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The Retinitis Pigmentosa-Linked Mutations in Transmembrane Helix 5 of Rhodopsin Disrupt Cellular Trafficking Regardless of Oligomerization State

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Abstract

G protein-coupled receptors can exist as dimers and higher-order oligomers in biological membranes. The specific oligomeric assembly of these receptors is believed to play a major role in their function, and the disruption of native oligomers has been implicated in specific human pathologies. Computational predictions and biochemical analyses suggest that two molecules of rhodopsin (Rho) associate through the interactions involving its fifth trans-membrane helix (TM5). Interestingly, there are several pathogenic loss-of-function mutations within TM5 that face the lipid bilayer in a manner that could potentially influence the dimerization of Rho. Though several of these mutations are known to induce misfolding, the pathogenic defects associated with V209M and F220C Rho remain unclear. In this work, we utilized a variety of biochemical and biophysical approaches to elucidate the effects of these mutations on the dimerization, folding, trafficking, and function of Rho in relation to other pathogenic TM5 variants. Chemical cross-linking, bioluminescence energy transfer, and pulsed-interleaved excitation fluorescence cross-correlation spectroscopy experiments revealed that each of these mutants exhibits a wild type-like propensity to self-associate within the plasma membrane. However, V209M and F220C each exhibit subtle

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Author Contributions

B.J., A.W.S., and J.P.S. conceived and designed the experiments. B.J., A.W.S., P.M., E.G., M.P., C.K., W.D.C., J.P.S., and F.J.R. conducted the experiments. B.J., A.W.S., and J.P.S. wrote the manuscript. B.J. and A.W.S. coordinated and oversaw the research project. All authors discussed the results and commented on the manuscript.

Notes

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defects in cellular trafficking. Together, our results suggest that the RP pathology associated with the expression of the V209M and F220C mutants could arise from defects in folding and cellular trafficking rather than the disruption of dimerization, as has been previously proposed.

GRAPHICAL ABSTRACT:

Rhodopsin (Rho) is a light-sensing seven-transmembrane helical receptor composed of the apoprotein opsin and a covalently bound 11-*cis*-retinal chromophore. Its major function involves the absorption of light in a manner that is coupled to the initiation of visual signal transduction.^{1–3} However, Rho is also an important structural protein, and its proper expression and folding are critical for the structural development of photoreceptor rod outer segments (ROS). In Rho knockout mice (*Rho*^{−/−}), ROS formation is completely ablated, and in heterozygous knockout mice (*Rho*^{+/-}), where the level of expression of Rho is reduced by half relative to that of the wild type (WT) mice, the length of ROS is reduced by 50%.^{4,5} The misfolding of destabilized Rho mutants, and the corresponding disruption of the cellular trafficking of Rho, also results in the malformation of ROS.^{6,7}

Proper oligomeric organization of G protein-coupled receptors (GPCRs) plays a significant role in the molecular basis of numerous human pathologies.^{8–10} Compelling biochemical and pharmacological evidence indicates that Rho forms dimeric and oligomeric complexes within biological membranes.^{11–14} Two modes of its association have been proposed on the basis of crystal packing within the high-resolution structures of dark state Rho, photoactivated Rho, and rod opsin.^{15–17} These proposed modes of oligomerization are also consistent with its organization within the native ROS disc membranes.^{18,19} On the basis of these observations, dimerization is believed to arise through interactions between transmembrane helices (TM) 4 and 5 and perhaps also through TM1, TM2, and cytoplasmic helix H8. A similar manner of oligomerization state also has been observed in other Rho-like family A GPCRs.^{20–23}

Five point mutations on the outer surface of TM5 have been identified in human patients with retinal degenerative disease such as autosomal dominant retinitis pigmentosa (adRP), a retinopathy that leads to progressive loss of vision due to degeneration of rods and in some cases cones.^{24–27} RP mutations are divided into seven classes based on their pathological mechanism. Most RP-linked mutations compromise the folding of Rho (class II) in a manner that reduces its expression level and/or disrupts its transport from the endoplasmic reticulum (ER) to the plasma membrane. Of the five known RP mutations in TM5, H211R, P215L, and C222R exhibit severe class II defects.^{28,29} Alternatively, the alterations caused by V209M and F220C, which are associated with mild RP phenotypes,³⁰ were not reported to affect

membrane trafficking. This leads to the question of what is the mechanism of the RP pathology caused by these mutants.

At least three different mechanisms could account for the basis of the retinopathy linked with the V209M and F220C mutations: (1) a mild impairment of protein folding and its membrane trafficking, (2) a deleterious effect on Rho function, and/or (3) disruption of Rho dimerization.

By using a combination of immunoblotting, fluorescence microscopy, and flow cytometry, we provide a strong quantitative indication that V209M and F220C Rho exhibit minor defects in cellular expression and trafficking. We also show that these mutations have a negligible effect on the absorbance spectra of Rho and on its ability to activate G_i signaling. Nevertheless, F220C does slightly retard chromophore release following photoactivation and exhibits decreased thermal stability. Finally, by using a combination of chemical cross-linking, bioluminescent resonance energy transfer (BRET), and fluorescence correlation spectroscopy (FCS and FCCS), we show that these mutations do not disrupt the native oligomerization state of Rho in cellular membranes. These results obtained in the live cell plasma membrane are inconsistent with the previously reported observation that V209M and F220C reconstitute into lipid vesicles as monomers, suggesting their disruptive effect on Rho dimerization.³¹ Taken together, by combining state-of-the-art biochemical and biophysical techniques, we found that subtle defects in the conformational stability and cellular trafficking of V209M and F220C Rho give rise to the RP phenotype associated with these mutations. Thus, our results add significant information to our understanding of the mechanistic basis for these two RP-linked enigmatic Rho mutations.

EXPERIMENTAL PROCEDURES

Chemical Reagents.

n-Dodecyl β -D-maltoside (DDM) was purchased from Affymetrix Inc. (Maumee, OH). 11-*cis*-Retinal was a generous gift from R. Crouch (Medical University of South Carolina, Charleston, SC). Coelenterazine h was obtained from Nanolight (Pinetop, AZ), and a 5 mg/mL stock solution prepared in ethanol was immediately used or stored at -80°C . GTP γ S and 9-*cis*-retinal were purchased from Sigma (St. Louis, MO). EDTA-free protease inhibitor cocktail tablets were obtained from Roche (Basel, Switzerland). DSP and DSG cross-linkers were obtained from Thermo Fisher Scientific (Waltham, MA). Mouse mAb anti-ATPase-(Na^+/K^+) $\alpha 5$ was a generous gift from Y. Imanishi (Case Western Reserve University, Cleveland, OH) and originally obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Anti-HA antibodies (2–2.2.14) conjugated to Dylight 550 (DL550) or AlexaFluor 647 (AF647) were purchased from Thermo Fisher Scientific (Waltham, MA).

Constructs.

