Mutations in Rhodopsin Transmembrane Helix 5 and Dimerization Interface

Margaret Pinkevitch
mvp19@zips.uakron.edu

Please take a moment to share how this work helps you through this survey. Your feedback will be important as we plan further development of our repository.

Follow this and additional works at: http://ideaexchange.uakron.edu/honors_research_projects

Part of the Biochemistry Commons, and the Other Biochemistry, Biophysics, and Structural Biology Commons

Recommended Citation
Pinkevitch, Margaret, "Mutations in Rhodopsin Transmembrane Helix 5 and Dimerization Interface" (2018). Honors Research Projects. 693.
http://ideaexchange.uakron.edu/honors_research_projects/693

This Honors Research Project is brought to you for free and open access by The Dr. Gary B. and Pamela S. Williams Honors College at IdeaExchange@UAkron, the institutional repository of The University of Akron in Akron, Ohio, USA. It has been accepted for inclusion in Honors Research Projects by an authorized administrator of IdeaExchange@UAkron. For more information, please contact mjon@uakron.edu, uapress@uakron.edu.
Mutations of Rhodopsin Transmembrane Helix 5 and Dimerization Interface

Margaret Pinkevitch

3150:497

April 27, 2018
Mutations in Rhodopsin Transmembrane Helix 5 and Dimerization Interface

Margaret Pinkevitch

University of Akron

Abstract: G protein coupled receptors (GPCRs) are membrane proteins with many functional roles. This family of receptors is expansive, with several subfamilies. Here, a member of subfamily A, rhodopsin (Rho) is studied. Mutations in rhodopsin have been linked to the disease retinitis pigmentosa (RP). Some of these mutations have been found to disrupt the dimerization/oligomerization of Rho at the TM4/TM5 proposed dimerization interface, causing pathogenesis of RP. In this study, two mutations of transmembrane helix 5 were investigated for changes in dimerization and stability, cell trafficking, and signaling. It was found that the V209M and F220C mutations did not result in disruption of dimerization of Rho.

Abbreviations: GPCR, G protein-coupled receptor; Rho, rhodopsin; RP, retinitis pigmentosa; TM, transmembrane helix/helices; GDP, guanine diphosphate; GTP, guanine triphosphate; PIE-FCCS, Pulsed-interleaved excitation fluorescence cross-correlation spectroscopy; GFP, green fluorescent protein.

Introduction

Heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors, or GPCRs, are a large family of cell surface receptors responsible for cell signaling. Understanding GPCRs is integral for modern medicine, as GPCR signaling regulates bodily functions including sensation, growth, and hormonal responses. There are several subfamilies of GPCRs categorized
by intracellular response activation to variant extracellular stimuli. Some extracellular stimuli GPCRs respond to include light and small molecules such as hormones, cytokines, neurotransmitters, and other ligands. GPCRs have 7 transmembrane α-helices that associate with heterotrimeric G proteins: α, β, and γ. The inactive $G_\alpha$ binds GDP and is complexed with β and γ. When ligand binds the GPCR and becomes activated, $G_\alpha$ exchanges GDP for GTP and dissociates from βγ. The G proteins activate or inhibit various cell signaling pathways. Subfamily A accounts for about 90% of GPCRs, rhodopsin belongs to this subfamily.

Rhodopsin (Rho) was the first to have structure resolved through X-ray crystallography and serves as a model for structural study of GPCRs, as GPCRs are highly conserved. Rhodopsin is a protein found in the retina that is essential for vision in low light conditions. Mutations in rhodopsin have been found to result in retinitis pigmentosa, a degenerative disease of the retina. Several Class II mutations have been identified, in which protein misfolding or instability causes RP pathogenesis. Understanding the pathophysiology of mutations of rhodopsin could help in targeting the disease for treatment.

Research evidence shows that GPCRs can form dimers and oligomers. However, GPCR dimerization functionality and stability is still unclear. Rhodopsin transmembrane helices 4 (TM 4) and 5 (TM 5) interactions are thought to be a site of dimerization interface. The goal of this project was to introduce site-specific mutations of specific amino acids involved in the proposed TM4/TM5 rhodopsin dimerization interface. In this study, the V209M and F220C mutations in Rho were investigated for their effects on dimerization.

PIE-FCCS (or pulsed-interleaved excitation fluorescence cross-correlation spectroscopy) was used in the determination of oligomerization state of the Rho mutants. Use of PIE-FCCS allows for study of rhodopsin interactions in a live cell membrane. In this study, mammalian
cells are used to study murine rhodopsin oligomerization. This technique counts photons, with time delayed laser pulses at two wavelengths. The laser emits the pulses to excite different fluorescently tagged proteins such as mCherry (red at 561 nm) and eGFP (green at 488 nm), as used in this study. The cross-correlation data collected would allow for the determination of potential oligomerization states of Rho.\textsuperscript{10,11}

My role in this project was focused on the culturing of mammalian cells and transfection of cells with mutant or wild-type plasmid DNA. The cells cultured were Cos-7 (African green tree monkey). Transfected cells were then analyzed via PIE-FCCS to determine if rhodopsin dimerization occurred.

