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INVITED REVIEW

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Molecular determinants of exocytosis

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Abstract Membrane fusion processes occur throughout the cell. Among those, exocytosis of secretory organelles is probably one of the fastest fusion events animal cells can achieve. Structural and functional studies have provided a conceptual framework and a starting point for our mechanistic understanding of how SNARE proteins may contribute to the final step in exocytosis.

Keywords Exocytosis · Membrane fusion · SNARE proteins

Introduction

The secretion of neurotransmitter content at presynaptic nerve terminals can occur with a minimal delay of 200 µs [29, 42], implying that the underlying protein machinery is brought to a point that requires only few additional molecular steps to make vesicles fuse with the plasma membrane. A detailed molecular understanding of such presynaptic events has advanced rapidly over the last 10 years, thanks to a fruitful combination of electrophysiology and molecular biology.

This review focuses on the molecular determinants that govern the final membrane merger. It summarizes the significant progress over the last years and highlights recent studies in yeast that challenge the currently favored hypotheses for membrane fusion.

The "zipper" model of membrane fusion

The heart of the exocytotic fusion machinery is likely be constituted by SNARE proteins [soluble-*N*-ethyl-maleimide sensitive factor (NSF) – attachment protein receptor], which comprise a superfamily of small and mostly membrane-bound proteins. Eukaryotic cells possess different sets of SNARE proteins promoting the various intracellular fusion events. To date, the best characterized SNAREs are involved in neuronal exocytosis. They include the plasma membrane protein syntaxin 1A and SNAP-25 together with the vesicle protein synaptobrevin (also referred to as VAMP). Both syntaxin and synaptobrevin are anchored to the membrane by carboxy-terminal transmembrane domains while SNAP-25 is attached to the membrane by palmitoyl modifications. A hallmark of SNARE proteins is that they contain heptad-repeat regions (termed *SNARE-motifs*) which reside at the membrane-proximal regions of the proteins and which can assemble into helical bundles involving coiled-coil interactions (see for review [10, 23]). It is assumed that membrane bridging interactions between SNARE-proteins form a four-helix bundle, the SNARE core complex, similar to a complex in the *cis*-configuration when all proteins are on the same membrane (Fig. 1). Indeed, electron paramagnetic resonance and X-ray crystallography have shown that the core complex comprises four parallel oriented helices, with one helix from synaptobrevin, one from syntaxin and two from SNAP-25 [40, 50]. The assembled structure thus forms a long helix bundle (100–120 Å) with a left-handed superhelical twist.

The "zipper" model of SNARE function in membrane fusion hypothesizes that SNARE proteins "zip" from their membrane distant amino terminal ends toward the membrane-proximal carboxy termini (Fig. 2). It is possible that the conformational changes that have been observed to occur during complex formation of the cytosolic parts of these proteins [13] may provide the energy to overcome repulsive electrostatic forces between the vesicle and target membrane [20, 26]. The most probable route of SNARE-mediated membrane fusion comprises a series of events that requires both protein-protein and protein-lipid interactions. Once the amino termini have found each other, they may partially zip together, probably establishing a stalk-like membrane merger that reflects the fusion of proximal, but continuity of distal,

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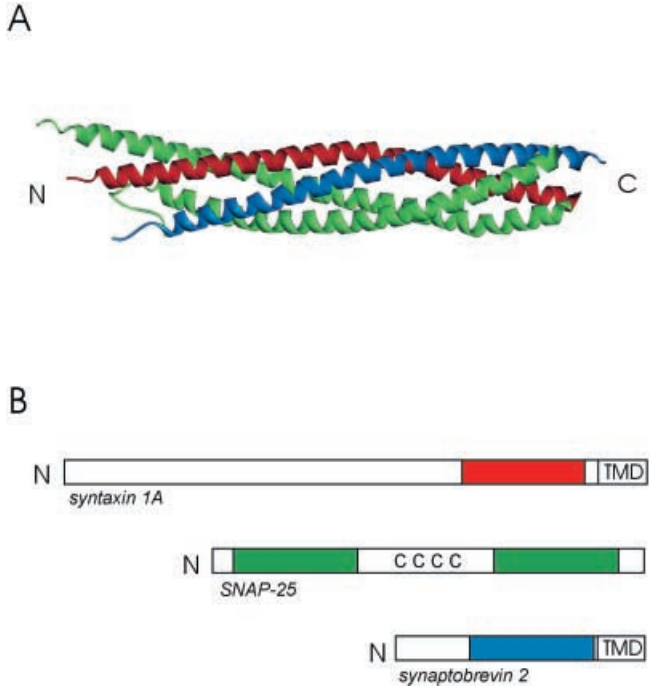