The wild type rod opsin-EGFP and rod opsinmCherry vectors were described in a previous study and used here without modification.³²

The RP-causing rod opsin mutants (V209M, P215L, F220C, and C222R) were constructed by using Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) following the manufacturer's protocol. Src₁₆-EGFP/mCH and Src₁₃-GCN4-EGFP/mCH plasmids were obtained from the Groves lab at the University of California (Berkeley, CA).

The RP-causing rod opsin mutants (V209M, P215L, F220C, and C222R) were subcloned into a pcDNA3.1(+) vector according to the manufacturer's protocol. The resulting constructs were used for cross-linking, protein purification, and functional experiments. cDNA was amplified by polymerase chain reaction; EcoRI and NotI restriction sites were introduced at the 5' - and 3' -ends, respectively, by using the following primers: forward primer, GTGGGGAATTCGCCATGAACGGCACAGAGGG; and reverse primer, TCTGGGCGGCCGCTCAGGCTGGAGCGACCTGA. DNA sequencing was performed to confirm the composition of each construct.

Constructs of mouse opsin fused to Venus (mOpsin-Venus) and *Renilla* luciferase (mOpsin-RLuc) in the pcDNA3.1Zeo vector were a generous gift from N. A. Lambert (Georgia Regents University, Atlanta, GA). These constructs were used for the BRET assay.

Flow cytometry analysis was performed with the use of Rho constructs bearing an N-terminal influenza hemagglutinin (HA) tag. To construct these vectors, Gibson assembly was used to integrate mutant Rho genes into a modified pcDNA5 vector containing an N-terminal HA tag and an IRES Dasher GFP element downstream of the Rho stop codon.

HEK-293 Cell Culture.

HEK-293 or HEK-293 GnTI⁻ cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 5 µg/mL plasmocin (InvivoGen, San Diego, CA), and 1 unit/mL penicillin with 1 µg/mL streptomycin (Life Technologies, Carlsbad, CA) at 37 °C under 5% CO₂. HEK-293 and HEK-293 GnTI⁻ cells were obtained from ATCC (CRL-3022). HEK-293 GnTI⁻ cells do not have N-acetylglucosaminyl-transferase I (GnTI) activity; thus, they lack the ability to synthesize complex glycans. Consequently, opsin expressed in these cells is homogeneously N-glycosylated.

Expression of WT and Mutant Rod Opsins in HEK-293 Cells.

Transfection of HEK-293 GnTI⁻ cells with WT rod opsin and RP-causing rod opsin mutants (V209M, P215L, F220C, and C222R) cloned into the pcDNA3.1(+) vector (Clontech, Mountain View, CA) was performed using polyethylenimine.^{33,34} 9-*cis*-Retinal was added 24 h post-transfection to a 10 µM final concentration to one plate, while a second plate was kept without retinal. Cells were cultured overnight in the dark at 37 °C in 5% CO₂ with 90% humidity. Forty-eight hours post-transfection, cells were washed with phosphate-buffered saline (PBS), harvested, and pelleted at 800g. The cell pellet was resuspended in the desired buffer and either solubilized with DDM or first used for cross-linking and then solubilized with DDM and processed as described below.

Membrane Localization of Rod Opsins in HEK-293 Cells.

To evaluate the membrane localization of WT rod opsin and RP-causing mutants (V209M, P215L, F220C, and C222R), we expressed them as fusion proteins with Venus in HEK-293 or HEK-293 GnTI⁻ cells. A TCS SP2 confocal microscope (Leica Microsystems Inc., Bannockburn, IL) was used to image those cells.³⁵ Additionally, immunolocalization of Na⁺/K⁺ ATPase was applied as a plasma membrane localization marker.³⁶

Quantification of Cellular Trafficking by Flow Cytometry.

The cellular localization of Rho variants was quantitatively assessed using flow cytometry in a manner that has been described previously.³⁷ Briefly, HEK-293 cells were transiently transfected with Rho constructs bearing an N-terminal HA tag using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Sixteen hours post-transfection, transfected cells were harvested and expanded into two 6 cm culture dishes. Twenty-four hours post-transfection, 9-*cis*-retinal was added to one set of dishes to a final concentration of 5 μ M, and an equal volume of dimethyl sulfoxide (DMSO) was added to the other set of plates for the sake of comparison. Forty hours after transfection, intact cells were trypsinized and then immunostained with a fluorescent anti-HA primary antibody (Dylight 550) for 30 min at room temperature. Cells were fixed using the Fix & Perm kit (Invitrogen) to cross-link the antibody to the surface antigen and then washed twice with PBS containing 5% FBS and 0.1% sodium azide to remove the fixative and excess antibody. Following the washes, cells were permeabilized using the Fix & Perm kit (Invitrogen) and stained with an AlexaFluor647 conjugate of the same anti-HA antibody to label the intracellular opsin. The cells were again washed twice with PBS containing 5% FBS and 0.1% sodium azide and analyzed by flow cytometry using a BD LSRII cytometer (BD biosciences, San Jose, CA). The lasers were set such that the relative intensities of the two fluorescent antibodies were nearly equal. Controls indicated no significant spillover among the dasher GFP, DL550, and AF647. To compare the trafficking patterns, the analysis was limited to positively transfected cells expressing the indicated variants, which were identified on the basis of bicistronic Dasher GFP expression. The contours showed the density profile of >1000 cellular measurements from each transfection.

Pigment Reconstitution and Purification by 1D4 Immunoaffinity Chromatography.

Forty-eight hours post-transfection with WT rod opsin and RP-causing mutant (V209M and F220C) constructs, HEK-293 cells were harvested and centrifuged at 800g and the pellet was resuspended in a buffer consisting of 20 mM Bis-tris propane (BTP), 120 mM NaCl, and protease inhibitor cocktail (pH 7.5). For pigment reconstitution, 11-*cis*-retinal was added to the cell suspension from a DMSO stock solution to a final concentration of 10 μ M, which then was incubated in the dark for 2 h at 4 °C on a rotating platform. Then *n*-dodecyl β -D-maltopyranoside (DDM) was added to the cell suspension to a final concentration of 20 mM and incubated for 1 h at 4 °C on the rotating platform, which then was centrifuged at 100000g for 1 h at 4 °C. WT Rho and mutants were purified from the supernatant by immunoaffinity chromatography with an anti-Rho C-terminal 1D4 antibody immobilized on CNBr-activated agarose.³⁸ The supernatant was mixed with 200–300 μ L of a mixture of 6 mg of 1D4/mL of agarose resin and incubated for 1 h at 4 °C on the rotating platform. The

beads were then transferred to a column and washed with 10–15 mL of buffer composed of 20 mM BTP, 120 mM NaCl, and 2 mM DDM (pH 7.5). Proteins were eluted with the same buffer containing 0.6 mg/mL 1D4 (TETSQVAPA) peptide.

Ultraviolet–Visible (UV–vis) Spectroscopy of Rho Samples.

