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3055-Pos Board B210

Tryptophan Point Mutants of HET-C2, a Fungal Glycolipid Transfer Protein, Provide Insights into Glycolipid Binding/Transfer and Membrane Interaction

Roopa Kenoth[1], Xianqiong Zou[1], Dhirendra K. Simanshu[2], Xiuhong Zhai[1], Helen M. Pike[1], Julian G. Molotkovsky[3], Dinshaw J. Patel[2], **Rhoderick E. Brown**[1].
[1]University of Minnesota, Austin, MN, USA, [2]Memorial Sloan-Kettering Cancer Ctr, New York, NY, USA, [3]Russian Academy of Sciences, Moscow, Russian Federation.

Fungal glycolipid transfer protein (GLTP), HET-C2, forms a GLTP-fold, but with higher binding/transfer preference for simple neutral glycosphingolipids than broad-selectivity mammalian GLTPs (Kenoth et al., 2010, *J. Biol. Chem.* 285, 13066-13078). In human GLTP, three Trp residues function in glycolipid binding (Trp96), membrane interaction (Trp142) and protein fold stabilization (Trp85). HET-C2 contains two Trp residues. Trp109 is structurally homologous to GLTP Trp96, consistent with function as a stacking plate that orients the ceramide-linked sugar during hydrogen bonding with Asp66, Asn70, and Lys73. HET-C2 Trp208 resides on the surface in a different location than either Trp142 or Trp85 of GLTP. In this study, we generated single-Trp mutants of HET-C2 to assess potential functional roles. Phe149, a structural homologue of GLTP Trp142, was also investigated for potential function in membrane interaction. W208F-, W208A- and F149Y-HET-C2 retained >90% activity and 80-90% intrinsic Trp intensity; whereas F149A-HET-C2 transfer activity decreased to ~60% but displayed ~120% intrinsic Trp intensity. W109Y/F149Y-HET-C2 was nearly inactive and displayed ~8% intrinsic Trp intensity. Thus, neither W208 nor F149 is essential for activity and most Trp emission intensity (~90%) originates from Trp109. With wtHET-C2, incubation with POPC vesicles containing glycolipid decreases Trp fluorescence intensity (25-30%) and blue-shifts Δ_{max} (6-7 nm). For HET-C2 mutants involving Trp208 or F149, the intensity changes and Δ_{max} blue-shifts induced by vesicles containing glycolipid become elevated and similar to human GLTP (~30-40% intensity decrease and ~12 nm Δ_{max} blue-shift). Only W109Y/F149Y-HET-C2, which lacks Trp109, deviated from the pattern, showing that the Trp Δ_{max} blue-shift is diagnostic for glycolipid binding in the HET-C2 GLTP-fold; whereas, the Trp intensity decrease reflects both glycolipid binding and nonspecific bilayer interaction [Support: NIH/NIGMS-GM45928, NIH/NCI-CA121493, Russian Fdn. Basic Research 012-04-00168, Hormel Fdn.]

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Elucidation of Calcium Ion and Phospholipid Binding Profiles of Multiple Dysferlin Isoforms

Anne M. Rice[1], Anne Hinderliter[1], R. Bryan Sutton[2].
[1]University of Minnesota-Duluth, Duluth, MN, USA, [2]Texas Tech University Health Center, Lubbock, TX, USA.

Dysferlin is a large type II peripheral membrane protein associated with the sarcolemma and T-tubular system of muscle fibers. It contains seven C2 domains and two DysF domains and has been implicated in membrane repair. This repair is thought to be mediated through calcium-triggered exocytosis of intracellular vesicles, which leads to the formation of a membrane patch. Due to dysferlin's structural similarity to synaptotagmin1 it is assumed that its role in the overall scheme of membrane repair is to act as a calcium and membrane sensor. The C2A domain is the first of the seven C2 domains and is assumed to be the protein's main calcium and membrane sensor.

The C2A domain exists in nature as a combination of two different isoforms derived from alternative readings of the first exon sequence: the canonical C2A (WT) isoform derived from exon 1, and the C2Av1 (V1) isoform derived from exon 1'. The two different isoforms of the C2A domain were studied to probe the calcium and lipid binding affinities of dysferlin. Using isothermal titration calorimetry (ITC) the calcium and lipid binding profiles of the two constructs were established. These profiles indicate a difference in the phospholipid dependence of calcium binding between the two constructs as well as a uniform calcium dependence on the phospholipid binding. Initial approximations of the constructs' binding parameters were estimated individually and can be fit globally using a partition function approach.

