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

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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

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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

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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

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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

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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