Fig. 1A, B Structure of the SNARE core complex. **A** Backbone ribbon drawing of the SNARE complex comprising all four SNARE motifs as revealed by X-ray crystallization: syntaxin *red*, SNAP-25 *green* and synaptobrevin *blue*. The helices are oriented in a parallel fashion indicated by the common N- and C-terminal orientation. **B** SNARE domains residing within the individual proteins are illustrated by the *colored bars*. SNAP-25 harbors four palmitoylated cysteine residues within the linker region connecting the SNARE motifs. (*TMD* Transmembrane domain)

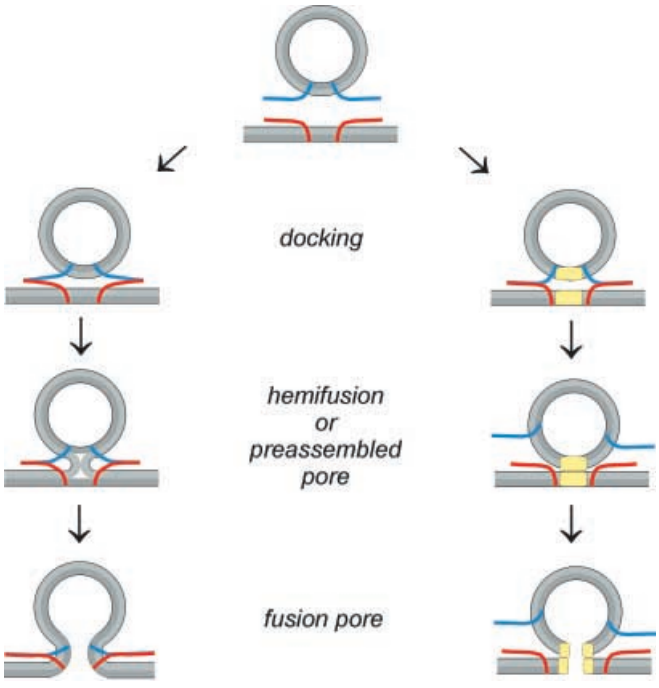

Fig. 2 Hypothetical models for protein-mediated membrane fusion. Vesicular SNAREs (*blue*) interact with the accepting SNARE protein (*red*) on the target membrane. SNARE proteins partially zipper to transfer the vesicle into a readily releasable state leading to hemifusion between both membranes (*left side*). Further assembly triggered by a calcium-dependent step may lead to the completion of fusion. Alternatively, membrane fusion is promoted by a proteinaceous pore as suggested by studies in yeast (*right side*). Trans-SNARE pairing is an intermediate step followed by pairing of V0 units (*yellow*) of the vacuolar V-ATPase present in both membranes. Opening of the channel-like pore that enlarges upon invasion of lipids between dispersing subunits leads to membrane merger

leaflets (*hemifused state*; [25, 60]. While hemifused intermediates have often been considered as unproductive, dead-end scenarios of membrane fusion [24], recent studies on viral fusion events strengthen the view that hemifusion is indeed a bona fide state that can progress to full merger of membranes [35, 41]. Organelles docked with the plasma membrane await the calcium stimulus that could trigger complete assembly of SNARE complexes, which destabilizes the transition state, leading to local membrane breakdown and yielding a fusion pore that initiates release of the vesicular contents.

Although it is conceivable that this process may meet the requirements for the speed and timing of neuronal signaling, to this end direct experimental support for SNARE-driven membrane fusion on a millisecond time scale has not been presented. In fact, the overall structural similarities between SNARE complexes and fusogenic viral proteins (acting on the time scale of seconds to minutes) have been most influential in developing the conceptual framework for a general model that employs proteins to fuse membranes in a zipper-like fashion [48]. A unifying concept could be that fusion proteins generally use coiled-coil interactions because they provide the simplest structural motif to promote conformational transitions from less stable to highly stable protein-protein interactions, releasing sufficient energy to drive the fusion of two membranes. Admittedly, the mechanistic parallels between SNARE proteins and viral fusion proteins are stunning but they can lend at best only indirect support for the hypothesis that SNARE proteins operate in a similar way to drive membrane fusion in neurons.