By determining dysferlin's binding profile we can better characterize its function in the scheme of membrane repair, as well as form a better understanding of the intramolecular communication that tunes its physiological response.

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Focusing the Glycolipid Specificity of Human Glycolipid Transfer Protein by Designer Mutation

Xiuhong Zhai[1], Yongguang Gao[1], Borja Ochoa-Lizarralde[2], Valeria R. Samygina[2], Helen M. Pike[1], Julian G. Molotkovsky[3], Lucy Malinina[2], Rhoderick E. Brown[1].
[1]University of Minnesota, Austin, MN, USA, [2]CIC bioGUNE, Derio, Spain, [3]Russian Academy of Sciences, Moscow, Russian Federation.

Human glycolipid transfer protein (GLTP) fold represents a novel structural amphitropic motif for lipid binding/transfer and reversible membrane translocation. GLTP can transfer various glycosphingolipids known to serve as key regulators of cell growth, division, surface adhesion, and neurodevelopment. Previously, we reported structure-guided engineering of point-mutated GLTP with enhanced selectivity for sulfatide (Samygina et al., 2011, *Structure* 19, 1644-1654). Herein, we extend the approach to characterize novel designer GLTPs capable of focused glycolipid binding and transfer. Structure-guided mutations were directed to three residues, Lys55, Lys87, and Leu92. Different glycolipids (GlcCer, GalCer, LacCer, sulfatide, disulfatide) were analyzed to determine mutant GLTP glycolipid transfer specificity using an established fluorescence resonance energy transfer assay involving anthrylvinyl-labeled glycolipid and 3-perylenoyl-labeled phosphatidylcholine. Assessment of glycolipid binding by the designer mutants relied on intrinsic changes in tryptophan fluorescence originating from Trp96, i.e. GLTP Trp signature fluorescence response. The response involves large decreases (up to 40%) in emission intensity accompanied by substantial blue-shift (up to 13 nm) of the emission wavelength maximum. Insights into the molecular basis for enhanced glycolipid selectivity was provided by X-ray diffraction structural determination of certain designer mutants complexed with glycolipid. The development of designer GLTPs with enhanced specificity for select GSLs provides a potential new therapeutic approach for targeting GSL-mediated pathologies [Support: NIH/NIGMS-GM45928 & NIH/NCI-CA121493, Russian Foundation for Basic Research 012-04-00168, Spanish Ministerio de Ciencia e Innovacion (MICINN BFU2010-17711), and Hormel Foundation]

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Several Asparagine Residues Flanking a Hydrophobic Helix are required to Block Interconversion between Transmembrane and Non-Transmembrane Configurations

James LeBarron, Erwin London.
Stony Brook University, Stony Brook, NY, USA.

Proteins that are embedded in cellular membranes include polar residues at both ends of their transmembrane (TM) helices or in loops that connect their TM helices. These polar residues can prevent the interconversion of these helices from the TM state to a non-TM configuration, with the helices located near the water-lipid interface. The energetic cost of segment movement containing polar residues through the hydrophobic membrane is thought to be too large to allow rapid TM to non-TM interconversion. We have investigated how TM/non-TM interconversion is influenced by an increasing number of asparagine residues in the juxta-membrane regions flanking both sides of simple hydrophobic helical peptides. The central hydrophobic region of these peptides includes a Trp whose fluorescence reflects the configuration of the peptide in the bilayer, and an Asp residue whose protonation state can be controlled via pH. The ends of the helices contained two Lys as well as a variable number of asparagine residues. At low pH the Asp was uncharged and the TM configuration of the helix was favored, while at high pH the uncharged Asp favored the non-TM configuration of the helix. We find six asparagine residues block non-TM to TM interconversion (insertion) for at least 30 minutes, but do not block the reverse which takes on the order of seconds. However, two asparagines allowed interconversion between TM and non-TM states, in either direction, on the order of seconds. This shows that a significant number of polar residues is needed to block interconversion between the TM and non-TM state, and from the difference in behavior when Asp is charged or uncharged implies that the hydrophobic sequence of the peptide is likely to influence this process.

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A Buttressed Unilamellar Membrane System for Studying Lipid-Protein Interactions

Sungsoo Lee, Marc Llaguno, Qiu-xing Jiang.
University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA.

