Short Hydrocarbon Chain Lipids Enable Class A G-Protein-Coupled Receptor Isolation in its Active Conformation: Eliminating the Need for Detergents

Amanda N. Narango1, John Katsaros2, Anne S. Robinson1,3
1University of Delaware, Newark, DE, USA, 2Oak Ridge National Laboratory, Oak Ridge, TN, USA, 3Tulane University, New Orleans, LA, USA.

G protein-coupled receptors (GPCRs) represent the largest family of integral membrane receptors and are involved in many intracellular communications in response to diverse external stimuli. Despite advances in GPCR biophysical characterization, a major challenge continues to be the isolation of large quantities of membrane protein in functional form. Here we report the purification of a GPCR, namely the human adenosine receptor A2A, using solely the short hydrocarbon chain lipids of membrane protein in functional form. Here we report the purification of a GPCR, namely the human adenosine receptor A2A, using solely the short hydrocarbon chain lipids of membrane protein in functional form. Here we report the purification of a GPCR, namely the human adenosine receptor A2A, using solely the short hydrocarbon chain lipid 1,2-dihexanoyl-snglyceryl-1-phosphocholine (DHPC, d6:0PC). After purification, the receptor remains folded and capable of binding activity and no detergent removal step was necessary. Additionally, this approach will enable the addition of longer chain lipids create biomimetic membrane systems (e.g. bilayered micelles) that can be oriented in the presence of a magnetic field, which are widely used in NMR studies.

Membrane Curvature Regulates the Oligomerization of Human β3-Adrenergic Receptors

Signe Mathiasen1, Asger Tonnesen1, Sune Christensen1,2, Juan Jose Fung3, Søren Gøgsig Faarup Rasmussen1, Ernesto Borrero2, Davide Provasi4, Marta Filizola4, Brian Kobilka3, Dimitrios Stamou1.

School of Medicine, 279 Campus Drive, Palo Alto, CA, USA, 4Department of Biomembranes in Nanomedicine, University of Copenhagen, Chemistry, Nano-Science Center, Lundbeck Foundation Center of Excellence, 1University of Delaware, Newark, DE, USA, 2Oak Ridge National Laboratory, UC Berkeley, CA, USA.

The topology of cell membranes attracts increasing attention as a potential regulator of numerous vital cellular functions such as protein sorting, association and localization [1,2,3]. To examine the influence of membrane shape on GPCR oligomerization we developed a fluorescence-based assay, employing a model membrane system with the prototypical GPCR, human β2-adrenergic receptor, β2AR, reconstituted in liposomes [4]. We monitored receptor oligomerization by intermolecular FRET between β2AR-Cy3 and β2AR-Cy5 in membranes of different curvature (75-400 nm in diameter) at the single proteoliposome level by the use of confocal microscopy.

Many membrane proteins are proposed to work as oligomers, however, the conclusion is sometimes controversial, as for β2-adrenergic receptor (β2AR), which is one of the best-studied family A G-protein-coupled receptors. This is due to the lack of methods for precise detection of oligomerization state of membrane proteins on living cells. Fluorescence and bioluminescence resonance energy transfer (FRET and BRET) have been widely used as a nondestructive indicator of interaction among target proteins in living cells. Although most studies use fluorescent proteins or luminescent proteins to label the target proteins, these methods have limitations for the quantitative analysis of protein oligomerization.

We have recently developed the novel coiled-coil tag-probe method to overcome these problems [1-3]. The coiled-coil tag-probe labeling method utilizes tight interaction between the heterodimeric coiled-coil peptides E3 (EIAAKE3) and K4 (KIAALK4). The E3 peptides were chemically synthesized and labeled at their N-termini with either the donor dye Alexa Fluor 568 or the acceptor dye Alexa Fluor 647 to obtain Alexa568-K4 (donor) or Alexa647-K4 (acceptor), respectively. The addition of the K4 probes can label the E3-tagged receptors expressed on living cells within 1 min. Here using the coiled-coil labeling method combined with in-cell fluorescence spectroscopy, we succeeded in determining the oligomerization states of several standard membrane proteins. Having validated the method, we examined the oligomeric states of β2ARs and influenza virus A M2 ion channel.

Electron Crystallography of Euglenoid Four-Transmembrane Protein Revealed the Linear Polymerization by a Combination of Three-Ways of Intermolecular Interaction

Hiroshi Suzuki1, Yasuyuki Ito1, Kazutoshi Tani1, Yuji Yamazaki2, Masami Uji1, Katsuhiko Mineta1, Sachiko Tsukita1, Yoshinori Fujiyoshi1.

1Nagoya University, Nagoya, Japan, 2Osaka University, Suita, Japan.