Given the obvious functional differences between viral fusion and neuronal exocytosis, it may not only be possible but also very likely that neurons have developed important extensions of this mechanism or even alternatives routes to ensure the synchronicity and regulation of nerve-evoked transmitter release. Clearly, the involvement of SNARE proteins in steps leading to vesicle-mediated neurotransmitter release has been documented beyond any reasonable doubt with a variety of different experimental approaches [4, 9, 28, 46, 56]. Furthermore, it is clear that vesicles can dock with the plasma membrane when the vesicular SNARE partner synaptobrevin is selectively proteolysed by clostridial neurotoxins or after genetic deletion of the syntaxin locus, indicating a post-docking role of SNARE proteins in exocytosis [22, 47]. Yet, the most fundamental question remains, namely whether these proteins indeed operate in the out-

lined mechanism as fusogenic motors or simply serve as workhorses to pull the vesicles close to the plasma membrane thus transferring them into a readily releasable state. Completion of fusion is then performed by an unknown downstream protein. This ambiguity leaves room for alternative concepts in membrane fusion as discussed below.

Pros and cons for SNARE-mediated membrane fusion

While it is largely accepted that SNARE complexes can exist in *trans*-complexes as well as in *cis*-complexes (after fusion in the same membrane) the "zipper" model postulates that proteins should partially assemble before fusion. Indeed biochemical studies mapping conformational changes that occur upon complex formation suggest that SNARE complexes can assemble only partially in such a way that part of the helical bundle is correctly folded, whereas the remainder of all four participating SNARE motifs are unstructured [15, 31].

Experiments on adrenal chromaffin cells, using membrane capacitance measurements, suggested that SNARE complexes of readily releasable organelles exist in a dynamic equilibrium between a loosely and a more tightly associated state [57]. Using an antibody directed against the amino terminal portion of SNAP-25 that is part of the SNARE motif and blocks stable SNARE complex formation in vitro, Xu et al. [57] provide evidence that unperturbed zippering is required for the fastest component of the exocytotic response, probably reflecting the exocytosis of readily releasable vesicles. Astonishingly, they also observed that a significant fraction of release comprising a subsequent slower phase of the exocytotic signal remains unchanged, indicating that impairment of SNARE complex assembly differentially affects kinetic components of exocytosis but does not block fusion completely. This is expected when two putative states of SNARE assembly, loosely and more tightly formed complexes, can support exocytosis. Assuming that antibodies bind less strongly to partially assembled SNAP-25 than to its free, uncomplexed form, an elevation of calcium may still initiate the progressive zippering of loosely associated SNAREs, rapidly displacing the antibody and allowing for exocytosis at a slower rate. In comparison, the profound inhibition of an even later sustained phase of exocytosis (reflecting the recruitment of new vesicles to secretion competence) is compatible with strong, nearly irreversible binding of the antibody to uncomplexed SNAP-25, preventing the new assembly of complexes.

Evidence for the existence of partially assembled *trans*-SNARE complexes before exocytosis has also been provided by experiments on the crayfish neuromuscular junction [21]. It was shown that the actions of the clostridial neurotoxins TeNT and BoNT/D were stimulus dependent, but those of BoNT/B were not. The former two toxins, in contrast to BoNT/B, require a free uncomplexed amino terminus of synaptobrevin for their binding and subsequent proteolysis. This suggests that the amino terminal coil-forming domain of synaptobrevin may form a complex without involving the coil domain of the carboxy terminus, which harbors the cleavage site of the toxins. Consequently one may propose that a partial complex probably involving the amino terminus of synaptobrevin with syntaxin and SNAP-25 may represent an intermediate that can explain the different effects of the neurotoxins.

In so-called "cracked" PC12 cells, in which the exocytosis of primed dense core granules (probably resembling docked vesicles) can be measured, soluble SNARE proteins competed successfully until the Ca^{2+}-triggering step, suggesting that the assembly of complexes occurred only during or after the arrival of the Ca^{2+} and could not be experimentally uncoupled from the membrane fusion process [11, 44]. Similarly, experiments by Neher and coworkers on chromaffin cells suggest that the very carboxy terminal end of SNAP-25 (targeted by BoNT/A at Q197-R198) is required in its intact form for the fastest step of the exocytotic response (≅50 ms) to reach its full speed [56]. Thus, assembly of *trans*-SNARE-complexes toward the extreme carboxy terminal part of the SNARE-motif appears to coincide with the last step in exocytosis. Taken together these studies suggest a close temporal link between zippering of SNAREs and the exocytotic event.

