calcein leakage assay and UV resonance Raman spectroscopy. Effect of small changes in the primary structure of the peptide on the membrane rupturing activity is discussed.

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457-Pos

Simultaneous Single-Channel Recording and Fluorescence Imaging of Calcium Flux Reveals the Behaviour of Individual Antimicrobial Peptide Pores

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We have used simultaneous single-channel recording and total internal reflection fluorescence (TIRF) microscopy to investigate the behaviour of antimicrobial peptides in artificial droplet-on-hydrogel lipid bilayers (DHB).

These pore-forming peptides play an important role in many organisms by providing resistance to infection. An improved understanding of their mechanism of action is essential in the development of new antibiotics.

Our study focuses on two peptides thought to follow different pore-formation mechanisms. Alamethicin is produced by the fungus *Trichoderma viride* and is understood to form barrel-stave pores. Magainin II is found in the skin of the African clawed frog *Xenopus laevis* and is thought to follow a toroidal-pore model.

Using a fluorescent calcium indicator we are able to detect the ion flux through individual alamethicin and magainin II pores and can monitor multiple pores at once. We observe multiple conductance states from single alamethicin pores and see that magainin II forms stable pores.

458-Pos

Peptide-Induced Domain Formation in Supported Lipid Bilayers: Direct Evidence By Combined Atomic Force and Polarized Total Internal Reflection Fluorescence Microscopy

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Direct visualization of the mechanism(s) by which peptides induce localized changes to the structure of membranes has tremendous potential for understanding the structure-function relationship in antimicrobial and cell-penetrating peptides. We have applied a combined imaging strategy to track the interaction of a model amidated antimicrobial peptide, PFWRIRIRR-amide, with bacterial membrane-mimetic supported phospholipid bilayers comprised of POPE:TOCL. Our in situ studies revealed rapid reorganization of the POPE:TOCL membrane into localized TOCL-rich domains with a concomitant change in the organization of the membranes themselves, as reflected by changes in fluorescent membrane probe order parameter, upon introduction of the peptide.

459-Pos

Time-Resolved, Single-Cell Study of the Attack of the Antimicrobial Peptide Ll-37 on Live E. Coli Cells

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Human LL-37 is an antimicrobial peptide whose amphiphilic helices selectively degrade bacterial membranes by a mechanism that is poorly understood. We are using single-cell, two-color fluorescence microscopy to directly observe the attack of rhodamine-LL-37 on live E. coli cells in real time. The cells express either periplasmic or cytoplasmic GFP. This enables quantitative correlation of the extent of LL-37 adsorption with leakage or lysing of GFP from the two different compartments, while simultaneously monitoring cell growth. At 15 uM, LL-37 lyses the periplasm to GFP and halts growth in 2-4 min, long before the cytoplasm lyses to GFP at 20-30 min. At 6 uM, rhodamine-LL-37 binding occurs in three distinct waves, with Wave 2 correlating in time with the halting of cell growth (t = 7-10 min). Wave 1 coats the cell periphery uniformly, but Wave 2 preferentially attacks the septal region and slowly spreads outward towards the poles. This suggests that the cell division machinery may be a target of LL-37-induced cell death. We will use FRET to discern the penetration depth of LL-37 during the different waves of attack and a variety of mutant strains to correlate the LL-37 attack with formation of the Z-ring and additional parts of the divisome. These methods will enable quantitative comparison of antimicrobial attack on real bacterial membranes with studies of lysing of synthetic lipid bilayers. They will be applicable to a wide variety of antimicrobial agents and bacterial species.