A UV–vis spectrophotometer (Cary 50, Varian, Palo Alto, CA) was used to record spectra from freshly purified rhodopsin samples, and their concentrations were quantified using an absorption coefficient ϵ_{500} of $40600 \text{ M}^{-1} \text{ cm}^{-1}$.³⁹

Photosensitivity of Rho Samples.

Immunoaffinity-purified WT Rho or RP-causing V209M and F220C mutant proteins were exposed to light for 5, 15, 30, 60, 120, or 300 s with a Fiber-Light illuminator (150 W lamp) (Dolan-Jenner, Boxborough, MA) through a band-pass wavelength filter (480–520 nm) from a distance of 15 cm. Immediately after each bleaching procedure, UV–vis spectra were recorded at room temperature. The decline in the absorption maxima was plotted as a function of time, and the half-lives ($t_{1/2}$) of conversion of the 11-*cis*-retinal chromophore to all-*trans*-retinal were calculated from these plots. All samples were measured in triplicate.

G_t Activation.

G_t was purified from ROS membranes as described previously.^{40,41} The abilities of the WT Rho and RP-causing mutants V209M and F220C to activate G_t were tested in the Trp fluorescence activation assay. G_t and Rho samples at a 10:1 ratio, with G_t at 250 nM and Rho at 25 nM, were added to the assay buffer composed of 20 mM BTP, 120 mM NaCl, and 2 mM MgCl₂ (pH 7.0), followed by their illumination for 30 s with a Fiber-Light illuminator through a band-pass wavelength filter (480–520 nm). G_t activation was recorded as the intrinsic Trp fluorescence increase from G_{td} due to guanylyl nucleotide exchange upon addition of 5 μM GTP γS 5 min post-illumination. The measurement was performed with a PerkinElmer LS 55 luminescence spectrophotometer. Excitation and emission wavelengths of 300 and 345 nm, respectively, were employed.^{42–44} In control experiments, no signals from Rho without G_t were detected. All samples were measured in triplicate.

Meta II Decay.

Chromophore release (Meta II decay) was measured with 25 nM purified Rho samples diluted in a buffer composed of 20 mM BTP, 100 mM NaCl, and 1 mM DDM (pH 6.0). Illumination was applied for 15 s to the samples with a Fiber-Light illuminator through a 420–520 nm band-pass filter immediately before the fluorescence measurements. Light exposure was conducted at a distance of 15 cm. Changes in the intrinsic Trp fluorescence were recorded for 60 min. An increase in the intrinsic Trp fluorescence correlates with the decrease in the protonated Schiff base concentration.⁴⁵ Meta II decay was recorded with a PerkinElmer L55 fluorescence spectrometer at 20 °C with the following slit settings: 8 at 295 nm for excitation and 10 at 330 nm for emission collection. All samples were measured in triplicate.

Thermal Stability.

WT Rho or RP-causing V209M and F220C mutant Rho samples diluted in a final volume 0.4 mL of 20 mM BTP, 120 mM NaCl, and 1 mM DDM (pH 7.5) were incubated at 55 °C in the dark, and their spectra were recorded every 2 min for 1 h. The absorbance at maximum wavelength at 498 nm was assumed to be 100% at the initial time point. The percentages of remaining pigments normalized to their initial concentrations were then plotted as a function of time, and these plots were used to calculate the half-lives ($t_{1/2}$) of chromophore release. All samples were measured in triplicate.

Cross-Linking of WT and Mutant Rod Opsins in Membranes.

HEK-293 GnTI⁻ cells were transiently transfected with WT rod opsin or RP-causing V209M and F220C mutant constructs cloned into the pcDNA3.1(+) vector. Forty-eight hours after transfection, cells from two 6 cm plates were harvested, washed with PBS, and suspended in 200 μ L of the buffer composed of 20 mM BTP and 120 mM NaCl (pH 7.5). A disuccinimidyl glutarate (DSG) cross-linker (2 mM) was added to half of the cell suspension, and the cross-linking reaction was performed for 2 h on ice. Alternatively, cells were suspended in 100 mM Na₂HPO₄ (pH 8.3) containing 150 mM NaCl. A disuccinimidyl glutarate (DSP) cross-linker (2 mM) was added to half of the cell suspension, and the cross-linking reaction was performed for 2 h on ice. Then 1 M Tris-HCl (pH 8.0) was added to the samples to a final concentration of 50 mM to stop the reaction, followed by membrane solubilization with 20 mM DDM. Cross-linked opsins together with non-cross-linked opsins were subjected to immunoblot-ting analysis and detected with an anti-Rho C-terminal 1D4 antibody and an HRP-conjugated anti-immunoglobulin by using a chemiluminescence assay (Thermo Scientific). Densitometric analysis with ImageJ was used to quantify the amount of opsin monomer and dimer.

Bioluminescence Resonance Energy Transfer (BRET) Assay.

On the first day, HEK-293 cells were plated into two 12-well plates at a density of $\sim 25 \times 10^4$ cells/mL. The plate was cultured at 37 °C with 5% CO₂ and 90% humidity. The next day, cells were transiently transfected either with both opsin-Rluc (donor) and opsin-Venus (acceptor) at a 1:5 donor:acceptor construct ratio or with only the opsin-Rluc construct using polyethylenimine.^{33,34} Twenty-four hours post-transfection, 9-*cis*-retinal at a final concentration of 10 μ M was added to one plate, which then was covered with aluminum foil and cultured overnight at 37 °C in 5% CO₂ with 90% humidity. The second plate was kept without retinal under the same conditions. The following day, 48 h post-transfection the culture medium was aspirated and replaced with 500 μ L of PBS/well. Cells were resuspended, and 200 μ L of the suspension was transferred to a white-walled opaque 96-well plate (Corning, Corning, NY). Cells treated with 9-*cis*-retinal were kept under dim red light. Each well of the 96-well plate was supplemented with 25 μ L of coelenterazine h diluted with PBS to a concentration of 25 μ M, followed by dual luminescence readings at 480 and 530 nm 5 s after each injection by the SpectraMax plate reader with the BRET1 Filter Set (Molecular Devices, Sunnyvale, CA). The BRET1 signal was calculated as the emission ratio at 530 nm compared to that at 480 nm. The net BRET signal was calculated as the

difference between the BRET1 signal obtained in cells co-transfected with a donor and an acceptor and emission at 480 nm obtained from cells transfected with a donor only.

Cos-7 Cell Cultures and Data Collection.