Direct support for SNARE-mediated membrane merger is provided by experiments on reconstituted SNARE bundles in artificial liposome systems. Using lipid and content mixing assays between liposomes Rothman and colleagues showed that formation of *trans*-SNARE complexes is essential and sufficient for membrane fusion in this system [53]. Moreover these results demonstrated that extension of the linker region between the SNARE motifs and the transmembrane domains significantly reduced fusion efficiency [32, 33]. This is compatible with the hypothesis that mechanical coupling between complex formation and force-consuming membrane movement must depend on the structural properties of intermittent elements. It is not yet known whether the kinetic properties of the observed fusion reaction acting on the time scale of minutes to hours can be adapted to the physiological requirements under appropriate conditions in vivo.

Taken together, in vitro and in vivo studies seem to provide converging lines of evidence suggesting that SNARE assembly and exocytosis coincide. Still, the challenge remains to substantiate these findings and to correlate distinct steps of the fusion process with biochemically defined counterparts. Methodological approaches that are capable of monitoring individual fusion events at high time resolution, such as fusion pore conductance measurements or amperometric detection of transmitter release, have so far not been employed to probe the question of SNARE engagement in the final fusion event. It seems safe to conclude that the maturation of core complex assembly is an essential step that determines readiness for exocytosis. From the physiolog-

ical viewpoint readiness seems to be a stage with many exits [52] but this does not exclude the possibility that all fusion events engage the same mechanism of SNARE-mediated membrane merger, just progressing at different rates.

However, there are also observations that are more difficult to reconcile with SNARE-mediated membrane fusion. While genetic deletion of syntaxin 1A in *Drosophila* has profound effects on the functioning secretory pathway, with complete loss of synaptic transmission [47], the function of vesicular SNARE proteins seem to be expendable. For instance, neurotransmitter release is not completely blocked in the absence of the predominant form of synaptobrevin in *Drosophila* [8, 12]. While evoked release is severely impaired, spontaneous fusion events can still be observed [43, 58]. Although the presence of additional, functionally partially redundant SNAREs on the vesicles cannot be excluded, these results may indicate that SNARE complex formation has a strong facilitating rather than an executive role in fusion. Consistent with this contentious suggestion, recent reports indicate that the complete assembly of SNARE core complexes might occur prior to neurotransmitter release and influences the supply of readily releasable vesicles [30, 54].

Evidence that complex formation primes the membranes for fusion rather than being directly responsible for the fusion reaction comes also from experiments on homotypic fusion between yeast vacuoles [51]. These studies suggest that SNAREs can be dissociated and prevented from reassembly without affecting the fusion efficiency, arguing that core complexes are no longer needed during membrane merger and that additional unknown proteins are involved. In particular the latter study has heavily fuelled the controversy on SNARE engagement in membrane fusion and has stimulated a series of experiments on vacuolar fusion to identify candidates that control fusogenic activity.

Candidates for membrane fusion that may be operational after SNAREs

Homotypic vacuole–vacuole fusion, in contrast to neuronal exocytosis, provides the advantage that genetic and biochemical approaches are more easily combined to study molecular events leading to fusion. Vacuole–vacuole fusion is a stage-defined assay that comprises a sequence of priming, docking and fusion reactions [55]. Docking is operational when clusters of vacuoles form and the subsequent fusion reaction is resistant to dilution of the assay system. Fusion is assayed by content mixing involving proteolytic enzymes (Proteinase A) and their target proteins [19]. Here, recent experiments have identified several biochemical events that follow the docking of vacuoles, possibly triggered by *trans*-SNARE association.

Three important downstream factors of *trans*-SNARE pairing in the vacuole system appear to be calmodulin, protein phosphatase 1 and the V0-subunit of the vacuolar H^+-ATPase. Calcium released from the vacuole is needed for the binding of calmodulin to the vacuole membrane, probably involving a multisubunit protein complex that also contains protein phosphatase 1 [37]. It has been suggested that Ca^{2+}/calmodulin-dependent dephosphorylation leads to membrane fusion. Recent experiments by Mayer and coworkers have identified V0, the membrane-integral sector of the vacuolar H^+-ATPase, as a target of calmodulin in yeast vacuoles. The authors propose that V0-subunits assemble into gap-junction-like channels that connect the fusing membranes (Fig. 2). Surprisingly, these findings put oil onto an old fire, reopening the discussion that a proteinaceous fusion channel rather than an external scaffold of proteins (around a lipidic pore) forms the heart of the fusion mechanism [27, 36].

