

recent progress in understanding mechanisms of vesicle fusion, the molecular mechanisms of synaptotagmin C2AB membrane docking remain incompletely characterized. For example, the two C2 domains of Syt1 are reported to cooperatively insert into target membranes, but specific interdomain contacts have not been identified. To test whether the two C2 domains from Syt7 interact on a planar lipid bilayer, lateral diffusion constants of fluorescent-tagged C2A, C2B, and C2AB domains from human Syt7 were measured on PC:PS (3:1) bilayers using total internal reflection fluorescence microscopy with single-particle tracking. The Syt7 C2AB tandem exhibits a lateral diffusion constant half the value of the isolated single domains, and does not change when additional residues are engineered into the C2A-C2B linker. This is the expected result if C2A and C2B are separated when membrane-bound; theory predicts that C2AB diffusion would be faster if the two domains interact. Furthermore, ensemble stopped-flow measurements of membrane dissociation kinetics also support an absence of interdomain interactions, as EDTA-induced dissociation kinetics of the C2AB tandem are similar to the isolated C2A domain and remain unchanged when rigid or flexible linker extensions are included. Together, the results suggest that the two C2 domains of Syt7 bind independently to membranes. Ongoing efforts seek to perform analogous measurements with Syt1, whose C2 domains have much shorter membrane-bound lifetimes.

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Single Molecule Diffusion Studies of PTEN: Insights into Membrane Binding

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PTEN, a tumor suppressor gene that encodes a dual specificity phosphatase that dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P₃), is one of the most frequent genes deleted or mutated in a wide variety of tumors. PTEN acts as an antagonist to phosphoinositide-3-kinase (PI3K) signaling, thereby affecting various cellular processes such as cell proliferation and survival. The activity of PTEN is regulated by dynamic shuttling between the cytoplasm and the plasma membrane. The membrane association of PTEN strongly depends on the composition and lateral distribution of the lipids in the membrane. Several biophysical techniques have been used to characterize PTEN-membrane interaction. Here, we use single-molecule total internal reflection fluorescence microscopy (TIRFM), which is a powerful tool to study the molecular mechanism of membrane targeted proteins. The single-molecule TIRFM allows us to observe single PTEN molecules as they dynamically associate/dissociate and laterally diffuse along the lipid bilayer membrane. PTEN lipid binding is investigated on supported lipid bilayers of binary and ternary lipid mixtures of PC with physiological relevant levels of PS, PI(4,5)P₂ and PI. We tracked individual PTEN molecules and statistically determine the lateral diffusion and dwell time of PTEN on heterogeneous lipid bilayers. We find significant differences in PTEN dynamic behavior when bound to the different membrane environments. Furthermore, to gain insight into the molecular mechanisms of PTEN membrane association, we compared the lipid binding of wt PTEN, an N-terminally truncated PTEN-(Δ1-15 AA) that lacks the PI(4,5)P₂ binding site, and the recently discovered, PTEN-L with a 173 AA N-terminal extension. We find profound differences in the dynamic behavior of these PTEN derivatives at the membrane. It has recently been suggested that PTEN associates as a dimer with the membrane. We find that the tendency to form membrane bound dimers varies among these PTEN variants.

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A Single-Molecule Imaging Based Method for Estimating Subunit Stoichiometry of Purified Membrane Protein Complexes in Liposomes

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Estimating the number of subunits of a purified novel membrane protein is challenge. There are various methods for determining stoichiometry accurately in detergent, but often we are interested in the protein structure in the native solvent environment - the lipid bilayer. Here we describe a robust and widely applicable method to estimate the number of subunits in a purified membrane protein sample using single molecule fluorescent imaging. First, the multimeric protein complex is solubilized in detergent and purified. Second, it is quantitatively labelled with Cy5-maleimide and reconstituted into liposomes made of