Cos-7 cells were obtained from ATCC (CRL-1651) and cultured in DMEM + GlutaMAX (Life Technologies) with 10% fetal bovine serum (FBS, Life Technologies, Hyclone) and 1% penicillin/streptomycin (BioReagent, Sigma-Aldrich, St. Louis, MO). Cultures of $\sim 10^6$ cells/mL were incubated at 37 °C in 100 mm $\times$ 20 mm tissue culture plates (Falcon, Corning Inc.) until being split upon reaching ~ 80 –90% confluency. Cell stocks were used up to passage number 18. Prior to PIE-FCCS experiments, cells were split into 35 mm $\times$ 10 mm γ -irradiated glass bottom culture dishes (MatTek, Ashland, MA). Transfection was performed for each culture dish with 2.5 μ L of Lipofectamine 2000 (Life Technologies), and ~ 180 ng of each fluorescent protein plasmid in 31.25 μ L of DMEM with reduced phenol red. Around 1 h prior to being imaged, cells were washed with PBS and then phenol red-free Opti-MEM I medium (Life Technologies).

Pulsed-Interleaved Excitation Fluorescence Cross-Correlation Spectroscopy (PIE-FCCS).

PIE-FCCS was performed on a custom-built instrument described in previous publications.^{32,46,47} Two excitation beams, with wavelengths of 488 and 561 nm, were isolated and filtered (LL01-488-12.5 and LL02-561-12.5, Semrock, Rochester, NY) from a 5 ps pulse duration, 10 MHz repetition rate supercontinuum fiber laser (SuperK NKT Photonics, Birkerød, Denmark). The isolated laser beams were coupled into single-mode optical fibers of different lengths to induce an optical delay (50 ns) between the pulse trains. The beams were then overlapped and directed into the microscope light path with a dichroic mirror (zt488/561rpc, Chroma Technology Corp., Bellows Falls, VT).^{32,46} After exiting the sample, the emitted radiation passes through a laser-blocking filter (zet488/561m, Chroma Technology Corp.) and a 50 μ m confocal pinhole. After the pinhole, the red signal was split from the green by a long-pass filter (FF560-FDi01-25 $\times$ 36, Semrock). Two band-pass-filtered (FF01-621/69-25 and FF01-520/44-25, Semrock) SPAD detectors (Micro Photon Devices, Bolzano, Italy) with a 30 ps timing resolution then measured single-photon counts. A four-channel routed time-correlated single-photon counting (TCSPC) device (PicoHarp 300, PicoQuant, Berlin, Germany) was used to record the data. Data were then time-gated within 50 ns windows following each laser pulse arrival: the 621 nm-filtered detector after 561 nm excitation and the 544 nm filter after 488 nm excitation. This PIE time gate eliminates cross-talk contamination in the fluorescence signals.^{32,46,48}

The time-tagged photons arriving in the 621 and 544 nm detectors are used to compute fluorescence intensity fluctuation traces as functions of time, $F_i(t)$, where i denotes red or green photons. Autocorrelation [$G_R(t)$ and $G_G(t)$] and cross-correlation [$G_{RG}(t)$] functions of the single-cell data are then calculated by

$$G_R(\tau) = \frac{\langle \delta F_R(t) \times \delta F_R(t + \tau) \rangle}{\langle F_R(t)^2 \rangle}$$

$$G_G(\tau) = \frac{\langle \delta F_G(t) \times \delta F_G(t + \tau) \rangle}{\langle F_G(t)^2 \rangle}$$

$$G_{RG}(\tau) = \frac{\langle \delta F_R(t) \times \delta F_G(t + \tau) \rangle}{\langle F_R(t) \rangle \times \langle F_G(t) \rangle}$$

where τ is the lag time and $\delta F(t) = \langle F - \bar{F}(t) \rangle$ (brackets indicate average over all times).

Using a least-squares method, the resulting curves were fit to the following two-dimensional diffusion with a triplet state model:

$$G_i(\tau) = \frac{1}{N_i} \times \frac{1 - T + T^{-\tau/\tau_{T,i}}}{1 - T} \times \frac{1}{1 + \tau/\tau_{D,i}}$$

where N_i is the average number of molecules in the detection radius with a τ_D dwell time and T is the fraction of molecules in the triplet state with a relaxation time τ_T .^{32,46,48,49} Of the resulting data, only those from cells with molecular densities between 100 and 2000 molecules/ μm^2 were used to help avoid errors from detector limitations and in the interpretation of the cross-correlation data.

RESULTS

Influence of RP-Causing Mutations on the Expression and Trafficking of Rho.

Several point mutations on the outer surface of TM5, including V209M, P215L, F220C, and C222R, have been associated with retinitis pigmentosa (RP) (Figure 1A,B).

To determine the manner in which these defective variants influence the biogenesis and assembly of rod opsin, we surveyed their effects on the production and processing of Rho in HEK-293 cells. We produced these mutants in the context of the mouse rod opsin cDNA, which is 95% identical to human Rho and differs by only one amino acid residue in TM5 [residue 218 (see the sequence comparison in Figure 1A)]. The immunoblotting with a specific anti-Rho antibody and flow cytometry measurements confirmed the expression levels and cellular trafficking profiles of the mouse and human rod opsin proteins are similar in HEK-293 cells (Figure S1A,B). Moreover, mouse and human Rhos are organized into similar nanodomains in the native rod outer segment membranes.⁵⁰ Thus, no major differences in biochemical or biophysical properties between mouse and human Rhos were expected. We first compared the transient expression levels of each mutant in a glycosylation-deficient cell line of HEK-293 cells (GnTI⁻), which simplifies the banding pattern of the Rho protein. As we found, the V209M and F220C mutants exhibited only a slight decrease in expression level ($83 \pm 5\%$) compared to that of WT ($88 \pm 9\%$) (Figure 2A). However, P215L and C222R mutants exhibited dramatic reductions in expression levels as compared to that of WT (40 ± 11 and $12 \pm 3\%$, respectively). Additionally, the P215L mutant appeared on the immunoblot as several bands (indicated with a star), which may

suggest a different glycosylation pattern of this particular mutant as compared to that of WT protein or susceptibility to degradation due to instability. In the case of C222R, only the rod opsin dimer and higher-order oligomers, not monomers, were detected on the immunoblot, indicating that this mutation causes extensive aggregation (Figure 2A). To determine whether the mutants are properly targeted to cellular membranes, we used immunofluorescence in conjunction with fluorescence microscopy to evaluate their cellular localization. Each mutant accumulated at detectable levels within the cell. WT rod opsin and both V209M and F220C mutants exhibited appreciable co-localization with a plasma membrane marker Na/K ATPase) in a manner that confirms these mutants were targeted to cellular membranes (Figure 2B). However, both P215L and C222R rod opsins exhibited heightened accumulation within the cell, which suggests that these variants were likely retained within the ER (Figure 2B). In agreement with previous reports,^{28–30} these results indicate that P215L and C222R mutations cause severe Rho misfolding in a manner that prohibits its proper membrane localization.