Several observations implicate that V0 can act as a fusogenic mechanism in the homotypic fusion of yeast vacuoles. First, the V0-subunit when reconstituted in liposomes seems to control the flux of small molecules (e.g., choline) across the membrane in a Ca^{2+}/calmodulin-dependent fashion. Along the same lines, the V0-subunit of the vacuolar ATPase from the *Torpedo* electric organ (also referred to as mediatophore) has also been reported to govern permeability changes for acetylcholine [5, 6]. Second, fusion between vacuoles appears to be sensitive to the V-ATPase inhibitor DCCD. This was also observed in the absence of H^+-pump activity, suggesting that the V0 sector may have a function in fusion independent of proton pumping. And third, there is some albeit very indirect evidence suggesting that V0–V0 *trans*-complexes may form between opposing vacuoles. The latter structure is speculated to represent a V0 dimer of two proteolipid hexamers forming a closed-off channel that opens in a Ca^{2+}-triggered manner upon fusion [2].

The results on yeast vacuolar fusion and in particular the proposed sequence of events from intermediate *trans*-SNARE pairing via Ca^{2+}/calmodulin and dephosphorylation to the V0-subunit are difficult to reconcile with the properties of other systems. For instance, in neuronal exocytosis calmodulin has been implicated as a regulatory element [4, 18] rather than as the main calcium sensor. Furthermore it seems very unlikely that dephosphorylation can interpose during the short delay between the calcium-dependent triggering step and neuronal exocytosis. These unresolved differences may leave us with the unsatisfactory presumption that the properties of membrane fusion are fundamentally different in yeast vacuolar fusion and in other systems. Before agreeing upon such a conclusion it seems legitimate to speculate about alternative explanations in order to preserve the original idea that all eukaryotes engage a common mechanism for fusion.

What if the zippering of SNAREs induces the initial opening of a fusion pore by destabilizing the proteolipid ring of the V0-subunit rather than the postulated hemifused intermediate. A potential link between the V0-subunit and SNARE proteins has been implicated by former

studies on mammalian SNAREs [17] as well as by the recent study on V0-subunits in yeast [38]. Obviously, this speculation would counter the observations by Ungermann et al. [51] suggesting that *trans*-SNARE pairing and vacuolar fusion can be uncoupled. However, it is noteworthy that from the sequence of well-defined steps leading to vacuole fusion the fusion step itself is the least understood. Since this assay relies on the exchange of rather large molecules such as enzymes (Proteinase A; mol. wt.=42,000; [34] it is not yet known whether, during *trans*-SNARE pairing, small-sized fusion pores form before a measurable signal is generated. Thus it cannot be completely ruled out that potential downstream effectors, such as calmodulin and protein phosphatase 1, operate post fusion and are relevant to fusion pore expansion rather than its initial opening. The latter scenario would be compatible with studies on organelle exocytosis showing that fusion pore dilation is facilitated by calcium (maybe Ca^{2+}/calmodulin dependent) and dephosphorylation [14, 45].

Still, it is not yet known whether pore-like properties are indeed established by V0–V0–*trans* complexes between vacuoles and whether proteolipids can disperse radially to promote the enlargement of pores and the fusion of vacuoles. As pointed out by Mayer and coworkers, it also unclear why null mutants of V0 have a milder phenotype compared with genetic deletions of SNAREs in yeast that are lethal [39, 49]. It would be a masterpiece of evolutionary opportunism if the V0-subunit, despite its relationship with the highly specialized and conserved mitochondrial ATPase [1, 16], has evolved to perform a double role both in organelle acidification and membrane fusion.

Perspective

We are only beginning to elucidate the molecular steps leading to the fusion of secretory organelles. It is the conservation of the fusion mechanism that gives us the confidence to unravel the structural components by comparative analyses from viral fusion to neuronal exocytosis. To date, zippering of SNAREs seems to be the most comprehensive model to explain membrane fusion. The bulk of physiological and biochemical data suggest that SNAREs do not become expendable even when late stages of fusion competence have been reached. Still more experiments are needed to constrain the controversy. Interesting and similarly wide-ranging questions relate to the postulated calcium-dependent control of the SNARE–complex assembly. Do Ca^{2+}-binding proteins, such as synaptotagmin, control the fusogenic activity of the SNAREs? How do changes in the lipidic environment affect neuronal exocytosis? They may influence the postulated hemifused intermediates as well as a putative proteinaceous pore that may open radially by invading lipid molecules between the dispersing subunits [3, 59]. Addressing these important issues is a major task for future studies.

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