details have emerged regarding the mechanism of GPCR-Gαβγ complex formation during signaling initiation because of the intrinsic instability of such complexes both *in vivo* and *in vitro* in the presence of detergent micelles. To overcome this limitation, we examined the utility of a range of membrane mimetics for stabilizing GPCR-Gαβγ complexes using the model system pair rhodopsin-transducin responsible for visual transduction. This system was chosen for its spectroscopic properties that can be easily followed as a measure of complex stability. Our results support the dependence of GPCR-Gαβγ complex formation on membrane morphology and nonspecific electrostatic interactions between membrane components and membrane protein binding molecules.

159-Plat
Quantitative FRAP Analysis Demonstrates that Raft Protein Clustering Alters N-Ras Depalmitoylation, Membrane Interactions and Activation Pattern
Sharon Eisenberg[1], Haguy Wolfenson[1], Ronen Berkovich[1], Ian A. Prior[2], Marcelo Ehrlich[1], Joseph Klafter[1], Michael Urbakh[1], **Yoav I. Henis**[1].
[1]Tel Aviv University, Tel Aviv, Israel, [2]University of Liverpool, Liverpool, United Kingdom.
The trafficking, membrane localization and lipid raft association of oncogenic Ras proteins dictate their isoform-specific biological responses. Accordingly, their spatiotemporal dynamics are tightly regulated. While extensively studied for H- and K-Ras, such information on N-Ras, an etiological oncogenic factor, is limited. Here, we report a novel mechanism regulating the activation-dependent spatiotemporal organization of N-Ras, its modulation by biologically-relevant stimuli, and isoform-specific effects on signaling. We applied patch/FRAP, FRAP beam-size analysis and a novel quantitative FRAP analysis of the diffusion and membrane binding kinetics of non-integral membrane proteins to investigate N-Ras membrane interactions. Clustering of raft-associated proteins, either glycosylphosphatidylinositol-anchored influenza hemagglutinin (HA-GPI) or fibronectin receptors, selectively enhanced the plasma membrane-cytoplasm exchange of N-Ras-GTP (preferentially associated with raft domains) in a cholesterol-dependent manner. EM analysis showed N-Ras-GTP localization in cholesterol-sensitive clusters, from which it preferentially detached upon HA-GPI crosslinking. HA-GPI clustering enhanced the Golgi-accumulation and signaling of EGF-stimulated N-Ras-GTP. Notably, the crosslinking-mediated enhancement of N-Ras-GTP exchange and Golgi accumulation strictly depended on depalmitoylation. We propose that the N-Ras activation pattern (e.g., by EGF) is altered by raft protein clustering, which enhances N-Ras-GTP raft localization and depalmitoylation, entailing its exchange and Golgi accumulation following repalmitoylation. This mechanism demonstrates a functional signaling role for the activation-dependent differential association of Ras isoforms with raft nanodomains.

160-Plat
Impact of Charcot-Marie-Tooth Type 2B Disease-Associated Rab7 Mutations on Signaling and Axonal Trafficking of NGF/TrkA
Kai Zhang[1], Yasuko Osakada[1], Liang Chen[2], Liming He[3], Bianxiao Cui[1], Chengbiao Wu[3].
[1]Stanford University, Stanford, CA, USA, [2]Lawrence Berkeley National Laboratory, Berkeley, CA, USA, [3]University of California at San Diego, La Jolla, CA, USA.
Charcot-Marie-Tooth type 2B (CMT-2B) is a neurodegenerative disease characterized by terminal axonal death. Genetic analysis from human CMT-2B patients revealed four missense point mutations (L129F, K157N, N161T, V162M) in their genes encoding a small GTPase Rab7, a marker for late endosomes in the degradation pathway. The exact mechanism of how Rab7 mutants cause CMT-2B remains poorly understood. Here, we analyzed the effect of Rab7 mutants on the signaling and axonal transport of a nerve growth factor (NGF) receptor - TrkA. Fluorescent protein-engineered Rab7 and TrkA were transfected in rat embryonic dorsal root ganglia neuronal cells. Axonal transport of Rab7- and TrkA-containing endosomes was followed by time-stamped live cell fluorescence microscopy. We found that TrkA moves roughly twice as fast as Rab7s, among which CMT-2B associated Rab7 mutants outpace wt-Rab7. Curiously, endosomes co-transfected with both Rab7 and TrkA move even slower than those with singly transfected Rab7. Western blot analysis from Rab7/TrkA-cotransfected PC12 cells showed that the level of phosphorylated TrkA is lower in Rab7 mutants that that in wt-Rab7. Our results suggested that Rab7 mutants can potentially contribute to CMT-2B by dis-regulating NGF-TrkA signaling via perturbing the balance between retrograde and anterograde axonal transport processes. These results imply that axonal transport can be a potential treatment target for CMT-2B neurodegeneration.