To quantify the effects of these mutations on the cellular trafficking of rod opsin, we employed selective immunostaining in conjunction with flow cytometry as described previously.³⁷ Briefly, Rho mutants were transiently expressed in HEK-293 cells prior to labeling of the mature rod opsin protein at the plasma membrane using a DyLight550-labeled antibody. Following fixation and permeabilization, the remaining intra-cellular rod opsin was then differentially labeled with an Alexafluor647-labeled antibody. Analysis of cellular fluorescence profiles of positively transfected cells, which were marked by bi cistronic GFP expression, was then performed by flow cytometry. Consistent with expression level measurements, significant reductions in total protein levels were apparent for P215L ($62 \pm 2\%$) and C222R ($60 \pm 2\%$) mutants (Table 1). Moreover, these mutants exhibited dramatic reductions in plasma membrane staining levels (Table 1 and Figure 3A,B) as compared to that of WT rod opsin. Deficiencies were less pronounced for V209M, which exhibited a $39 \pm 2\%$ reduction in the total protein level and a $55 \pm 1\%$ reduction in protein levels at the plasma membrane (Table 1 and Figure 3C). In contrast, the trafficking of F220C rod opsin was quite similar to that of WT (Figure 3D), and any differences in the total F220C mutant levels were within the error of the measurement (Table 1). Nevertheless, the results showed a slight reduction [$14 \pm 9\%$ (Table 1)] in the level of accumulation of F220C rod opsin at the plasma membrane.

Treatment with $5 \mu\text{M}$ 9-*cis*-retinal significantly enhanced the accumulation of V209M and F220C mutants at the plasma membrane (Figure 3E,F and Table 1), while the effect of 9-*cis*-retinal on the accumulation of WT isorhodopsin at the plasma membrane was relatively modest (Figure S2). Altogether, these results provide additional evidence that V209M and F220C mutations induce misfolding.^{51,52} By comparison, addition of 9-*cis*-retinal markedly increased intracellular P215L and C222R levels, but corresponding gains in the level of plasma membrane staining were too modest to quantify (Table 1). Taken together, these results demonstrate that P215L and C222R are irreversibly misfolded. V209M appears to cause moderate defects in cellular expression and trafficking, and its stabilization by retinal largely compensates for these defects. Finally, the F220C mutation causes only minor reductions in the level of accumulation of rod opsin at the plasma membrane.

Functional Characterization of RP-Causing Rho Mutants.

In addition to the effects of the RP-linked mutations described above on rod opsin expression and trafficking, it is possible that they also impact the Rho function. Because of the strong indication that P215L and C222R are irreversibly misfolded, we concentrated on the V209M and F220C RP-associated mutants. To determine if these Rho mutants can function as normal visual receptors *in vitro*, WT rod opsin and the mutants were expressed in HEK-293 cells, regenerated with the 11-*cis*-retinal chromophore, and purified by affinity chromatography. UV-vis absorption spectra revealed the presence of absorption maxima at 498 nm in WT and both mutants, which is a characteristic of properly folded dark state Rho (Figure 4A). Upon illumination, the absorbance at 498 nm declined and the maximum absorption shifted to 380 nm in all proteins, indicating that both V209M and F220C mutants are capable of a light-dependent transition to the active Meta II state (Figure 4A, insets, dashed lines). The half-lives of Meta II formation were very similar in all samples: 4.7 ± 1.4 , 4.1 ± 1.2 , and 4.1 ± 1.2 s for WT, V209M, and F220C Rho, respectively (Figure 4B). Additionally, both RP mutants were able to activate the GDP $\rightarrow$ GTP nucleotide exchange in transducin after light illumination in a manner that is similar to that of WT Rho (Figure 4C). Nucleotide exchange was monitored by an increase in the intrinsic Trp fluorescence associated with the GTP γ S-induced dissociation of the Rho-G_t complex. The calculated rates of G_t activation were nearly identical for all three samples: $(3.4 \pm 0.3) \times 10^{-3}$, $(3.2 \pm 0.3) \times 10^{-3}$, $(3.6 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$ for WT, V209M, and F220C Rho, respectively (Figure 4C). The half-lives of chromophore release (or Meta II decay) were also comparable for WT Rho and the V209M mutant (22.3 ± 1.7 and 23.9 ± 2.7 min, respectively). However, the release of the chromophore was slightly slower, 29.3 ± 0.9 min ($p = 0.003$), for F220C Rho (Figure 4D).

Incubation of these proteins at 55 °C in the dark indicated similar thermal stability of WT and the V209M mutant with specific half-lives ($t_{1/2}$) of 17.4 ± 1.6 and 18.0 ± 2.3 min, respectively. In contrast, the F220C Rho mutant was less thermally stable [$t_{1/2} = 11.0 \pm 0.7$ min ($p = 0.005$)] (Figure 4E), which may account for its reduced level of accumulation at the plasma membrane (Table 1). Together, the minor effects of the F220C mutation on the function and stability of Rho could potentially account for its mild RP phenotype. In contrast, the V209M mutation has no apparent impact on the function of Rho but has a significantly heightened propensity to misfold and mistraffic in the cell (Figure 3C and Table 1). Thus, these results highlight the variability in the nature and severity of the pathogenic defects caused by RP-associated mutations.

Effects of RP-Causing Mutants on the Dimerization of Rho within Cellular Membranes.

The mutations characterized above fall within TM5, which is likely to mediate the formation of Rho dimers.^{19,32} We therefore sought to determine whether their attenuated expression and trafficking arise from the disruption of native Rho dimers. The low expression levels of P215L and C222R rod opsin prevented reliable measurements of dimerization within cellular membranes. Thus, we restricted our dimerization analysis to V209M and F220C rod opsin, which were previously found to disrupt Rho dimerization *in vitro*.³¹ To determine whether the V209M and F220C mutations disrupt Rho dimerization within cellular membranes, we first employed chemical cross-linking of V209M and F220C RP mutants

and compared them with WT rod opsin after their expression in HEK-293 GnTI⁻ cell membranes. We utilized both a short disuccinimidyl glutarate (DSG) cross-linker (7.7 Å spacer arm) and a longer, cleavable dithiobis(succinimidyl propionate) (DSP) cross-linker (12 Å spacer arm) for the cross-linking reaction, each of which cross-links primary amines. The DSP cross-linker additionally contains a cleavable disulfide (thiol) bond in the spacer arm that could be used to cleave the cross-linked residues. Two receptor molecules could be connected through the cross-linker only if they are in the proximity of one another within the membrane. For WT and each of the mutants, we found that the band intensity of the Rho dimer increased following incubation with either the DSG or DSP cross-linker (panel A or B of Figure 5, respectively) with no apparent differences between the samples. DSP-cross-linked dimers could be reduced with dithiothreitol (DTT), resulting in a decreased intensity of the dimer band, confirming the specificity of the cross-linking reaction (Figure 5B). These results suggest that neither V209M nor F220C mutations perturb the oligomerization of Rho.

