the plasma membrane of neuroendocrine cells. Previous studies have shown that syntaxin-1A is found in clusters that are different from lipid rafts in neuroendocrine plasma membranes. However, the interactions that promote these clusters have been largely unexplored. Here, we have reconstituted syntaxin-1A into lipid model membranes and show that cluster formation of syntaxin depends on cholesterol in a lipid system that lacks sphingomyelin and therefore does not form typical liquid-ordered phases that are commonly believed to represent lipid rafts in cell membranes. The cholesterol-induced clustering of syntaxin is found to be reversed by as little as 1 mol % of the regulatory lipid phosphatidylinositol-4,5-bisphosphate (PIP2), and PIP2 is shown to bind specifically and electrostatically to syntaxin, presumably mediated by the positively charged juxtamembrane domain of syntaxin. Possible consequences of these results to the regulation of SNARE mediated membrane fusion are discussed.

1837-Pos Board B681

Lipid Mixing and Content Release in Single-Vesicle, SNARE-driven Fusion Assay with 5 ms Time Resolution

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A single-vesicle, fluorescence based SNARE-driven fusion assay enables simultaneous measurement of lipid mixing and content release with 5 ms/frame, or even 1 ms/frame, time resolution. The v-SNARE vesicles, labeled with lipid and content markers of different color, dock and fuse with a planar t-SNARE bilayer supported on glass. A narrow (< 5 ms duration), intense spike of calcein fluorescence due to content release and dequenching coincides with inner-leaflet lipid mixing within 10 ms. The spike provides much more sensitive detection of productive hemifusion events than do lipid labels alone. Consequently, many fast events that were previously thought to be prompt, full fusion events are now re-classified as productive hemifusion events. Both full fusion and hemifusion now occur with a time constant of 5-10 ms. At 60% DOPE lipid composition, productive and dead-end hemifusion account for 65% of all fusion events. However, quantitative analysis shows that calcein is released into three-dimensional space above the bilayer (vesicle bursting), rather than the thin aqueous space between bilayer and glass. Evidently at the instant of inner-leaflet mixing, flattening of the vesicle increases the internal pressure beyond the bursting point. In the future, additional height of the aqueous space may enable proper content release. To achieve this goal, single vesicle-vesicle fusion will be tested by tethering v-SNARE vesicles onto supported lipid bilayers. Also GUVs with t-SNARE proteins will be ruptured onto polymer cushion to create a floating target membrane for v-SNARE vesicles to fuse with.

1838-Pos Board B682

Visualizing Viral Fusion At The Single-particle Level

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Specific fusion of biological membranes is a central requirement of many cellular processes and is the key event in the entry of enveloped viruses into cells. Though many biochemical and biophysical studies have contributed to an understanding of the mechanisms underlying fusion, important questions remain about the sequence and orchestration of events underlying the process. Conventional fusion assays are generally limited to observation of ensembles of multiple fusion events, making more detailed analysis difficult. We have developed an in vitro two-color fluorescence assay that enables us to monitor the kinetics of individual fusion events. The resulting 'molecular movies' allow us to dissect the reaction kinetics at a level of detail previously inaccessible. Analysis of lipid and content mixing trajectories of single viral particles provides further evidence of a hemifusion intermediate preceding pore formation. Distributions of the lag times of events reveal multiple long-lived kinetic intermediates leading to hemifusion followed by a single rate-limiting step to pore-formation. We interpret the series of intermediates preceding hemifusion as the result of multiple copies of the trimeric hemagglutinin fusion protein participating in a single fusion event.