Recent studies have demonstrated that annular lipids can directly change the voltage-gating of voltage-gated ion channels. We recognized that it was a challenge to insert membrane proteins into a bilayer that mimics the eukaryotic cell membranes in current model systems, especially the membranes that contain sphingomyelin and cholesterol. A new system is needed to overcome this technical barrier. In this study we developed a stable unilamellar vesicle system that is supported by unidirectionally inserted membrane proteins, and offers the capability of controlling lipid composition with relative ease. A voltage-gated potassium channel, KvAP, was used as a model system, and was selectively anchored onto the surface of micron-sized beads. Our data suggested that with a high surface density of channel molecules, it was feasible to introduce various lipids and form continuous membranes around the beads. The unilamellar nature of the bilayers was demonstrated by cryo-electron microscopic

observations, and the functionality of the channels in the bead-buttressed unilamellar membrane (bBUM) system was examined by electrical recordings of voltage-gated activities. We anticipate that this novel membrane system will provide a new technique to study how lipids influence membrane proteins.

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Toward Understanding the Role of Angiomotin Lipid Binding in Cellular Proliferation and Migration

Ann C. Kimble-Hill[1], Ilse Jimenez-Segovia[1], Millicent A. Firestone[2], Thomas D. Hurley[1], Clark D. Wells[1].
[1]Indiana University School of Medicine, Indianapolis, IN, USA, [2]Center for Integrated Nanotechnologies, Los Alamos National Laboratory, Los Alamos, NM, USA.

The Angiomotin (Amot) family of adaptor proteins directly integrates the signaling that controls cellular differentiation and cell growth. Amot family members bind core polarity proteins that control the organization of the apical domain of epithelial cells as well as Yap, a transcriptional co-activator that appears to be the key regulator of cell growth. A critical feature of all Amot proteins is a novel lipid binding domain, the Amot coiled-coil homology (ACCH) domain, which confers its association with membranes and affects membrane curvature. This domain, while sharing some features of BAR domains, i.e. a predicted coiled coil fold of approximately 240 residues, also has unique properties including the ability to selectively bind monophosphorylated phosphatidylinositols (PI). Similar binding of PI has been reported in other protein domains, including FYVE, PX and PH domains where PI binding is mediated through basic residues within a loop rich in lysines, arginines, as well as hydrophobic amino acids. The ACCH domain is predicted to have a coiled-coil fold and is rich in lysines.

One of our goals is to delineate which amino acids contribute to PI binding, thereby suggesting possible routes to modulate the increased Amot80 ACCH domain activity that is associated with ductal hyperplasia, and later breast cancer. Site-directed mutagenesis was employed to probe the specific contributions of selected lysines and arginines toward lipid head-group binding. The effect of the mutation was then analyzed using liposome sedimentation, FRET, and SAXS to monitor lipid binding. As the ACCH domain lacks tryptophan residues, protein association with liposomes was followed by monitoring changes in protein fluorescence associated with the proximity of tyrosines 11, 47, or 118 to lipid.

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Assembly and Activity of Respiratory Complex II in Nanolipoprotein Particles

Christine Schwall, Nathan Alder.
University of Connecticut, Storrs, CT, USA.

Mitochondrial respiratory Complex II (succinate:ubiquinone oxidoreductase) couples the citric acid cycle to the electron transport chain by oxidizing succinate in the matrix and passing electrons to ubiquinone in the inner membrane. Complex II is comprised of a soluble catalytic heterodimer (Sdh1/Sdh2) and a membrane-bound heterodimer (Sdh3/Sdh4). The structure of Complex II is well established; however, little is known about how the lipid environment regulates holocomplex assembly and activity. To address this question, we reconstituted Complex II from native biomembranes into nanoscale phospholipid bilayers (nanodiscs) containing a defined lipid content. We found that the dimeric phospholipid cardiolipin, the signature lipid of energy-conserving membranes, is critical for Complex II stability and function. First, the presence of cardiolipin in the bilayer promoted the interaction of the soluble and membrane-bound dimers. Second, cardiolipin was essential for enzymatic activity of the reconstituted complex and for curtailment of reactive oxygen species production. The function of cardiolipin could be partially compensated by the presence of phosphatidylglycerol, another phospholipid with an anionic headgroup; moreover, reducing the acyl chain lengths of cardiolipin used for reconstitution prevented its stimulatory effect on Complex II activity. Hence, both the headgroup and hydrocarbon chains of cardiolipin play important roles. Using this experimental platform, we have employed site-specific fluorescence labeling to address which structural elements of Complex II membrane subunits undergo conformational dynamics during assembly. Our results indicate that Sdh3 matrix-facing helix I, which interacts extensively with the catalytic dimer in the holocomplex, undergoes structural changes when reconstituted with Sdh4, but not Sdh4 homologs, suggesting that this helix may act as a conformational switch for downstream assembly steps (e.g., recruitment of the soluble dimer). Taken together, we show that Complex II is structurally dynamic during assembly and that its function is highly lipid-dependent.