In Euglena gracilis, cell membranes on the ridge regions of the striped surface structure are covered with paracrystalline arrays, mainly composed of the integral membrane protein, called IP39. It has recently been cloned as a putative four-membrane-spanning protein with a conserved sequence motif of PMP-22/EMP20/Claudin superfamily. Here, we report the three-dimensional structure of Euglena IP39 at 7 Å resolution determined by image-based electron crystallography of two-dimensional (2D) crystals. The 2D crystal array of IP39 appears to be a striped pattern of the antiparallel double-rows, in which the trimeric units of IP39 are longitudinally polymerized, resulting in continuously extending zigzag lines. Each protomer shows an overall cylindrical density (approximately 20 Å in diameter) with ‘nook’ and ‘pointed’ shapes at the respective sides of the membrane. Structural analysis of another 2D crystal bound with anti-phosphotyrosine Fab fragments reveals that the ‘nock’-like protruded structure is facing to the cytoplasmic side. A four-helical bundle model is consistently fitted to the EM density map and shows that the transmembrane regions are mainly involved in the intermolecular interactions to form the linear strands. The polymerization pattern of IP39 in the 2D crystal, however, exhibits unexpected interactions between respective protomers. In the trimeric unit of the single strand, one of the three protomers is likely to be rotated at approximately 180 degree in the opposite direction to the others, indicating that there are at least three different ways of possible intermolecular interactions in the transmembrane regions between neighboring protomers. A combination of such multiple interactions would be important for the continuous linear polymerization, thus providing important implications in the strand formation of other four-transmembrane proteins of this family in the lipid environment.

X-Ray Structure of the Cx26 Gap Junction Channel and Comparison with the Cryo-EM Structure of Cx43

Brad Bennett1,2,3, Michael Purdy1,2,3, Kent Baker1, William McIntire1,2, Raymond Stevens3, Qinghai Zhang2, Mark Yeager1,3,4.

1University of Virginia, Charlottesville, VA, USA, 2The Scripps Research Institute, La Jolla, CA, USA.

Intercellular gap junction channels are formed by the end-to-end docking of connexin (Cx) hexamers that traverse apposing cell membranes. Each Cx subunit has four transmembrane (TM) α-helices and two extracellular loops (E1 and E2). Three-dimensional crystals of recombinant, purified Cx26 were grown using a new class of detergents designated facial amphiphiles (FAs), which
have a cholate backbone with polar groups extending from one face and a short alkyl chain extending from the opposite face. FA-solubilized Cx26 crystallized into the top leaflet asymmetric unit, and the crystals diffracted isotypically to 3.3-Å resolution. Using a molecular replacement search model based on a cryoEM map of Cx43 at 5.7-Å resolution [Fleishman et al., Mol. Cell 15: 879-888 (2004)], we solved the structure independently from a previously reported 3.5-Å resolution X-ray structure [2ZW3, Maeda et al., Nature 458: 597-602 (2009)]. The overall R/|R|free values and completeness were 0.311/0.328 and 98.9%, respectively, and the Molproportion score was 2.07 (100th percentile). The RMS differences between 2ZW3 and our structure were 0.9 and 1.7-Å for the main-chain and side-chain atoms in the TM helices and 1.3 and 1.9-Å for the main-chain and side-chain atoms in E1 and E2. Although the topology and fold recapitulated 2ZW3, the maximum differences were significant: 2.7 and 5.9-Å for the main chain and side chain atoms in the TM helices and 3.9 and 7.6-Å for the main-chain and side-chain atoms in E1 and E2. We generated an electron density map at a resolution comparable to the cryoEM structure of the authentic Cx43 channel. The similarity of the maps suggests that detergent-solubilized Cx26 that crystallized as a dodecamer represents the authentic gap junction channel.

217-Plat
Hydrogen Bonds at the Docking Interface are Critical for Functional Gap Junction Channel Formation of Cx26 and Cx32
Xiaopeng Li, Tomonori Masaoka, Toshiyuki Kihara, Donglin Bai
The University of Western Ontario, London, ON, Canada, Osaka Univ., Osaka, Japan.

Gap junctions are unique intercellular channels formed by proper docking of two hemichannels from adjacent cells; each hemichannel is a hexamer of connexins - the gap junction proteins encoded by 21 homologous genes in human genome. Docking of two hemichannels to form a functional gap junction channel is only possible between the hemichannels formed by compatible connexins. The underlying docking mechanism for compatibility is not clear. Based on the crystal structure of Cx26 gap junction channel, we developed homology structural models and generated a series of mutants on extracellular domain 2 (E2). According to the model, 36 hydrogen bonds (HBs) were indentified at E2-E2 interface between a pair of Cx32/Cx26 hemichannels. The HB-forming residues between E2 domains are conserved in Cx32/Cx26 and heterotypically compatible connexins, but not in non-compatible connexins. Asp175 of Cx32 was a pivotal residue forming 3 HBs with K168, T177 and D179 of inter-docked Cx26. Cx32 mutations, N175Y or N175H, destroy 3/3 or 2/3 HBs, respectively, at the E2-E2 docking interface. Experimentally, two mutants failed to form putative gap junction plaques and were unable to form homotypic functional gap junction channels. Morphological and functional tests of various combinations of these mutants with designed mutant and wild-type Cx26 revealed that the hydrogen bonds at the E2 docking interface are critical for docking compatibility in the gap junctions formed by Cx26 and/or Cx32. Restoring more hydrogen bonds at the docking interface was able to rescue the function of Cx32N175H, but not N175Y using designed mutant or wild-type Cx26. Our results demonstrate that HBs at the E2-E2 docking interface are key factors for heterotypic docking compatibility between Cx32 and Cx26 hemichannels and possibly other hemichannels formed by compatible connexins.

