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Regulated Membrane Fusion: Molecular Mechanisms and Machinery

Goal

To decipher the cascade of reactions that confers regulated exocytosis, revealing the stage-specific molecular mechanisms that each individual component (proteins and lipids) plays along the reaction pathway. Structural alterations in the fusion machinery shall be linked to dynamic architectural changes in the lipid bilayers during membrane merger (fusion pore formation).

Background

Neurotransmitter release is the paradigm for regulated exocytosis, characterized by unprecedented temporal and spatial precision. The core machinery mediating intracellular membrane fusion consists of compartment-specific cognate v/t-SNARE pairs. The pairing of v-SNAREs (VAMP2) on a synaptic vesicle with their cognate t-SNAREs (syntaxin1/SNAP-25) on the plasma membrane (SNAREpin formation) is sufficient to drive membrane fusion. Actually, the assembly of cognate SNARE motifs - coiled-coiled regions with 15 layers of hydrophobic amino acids and a central ionic layer - into a four-helix bundle provides the driving force for membrane merger. This protein folding process starts at the membrane distal N-terminal end and sequentially progresses towards the membrane proximal C-terminal end of the SNARE complex thereby bringing the two membranes into close proximity. Remarkably, this assembly process is characterized by inherently built-in breakpoints, which are localized to the ionic layer in the center of the SNARE motif and the transitions to the subsequent linker region and to the transmembrane domain. These natural turning points seem to be superior sites for regulatory components that either slow down or speed up distinct steps. Thereby, defined vesicle pools of distinct sizes can be tailored to the specific needs of a secretory cell. Regulated exocytosis may be relatively slow, for example, by incorporating significant amounts of glucose transporters into the plasma membrane (to regulate the blood glucose level) or occur instantly e.g. releasing a single quantum of neurotransmitters (to propagate signals between neurons in the central nervous system). It has been shown that *in vitro* a single SNARE complex is sufficient to overcome the repulsive forces, which prevent spontaneous membrane fusion. However, in a physiological environment it is more likely that several SNARE complexes form macromolecular assemblies to build fusion pores of a defined size. Fusion pores are well characterized by electrophysiological methods, but their morphological architecture remains obscure

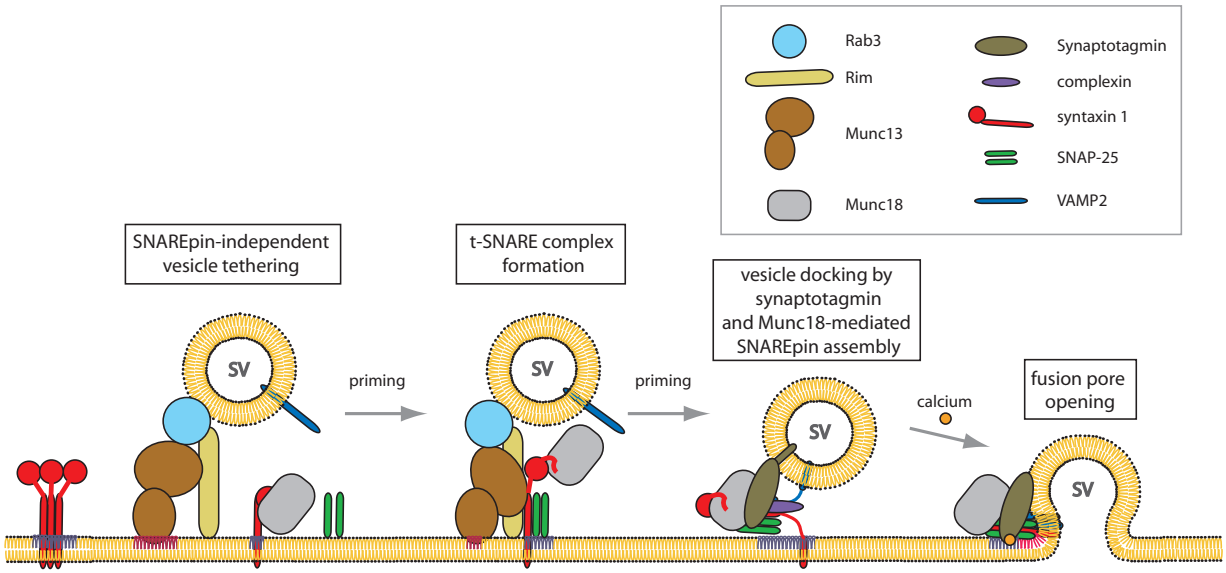

Fig. 1: *Components and reaction steps involved in regulated exocytosis at the neuronal synapse. Please note that only functionally relevant components are shown at the distinct reaction steps. Protein-lipid interactions are depicted by colored lipids.*