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Functional and Structural Measurements of HIV gp41 Fusion Protein Constructs

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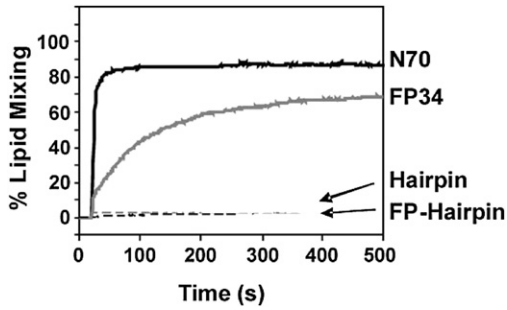
The initial step of HIV infection is fusion between the viral and target cell membranes. Fusion is mediated by the HIV gp41 protein and its N-terminal "fusion peptide" (FP) which binds to target cell membranes. Shorter constructs of gp41 that contain the FP usually catalyze vesicle fusion and such fusion at physiological pH was measured for three different gp41 constructs which differed in their numbers of N-terminal gp41 residues. "FP34" and "N70" were respectively models of the FP and "pre-hairpin intermediate" gp41 conformation while "FP-hairpin" was a model of the final "six-helix-bundle" gp41 structure. N70 induced rapid fusion, FP34 induced moderate fusion, and FP-hairpin induced no fusion and even arrested fusion induced by FP34. The data therefore suggest that the six-helix bundle conformation stops membrane fusion. In related work, solid-state nuclear magnetic resonance measurements probed the membrane locations of three different FP constructs with very different fusion rates. There was a positive correlation between fusion rate and depth of membrane insertion for the FP in either helical or β strand conformation. The key determinant of fusion rate may therefore be FP membrane location rather than conformation.



1840-Pos Board B684

SNARE-Mediated Adhesion Kinetics in Giant Membrane Systems

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Giant membrane *in vitro* systems are powerful and relatively unexplored model systems to probe mechanisms of SNARE-mediated adhesion and fusion. We present results of a joint experimental-modeling study of SNARE-mediated adhesion kinetics of giant unilamellar vesicles (GUVs) with supported bilayers (SBLs). The large size of GUVs allows control of physical parameters such as tension and direct observation of adhesion kinetics unavailable in small unilamellar vesicle (SUV) systems. In the gravity-imposed contact zone between v-SNARE GUVs and t-SNARE SBLs, reflection interference contrast microscopy (RICM) revealed initial membrane separation ~50 nm, consistent with Helfrich theory predicting intermembrane repulsion from thermal undulations. Tight adhesion in several discrete patches within the contact zone then develops over ~10 min, with total patch area growing linearly in time. We mathematically modeled the adhesion kinetics, which comprises two stages. (a) Patch nucleation kinetics. Nucleation is limited by the mean membrane separation which exceeds the reach of cognate SNAREs in apposing membranes. Complexation requires fluctuations to bring cognate SNAREs into proximity and the nucleation rate depends on tension and SNARE density. (b) Patch growth. Once nucleated a patch encourages further complexation because the membrane separation is within SNARE reach. We find two classes of patch growth kinetics. (i) Mobile SNARE complexes. Complex osmotic pressure then drives patch growth and patch area grows quadratically in time. (ii) Immobile SNARE complexes. Patches of tightly clustered SNARE complexes are predicted: patch growth, limited by SNARE diffusion, is linear in time. The experimental data are consistent with model (ii). The SNARE complex density in the patch inferred from the areal growth data suggests that complexes were shoulder-to-shoulder as expected in clusters generated by SNARE-SNARE interactions.

1841-Pos Board B685

Relative Rearrangements Of Synaptotagmin3 C2A And C2B Domains Are Influenced ay Calcium, Lipids And SNARE Proteins