Platform: Membrane Physical Chemistry II

218-Plat
Bilayer Asymmetry, Cholesterol Content, and Ligand Binding Influence Membrane Protein Sequestering in Raft-Mimicking Lipid Mixtures
Noor F. Hussain, Yifan Ge, Amanda P. Siegel, Rainer Jordan
1Indiana University-Purdue University Indianapolis, Indianapolis, IN, USA, 2Technical University Dresden, Dresden, Germany.

Lipid heterogeneities and membrane proteins are widely considered to show an important functional relationship in plasma membranes. However, the analysis of this intriguing relationship is challenging, due to the small size and transient nature of lipid heterogeneities in the plasma membrane environment. Recently, we presented a powerful model membrane platform that allows the thorough analysis of membrane protein sequestering and oligomerization in well-defined heterogeneous lipid environments using confocal fluorescence intensity analysis paired with a photon counting histogram (PCH) method (1). By applying this experimental approach, here we show that bilayer asymmetry has a significant influence on the sequestering of α1β3 and αβ2 integrins. Our experiments demonstrate a higher affinity of integrins for the liquid-disordered (lα) phase in the presence of bilayer-spanning liquid-ordered (lβ)-lα phase separations. In contrast, a preference for the lβ phase is observed in an asymmetric bilayer with lβ-lα phase separations, which are exclusively located in the top leaflet. Importantly, PCH analysis shows that the observed changes in integrin sequestering are not caused by altered receptor oligomerization states. In another set of experiments, we also demonstrate that changes in cholesterol may have a profound impact on integrin sequestering without altering receptor oligomerization state. The obtained results are discussed in terms of potential changes in lipid packing density and hydrophobic thickness within the model membrane. The experimental model membrane approach is also applied to explore the functionally important relationship between the sequestering, level of dimerization, and ligand binding of the GPI-anchored urokinase receptor.


219-Plat
Steric Pressure between Proteins Opposes Membrane Phase Separation
Christine S. Scheve, Paul A. Gonzales, Noor Momin, Jeanne C. Stachowiak.
University of Texas at Austin, Austin, TX, USA.

From endocytosis to intercellular signaling, biological processes require precise and rapid assembly of protein complexes on membrane surfaces. How is this accomplished within the complex and crowded environment of cellular membranes? Lipid rafts, defined as phase-separated lipid domains enriched in cholesterol and saturated lipids, are thought to locally organize specific proteins, assisting the assembly of protein complexes. However, the extent to which lipid rafts can concentrate proteins has not been experimentally measured. Using a reconstituted system, we varied the density of proteins bound to the surfaces of lipid domains. Surprisingly, we found that when membrane-bound proteins became crowded, steric pressure arising from collisions between proteins destabilized lipid domains (Figure). These results demonstrate that protein-protein steric pressure creates a significant energetic barrier to the stability of phase-separated biological membranes. Comparison with a simple analytical model reveals that domains are destabilized when steric pressure exceeds the approximate enthalpy of membrane mixing, a threshold that larger membrane-bound proteins reach more efficiently. These results provide a new perspective on the role of lipid rafts as organizers of membrane proteins and suggest that a dynamic balance of membrane surface pressures governs the stability of phase-separated cellular membranes.

220-Plat
Controlling Bilayer Curvature and Membrane Protein Density using Droplet Interface Bilayers
Oliver K. Castell, Linda C.M. Gross, Brid Cronin, Mark I. Wallace.
Oxford University, Oxford, United Kingdom.

A Droplet Interface Bilayer (DIB) forms when nanolitre aqueous droplets are contacted together in an oil solution in the presence of phospholipids: A lipid monolayer forms at each oil-water interface, and by bringing together two monolayers a bilayer is created. We have recently extended this methodology to modulate both two-dimensional protein concentration[1] and membrane curvature. Manipulation of the axial position of the droplet relative to a hydrogel substrate controls the size of the bilayer formed at the interface; this enables the surface density of integral membrane proteins to be controlled. We are able to modulate the surface density of integral membrane proteins over a range of 4 orders of magnitude within a timeframe of a few seconds. By imaging DIBs on curved substrates we have observed correlation of lipid domain formation with sites of intrinsic curvature. This technique provides a new method for dictating the curvature of artificial bilayers, enabling single molecule measurements on the role of curvature in membrane organization and function.


221-Plat
Studying Bending Rigidity of Model Vesicles and Cell Plasma Membrane using Lipid Nanotubes
Rachel Pooley, Kevin D.M. Jeffries, Owe Orwar, Aldo Jesorka.
Chalmers University of Technology, Gothenburg, Sweden.

Mechanical properties of artificial lipid membranes have been examined by variety of techniques, like X-ray diffraction, NMR, micropipette aspiration or fluctuation analyses. However, new methods able to study membranes in most natural environment and with potential to approach cell membranes directly are desirable.