Interfacial Protein-Lipid Interactions I

460-Pos

Membrane Diffusion of PH Domain-PIP3 Complexes: the Effects of Target Lipid Stoichiometry on Diffusion Constant Probed Using Single-Molecule Fluorescence Microscopy

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Pleckstrin homology (PH) domains are recruited to specific membrane surfaces during a variety of cell signaling events, including those occurring at the leading edge of chemotaxing cells. This recruitment is often driven by molecular recognition of specific phosphatidylinositol phosphate lipids, such as phosphatidylinositol-(3,4,5)-trisphosphate (PIP3). Using single-molecule fluorescence microscopy, we have recently shown that when bound to PIP3 on the surface of a supported lipid bilayer, the PH domain of GRP1 diffuses at the same rate as an individual phospholipid molecule diffusing in the same type of bilayer. More generally, we hypothesize that protein lateral diffusion constant will decrease as the number of lipid molecules tightly bound to the protein increases. Here, we probe the effects of PIP3 stoichiometry on the diffusion constants of constructs containing one, two, or three GRP1 PH domains coupled by flexible linkers. To a first approximation, we find that the lateral diffusion constants of these engineered PH domain constructs are inversely proportional to the number of bound PIP3 molecules. This observation suggests that the frictional contributions of multiple, tightly bound lipids are additive, at least when the binding sites are located on separate domains. At the meeting we will present our latest diffusion constant measurements, which will shed light on the mechanisms of peripheral membrane protein diffusion, and will provide useful calibration points in molecular dynamics simulations of proteins docked to membranes. Overall, single molecule diffusion methods provide a new, much needed window into the lipid interactions of membrane targeting proteins.

461-Pos

The Autism-Related H93R PTEN Mutant Shows Enhanced Plasma Membrane Binding But Reduced Activity

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The tumor suppressor, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), is a phosphoinositide (PI) phosphatase specific for the 3-position of the inositol ring. PTEN has been implicated in autism for a subset of patients with macrocephaly. Various studies identified patients in this subclass with one normal and one mutated PTEN gene. We characterize the binding, structural properties, activity and subcellular localization of one of these autism-related mutants, H93R PTEN, using fluorescence quenching, SPR and ITC. The membrane association of the mutant protein with solid-supported membranes (tBLMs) is investigated with neutron reflection. We observe that H93R PTEN shows enhanced binding to phosphatidylserine (PS)-containing membranes, in contrast to *wt* PTEN. On the other hand, binding to membranes that contain $PI(4,5)P_2$, a requirement for allosteric activation of PTEN, was strongly reduced for the H93R mutant. H93R and *wt* PTEN share the same secondary structure. However, while α-helical content increases in *wt* PTEN upon binding to $PI(4,5)P_2$, this is not observed for the H93R mutant. Consistent with the increased affinity of H93R PTEN to PS, we find in cell-based studies that the association of the mutant with the plasma membrane is strongly enhanced in comparison to *wt* PTEN. Unexpectedly, this does not enhance $PI(3,4,5)P_3$ turnover, but instead reduces enzyme activity significantly. We hypothesize that the tight binding of H93R PTEN to PS prevents $PI(4,5)P_2$ from interacting with the protein, thereby inhibiting allosteric activation, which is a requirement for binding to and turnover of the substrate, $PI(3,4,5)P_3$.

