Secretory granules transit through a series of highly regulated associations and dissociations of molecular interactions enroute to membrane fusion and exocytotic release of the granule content. The ability to sequence these protein-protein interactions as they occur in living cells in real-time, with high spatial resolution, is paramount to furthering our understanding of how they relate to the functional state of the secretory granule as it transits the regulated exocytotic pathway. In the present study we have taken both an experimental and a theoretical approach to gain a quantitative understanding of the effects of evanescent illumination on sensitized-emission FRET calibrations and measurements, under a variety of conditions that mimic differing subcellular localizations of interacting molecules. Our results demonstrate that the TIRF-FRET method is straightforward for simple situations in which both donor and acceptor are on the same molecule and localized to the plasma membrane. By comparison when donor and acceptor molecules are localized to multiple intracellular compartments and where one compartment may be mobile, additional considerations must be taken into account. Our results define several of the parameters that are critical to the quantitative application of this method in living cells. Moreover, we demonstrate use of TIRF-FRET to visualize and quantify a specific set of bi-molecular interactions on insulin secretory granules in Min6 cells as they occur in time and subcellular space within the cell and we correlate these to the secretory event. This work supported by NIH, NINDS 039914 and NIDDK 053978.

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Patterning Single Cell-Electrode Pairs for Electrochemical Measurement of Quantal Exocytosis on Microchips

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We are developing transparent multi- electrochemical electrode arrays on microchips in order to automate measurement of quantal exocytosis. Design goals are that one and only one cell be positioned directly over each electrode and working electrodes have μm-scale dimensions in order to resolve pA-level currents. Patterning of cell-adhesion molecules in register with electrodes using conventional photolithographic approaches is problematic because organic solvents can disable sensitive biomolecule films. We report the parylene ''dry liftoff'' approach pioneered by Ilic and Craighead (Biomed Microdev 2: 317, 2000) can be used to pattern single cell-electrode pairs on the chip. A 1 μm-thick parylene C film is deposited on the multi-electrode array and S1813 photoresist is spin coated onto the device and patterned. The unprotected parylene over the electrodes is then removed using Reactive Ion Etch. Poly-l-lysine (PLL) is then added to promote cell attachment. Chromaffin cells are loaded on the chip in standard culture media and left in an incubator overnight. Finally, the parylene film is peeled off to remove excess cells and PLL, leaving tightly adhered chromaffin cells at the desired locations. Importantly, we find that promoting cell attachment with PLL films does not passivate the electrochemical electrodes. Experiments are in process to explore an alternative approach whereby PLL is patterned using the dry liftoff approach but cells are added after peel off of the parylene. With this approach, cell attachment to inactive areas of the chip is blocked by using ''cytophobic'' materials such as Teflon AF. This alternative approach may allow efficient targeting of cells at lower cell densities as cells migrate from cytophobic areas to the electrode binding sites (Supported by NIH BRP grant RO1 NS048826).

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Endophilin N-BAR Domains-induced Membrane Remodeling Revealed by Molecular Dynamics Simulations

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Endophilin N-BAR domains play a critical rule in membrane remodeling (e.g., endocytosis, synapses) due to their membrane sculpting abilities. Presently, roles of the amphipathic helices and the positively charged concave surface on the crescent dimer in membrane remodeling are still not well understood. In addition, the endophilin N-BAR domain has one additional inserted helix on each of the monomers, thus making it unique in the entire BAR superfamily. Both the structure and the function of this additional helix are unknown up to now. Interestingly, the tubulated structures of endophilin N-BAR domains are much larger than the corresponding amphiphysin N-BAR domains. It is important to investigate the effect of the inserted helices in order to fully understand the mechanism of endophilin N-BAR domain protein driven liposome tubulation. Large scale all-atom molecular dynamics simulations are used to examine the details of the endophilin mediated membrane remodeling process. By comparing the results of different possible arrangements of the protein and membrane, we predict the optimum location of the additional helix. These results will facilitate in understanding the overall mechanism of endophilin N-BAR domains membrane oligomerization and remodeling.