and controversial models are highly debated. Overall, neurotransmitter release requires several reaction steps (see Fig. 1). In an initial event synaptic vesicles are selectively *tethered* at the active zone of the nerve terminal. This step seems to occur independently of SNAREpin formation but involves small GTP binding proteins – Rab proteins – like Rab3/Rab27 on the synaptic vesicles and its effectors, like Rim1 at the active zone of the presynaptic plasma membrane. Subsequently, *vesicle priming* mediates t-SNARE complex assembly and initial SNAREpin formation resulting in a pool of *docked vesicles*, which is then converted into a *readily-releasable vesicle pool*. These reactions involve regulatory proteins like Munc13-1, Munc18-1, and the incorporation of the calcium responsive machinery - synaptotagmin 1 and complexin. The *readily-releasable pool* can now instantly respond to a Ca^{2+} trigger, which involves local membrane perturbations mediated by the Ca^{2+}-dependent interaction of synaptotagmin 1 with anionic membrane lipids and the release of the complexin clamp. Different types of regulated exocytosis use distinct synaptotagmin isoforms or other putative calcium sensors such as Doc2 and ferlin proteins, which are characterized by the presence of at least two C2 domains. In summary, the orderly interplay of these components with the local lipid environment precisely controls membrane merger.

Research Highlights

By reconstituting preassembled t-SNAREs into giant unilamellar vesicles (GUVs) and v-SNAREs into small unilamellar vesicles (SUVs) mimicking the plasma membrane and synaptic vesicles, respectively, we have tested which regulatory components are necessary to confer fast Ca^{2+}-synchronized membrane fusion *in vitro* (see Fig. 2). A biochemical docking assay revealed that SNAREs when reconstituted at their physiological protein - lipid ratios are not sufficient to confer efficient vesicle docking. In contrast, the presence of membrane-anchored synaptotagmin 1 in SUVs and its interaction with the t-SNARE or PIP_2 on the GUV surface mediates potent vesicle docking. Such a mechanism ensures the presence of the calcium sensor and the orderly formation of SNAREpins. Docking occurs calcium-independent, and results in slow Ca^{2+}-independent membrane fusion. Ca^{2+} addition confers a fast fusion burst, but of low amplitude (see Fig. 2B). To obtain potent calcium-synchronization, complexin, a small cytosolic protein, needs to be added to the reaction. In the absence of synaptotagmin, complexin weakly stimulates the fusion reaction

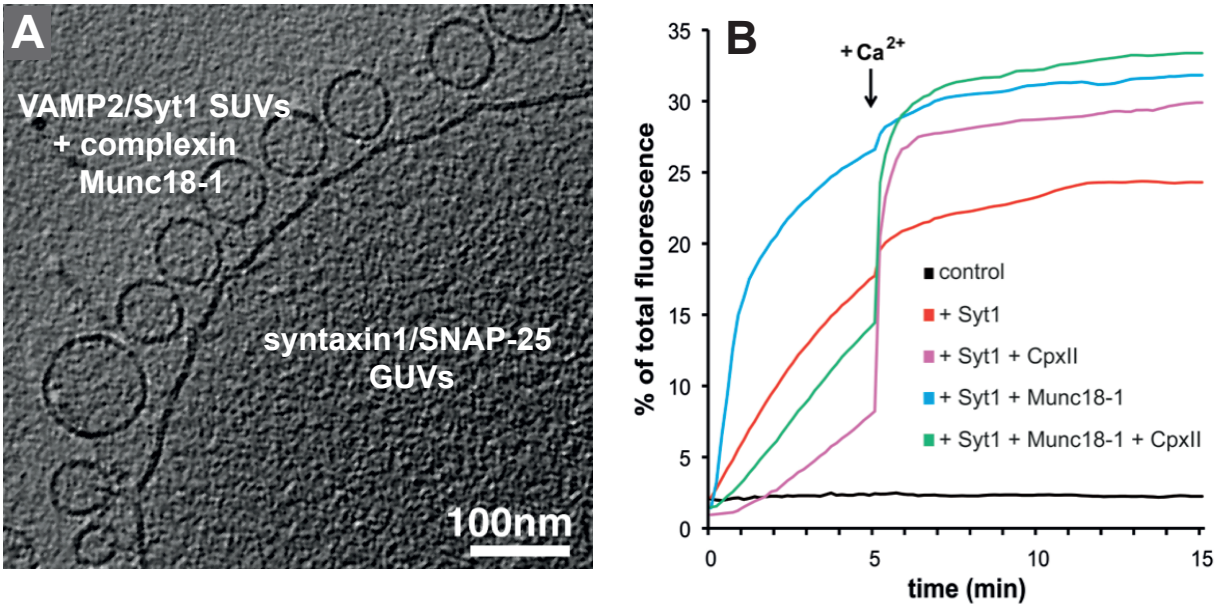

Fig. 2: *A) Cryo-electron micrograph of VAMP2-SUVs bound to syntaxin1/SNAP-25-GUVs by synaptotagmin 1 (Syt1) and arrested by complexin in the presence of Munc18-1, but in absence of Ca^{2+}. SNAREpins together with regulatory components cause the formation of local membrane protrusion pointing towards the SUV membrane, likely reducing the activation energy for lipid bilayer merger (EM-studies were conducted together with John Briggs' group at EMBL). B) Membrane fusion measured by lipid mixing. The data show the fusion arrest by complexin, the stimulatory function of Munc18-1 and the calcium synchronization by synaptotagmin 1. Please note that SNAREs by themselves do not confer significant fusion under the conditions employed.*

