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Regulated Membrane Fusion: Molecular Mechanisms and Machinery

Goal
To solve the entire reaction cascade mediating regulated exocytosis on a functional and structural basis. Using neurotransmitter release as a model system, the physiological events shall be reconstituted in an in vitro liposome fusion assay employing purified proteins and defined lipid species to reveal the concerted interplay of the components at distinct reaction steps.

Background
Regulated exocytosis at the neuronal synapse is a multistep process starting with synaptic vesicle tethering at the active zone followed by vesicle priming and culminating in fast calcium-triggered membrane merger. Distinct protein-protein interactions forming macromolecular assemblies control individual steps and sequentially bring the two membranes into close proximity creating a hemifusion intermediate, which finally leads to fusion pore opening and dilation. The key protein machinery has been identified: vesicle tethering: Rab3, Rim1; priming: Munc13-1 and Munc18-1; calcium regulation: synaptotagmin1 and complexin; fusion machinery: SNARE proteins (syntaxin1, SNAP-25, VAMP2). In addition, specific lipids such as phosphoinositides and diacylglycerol play distinct functions in the assembly reaction (see Figure 1).

In more detail, following the initial tethering step, Munc13-1 and Munc18-1 control the initial pairing of syntaxin1 and SNAP-25 on the plasma membrane with VAMP2 on synaptic vesicles, thereby contributing specificity to membrane trafficking. Subsequently, the trans SNARE complex (SNAREpin) starts to zipper into a four-helix bundle. This protein folding reaction provides the driving force for membrane fusion. Complexin stabilizes a half-zippered SNAREpin but blocks the zipping of the membrane proximal SNARE motifs. Synaptotagmin1, the calcium sensor contributes to this block in an ill-defined manner and in the presence of calcium confers fast synchronized membrane fusion. How these proteins are exactly arranged in the distinct prefusion intermediates and how they affect the lipid environment and vice versa are key questions in cell biology. Furthermore, the opening and structure of fusion pores remain obscure events.

Reconstituted membrane fusion assays, including purified proteins and liposomes are instrumental to reveal structural and functional contributions of individual components in a well-defined lipid bilayer system.
Research Highlights

By reconstituting preassembled syntaxin1/SNAP-25 into giant unilamellar vesicles (GUVs, mimicking the plasma membrane) and VAMP2, together with synaptotagmin1 into small unilamellar vesicles (SUVs, mimicking synaptic vesicles) and adding complexin and Munc18-1, we were able to functionally reconstitute the equivalent of a readily releasable pool of vesicles, which fuses in a calcium-synchronized manner. Using cryo electron tomography in collaboration with John Briggs at the EMBL, we could demonstrate that this late prefusion intermediate results in the formation of membrane protrusions on GUVs, which likely lower the activation energy for membrane fusion, permitting calcium-triggered fusion pore opening on a sub-millisecond time scale.

To allow such a fast response, the prefusion intermediate relies on weak protein-protein and protein-lipid interactions. Considering the membrane constraints and the weak interactions, it is technically challenging to identify the relevant binding sites. To determine interaction sites on a single amino acid level, we started to scan the accessory helix of complexin, which contributes to the fusion-clamp. Using a site-specific photo-crosslinking approach, we introduced the unnatural photo-activatable amino-acid p-benzoyl-l-phenylalaine (BPA) at every position covering amino acids 23-49 of complexin II (see Figure 2). Most of these substitutions, with the exception of E23 had only minor effects on the clamping function of complexin in the reconstituted fusion assay. However, upon photo-activation, crosslinks to the nearest neighbor impaired significantly calcium triggered lipid mixing. The systematic analysis of the crosslink products revealed that the accessory helix of complexin provides two interacting surfaces: one providing a binding site for the membrane proximal region of VAMP2, the other one an interaction site for the C-terminal region of the second SNARE motif of SNAP-25. Thus, the interactions with the C-terminal regions of VAMP2 and SNAP-25 apparently block final SNAREpin zippering. Considering the weak interaction, the simultaneous temporal C-terminal “inactivation” of two SNAREs could provide an efficient clamp not compromising the fast calcium trigger. Indeed, independent quadruple mutants of the relevant complexin residues covering the VAMP2 and SNAP-25 binding sites separately resulted in a significant loss of the fusion clamp. The role of E23 in fusion clamping could be confirmed in vivo in living neurons (collaboration with Christian Rosenmund, Charite-Universitätsmedizin Berlin).
This photo-crosslinking approach will now be extended to other proteins of the fusion machinery.

To identify further functional interaction sites in the fusion machinery we also introduced mutations at distinct positions of synaptotagmin1 and SNAP-25. In synaptotagmin1, we could show that the positively charged amino acids R398/R399, which are opposite to the calcium-binding site, play a postdocking role in membrane fusion. R398/R399 interact with the SNAREs and presumably directly couple the calcium-dependent synaptotagmin1-lipid interaction to rearrangements in the SNARE machinery. Collaborations with the groups of Matthijs Verhage at the University of Amsterdam and Jakob Sorensen at the University of Copenhagen demonstrated the physiological relevance of this interaction in neuronal cultures. Along the same line we also probed the other side of the interaction by introduced mutations in both helices of SNAP-25. Our in vitro experiments in combination with electrophysiological measurements in neurons (in collaboration with Jakob Sorensen), revealed that interactions between SNAP-25 and synaptotagmin1 are involved in vesicle priming, clamping spontaneous release and stimulating evoked neurotransmission.

Using GUVs containing syntaxin1 instead of preassembled t-SNAREs, we were also able to analyze earlier priming steps in SNARE complex assembly, which are controlled by Munc18-1. We could demonstrate that Munc18-1 phosphorylation at Y473 shuts off neurotransmitter release without affecting vesicle docking. This phosphorylation selectively impairs VAMP2 binding. A collaboration with Matthijs Verhage demonstrated that this tyrosine phosphorylation impairs the formation of the readily releasable, “sucrose-sensitive” vesicle pool.

We also established a single vesicle fusion assay using SUVs immobilized on a cover slip and FRET based lipid- and content-mixing using TIRF microscopy (see Figure 3). This assay will now allow us to resolve the role of regulatory components in vesicle docking und subsequent membrane fusion at the single vesicle level.

Overall, we have set up in vitro reconstituted lipid- and content-mixing assays at the biochemical and microscopic level. Crosslinking approaches have been developed to detect direct interactions.
at the single amino acid level allowing us to probe the local environment of distinct components in a stage-specific manner. However, we still need to establish the mass spectrometry analysis to identify the interacting peptides/residues. Collaborations with groups using electrophysiology with its exquisite temporal resolution in living cells (neurons, chromaffin cells) will demonstrate the physiological relevance. Cryo-electron microscopy employing single particle analysis and tomography using direct electron detectors should provide high-resolution structural insights into the structural organization of fusion machinery assembly intermediates and fusion pore dynamics. Thus, systematic functional and structural analyses of the membrane fusion machinery should be in reach.

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Figure 3: Single vesicle fusion assay using FRET-based lipid mixing and TIRF microscopy. (A) SUVs containing e.g. t-SNAREs and a lipid-coupled donor fluorophore (e.g. an Atto dye) are immobilized on a cover slip using a lipid-biotin/streptavidin/biotin-PEG sandwich. SUVs containing v-SNAREs and the acceptor fluorophore are added in the presence/absence of regulatory components. (B) Reactions are analyzed by TIRF microscopy. (C) Docking can be monitored by the colocalization of the donor and acceptor dyes. Fusion results in increased FRET and quenching of the donor fluorophore.