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Chronic Palmitate Exposure Inhibits Insulin Secretion By Dissociation of Ca^{2+}-Channels From Secretory Vesicles

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Type-2 diabetes, characterized by insufficient insulin secretion, shows a strong correlation with obesity and elevated plasma levels of free fatty acids (FFA). Long-term exposure of pancreatic islets to FFAs results in marked suppression of glucose-induced insulin secretion. Although the latter effect has been extensively characterized, the cellular mechanisms remain enigmatic. We have examined the effect of long-term exposure of pancreatic β-cells to palmitate using a combination of elecrophysiology and evanescent field microscopy. Here we show that rapid exocytosis in β-cells requires discrete microdomains of Ca^{2+}-entry close to the secretory vesicles and that this arrangement becomes disrupted following palmitate exposure. This culminates in the selective suppression of insulin release during brief (<50 ms) action potential-like stimulation whereas exocytosis evoked by unphysiologically long (>300 ms) pulses is unaffected. Additionally, inclusion of the slow Ca^{2+}-buffer EGTA (10 mM) in the electrode solution reversed the restored secretion observed during long pulses. Prolongation of the β-cell action potential by pharmacological maneuvers which expand the $[Ca^{2+}]_i$ microdomains corrects the FFA-induced secretion defect in both mouse and human islets. We propose that the FFA-induced dissociation of Ca^{2+}-entry from vesicles in β-cells selectively impairs the readily-releasable pool of vesicles but leaves vesicle docking with the membrane unaffected. This finding may represent an evolutionarily preserved mechanism to abate insulin secretion during nutrient deprivation when normoglycaemia is maintained by mobilization of lipids from fat depots.

Endoplasmic Reticulum & Protein Trafficking

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Conformational transition of the Sec translocon induced by channel partner: A molecular dynamics study

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Sec translocon is a highly conserved heteromeric membrane protein complex, which functions as a protein-conducting channel. In bacteria, the Sec translocon (SecYEG) achieves the translocation of polypeptides across the membrane by binding of the channel partner, SecA ATPase. However, little is known about the atomically detailed mechanism on the translocation. Recently, a new crystal structure of the SecYE translocon bound with an anti-SecY Fab fragment has been determined. It contains a large hydrophobic crevasse open to the cytoplasm (the pre-open form) and differ from the crystal structure of SecYEβ from *Methanococcus jannaschii* in the closed form, suggesting that the binding of a channel partner induces a large conformational change of the Sec translocon in the initial step of the polypeptide translocation. To investigate the role of channel-partner binding to the SecYE translocon, we performed all-atom molecular dynamics simulations of SecYE with and without a Fab fragment in explicit membrane. During a 100-ns simulation, SecYE undergoes a large conformational transition toward the closed form in the absence of a Fab fragment, whereas the structure keeps the widely opened crevasse in the simulation of SecYE with a Fab fragment. In the transition, protein-lipid interaction around the lateral gate region of SecYE is changed greatly, indicating that there is a competition of interactions between the protein and phospholipid molecules, which is controlled by the binding of the channel partner to the SecYE transolon.

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Simulations of Multi-protein Complexes: Structure, Binding Affinity, and Dynamics of Vps27/hse1 Bound to Membrane-tethered Ubiquitin

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Protein-protein interactions play an essential role in many cellular functions. While biophysical and structural characterizations have traditionally focused on strong binary complexes, the biological importance of weakly bound multi-protein complexes is increasingly recognized. Such complexes typically contain various proteins, with different folded domains held together in part by flexible linkers. Further increasing the complexity, many multi-protein