likely by stabilizing newly formed partially zippered SNARE complexes. However, in the presence of synaptotagmin, complexin suppresses/clamps the Ca^{2+}-independent reaction and shifts the reaction towards fast Ca^{2+}-synchronization. These different functions could be assigned to distinct protein domains. SNARE binding and the fusion stimulation by complexin require the central helix of complexin. The clamping function of complexin depends on its amino- and carboxy-terminus. A recent SNAREpin-complexin crystal structure indicates that the amino-terminus of complexins competes with VAMP2 for binding to the membrane proximal region of the t-SNARE, thereby blocking SNAREpin zippering. To which degree complexin also interacts with synaptotagmin remains to be shown. Ca^{2+}-independent vesicle docking by synaptotagmin requires the polybasic motif of the C2B domain. Membrane fusion at physiological Ca^{2+} concentrations strictly depends on the known Ca^{2+} binding site on the tip of the C2B domain.

By cryo-electron tomography, in collaboration with John Briggs' laboratory at the EMBL, we could show that the clamped pre-fusion state induces a local protrusion in the GUV membrane at the vesicle-docking site (see Fig. 2A). Such a local high curvature protrusion likely generates a metastable state, which lowers the activating energy for membrane fusion and thus allows instant lipid bilayer merger upon Ca^{2+} triggering.

In addition, we could demonstrate that following vesicle docking by synaptotagmin 1, Munc18-1 profoundly accelerates SNARE complex assembly. On a mechanistic level, Munc18-1 binds the membrane proximal region of VAMP2, which facilitates initial SNAREpin formation or/and helps to transduce force generated by SNAREpin zippering to the lipid bilayer. This reaction involves helix 12 exposed on the surface of Munc18-1 and neighboring regions, which seems to function as a folding template for VAMP2/SNAREpin assembly (see Fig. 3). Remarkably, conversion of a proline residue at the amino-terminus of helix 12 into an alanine residue extends helix 12 and switches Munc18-1 into an activated conformation further stimulating membrane fusion. This activated Munc18-1 likely favors an open conformation of syntaxin 1 promoting SNAP-25 binding and thus allowing fast and efficient-SNARE com-

plex assembly. Physiological reactions that would promote such a switch reaction still need to be identified. Thus, domain 3a and helix 12 seem to function as the central hub of Munc18-1 controlling SNARE complex assembly. Overall, the reconstituted reaction pathway may reflect the generation of a readily-releasable pool of synaptic vesicles at the neuronal synapse, which can fuse with the plasma membrane within less than 1 msec.

Future studies shall reveal how structural changes in the membrane fusion machinery or the assembly of the fusion machinery into macromolecular complexes locally affects the lipid bilayer architecture, dictating the subsequent membrane merger reaction. These studies will be conducted in collaboration with John Briggs' group. Having established the function of synaptotagmin and complexin, the molecular mechanisms of other calcium sensors such as Doc2 and ferlins will be analyzed. In addition to these late steps in the fusion reaction, the early vesicle tethering events and the subsequent vesicle priming reactions at the active zone will be reconstituted. These studies will also focus on the interplay of regulatory proteins with distinct lipids using biochemical and microscopic single vesicle docking/fusion assays.

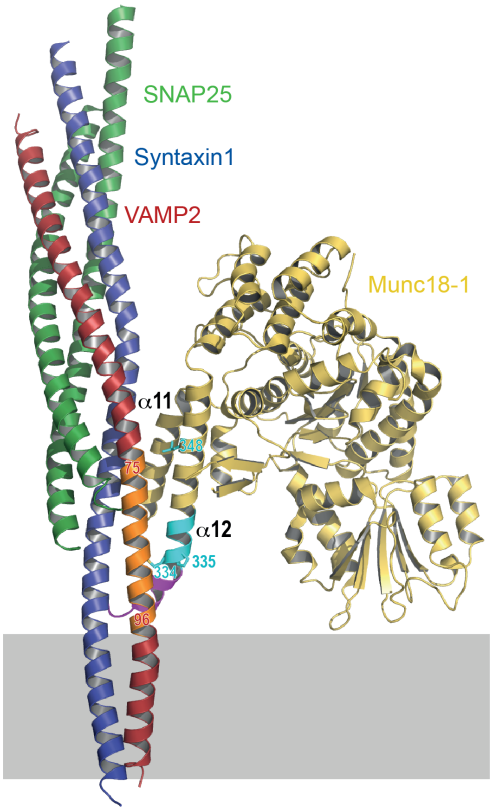

Fig. 3: *Structural model for the interaction of Munc18-1 with a fully assembled post fusion v-/t-SNARE complex. The model is based on published crystal structures of Munc18-1 and the SNARE complex and was developed together with Klemens Wild in Irmi Sinning's laboratory. The model predicts an interaction of domain 3a of Munc18-1 with the membrane proximal region of VAMP2. Such an interaction could initiate v-/t-SNARE complex formation or/and help to transduce the force generated by SNAREpin zippering to the lipid bilayer favoring membrane fusion.*

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