To probe the dimerization propensity of V209M and F220C rod opsin within the context of cellular membranes, we also performed a luciferase-based bioluminescence resonance energy transfer (BRET) assay as was described previously.³² Mouse opsin WT and RP mutants were prepared as fusions with Renilla luciferase (Rluc) (donor) or Venus fluorescent protein (acceptor). Then opsin-Rluc (donor) and opsin-Venus (acceptor), either WT or the mutants, were transiently co-expressed in HEK-293 cells at increasing donor:acceptor ratios to assess the specificity of the receptor–receptor interaction. As expected,³² a comparison of WT and the mutants revealed an increase in the intensities of the BRET signals in response to the increased receptor concentration, which indicates the formation of homodimers (Figure S3). BRET signals for WT as well as V209M and F220C mutants were measured at the employed donor:acceptor ratio of 1:5, assuring the protein density at which the BRET signal is not yet saturated. Recorded BRET signals for WT and each mutant were comparable in the presence and absence of 9-*cis*-retinal (Figure 6A), which suggests these variants are each capable of dimerizing. To demonstrate the selectivity of rod opsin–rod opsin interaction, in a control experiment, we assessed the BRET signal in cells co-expressing WT rod opsin-Rluc (donor) and unrelated protein Kras-Venus (acceptor) (Figure 6B). The intensity of the BRET signal detected in cells expressing WT rod opsin-Rluc and Kras-Venus was ~5-fold lower than the intensity of the BRET signal obtained for WT rod opsin, which confirms this signal detects Rho dimerization. The disruption of rod opsin dimers by DDM also resulted in a gradual decrease in the intensity of the BRET signal (Figure 6C). Thus, the similarity in the observed BRET signals in cells expressing V209M and F220C mutants and the BRET signal observed for WT rod opsin and 9-*cis*-retinal-bound isoRho suggests that these mutants do not significantly perturb the dimerization.

To further investigate the role of dimerization of rod opsin RP mutants, we turned to fluorescence correlation spectroscopy (FCS), which can measure the density and mobility of molecules at low concentration.^{47,53} This allows us to quantify the diffusion and expression level of rod opsin in our model cell line, which is not possible with the BRET assay described above. We also perform a two-color FCS experiment called PIE-FCCS, which can assess the stable association of rod opsin into dimeric complexes (Figure 7A,B).³² Fluorescent rod opsin mutants were prepared as fusions with EGFP or mCherry and

transiently expressed in Cos-7 cells (Figure 7B,E). The FCS data were used to quantify the membrane density of the receptors, which varied between 500 and 2000 molecules per square micrometer. To quantify dimerization, we compared the amplitude of the cross-correlation to the amplitudes of the autocorrelation functions (Figure 7B, Figure S4, and Table 2) to calculate the fraction correlated, f_c . Strong dimerization for a Src13-GCN4 dimer control had a measured f_c value of 0.13, while the Src16 monomer control remained closer to zero at 0.01 (Figure 7C). The f_c distribution of WT rod opsin had a median value of 0.11, which indicated dimerization as reported previously.³³ The f_c distributions of the V209M and F220C mutants were statistically identical to that of WT, with median values of 0.11 and 0.12, respectively, indicating that the dimerization affinity in live cells was not affected by the mutations. The effective diffusion coefficients, D_{eff} , of WT rod opsin and the V209M and F220C mutants with median values of 0.52, 0.58, and 0.52 $\mu\text{m}^2/\text{s}$, respectively (Figure 7D), showed only modest differences, consistent with the idea that the mutations do not effect dimerization. Taken together, the results obtained from cross-linking, BRET, and PIE-FCCS experiments indicated that V209M and F220C mutations did not affect the dimerization of rod opsin in the cell plasma membrane.

DISCUSSION

More than one hundred point mutations associated with progressive retinal degeneration disorders such as adRP have been identified in the Rho gene.^{24–26,54} The molecular basis of disease has been identified for many of these mutations.⁵⁵ However, there are still several mutations known for which the pathogenic mechanism is not fully understood. A number of RP-linked point mutations, including V209M, H211R, P215L, F220C, and C222R, are located on the outer surface of TM5 at positions that could potentially disrupt the formation of native homodimers.^{18,19,30,56} Therefore, we aimed to elucidate if RP-linked mutations located at the Rho dimer interface affect receptor membrane trafficking and function or disrupt the self-association of Rho in a manner that leads to a disease phenotype. Of these mutations, P215L and H211R have been previously found to destabilize the native Rho structure.^{57–59} Thus, for these mutations, dimerization defects are not likely to constitute a primary cause of the disease. In fact, substitutions at H211 were found to cause significant structural perturbations as a result of the disruption of native H-bonding interactions.⁵⁷ For this reason, we excluded the H211R mutant from our studies. In the case of P215L, mutations of the proline are likely to influence a native kink in the trans-membrane α -helix in a manner that may destabilize the native fold.^{60,61} Arrest in the ER and/or improper membrane trafficking has also been previously reported for C222R. The addition of a non-native charged residue in C222R could potentially compromise the native tertiary structure or the translocon-mediated membrane integration of TM5 in a manner that may lead to enhanced misfolding and arrest in the ER.⁶² Therefore, the molecular basis of the disease phenotype associated with H211R, P215L, and C222R is unlikely to arise from aberrant Rho dimerization. Indeed, our results revealed that both P215L and C222R were expressed at much lower levels and did not travel properly to the plasma membrane in agreement with the previous reports.^{28,29} V209M and F220C, therefore, remained the only RP-linked mutations on TM5 for which the mechanism of the associated disease could not be explained. However, on the basis of comprehensive investigations of the effects of these mutations on

cellular trafficking, biochemical function, and Rho dimerization, we provide an explanation for the RP etiology associated with the V209M and F220C substitutions in the Rho gene.

Similar to other GPCRs, Rho exists as dimeric/oligomeric assemblies in the native membranes, and the functional significance of this supramolecular architecture has been demonstrated in multiple studies.^{12,18,19,56,63–66} The pathological consequences of disrupted receptor dimerization have also been demonstrated for many other GPCRs.^{8–10} Thus, defective dimerization in V209M and F220C Rho could potentially explain the RP pathology associated with these mutations. In recent work, the V209M and F220C mutants were shown to affect how detergent-solubilized monomers interact with POPC/POPG lipid vesicles.³¹ Both mutants reconstituted as monomers compared to WT Rho, which reconstituted as a dimer. These results led to the hypothesis that the mutations affect dimerization in cells. However, our results obtained from chemical cross-linking, BRET, and PIE-FCCS experiments indicate that neither V209M nor F220C mutants showed any difference in oligomerization state relative to WT rod opsin in the context of cellular membranes. The discrepancy between these experimental observations could potentially arise from the fact that *in vitro* reconstitution experiments may not fully reproduce the complexity of biological membranes. First, it should be noted that the lipid environment varies greatly between native plasma membranes and synthetic vesicles, which lack both the native lipid composition and asymmetry of cellular membranes. Another factor is the stability of the reconstituted proteins, which can be detrimental for destabilized variants. Indeed, our studies provide the first evidence that the F220C mutant decays faster at high temperature than WT Rho does, which is indicative of its lower stability.