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Synaptotagmins are able to bind negatively charged lipids in Ca2+ dependent manner and act as Ca2+ sensors. Together with SNARE and additional auxiliary proteins synaptotagmins coordinate Ca2+ triggered vesicle exocytosis, although the exact mechanism remains unclear. Synaptotagmins are transmembrane proteins comprised of a short intralumenal/extracellular sequence,

single transmembrane helix, long unstructured linker followed by two cytoplasmic domains, called C2A and C2B, which are separated by a short flexible linker. The reports of the intramolecular interactions between C2A and C2B domains in solution are inconsistent. In order to determine relative rearrangements of C2A and C2B domains in the presence of synaptotagmin ligands, lipids, SNARE proteins and SNARE complex, single molecule fluorescence resonance energy transfer (sm FRET) was used. C2A and C2B domains were each labeled with a single fluorophore, tethered to the surface and conformational dynamics monitored using smFRET. Results suggest that the relative rearrangement of Synaptotagmin III C2A and C2B domains in the presence and absence of calcium is dynamic and influenced by interactions with liposomes, SNARE proteins, and SNARE complex.

1842-Pos Board B686

Fusion Activity of HIV GP41 Fusion Domain is Related to its Secondary Structure in a Cholesterol-Dependent Fashion

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The HIV gp41 fusion domain plays a critical role in membrane fusion during the viral entry process. A thorough understanding of the relationship between the structure and activity of the fusion domain in different environments helps to formulate mechanistic models on how it might function in mediating membrane fusion. We examined the secondary structure of the fusion domain in small unilamellar vesicles composed of different lipid compositions by circular dichroism spectroscopy. The results show that the secondary structure switches from alpha helix below approx. 30% cholesterol to beta structure above this threshold. The lipid and content mixing activities of the fusion domain were examined by standard FRET fusion assays. Although the fusion activities increase with higher percentages of cholesterol, the helical conformation supports fusion in the absence of cholesterol and the beta-sheet conformation supports fusion in the presence of >30% cholesterol. Our results also show that the fusion domain has a higher activity in more negatively charged membranes, which can be explained by its higher binding affinity to such membranes.

1843-Pos Board B687

Membrane Fusion in Autophagy

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Macroautophagy is a pathway for the sequestration and degradation of cytosolic material when the cell is faced with stress conditions like starvation or infection. This cytosolic material is captured within a double-membraned vesicle (the autophagosome) which forms de novo and ultimately traffics to the lysosome for degradation. How membranes come together to form the autophagosome is unknown, but recent studies have suggested a possible role for the autophagosome-associated protein ATG8 in membrane tethering and fusion. Here we develop novel membrane platforms and protein-lipid coupling strategies to test fundamental aspects of ATG8 function, including the involvement of associated factors (ATG3 and ATG7) and the role of different lipid populations such as phosphatidylethanolamine. We compare the functionality of ATG8 to its mammalian homologue, MAP1LC3 and to a variety of mutants to establish which regions of the protein contribute to each activity. We utilize a range of different membrane dynamics assays including lipid-mixing, real-time membrane tethering, vesicle aggregation and cryo-electron microscopy. Our results suggest ATG8 may act as a ''molecular glue'', bringing membranes and possibly protein cargo together, but cast doubt on the physiologic relevance of ATG8 as a membrane fusion protein.

1844-Pos Board B688

Hemifusion of Giant Lipid Vesicles by a Small Transient Osmotic Depletion Pressure

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We observed hemifusion of giant unilamellar vesicles (GUVs) by injecting a small volume of 5% polyethylene glycol solution in the vicinity for a short time. The induced transient osmotic depletion attraction between two bilayers was smaller than the corresponding van der Waals force, yet hemifusion readily occurred for some lipid compositions. This might imply that the energy barrier for hemifusion is very low if the lipid compositions were primed for the reaction. The barrier for hemifusion is then the task of bringing two membranes into contact. We found that hemifusion is a stochastic event very much like pore formation by tension or by pore-forming peptides. This method provides an operational definition for hemifusogenicity which so far has been defined only qualitatively. Surprisingly the configuration of hemifusion depends on the lipid composition, ranging from a stalk-like hemifusion to a large hemifusion diaphragm. The formation of a large hemifusion diaphragm requires trans-bilayer lipid mixing in the participating GUVs. We also found that hemifusogenicity correlates with the water activity for the stalk phase in the lipid water mixture. The effect of cholesterol on hemifusogenicity was clearly demonstrated.