462-Pos

Unusual Thermal Stability of Human Secreted Phospholipase A2 Enzymes

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Analysis of the thermal stability of proteins in general and enzymes in particular is important for understanding their molecular mechanisms and for their analytical or industrial exploitations. While enzymes with extreme thermostability (resistance to irreversible inactivation at high temperatures) have been identified in hyperthermophilic microorganisms, their occurrence in mammals is rare. Here, we have studied the thermostability of two human secreted phospholipase A2 (PLA2) enzymes, namely, group IB PLA2 and group IIA PLA2. In both cases, the enzymes exhibited maximum phospholipid hydrolyzing activity between 60 and 70°C. [The target membranes contained 70% phosphatidylcholine (PC) and 30% phosphatidylglycerol (PG), and the buffer contained 50 mM Hepes, pH 7.4, and 2 mM CaCl2).] Parallel circular dichroism (CD) measurements identified sigmoidal temperature dependencies of the ellipticity at 222 nm with ''melting'' transition temperatures around 90-95°C. The activity rapidly declined at higher temperatures and was practically absent between 85 and 100°C. However, when the sample containing the enzyme and the substrate (PC/PG membranes) was ''cooked'' at 100°C for up to 30 minutes and then cooled to favorable temperatures (e.g., 40-50°C), the activity of the enzyme was gradually recovered, reaching even higher levels than those initially measured at those temperatures. This was accompanied with partial restoration of the CD spectra, hence the secondary structure of PLA2s. These facts indicate an unusual thermostability of human group IB and IIA PLA2s, which may be supported by a high content of intramolecular disulfide bonds in these enzymes (7 disulfides per 124-126 residues). We interpret these data in terms of partial thermal dissociation of the disulfides at near-boiling temperatures and their restoration upon cooling. Experimental verification of this hypothesis, as well as quantitative characterization of the thermodynamic and kinetic parameters of the thermostability of the enzymes, are in progress.

463-Pos

Interaction of Bacterial PI-Specific Phospholipase C With Lipid Bilayer Surfaces

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PI-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* is a 40 kDa enzyme that catalyzes the cleavage of GPI-anchored proteins and PI. PI-PLC binds to the membrane via the interfacial binding surface, a special region on the rim of the active site pocket. Hydrophobic and electrostatic interactions (via Trp and Lys residues, respectively) are proposed to play a role in membrane binding. To further examine these interactions, recombinant wild-type (WT) and mutant His$_6$-tagged PI-PLCs (W47A, W242A, W47A/W242A and K44A) were expressed in *E. coli* and purified. Trp emission of WT and mutant enzymes did not undergo a blue shift upon addition of phosphatidylcholine (PC) liposomes, suggesting that PI-PLC does not deeply penetrate the membrane during membrane association. Trp quenching upon association of WT/mutant PI-PLC with bilayers containing brominated lipids (70% egg PC, 30% 6,7-Br$_2$PC) was equal to that observed for pure egg PC, again suggesting no deep membrane penetration of the enzymes. Stopped-flow fluorescence studies showed first-order quenching of Trp fluorescence upon mixing of WT/mutant PI-PLCs with zwitterionic and negatively charged liposomes, consistent with the notion of a single binding event. The calculated dissociation constants revealed much weaker membrane association of W47A/W242A PI-PLC compared to WT enzyme, and ultimately suggest a role for these Trp residues in membrane association. Introduction of a PI substrate into egg PC liposomes resulted in 5-fold stronger association of WT and mutant PI-PLCs with the membrane surface. Lastly, Langmuir isotherms showed a change in the cross-sectional area per molecule of dimyristoyl-PC and dimyristoylphosphatidylglycerol monolayers upon injection of WT PI-PLC into the subphase, implying that PI-PLC association alters the surface packing of the monolayer. Changes in the area per molecule on PI-PLC binding were more substantial above the T$_m$ of these lipids. (Supported by NSERC).

464-Pos

Exploring Molecular and Supramolecular Aspects of Sphingomyelin-Containing Membranes Upon Action of Sphingomyelinase D

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Lipid-modifying enzymes play a vital role in the regulation of lipids as mediators of cell function. At the same time, the activity of these enzymes is highly affected by the lipid membrane structure. These processes at lipid membranes can be observed in situ through the application of different biophysical techniques. Thus, we are investigating an enzyme from spider venoms, which is termed sphingomyelinase D (SMD). SMD hydrolyses sphingomyelin (SM) into ceramide-1-phosphate (Cer-1-P). While SM is an integral constituent of many cell membranes, e.g., of red blood cells, Cer-1-P occurs in very low concentrations and is suggested to be a novel lipid second messenger. At present, the physiologically relevant mechanism following Cer-1-P formation by SMD is incompletely understood, but possibly related to the modulation of membrane properties.