Cumulatively, our observations suggest subtle changes in protein folding and stability likely reflect the pathogenic defect associated with the F220C Rho mutant rather than differences in oligomerization. In the case of V209M Rho, neither disrupted dimerization nor defective biochemical properties could account for the RP phenotype, because this variant is indistinguishable from WT Rho. However, our quantitative flow cytometry-based analysis of Rho trafficking to the cell membrane demonstrated that the V209M mutation leads to mild protein misfolding resulting in its substantial (~55%) trapping in the secretory pathway. This ER arrest could, however, be corrected by supplementation with excess isochromophore 9-*cis*-retinal.^{26,51} In fact, an unfavorable apparent free energy change for membrane insertion (>0.5 kcal/mol) calculated for both V209M and F220C Rho was shown before,³⁰ which supports our findings that these mysterious mutations may reduce the yield of the functional protein in a manner that reduces the level of cellular trafficking to the plasma membrane. Because proper expression and folding are critical for structural development of photoreceptor rod outer segments,^{4–7} such aberrant transport of these Rho mutants to the disc membranes could compromise the accurate architecture and stability of photoreceptors. To further test this hypothesis, future work should determine how cell type (cultured cells vs photoreceptors in the retina of the eye) and plasma membrane lipid composition affect the dimerization propensity and trafficking of WT Rho and RP-associated mutants.

Furthermore, V209M and F220C Rho are present in the general population at a frequency of >1 in 80000, which possibly is too frequent to be a cause of high-penetrance dominant RP.⁵⁴ Moreover, genetic studies have demonstrated that V209M and F220L Rho did not segregate

with disease in families.^{67–69} Thus, it is uncertain if these mutations are directly pathogenic or rather if the subtle defects in V209M or F220C Rho mutants found in this study might play a role in late onset disease or as disease modifiers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Location of RP-causing mutations at the surface of the Rho dimer. (A) Sequence alignment of mouse and human Rho. Only a sequence fragment composed of 181–240 amino acids that includes residues of TM5 is shown. TM5 is colored cyan in both Rho molecules. Mutations located in TM5 causing RP are colored: green for V209M, magenta for H211R, blue for P215L, red for F220C, and yellow for C222R. The symbols below the sequences indicate conserved amino acids (asterisks) and conservative mutations (colons). There is only one residue difference at position 218 between mouse and human Rho within helix TM5. (B) Three-dimensional model of the Rho dimer (Protein Data Bank entry 1N3M): side view (left) and top view (right). Mutations at the dimer interface causing RP are shown with colored spheres; green for V209M, magenta for H211R, blue for P215L, red for F220C, and yellow for C222R.

Figure 2.

Expression of RP-causing Rho mutants located at the dimer interface and their localization in HEK-293 GnTI⁻ cells. (A) Immunoblot indicating expression levels of WT, V209M, P215L, F220C, and C222R rod opsins transiently expressed in HEK-293 GnTI⁻ cells. Fifty micrograms of total protein cell lysate obtained 48 h post-transfection was loaded on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. Rho was detected with an anti-Rho C-terminal 1D4 tag antibody. GAPDH was the protein loading control. The representative immunoblot is shown (left). The expression levels of WT and RP-causing Rho mutants were quantified from three independent experiments as band intensities normalized to the GAPDH expression by using ImageJ (right). (B) Membrane localization of WT and RP-causing Rho mutants detected by Venus fluorescence in living cells. Immunolocalization of Na/K ATPase was used here as a plasma membrane localization marker. Nuclei were stained with DAPI. The merged image of all three panels shows co-localization of Rho and Na/K ATPase. The scale bar is 7.5 μ m.

Figure 3.

Effect of RP-causing mutations on the cellular trafficking of Rho. WT and rod opsin mutants were transiently expressed in HEK-293 cells, and the cellular trafficking of each mutant was quantitatively assessed using flow cytometry. Contours depict the distribution of cellular fluorescence intensities associated with the differential immunostaining of the mature protein at the plasma membrane and immature intracellular protein among cells expressing (A) V209M (green), (B) P215L (blue), (C) F220C (red), and (D) C222R (yellow) rod opsins. The distribution of intensities among cells expressing WT rod opsin is shown in black for

reference. Histograms depict the distribution of cellular fluorescence intensities associated with the immunostaining of mature (E) V209M (green) and (F) F220C (red) rod opsins at the plasma membrane in the presence and absence of 5 μ M 9-*cis*-retinal.

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Figure 4.

UV-vis spectra and biochemical properties of RP-causing Rho mutants located at the dimer interface. (A) UV-vis absorption spectra of WT Rho (black), V209M (green), and F220C (red) expressed in HEK-293 cells, regenerated with 11-*cis*-retinal, and purified by immunoaffinity chromatography are shown. Compared to that of WT, substitutions of V209 with M and F220 with C do not affect absorption maxima. (B) , Photosensitivity assay results for WT Rho (black) and RP-causing Rho mutants V209M (green) and F220C (red). Absorption spectra were recorded for WT, V209M, or F220C Rho after illumination with a Fiber-Light through a band-pass filter (480–520 nm) for different periods of time. The decline in absorption maxima at 498 nm was plotted as a function of illumination time. The $t_{1/2}$ of rhodopsin bleaching was calculated from these plots. (C) Results of a G_t activation assay of WT (black) and RP-causing Rho mutants V209M (green) and F220C (red) are shown by the time course of the intrinsic Trp fluorescence change at 345 nm due to guanylyl

nucleotide exchange. The pseudo-first-order kinetic rates (k) of G_t activation were derived from the function $A(t) = A_{\max}(1 - \exp^{-kt})$, where $A_{\max}$ is the maximal G_t fluorescence change and $A(t)$ is the relative fluorescence change at time t . (D) Chromophore release (Meta II decay) of WT Rho (black) and RP-causing mutants V209M (green) and F220C (red) is shown by the time course of changes in the intrinsic Trp fluorescence at 330 nm. (E) Thermal stability of WT Rho (black) and RP-causing mutants V209M (green) and F220C (red) after samples had been incubated at 55 °C. UV-vis absorption spectra were recorded every 5 min in the dark. The percentages of remaining pigments normalized to their initial concentrations were then plotted as a function of time. The $t_{1/2}$ of chromophore release was calculated from these plots. Each experiment was performed in triplicate.

Figure 5.