**Experimental Methods**

**Constructs:**

The rod opsin-EGFP and rod opsin-mCherry constructs were made by amplifying mouse opsin cDNA using PCR. EcoR1 and BamH1 restriction sites were added at the 5’ and 3’ ends. For opsin-EGFP, the forward primer: GTGGGGAATTCGCCATGAACGGCACAGAGGG and the reverse primer: TCTGGGGATCCGCTGGAGCCACCTGG were used to do this. For opsin-mCherry, the forward primer: GTGGGGAATTCGCCATGAACGGCACAGAGGG and the reverse primer: TCTGGGGATCCCGCTGGAGCCACCTGG were used. The pEGFP-N3 and pmCherry-N1 vectors (Clontech, Mountain View, CA) were used for cloning of respective amplified DNA. The Groves Lab at UC Berkeley provided Src\textsubscript{16}-eGFP/mCH and Src\textsubscript{13}-GCN4-eGFP/mCH plasmids used as negative and positive dimer controls, respectively.
Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) was used to construct the RP-causing rod opsin mutants according to the manufacturer’s protocol. The composition of each construct was confirmed by Sanger DNA sequencing (GeneWiz, Inc).

**Cos7 cell culture:**

The Cos7 cells used in these experiments were obtained from ATCC (CRL-1651). Cos7 cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM 1X + GlutaMAX, Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS, Life Technologies, Hyclone, Logan, UT) and 1% penicillin/streptomycin (BioReagent, Sigma-Aldrich, St. Louis, MO). Cultures were incubated at 37°C with CO2 at ~5-10%, with 100% humidity. Cos7 cells were cultured on 100×20 mm tissue culture plates (Falcon, Corning Inc., Corning NY). Cell cultures were split at ~80-90% confluency and were not split above passage 18.

**Cos7 transfection and prep for imaging:**

Cos7 cells were split into 35×10 mm gamma-irradiated glass bottom culture dishes (MatTek, Ashland, MA) for transfection and imaging. For transient transfection, Lipofectamine 2000 transfection reagent (Life Technologies) was used (2.5 µL) along with DMEM media with reduced phenol red (~31.25 µL). About 180 ng of each fluorescent plasmid were used in each dish. This procedure was done the day prior to imaging, allowing for ~24 hours of incubation.

In preparation for imaging, transfected cells were washed with PBS and then Opti-MEM I media without phenol red (Life Technologies). This was done approximately 1 hour prior to PIE-FCCS imaging and data collection.
Figure 1: Shows the custom scope set-up for PIE-FCCS experiments. Taken from reference 12.

**Pulsed-interleaved excitation-fluorescence cross-correlation spectroscopy (PIE-FCCS):**

PIE-FCCS experiments were conducted on a custom-built instrument, with procedure as described by Smith (2015). A scheme of this spectroscopy is shown above (Figure 1). There are two pulsed excitation beams at 488 nm and 561 nm (LL01-488-12.5 and LL02-561-12.5, Semrock, Rochester, NY), ideally targeting eGFP and mCherry fluorescent proteins, respectively. These beams are split and filtered, coming from a supercontinuum fiber laser (SuperK NKT Photonics, Birkerød, Denmark). The laser pulses are temporarily separated by ~50 ns to prevent false-positives in cross-correlation. The two beams are then overlapped by a dichroic beamsplitter (LM01-503-25, Semrock) and redirected by a dichroic mirror (zt488/561rpc, Chroma Technology Corp., Bellows Falls, VT) to the microscope, where the excitation beams are direct to the objective.
and resulting emission signals are collected, then passes through a laser-blocking filter (z488/561m, Chroma Technology Corp., Bellows Falls, VT) and then directed through the 50µm pinhole (Thorlabs). A long-pass filter (FF560-FDi01-25x36, Semrock) then splits the emission beam(s) into red and green signals, two bandpass filtered (FF01-621/69-25 and FF01-520/44-25 Semrock) SPAD detectors (Micro Photon Devices, Bolzano, Italy) where photon counts are measured. A time-correlated single-photon counting (TCSPC) device (Picoharp 300, PicoQuant, Berlin, Germany) was used to record and process the raw fluctuating fluorescence data.