Our results show a strong dependency of SMD activity on the phase state of the substrate. SMD is one order of magnitude more active towards fluid- than gel-phase liposomes. The effect of SMD on fluid-phase giant unilamellar vesicles (GUVs) is observed by confocal fluorescence microscopy. GUVs composed of lauroyl-SM show a drastic shrinking and buckling accompanied by the multiple formation of membrane tubes, which are up to 80 μm long. Generalized-polarization measurements using the probe LAURDAN exhibit a macroscopic domain formation upon the hydrolysis of lauroyl-SM. This finding points to the induction of membrane curvature by lipid sorting in the simple, binary system of SM/Cer-1-P. SMD activity on GUVs composed of oleoyl-SM also shows tube formation followed by the immediate and complete disintegration of the vesicular membrane structure. The consequences of SMD activity and Cer-1-P formation on cellular systems are currently being examined. This will indorse the correlation between enzymatic activity and membrane structure influencing the regulation of physiological processes.

465-Pos

Heterogeneous Dielectric and Hydrogen Bonding Environment of Transmembrane Peptides

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A complex set of hydrogen bonding and hydrophobic interactions between the protein side chains and cellular membrane components is primary responsible for such important biophysical processes as initial protein binding/docking to cellular membranes, membrane insertion, folding, and the final adaptation of the correct transmembrane position. Although general concepts of membrane protein folding and thermodynamic stability are beginning to emerge, the experimental data on the transmembrane profile of the effective dielectric constant and the local hydrogen bond network formed by membrane protein side chains remain severely limited. Here we describe the use of an arsenal of modern spin-labeling EPR methods to profile heterogeneous dielectric and hydrogen bonding environment along a series of the alpha-helical chain of the alanine-rich WALP peptide that adopts a transmembrane orientation. Firstly, we have employed a recently described pH-sensitive cysteine-specific spin-label IMSTL (methanethiosulfonic acid *S*-(1-oxyl-2,2,3,5,5-pentamethylimidazolidin-4-ylmethyl) ester) to label a series of WALP cysteine mutants. EPR titrations of such peptides reconstituted into anionic lipid bilayers yield the magnitude of relative changes in the effective dielectric constant across the bilayer in the vicinity of the peptide alpha-helix. Secondly, perdeuterated and ^{15}N-substituted nitroxides in combination with High Field EPR at 130 GHz (D-band) were used to assess local polarity and formation of hydrogen bonds for the same series of spin-labeled WALP mutants. Finally, the nature of the hydrogen bonds observed by EPR was ascertained by a series of HYSCORE X-band measurements. It was concluded that such combination of EPR techniques significantly expands the capabilities of spin-labeling methods in studies of membrane proteins as demonstrated by deriving profiles of heterogeneous dielectric and hydrogen bonding environment along a typical transmembrane alpha-helix. Supported by NSF-0843632 to TIS and NIH 1R01GM072897 to AIS.

466-Pos

Molecular Mechanisms of Peptide Translocation Across Micelles: A Case Study of Cell-Penetrating Peptide and Antimicrobial Peptide

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We investigated the molecular mechanisms of short peptides across plasma membranes by studying their varying ability in permeating through membrane-mimetic systems. Three short peptides are selected for this study: penetratin is a cell-penetrating peptide and temporin A and KSL are antimicrobials (AMP). Their detailed interactions with SDS and DPC micelles, sometimes associated with their conformational changes, which govern their ability in translocation, are revealed by all-atomistic molecular simulations with explicit solvent models and free energy calculation of peptide insertion. We found that penetratin undergoes conformational changes upon binding and insertion and it causes less structural disturbance in the self-assembly of lipid molecules in micelles, which is in accordance with its macroscopic non-invasive behavior when it passes through membranes of mammalian cells. In contrast, temporin A and KSL peptides tend to destroy the self-assembly of lipid micelles through strong hydrophobic interaction in the former and electrostatic interactions in the latter. Our investigation addresses some of the speculation regarding the molecular mechanisms of the toxicity in short peptides.