»Membrane Fusion« was the topic of the 82nd International Titisee Conference held from October 25 to 29, 2000 in Titisee, Germany. Fusion of model membranes, viral and eukaryotic fusion mechanisms, reconstitution of eukaryotic membrane fusion in vitro, the structure of fusion proteins and electrophysiological approaches were among the subjects of the conference, to which the scientific organizer, Professor Dr. Reinhard Jahn, Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany, had invited some 25 leading scientists. Since proteins and lipids play cooperative roles in cellular membrane fusion, it is important to establish closer contacts between the different fields. The objective of this conference was therefore to bring together these different groups of scientists, and to promote the discussion between them, which could lead to new ideas and concepts.

Mechanisms of membrane fusion

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Fusion of lipid bilayers, which encoat cells, organelles, vesicles and viruses, is a fundamental process in cellular life. In eukaryotic cells, vesicles bud from one compartment and fuse with their target compartment to transport membrane-impermeable molecules within the cell. Signal transmission in the central nervous system depends on the fusion of transmitter-loaded vesicles with the plasma membrane and certain viruses have to fuse their envelopes with cellular membranes before being able to release their proteins and genome into the host cell.

Membranes do not fuse spontaneously. In fact, repulsive forces between them must be overcome before fusion can take place. Biological membranes require specialized proteins to achieve this goal. Several proteins essential for intracellular fusion have been identified to date, although their precise role in the fusion event is still a matter of discussion.

When enveloped viruses infect cells, the merger of the two phospholipid bilayers is fundamentally similar to that which takes place in intracellular fusion events. However, fusion is enforced from one side only, the viral membrane. Many of the viral proteins mediating this fusion event have been studied intensively, but their exact functional mechanism is still not completely understood.

Experimentally, it is possible to fuse phospholipid bilayers without the participation of proteins. Such fusion reactions are traditionally studied by physicists and are described in terms of lipid phase transitions, non-bilayer phases, monolayer and bilayer bending and curvature. In contrast, the study of the fusion proteins involved in cellular membrane fusion events is a relatively young discipline propagated mainly by cell biologists and molecular biologists, who were recently joined by structural biologists.

Basic concepts for fusion of phospholipid membranes

The first concepts of membrane fusion were developed by physicists on the basis of theoretical studies in combination with biophysical measurements of protein-free phospholipid mono- and bilayers. The different steps leading to membrane fusion were formulated as adhesion or docking of the membranes, a local increase in membrane curvature, destabilization of the bilayers, the formation of a fusion intermediate and, finally, the formation of a fusion pore. This series of events is still considered the most probable route for membrane fusion.

The intermediates that form during fusion have not been conclusively identified to date and have been a matter of discussion for many years. In 1978, Gingell and Ginsberg (1) formulated the hypothesis of a »stalk« structure as fusion intermediate (*Figure 1*). Such a structure is in agreement with most experimental data and is currently considered the most probable fusion intermediate. The expansion of the stalk leads to the touching of the distal monolayers (local hemifusion) and, upon further expansion, to an extended hemifusion structure from which an initial fusion pore develops. Early fusion pores of cellular membranes and pure lipid membranes show similar characteristics (2–4).

According to this model, the fusion intermediate contains highly curved non-bilayer structures. The nature of the lipid molecules can hence be assumed to play a pivotal role. Depending on their three-dimensional form, different lipids should either hinder or promote the formation of fusion intermediates. This has been confirmed experimentally; lipids with an inverted cone shape like long-chain phosphatidylethanolamines hinder fusion, while lipids with a regular cone shape like lysolipids promote it (5).

Several steps in the pathway to fusion require the input of energy. For pure lipid bilayers, this energy

has to be provided, for example, by applying pressure. In the case of biological membranes, proteins provide the energy for fusion.

Modelling the influence of proteins on fusion

To gain a better understanding of how proteins influence the process of membrane fusion, theoretical models for the behaviour of proteins in lipid membranes have been developed.

One of these models describes membranes as elastic surfaces which can easily be deformed. A protein can change the curvature of the membrane since the lipid bilayer has a tendency to bend away from the protein polymer. In addition, proteins, which are described as stickers in the membrane, tend to form clusters in this model. This could also account for a change in membrane morphology. Modelling membranes as elastic surfaces using statistical mechanics of surfaces leads to descriptions of membrane behaviour on a micrometer scale. However, this model cannot explain how proteins influence membrane properties on the molecular level.