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Purification and Characterization of the Tetrameric Potassium Channel Kcsa in "Native Nanodiscs"

Martijn C. Koorengevel, Stefan Scheidelaar, Tim R. Dafforn, Antoinette Killian.
Utrecht University, Utrecht, Netherlands.

We explored a new discovery in membrane research of amphipathic copolymers of styrene and maleic acid (SMA) that have the unique potential to directly extract proteins from membranes in the form of "native nanodiscs" without the need for detergent [1,2]. *E-coli* cells overexpressing a His-tagged version of the potassium channel KcsA were incubated with SMA and the conditions were optimized for extraction of the protein. After solubilization of the membrane, we found that KcsA indeed could be purified on a Ni^{2+}-NTA column in the form of nano-sized discs, as was confirmed by negative stain transmission electron microscopy (TEM) experiments. SDS-PAGE analysis showed that the protein in these nanodiscs is present as a tetramer, running at the same position as when subjected to SDS-PAGE from its native membrane or after purification in detergent.

Presently we are comparing the stability of this tetramer in the nanodiscs with that of purified KcsA in *n*-dodecyl β-D-maltoside (DDM) micelles and KcsA in native membranes. This is done by comparing the effects of heat-incubation and exposure to small alcohols using gel shift assays. Furthermore, to gain information about possible preferential lipid interactions of KcsA, the lipid composition of the purified, KcsA containing nanodiscs is being analyzed and compared to that of the total lipid composition in *E-coli*. Results of these studies will be presented.

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2) Orwich-Rydmark M, Lovett JE, Lindholm L, Graziadei A , Hicks M, Watts A (2012) Nanoletters 12, 4687-4692

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Determining the Role of NS4B in Membrane Remodelling during Hcv Replication

Esther von Schulthess, Chris McCormick, **Philip T.F. Williamson**.
University of Southampton, Southampton, United Kingdom.

Hepatitis C is an RNA virus that replicates in association with intracellular membranous structures call membranous webs (MWs). Viral protein NS4B is a key organizer of replication, one crucial function being the induction of MWs. The mechanisms of MW formation are unknown, but it clearly involves induction of membrane curvature, which may require NS4B oligomerisation and possibly hydrophobic wedging. NS4B is known to oligomerise, and the N-terminal amphipathic helix AH2 has been implied as a major determinant of self-association. In order to understand the process of MW induction, we aimed to determine AH2's capacity to remodel membranes by studying the interaction of AH2 with membranes mimicking those found within the cell using 2H and 31P solid-state NMR. Our results show changes in membrane morphology, induced by AH2 in negatively charged vesicles, an effect not observed in neutral bilayers indicating a requirement for negatively charged lipids. Chemical cross-linking studies of AH2 in lipids vesicles confirms AH2 homo-oligomerisation and suggests a charge dependency; with larger oligomers observed in neutral lipid bilayers compared to negatively charged lipid bilayers and lipid mixtures mimicking cellular membranes. These results suggest that AH2 plays a crucial role in NS4B's capacity to alter membrane morphology.

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Phosphorylation of the Amphipathic Helix Changes the Lipid Binding Capacities of PICK1

Rasmus Herlo, Ina Ammendrup-Johnsen, Kenneth Lindegaard Madsen, Dimitrious Stamou, Ulrik Gether.
University of Copenhagen, Copenhagen, Denmark.

PICK1 (Protein Interacting with C-kinase 1) is a functionally important protein, which is distributed mainly in testis, pancreas and brain. It has been shown to play a central role in regulation of dense core vesicles from the golgi apparatus and trafficking of ionotropic glutamate receptors.

PICK1 contains a N-terminal PDZ-domain, which we have earlier demonstrated to be important for interaction with a large number of proteins, including several important receptors and transporters. In addition, it has a BAR (Bin/Amphiphysin/Rvs) domain in the C-terminal end. BAR domains are generally believed to either recognize or induce curvatures of lipid membranes, but as we have demonstrated, proteins of the N-BAR family (incl. PICK1) binds lipids and recognizes membrane curvature (MC) through an associated amphipathic helix (AH) rather than through the BAR domain itself.

Here we show that the lipid binding AH of PICK1 contains a phosphorylation-site, which, through PKC activation, is responsible for an altered cellular