E. coli polar lipids or 2:1 POPE/POPG, doped with 0.3% of AF488-NHS ester labelled POPE. Following multiple freeze/thaw cycles to form multilamellar vesicles, the proteoliposomes are extruded through polycarbonate filters of 30, 100 and 400 nm pore diameter resulting in reproducibly distinct size distributions. Reconstitution follows the Poisson distribution resulting in liposomes containing either 0, 1, 2 or more protein molecules. The apparent protein occupancy into liposomes depends on protein:lipid density, efficiency of fluorescent labelling, liposome surface area and the subunit stoichiometry of the protein complex. Liposome size distributions are determined by cryo-electron microscopy whereas the protein density and fluorescent-labeling is controlled during the reconstitution step. We measure the first three terms in the Poisson distribution: F0 (unoccupied liposomes), F1 (single occupancy) and F2 (double occupancy) by single molecule imaging of the fluorescent proteoliposomes to measure protein/lipid co-localization and photobleaching of protein conjugated fluorophores. We test our model with a CLC-ec1 engineered monomeric construct, the native CLC-ec1 homodimer and tetrameric KcsA K⁺ channel. The methods outlined in this study can be used to determine the subunit stoichiometry of unknown purified membrane protein complexes in a variety of liposome environments.

Mechanosensation

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Nanobiomechanics and Mechanotransduction of Sensory Neurons

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A detailed knowledge of mechanical parameters such as cell elasticity, stiffness and viscoelasticity is essential for understanding the mechanisms that control the mechanotransduction in mechanosensory neurons (MSN). Indeed, in order to tune and maximize their sensitivity, MSN should be neither too rigid nor too compliant; moreover they are expected to show different elasticity as a function of the typology of mechanical stimulus they should record. However a precise correlation between MSN mechanical properties and mechanotransduction mechanism is still missing, and the sensory mechanical transduction, necessary for the senses of touch and pain, remains poorly understood.

Indentation measurements by atomic force microscopy (AFM) enable to investigate and quantify in vitro the softness of living MSN thanks to its ability to measure low forces (pN) and nanometer scale displacement. Moreover, the integration of AFM with fluorescence microscopy opens up the possibility to relate the involvement or activation of either cytoplasmatic structures or transmembrane proteins with variations of cell mechanical properties and, as result, their role in the modulation of mechanosensory neurons activity.

In this study we performed AFM indentation measurements to evaluate the mechanical properties of wild type and genetically modified proteins of the stomatin system of dorsal root ganglia (DRG). We found a decrease of cell elasticity in DRG neurons where stomatin system is genetically modified. The role of cell elasticity in mechanotransduction regulation of mechanosensory neurons is discussed.

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Cross-Linked Matrix Rigidity and Soluble Factors Induce Differentiation via Distinct but Overlapping Pathways

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Biophysical Eng'g Lab, University of Pennsylvania, Philadelphia, PA, USA. Stem cell differentiation is regulated by both soluble factors and the physical properties of extracellular matrix, but the extent to which differentiation pathways are distinct or overlap is often unclear. Here, the micromechanical stiffness of the collagenous bone surface together with broad compositional correlations with collagen-I across many soft tissues suggests enzymatic cross-linking of matrix correlates with nucleoskeletal protein lamin-A, with a retinoid receptor RARG, and with induction toward osteogenesis. Collagen films just 2 nm thick on mica were stiffened or not by transglutaminase cross-linking and used as minimal culture substrates for Mesenchymal stem cells (MSCs). Cells pulling on pristine nano-films visibly deformed and aligned with the collagen fibrils, but on cross-linked films, cells spread isotropically as if adhering to a substrate of greater effective stiffness. Cell nuclei also spread and stiffened, with an increase of lamin-A, nuclear localization of RARG, and upregulation of key early and late osteogenic factors. RARG antagonists also increased lamin-A, and enhanced osteogenesis on rigid substrates in vitro as well as in xenografts of MSCs in mice. A model of the underlying Mechanochemical Gene Circuit couples the sensitivity of stem cells to both insoluble