complexes assemble at or near membranes. We develop coarse-grained models and effective energy functions for simulating such large multi-protein complexes with low to intermediate binding affinities ($K_d > 1$ μM). The models are validated against structure and binding-affinity data for a broad range of binary protein complexes. Using replica exchange Monte Carlo simulation techniques, we apply our model to study the assembly, energetics, and dynamics of the complex between Vps27/Hse1 and membrane-tethered ubiquitin. The yeast Vps27/Hse1 complex and the homologous mammalian Hrs/STAM complex deliver ubiquitinated transmembrane proteins to the ESCRT endosomal protein-sorting pathway that is important in many biological processes. Vps27 and Hse1 contain several folded domains and flexible linkers. We find that the membrane-tethered ubiquitin binds preferentially to the UIM domains of Vps27. However, the simulations also show that ubiquitin interacts with other domains. The observed multiple specific and non-specific ubiquitin-Vps27 interactions greatly enhance the overall binding affinity. In the complex, the structure of Vps27/Hse1 is highly dynamic and flexible, reflecting the ability of Vps27/Hse1 to bind to a diverse set of ubiquitinated protein targets. The models developed here can easily incorporate additional experimental information (e.g., from fluorescence, scattering, electron microscopy), and hold promise for simulations of other large multi-protein complexes.

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Modulation of Membrane Mechanical Properties by Sar1, a Vesicle Trafficking Protein

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The trafficking of cargo in cells involves dramatic changes in membrane shape and topology. Though trafficking is widely studied and the identities and biochemical interactions of the responsible proteins are well mapped, remarkably little is known about the mechanics involved. We focus on Sar1, the key regulator of the coat protein complex II (COPII) family that ferries newly synthesized proteins from the ER to the Golgi. Sar1 is the only member of the COPII coat that interacts directly with the ER lipid bilayer membrane. It has an amphipathic N-terminal helix; when Sar1 is GTP-bound, the helix is exposed and the hydrophobic hemi-cylinder can insert into the bilayer. To investigate whether Sar1 has a physical role beyond merely localizing the other COPII proteins, we directly measure the force involved in membrane deformation as a function of its presence or absence, using optical traps and membrane-bound microspheres to pull tethers from lipid membranes. The lipid composition and large available surface area mimic the composition and geometry of the ER. Measurements of tether forces and radii allow extraction of the membrane bending modulus, the material parameter that dictates the energy required for deformation. We find that the bending modulus measured in the presence of Sar1 with a non-hydrolyzable GTP analogue, at concentrations sufficient for dense membrane coverage, is half that measured without Sar1 or with Sar1-GDP. These results reveal a paradigm-altering insight into COPII trafficking: Sar1 actively alters the material properties of the membranes it binds to, lowering the energetic cost of curvature generation.

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Blocking helix formation without blocking organellar localization in Plasmodium falciparum

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Transit peptide (TP) recognition in mitochondria and chloroplast localization is well described and requires a receptor to recognize the TP bound as an amphipathic alpha helix. This functional interaction leads to organellar import of the payload protein. *Plasmodium falciparum* (*Pf*), the causative agent of malaria, contains an organelle called the apicoplast. The apicoplast is evolutionarily related to the chloroplast, is essential to the metabolism of *Pf*, and contains numerous putative drug targets. As in chloroplasts, nuclear-encoded apicoplast proteins must be post-translationally targeted to the apicoplast. In contrast to chloroplast localization, molecular details of TP recognition in *Pf* are currently unknown. To assess if apicoplast TPs must form helical intermediates for proper organellar localization, we have examined the TP of *Pf* acyl carrier protein by circular dichroism (CD), nuclear magnetic resonance (NMR), and epifluorescent microscopy of mutant TP-GFP fusions. CD and NMR of acyl carrier protein with its TP in solution are consistent with the presence of a small population of helix in the TP. However, structure-disrupting proline mutations are correctly targeted to the apicoplast when observed *in vivo*. This observation contradicts the theory that apicoplast TP recognition occurs via a mechanism similar to chloroplast TPs, and instead suggests that the dominant population of disordered TP may be the active form and that *Pf* has evolved a distinct solution to the problem of organellar targeting.

