to be enriched in peripherin-2 and to mediate fusion (21). This system does not allow us to exclude the possibility that endogenous MDCK cell proteins, including those involved in intracellular transport are not contributing to the fusion signal. Thus we also employed liposomes containing purified recombinant peripherin-2. These studies demonstrated that wt peripherin-2 promotes fusion with the same rod outer segment plasma membrane vesicles. In both sets of fusion assays, the wild type protein promoted equivalent fusion levels (5–7% total fusion, calculated as the total amount of fusion/mg of peripherin-2). The P296T mutant showed an extent of fusion that was about twice as much as the wt protein, indicating a significant enhancement of the fusogenic capacity consistent with previously published results (21). The substitution of the proline at position 296 with threonine, a more polar amino acid with a hydroxyl side group, increased the hydrophilicity of this region of the protein, possibly contributing to the formation of a fusion-active coiled-coil domain (52). In contrast, substitution with alanine, leucine, or glutamate decreased fusion activity of peripherin-2. Because the compact structure of the amino acid proline indicates a branch point in the secondary structure of a polypeptide sequence, we speculate that the threonine substitution leads to structural change in the protein to a more fusion-active form. This metastable form could place the fusion peptide region in a position where it is able to interact more freely with the opposing membrane. Other well characterized fusion proteins, such as the HIV gp41 protein, are known to undergo activating conformational changes from a native state to a prefusion state and finally to a fusion-active structure (57). If peripherin-2 undergoes a similar activating conformational change to a fusion-active state, the structure of the P296T mutant may mimic the activated conformation of peripherin-2.

The fundamental observation in this work is that normally assembled peripherin/*rds* mutated at position Pro^{296} shows a decrease in fusogenic function. In summary, these studies show that peripherin-2,k a tetraspanin fusogen, requires not only a fusion peptide domain but also a proline upstream of this domain for normal fusion activity. These results lend further support to the growing hypothesis that the C-terminal domain of peripherin-2 plays a critical role in fusogenic function and not simply as a structural determinant. Furthermore, these studies provide a biochemical basis for the differences observed in peripherin-2 retinal degenerative diseases. Moreover, they provide a paradigm to address the role of other tetraspanins in fusion and how alterations in this function may lead to degenerative disease phenotypes.

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