Results and Discussion

Constructs:

The suspected RP-causing mutants constructed were V209M and F220C. These mutations are located on TM5, and are illustrated in the figure below (Figure 2). Murine cDNA was used in constructs because of their homology to human Rho (~95%). The proposed dimerization interface implicates TM 4 and TM 5. Based on this, it was investigated whether the V209M and F220C mutations disrupted the dimerization interface and were causatively implicated in pathogenesis of retinitis pigmentosa. These constructs were transiently transfected into Cos7, mammalian cells. The expression of these constructs was observed through PIE-FCCS experiments.
Figure 2: Shows the proposed TM4/TM5 dimerization interface of Rho. Amino acid sequence of TM5 of murine and human Rho is shown, along with locations of mutations V209M and F220C. The crystal structure shows TM5 locations of V209M and F220C mutations. Suspected RP-causing mutation V209M colored navy blue. Suspected RP-causing mutation F220C colored red.

**Cos7 cell transfection:**

The Cos7 cell line is an immortalized line of African green tree monkey kidney cells. These cells were chosen for these experiments for their large, uniform peripheral membrane; as the natural layout of the cells allows for ease of observing interactions in the plasma membrane in optical imaging. In Figure 3, transfected Cos7 cell images are shown for wild-type and mutations V209M and F220C.
Figure 3: This figure shows the fluorescent expression of mCherry and eGFP tagged proteins in wild-type Rho, V209M mutant Rho, and F220C mutant Rho. Cos7 cells were transiently transfected with respective constructs.

**Pulsed-interleaved excitation-fluorescence cross-correlation spectroscopy (PIE-FCCS):**

PIE-FCCS experiments enabled the quantification of dimerization affinities in the rhodopsin constructs. Transiently transfected Cos7 cells were observed with the custom scope, as mentioned previously and detailed in publication (10). Wild-type Rho, V209M mutant Rho, and F220C Rho were used and compared. Fluorescent tags were added to Rho, either mCherry (red) or eGFP (green). If dimerization occurred, cross-correlation would be observed in the PIE-FCCS data collected. Figure 4 shows representative PIE-FCCS curves, where wild-type in comparison to mutants V209M and F220C show no significant difference. These mutations did not disrupt the dimerization of rhodopsin.
Figure 4: This figure shows PIE-FCCS results as cross-correlation curves. The green indicates eGFP, red indicates mCherry, and blue for cross-correlation. These curves show that there was no observable difference between wild-type Rho and the RP-causing mutants: V209M and F220C. This means that dimerization was not disturbed because of these mutations.\textsuperscript{13}
Conclusion

In this project, rhodopsin mutations were investigated in whether their retinitis pigmentosa pathogenic effect was based on the disruption of rhodopsin dimerization. The transmembrane helices 4 and 5 (TM4 and TM5) have been proposed as a dimerization interface of rhodopsin previously.\textsuperscript{7,9} The RP-causing mutations V209M and F220C are located on TM5 of rhodopsin. Constructs were made, as described previously, of these mutations as plasmid DNA with fluorescent fusion proteins mCherry and eGFP. Mammalian cells were cultured, and then transiently transfected with plasmid DNA. Expression and cross-correlation in Cos7 cells was obtained and measured through PIE-FCCS experiments. There was no difference in rhodopsin dimerization found between wild-type rhodopsin and RP-causing mutants V209M and F220C, based on the data collected in this project. The mechanism of these mutations in retinitis pigmentosa remains unclear.

Acknowledgements

Thanks to the members of Jastrzebska and Smith laboratories. Laboratory training was provided by University of Akron graduate student William D. Comar. Running and processing data for PIE-FCCS experiments, as well as PIE-FCCS data for figures in this report were provided by University of Akron graduate student D. Paul Mallory. Funding and equipment were provided by Dr. Adam W. Smith of the Chemistry Department at the University of Akron. The supportive funding for this work was from the National Institutes of Health EY024451 (AWS), EY025214 (BJ) and P30EY11373 from the VSRC CORE grant.
References


(8) Gurevich, V. V.; Gurevich, E. V. *Trends Pharmacol. Sci.* **2008**, *29* (5), 234–240.


(13) Submitted: Mallory, D.P.; Gutierrez, E; Pinkevitch, M.; Klinginsmith, C.; Comar, W.D.; Roushar, F.J.; Schlebach, J.P.; Smith, A.W.; Jastrzebska, B. *Biochemistry* **2018**.
Appendix I- Safety

Laboratory Training: Laboratory equipment and protocol training were provided by University of Akron graduate student William D. Comar. Training included mammalian cell culture, transient transfection protocol, transformation, and other laboratory molecular biology experiments. The University of Akron graduate student D. Paul Mallory conducted PIE-FCCS experiments and collected data.

Biohazard Safety: Gloves were worn when working with biological substances. Sterile protocol was followed while working with live mammalian cell cultures. All glassware and pipette tips that encountered any biological substances were discarded in separate, biohazard waste containers. Liquid biological waste was bleached to prevent further growth before being disposed of. The Department of Environmental and Occupational Health and Safety disposed of biohazard waste containers.