Effect of RP-causing mutations on Rho cross-linking. (A and B) Effect of RP-causing mutations on the formation of DSG-cross-linked and DSP-cross-linked opsin rod dimers. Cross-linking reactions were performed with either a DSG or a DSP cross-linker within the membranes on the surface of HEK-293 GnTI⁻ cells transiently expressing WT rod opsin or RP mutants. Fifty micrograms of the total protein extracts was loaded on each lane of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and then immunoblotted onto a polyvinylidene fluoride membrane (left). Rho was detected with an anti-Rho C-terminal 1D4 tag antibody. The cross-linking experiments were repeated three times, and representative immunoblots are shown. Band intensities corresponding to the rod opsin dimer and monomer were determined by densitometric analyses from three independent experiments by using ImageJ. Efficiencies of DSG or DSP cross-linking are shown as the percentage of receptor dimer before and after cross-linking (right). The DSP cross-linker contains a cleavable disulfide bond in its spacer arm; thus, the specificity of the cross-linked dimer was tested by the dimer disruption with the DTT reducing agent.

Figure 6.

Effect of RP-causing mutations on Rho dimerization. (A) The effect of selected RP-causing mutations located within TM5 was tested with the BRET assay. The BRET signal was recorded in HEK-293 cells co-transfected with vectors expressing opsin•Rluc (donor) and rod opsin•Venus (acceptor) or rod opsin•Rluc (donor) only. Net BRET was calculated as the emission ratio at 530 nm to 480 nm (BRET1 signal) subtracted by the emission of the donor only at 480 nm. Net BRET = (530 nm/480 nm ratio – 480 nm). (B) The BRET signal recorded in HEK-293 cells co-transfected with vectors expressing WT opsin•Rluc (donor)

and Kras-Venus (acceptor) was used as a negative control. (C) The decrease in the intensity of the BRET signal with increasing concentrations of DDM is due to the disruption of opsin dimers. Each experiment was performed in triplicate.

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Figure 7.

Effect of RP-causing mutations on the dimerization of Rho in live cell membranes tested with PIE-FCCS. (A and B) Instrument scheme and data analysis performed for PIE-FCCS. Cos-7 cells co-transfected with EGFP- and mCherry-fused Rho constructs are illuminated with 488 and 561 nm excitation beams. Intensity fluctuations are then recorded for calculation of the correlation functions. Autocorrelation (ACF) and cross-correlation (CCF) plots generated from the data are fit to Brownian diffusion models described in the text. The maximum amplitude of the ACFs and that of the CCF are used to calculate f_c values, which quantify the degree of dimerization. Diffusion coefficients are calculated using the dwell times, τ_D , and excitation beam waist. (C) The relative cross-correlation values, f_c , from single-cell PIE-FCCS measurements are shown as gray circles for negative and positive dimer controls, Src₁₆ and Src₁₃-GCN4, respectively, WT rod opsin, and the RP-causing mutants. The f_c values of mutants did not show a significant change compared with that of WT. The number of single-cell measurements is displayed above the respective distributions. Overlaid box and whisker plots indicate the medians (red line), notches (95% confidence interval of the median), percentiles (box, interquartile; whiskers, all points except outliers), and outliers (red crosses). Differences among WT, V209M, and F220C distribution sets were not statistically significant as determined by the Kruskal–Wallis test. (D) Diffusion coefficients of the EGFP-labeled proteins show no significant changes between WT rod

opsin and mutants. (E) Membrane expression of WT, V209M, and F220C rod opsin mutants fused to EGFP or mCherry fluorescent proteins after transient transfection of Cos-7 cells. Scale bars are 10 μm .

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Table 1.

Cellular Trafficking of WT Rho and Rho Mutants

	with vehicle			with 5 μ M 9- <i>cis</i> -retinal		
	relative surface immunostaining ^a	relative intracellular immunostaining ^a	relative total immunostaining ^a	relative surface immunostaining ^b	relative intracellular immunostaining ^b	relative total immunostaining ^b
WT	–	–	–	1.27 $\pm$ 0.02	1.38 $\pm$ 0.01	1.33 $\pm$ 0.01
V209M	0.45 $\pm$ 0.01	0.75 $\pm$ 0.03	0.61 $\pm$ 0.02	2.7 $\pm$ 0.1	1.60 $\pm$ 0.06	1.95 $\pm$ 0.03
P215L	0.02 $\pm$ 0.01	0.68 $\pm$ 0.01	0.38 $\pm$ 0.02	– ^c	1.2 $\pm$ 0.2	1.2 $\pm$ 0.2
F220C	0.86 $\pm$ 0.09	1.01 $\pm$ 0.04	0.95 $\pm$ 0.05	1.6 $\pm$ 0.1	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1
C222R	0.01 $\pm$ 0.01	0.72 $\pm$ 0.05	0.40 $\pm$ 0.02	– ^c	1.09 $\pm$ 0.09	1.11 $\pm$ 0.09

^aIntensity measurements were normalized relative to the corresponding value of WT. Numbers reflect the average value from three biological replicates, and error values reflect the standard deviation.

^bIntensity measurements in the presence of 9-*cis*-retinal were normalized relative to the corresponding value for each mutant in the absence of 9-*cis*-retinal. Numbers reflect the average value from three biological replicates, and error values reflect the standard deviation.

^cReliable estimates of these ratios could not be determined because of the low intensity values associated with these measurements.

Table 2.

Quantification of Rho Dimerization in the Cellular Membrane

parameters extracted from PIE-FCCS data of WT Rho and mutants	WT Rho	V209M	F220C
f_c (mean) ^{ah}	0.114 ± 0.006	0.111 ± 0.006	0.115 ± 0.007
f_c (median) ^b	0.107	0.109	0.109
D_{eff} ($\mu\text{m}^2/\text{s}$) ^{ch}	0.525 ± 0.022	0.585 ± 0.021	0.551 ± 0.025
$\langle N_R \rangle$ ^{dh}	53 ± 3	39 ± 3	28 ± 3
$\langle N_G \rangle$ ^{dh}	45 ± 3	36 ± 2	31 ± 2
density (mol/ μm^2) ^{eh}	878 ± 46	676 ± 37	518 ± 41
η_R (cpm) ^{fh}	456 ± 10	486 ± 7	530 ± 9
η_G (cpm) ^{fh}	420 ± 15	390 ± 7	365 ± 7
$\tau_{D,R}$ (μs) ^{gh}	35 ± 1.4	29 ± 0.7	27 ± 0.8
$\tau_{D,G}$ (μs) ^{gh}	26 ± 1.4	23 ± 1.2	23 ± 1.1
$\tau_{D,X}$ (μs) ^{gh}	306 ± 32	277 ± 38	325 ± 57

^a Average f_c calculated using data shown in Figure 7.^b Median f_c found for same data set.^c Effective diffusion coefficients calculated using $\tau_{D,G}$, a parameter collected from FCS curve fits.^d Average molecular counts calculated by taking the inverse of autocorrelation amplitudes, $G_i(0)$.^e Effective molecular densities calculated by dividing the sum of both $\langle N_R \rangle$ and $\langle N_G \rangle$ by the product of π and the average beam waist radius squared.^f Molecular brightness calculated by dividing average counts per second of a channel by its respective $\langle N_i \rangle$ ^g Dwell times, $\tau_{D,i}$, calculated during the FCS data fit.^h Distribution of values displayed as means ± the standard error of the mean.