161-Plat
Lipid Membrane Association of the T Cell Antigen Receptor ζ Subunit: Affinities and Structure
Prabhanshu Shekhar[1], Kerstin Zimmermann[2], Mathias Lösche[1,3], Lawrence J. Stern[2], **Frank Heinrich**[1,3].
[1]Carnegie Mellon University, Pittsburgh, PA, USA, [2]University of Massachusetts Medical School, Worcester, MA, USA, [3]NIST Center for Neutron Research, Gaithersburg, MD, USA.
The T cell antigen receptor (TCR) is a cell surface protein complex that binds peptide antigen fragments presented by the Major Histocompatibility Complex (MHC). The interaction of TCR with MHC leads to T cell activation and initiation of an immune response,[1] but the molecular details of the transmembrane signaling process remain unknown. TCRζ is the 115 amino acid long cytosolic signaling domain of the CD3-T cell receptor complex that plays a central role in this process. TCRζ is predominantly unstructured[2] and carries several copies of the immunoreceptor tyrosine-based activation motif (ITAM). ITAMs become phosphorylated upon receptor engagement, constituting an early and obligatory event in the signaling cascade that blocks lipid association and lipid-associated conformational rearrangements[3] of TCRζ. We present a characterization of the affinity of TCRζ to tethered lipid bilayers by SPR and a structural study of its association with such membranes using neutron reflectometry (NR). We studied the binding of unphosphorylated TCRζ to highly charged stBLMs rich in anionic phosphatidylserine (PS) or phosphatidylglycerol (PG) and observed affinity constants of $K_d \approx 10$ μM. Minute amounts of phosphoinositides ($PI(4,5)P_2$) increase the membrane affinity of the protein by more than 10-fold. An NR characterization of TCRζ in the membrane-associated state shows the major portion of the protein interfacially associated with the bilayer surface and a minor protein penetrating the bilayer deeply. This is consistent with a model in which an α-helical structure is aligned on the membrane surface that may be connected via a hinge with another α-helical segment inserted into the bilayer.
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162-Plat
Membrane Protein Cluster Titration in Early Lymphocyte Signaling
Geoff P. O'Donoghue, Alex A. Smoligovets, Jay T. Groves.
UC Berkeley, Berkeley, CA, USA.
T cells are known to be sensitive to extremely small numbers of agonist peptides. Early T cell signaling originates in and is sustained by T Cell Receptor (TCR) microclusters. Recent reports indicate that T cell triggering thresholds are determined by the number of agonist peptide in individual TCR microclusters, a conclusion supported by the observation that 2D peptide-MHC kinetics are characterized by unexpectedly fast on-rates. Yet, questions remain regarding the interplay, if any, between signaling microclusters associated with the plasma membrane. We have studied the relationship between peptide number and protein cluster formation in early T cell signaling by direct, simultaneous observation of fluorescently labeled agonist peptides and the early T cell signaling molecule Zap70 genetically fused to EGFP in primary murine T cells. Our experiments replace the antigen-presenting cell with a supported lipid bilayer (SLB) chemically functionalized with peptide-MHC and ICAM-1. This approach allows us to titrate the density of triggering agonist peptide, while also providing a flat interface suitable for high-resolution TIRF imaging. By integrating micro-patterned chromium supports onto a cover glass we can also segregate the SLB into an array of spatially distinct micron-scale reaction centers. Using this method we measure antigen input vs. TCR triggering output in living primary T cells. These results have important implications concerning the role of cooperativity between adjacent TCR clusters in tuning the sensitivity of T cell triggering by exogenous agonist peptides.

Platform: Membrane Dynamics & Bilayer Probes

163-Plat
Nanoscale Interactions of Lipids and Proteins in Live Cell Membranes Revealed by STED Nanoscopy
Alf Honigmann, Veronika Mueller, Vladimir Belov, Stefan W. Hell, Christian Eggeling.
MPI Biophysical Chemistry, Goettingen, Germany.
The plasma membrane seems to be dynamically structured in regions of different composition and function. However, the spatial and temporal scale