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Direct Detection of Reconstituted, Snare-Mediated Fusion Pore Dynamics

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Exocytosis underlies release of neurotransmitters and hormones. Electrophysiological and electrochemical measurements from live cells have shown that the initial fusion pore is small (~1 nm diameter) and can repeatedly flicker before either dilating fully, or closing permanently. The fraction of flickering pores and flicker characteristics vary with stimulation strength, regulating the amount and size of released cargo. The molecular mechanisms regulating fusion pore dynamics are not well understood, partly because in vitro techniques with sufficient resolution have been lacking.

Here we present a novel assay that can directly report fusion pores formed between cells ectopically expressing "flipped t-SNAREs" (t-Cell) and nanodiscs (~17 nm flat bilayers stabilized with the membrane scaffold protein) reconstituted with the cognate v-SNAREs (v-NDs). Currents from a t-Cell-attached membrane patch are recorded. v-NDs that are placed into the patch pipette slowly diffuse to the patched cell surface where outward-facing t-SNAREs are present. Fusion of the v-ND with the cell patch results in a fusion pore connecting the cytoplasm to the pipette solution, whose expansion is prevented by the ND scaffold. This results in a long-lived fusion pore whose size fluctuations are directly related to the measured current fluctuations. These current measurements, reminiscent of single-channel recordings, provide high signal-to-noise ratios and are free of potential artifacts compared with time-resolved admittance and electrochemical measurements. 80-100% of the patched t-Cells had at least one opening (fusion event) with v-NDs, whereas dramatically fewer openings with much smaller amplitudes were observed in control experiments where fusion was inhibited using neurotoxins, protein-free NDs, wild-type cells, or in the presence of the soluble domain of the v-SNARE which competitively binds to available t-SNAREs. Typical openings had currents of a few pA, corresponding to a conductance of ~400 pS and a pore size of ~1 nm.

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Interference of Zippering of Snare Complexes by Alpha-Snap Arrest Fusion of Chromaffin Granule

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Neuronal exocytosis is mediated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Before fusion, SNARE proteins form complexes bridging the membrane followed by assembly toward the C-terminal membrane anchors, thus initiating membrane fusion. After fusion, the SNARE complex is disassembled by the AAA-ATPase NSF that requires the cofactor α-SNAP to first bind to the assembled SNARE complex. *In vitro*, SNARE-mediated fusion can be readily reconstituted, but it has been difficult to reproduce a docked state in which the SNAREs are arrested in a partially zippered state. Using both native and artificial vesicles we now show that α-SNAP on its own retards assembly of membrane-anchored SNARE complexes and can arrest SNAREs in a partially assembled *trans*-complex, preventing progression towards fusion, which can be rescued by active NSF. Intriguingly, the inhibitory effect of α-SNAP depends on the size of the vesicle, with inhibition being most profound in large vesicles. Our data suggest that binding of α-SNAP lowers the energy yield of the zippering reaction, inhibiting preferentially the fusion of large vesicles that exhibit low curvature strain and require more energy for overcoming the barrier for fusion.

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Comparative Study of the Snares Zippering with Single Molecule Resolution

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Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are ubiquitous in eukaryotes, and their assembly in a four-helix bundle drives membrane fusion. To monitor in real-time the folding of these proteins, we have developed a new versatile self-contained SNAREpin and have performed optical tweezers experiments on four representative sets of SNAREs: (i) Synaptic, (ii) GLUT4, (iii) early endosome, and (iv) yeast. (i) In neurons, SNAREs are tightly regulated to achieve fusion of the synaptic vesicles within one millisecond upon arrival of the action potential. (ii) GLUT4 is a highly effective glucose transporter responsible for most of glucose clearance in blood in response to insulin. (iii) Early endosome SNAREs are key players in membrane recycling between cell organelles. (iv) Yeast SNARE is believed to be representative of the proto-SNAREs that have expanded in metozoa during adaption to multicellular lifestyle. Despite the fact that biological context of the different sets of SNAREs is very different in respect to their regulation, as well as their time constrains for fusion and energetic costs, we have found that the structural and physical properties of their assembly is highly conserved. In all SNAREs investigated, assembly proceeds from the N to C terminal of the bundle and is punctuated by three sequential binary switches: the amino- and carboxy-terminal followed by a linker domain. Finally, the energetic and structural pathways of the assembly we have quantified provide details on how SNAREs drives fusion and are regulated within the SNARE superfamily.

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Towards Artificial Membrane Fusion: Ek-Peptides, the Coilied-Coil Zipper

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Membrane fusion is an essential step in synaptic-vesicle function. The components of the protein machinery that enable the process *in vivo* are known, but how they work is not yet understood. When two membranes come together, proteins anchored in either membrane need to interact to initiate the fusion. In our model pairing of the E- with the K-peptide (sequence example K: $Ac(KIAALKE)_3GWCONH_2$) should furnish this initial zipper by coiled-coil assembly. Heterodimer formation between the E- and K-peptide is detected by electron paramagnetic resonance (EPR) in situ, i.e. in buffer solution. First indication is the reduced mobility of a spin-labelled peptide when the partner is present (see Fig.). The absence of spin-spin interactions with distances < 0.8 nm in samples where both partners carry a spin-label puts constraints on the structure of the EK-heterodimer. Pulsed EPR-distance measurements complete the picture.

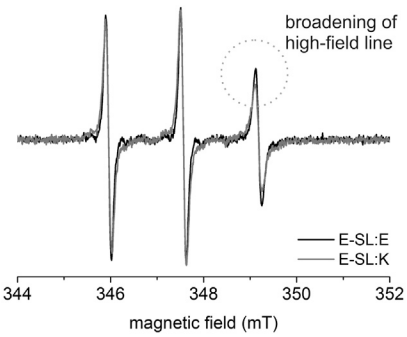

Fig. EPR spectra of the peptides E-SL with E (in black) and peptides E-SL with K (in red). Peptide E-SL has a spin label at the C-terminus. Conditions: 9 GHz, continuous wave EPR at 293 K, 100 kHz mod with 0.4 G_{pp} ampl.

Concluding, functionality of the zipper section can be tested and is confirmed by EPR. Since EPR is not limited by protein size and is applicable to vesicle solutions, also the final construct comprising membrane anchor, linker, and zipper can be tested in a membrane environment.

Membrane Structure II

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The Role of Phosphoinositol Lipids in Amot Membrane Association

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The Angiomotin (Amot) family of adaptor proteins binds core polarity proteins involved in polarization of the apical membrane and transcriptional co-activators as a regulator of cell growth and migration. The Amot coiled-coil homology (ACCH) domain has the unique property to selectively bind monophosphorylated phosphatidylinositols (PI) in a similar manner as FYVE, PX and PH domains. We endeavored to understand the physical properties of these PI containing membranes as an interface between the ACCH domain and the lipidic environment for membrane association. As a result, we suggest that the presence of the PI lipid induces a phase separation thereby creating an enriched nano- to micro- scaled ordered lipid domain. It is under this context that we then are able to discuss ACCH domain activity as a function of lipid content, as well as further design assays to ascertain the