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Mapping of the Signal Peptide-binding Domain of Escherichia coli SecA Using Förster Resonance Energy Transfer

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Identification of the signal peptide-binding domain within SecA ATPase is an important goal for understanding the molecular basis of SecA preprotein recognition as well as elucidating the chemo-mechanical cycle of this nanomotor during protein translocation. While recent studies have addressed this topic, the precise signal-peptide binding site on SecA remains controversial. The aim of the present study was to identify the SecA signal peptide-binding site using Förster Resonance Energy Transfer (FRET). FRET provides a more global view of the binding site and circumvents the common limitations of more genetic approaches where deletion and substitution mutagenesis can confound the correct interpretation of protein structure-function analysis. This study employs a collection of functional, monocysteine SecA mutants labeled with a donor fluorophore along with cysteine-containing, acceptor fluorophore-carrying PhoA signal peptides. Fluorescence anisotropy was utilized to determine equilibrium binding constants of 1.4 μM or 10.7 μM for the alkaline phosphatase signal peptide labeled at residue 22 or 2, respectively, for SecA, with a binding stoichiometry of one signal peptide bound per SecA protomer. Distance measurements determined for nine SecA mutants indicate that the signal peptide-binding domain encompasses a region proximal to residues 225–228, 371–375, 652–657, and 771–780 when mapped onto the recent NMR structure of SecA (Gelis, I., Bonvin, A., Keramisanou, D., Koukaki, M., Gouridis, G., Karamanou, S., Economou, A., and Kalodimos, C. (2007) Cell 131, 756–769). This places the signal peptide-binding domain within the heart of SecA, surrounded by and potentially responsive to domains important for binding nucleotide, mature portions of the preprotein, and the SecYEG channel component.

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Evaluating Protein Interactions & Organelle Dynamics in Saccharomyces cerevisiae: Spatial Distribution of Molecular Chaperone/Co-Chaperones Evident at a Sub-organelle Level in the Endoplasmic Reticulum

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BiP/Kar2 is a member of the Hsp70 family of chaperones that resides in the endoplasmic reticulum (ER) of *S. cerevisiae*. Biochemical and genetics experiments have demonstrated BiP's association with selective co-chaperones in multiple critical processes of the cell including translocation of protein into the ER, protein folding/maturation, and ER-associated degradation (ERAD). BiP's relative high cellular concentration combined with the low resolution of traditional immunofluorescence techniques has hindered the determination of protein localization effects. We hypothesize that the spatial heterogeneity of chaperones is regulated by co-chaperones, and this heterogeneity serves as a means of dictating cellular functions. To generate physiologically-relevant data of protein dynamics in *S. cerevisiae*, variants of green fluorescent protein (GFP) coupled with advances in confocal light microscopy techniques have allowed us to track multiple fluorescently-tagged proteins *in vivo.*

Dual expression strains composed of fusion proteins, BiP and co-chaperone Sec63, reveal that a heterogeneous spatial distribution is evident at the sub-organelle level. Secondary confirmation of our results has been performed using immunofluorescence techniques in multiple *S. cerevisiae* strains. Deconvolution of fixed cell images has allowed us to reconstruct and quantify the co-localization of BiP and Sec63 in three dimensions. We have captured the spatiotemporal effects of protein dynamics in live cells by monitoring ER membrane and luminal proteins, in addition to the nuclear pore complex; confirmed that the ER is continuous through Fluorescence Loss in Photobleaching (FLiP) experiments; and captured rapid diffusion of ER resident proteins. Integration of experimental data and computational design enables us to develop stochastic models of biological systems that accurately reflect spatiotemporal effects of molecular chaperone/co-chaperone interactions.

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Microtubule Network is Necessary to Direct and Maintain The Apical Localization of Slo1 Channels in Epithelial Cells

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The cytoskeleton plays a key role in different cellular processes such as cell motility, muscle contraction, mitosis and maintenance of cell shape. In polarized cells, microtubules are involved mainly in the apical targeting of proteins.