1845-Pos Board B689

Direct Visualization of Large and Protein-free Hemifusion Diaphragms

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Fusion of cellular membranes is an ubiquitous biological process requiring remodeling of two phospholipid bilayers. Very likely, merging of membranes proceeds via similar sequential intermediates. Contacting membranes form a stalk between the proximal leaflets which expands radially into a hemifusion diaphragm (HD) and subsequently open to a fusion pore. While considered to be a key intermediate in fusion, direct experimental verification of this structure is difficult due to its transient nature. Using confocal fluorescence microscopy we have investigated the fusion of giant unilamellar vesicles (GUVs) containing fluorescent membrane protein anchors and fluorescent lipid analogues in the presence of divalent cations. Time resolved imaging revealed that fusion was preceded by displacement of peptides and lipid analogues from the GUV-GUV contact region being of several μm in size. A detailed analysis of contact regions which could be stabilized at lower cation concentration showed that peptides were completely sequestered from this site as expected for an HD. Lateral distribution of lipid analogues in attached GUVs in three different experimental setups as well as fluorescence recovery after photobleaching (FRAP) measurements were consistent with the formation of an HD but not with the presence of two closely approached bilayers.

1846-Pos Board B690

Covalent Inhibition of HIV Membrane Fusion

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Peptide inhibitors consisting of sequences from the six helix bundle structure of the fusogenic portion (gp41) of the HIV envelope glycoprotein have been successfully implemented in preventing HIV entry. These peptides block entry by binding to regions of HIV gp41 that are transiently exposed during the fusion stage of viral entry. We have successfully designed and tested peptide analogs composed of chemical spacers and reactive moieties positioned strategically to facilitate the covalent attachment of the peptide to gp41. We have utilized the covalent peptide to show evidence for the trapping of a pre-six helix bundle fusion intermediate by a covalent reaction with a specific anti-HIV-1 peptide. This is the first demonstration in live cells of the trapping of an intermediate conformation of a viral envelope glycoprotein during the fusion process. The permanent specific attachment of the covalent inhibitor is projected to improve the long-term sustainability of peptide entry inhibitor therapy and to serve as an important tool in probing the conformational changes that occur to the envelope protein complex during viral entry.

1847-Pos Board B691

Leaky Fusion Between Intraluminal Vesicles Of Late Endosomes That Have An Unusual Lipid Composition As A Mechanism Of Endosomal Escape By Cell-penetrating Peptides

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It has recently been recognized that endocytosis/pinocytosis plays a major role in the delivery of various bioactive molecules such as proteins and nucleic acids by HIV Tat-derived peptide (TAT). In this work we explored the mechanism of TAT escape from endosomes using protein-free liposomes. We found that TAT induces both membrane leakage and fusion of liposomes mimicking late endosomal lipid composition and the extent increased with an increase in the content of bis(monoacylglycero)phosphate (BMP), which is a characteristic lipid of late endosomal membrane. The TAT-induced membrane fusion of BMP-containing liposomes was promoted by acidic pH and elevated Ca^{2+} concentration. In contrast, liposomes in which BMP was replaced by its structural isomer phosphatidylglycerol (PG) were much less affected by TAT. While there was no significant difference between BMP and PG in the binding affinity of TAT, effects of BMP and PG on the L_a to inverted H_{II} phase transition of egg PE suggested that BMP is more fusogenic than PG. In addition, modifications of liposome composition that inhibited TAT-induced lipid mixing (incorporation of either PEG-lipid or LPC) also inhibited TAT-induced leakage. Based on these results, we propose that TAT induced leaky fusion between BMP-containing bilayers of late endosomal membranes first deliver TAT into the intraluminal vesicles and then, upon vesicle fusion with the limiting membranes, release the peptide into cytosol.