A second model treats membranes as assembled bilayers containing water and amphiphilic molecules. This enables the simulation of membrane behaviour on the nanometer scale. However, this approach is limited by the high computing power required for modelling these large and complex systems. As discussed by Reinhard Lipowsky (Potsdam, Germany), a new method termed »dissipative particle dynamics« was developed to overcome this problem. This method simplifies the computer-simulated molecules and thus requires considerably less computing power. Hopefully, this will make modelling of such large systems feasible in the near future.

Thomas Heimburg (Göttingen, Germany) uses a different approach for the study of lipid bilayers containing proteins: he investigates the thermodynamics of such systems using heat capacitance measurements. Lipids alone exhibit phase transitions from a solid (gel) to a liquid (fluid) state in which the hydrophobic chains become disordered. In the solid state, mixtures of lipids have a tendency to separate and form domains. These domains disintegrate when the temperature is raised. The temperature at which this phase transition occurs depends on lipid composition, ionic strength, pH and similar conditions. It is also strongly influenced by the proteins which are integrated into the bilayer or associated with it. Interestingly, the observed phase transitions often occur in a physiologically relevant temperature range. It is therefore likely that such phase transitions play a role in vivo.

Thomas Heimburg and coworkers demonstrated that it is possible to model such phase transitions by Monte Carlo simulations (6). These simulations showed that peripheral membrane proteins tend to aggregate in the temperature range of the phase transition, while integral proteins aggregate in either the gel or the liquid crystalline phase. Such results provide a better idea of how proteins behave in the membrane and may contribute to improved concepts on membrane fusion.

Similar intermediates in proteinaceous membrane fusion and pure lipid fusion

Different lipid compositions have similar influences on intracellular and viral membrane fusion and on the fusion of pure lipid membranes. Thus, it is likely that membrane fusion catalysed by fusion proteins involves a lipidic intermediate, probably a stalk-like structure (5, 7).

In some cases, it was possible to isolate structures that can be interpreted as fusion intermediates. Some mutants of haemagglutinin, the viral fusion protein of influenza virus, reach the state of hemifusion. However, in these cases, the process does not progress to fusion. Thus, these states are end states rather than intermediates of fusion (»terminal hemifusion«)

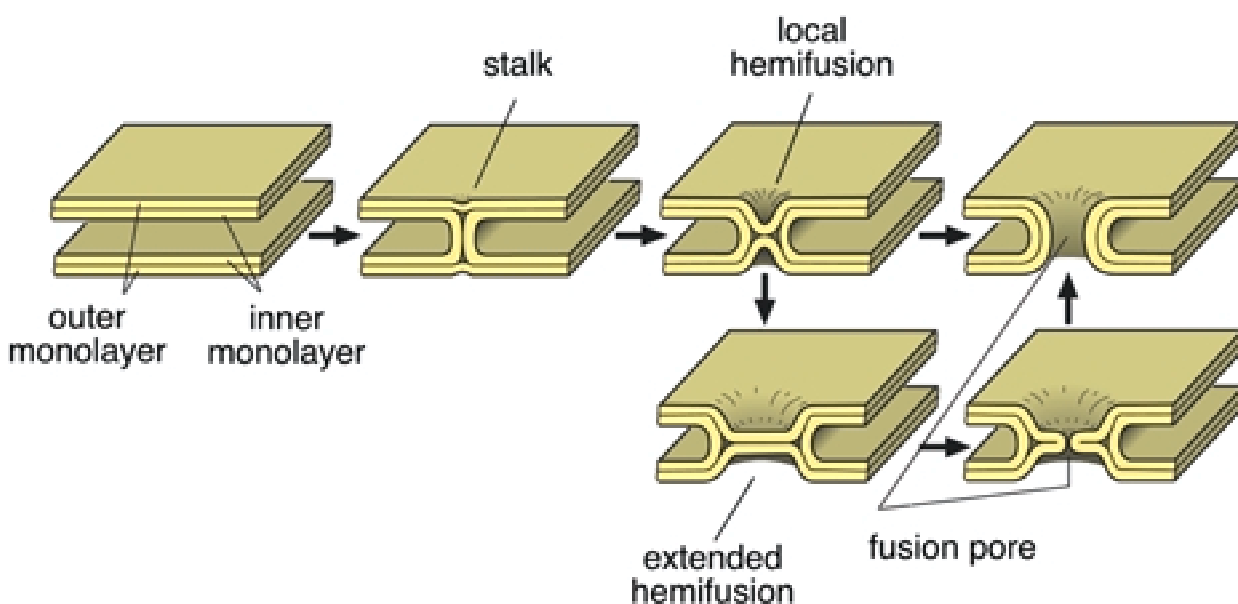

Fig. 1: Intermediates during fusion of two phospholipid membranes according to the »stalk hypothesis«

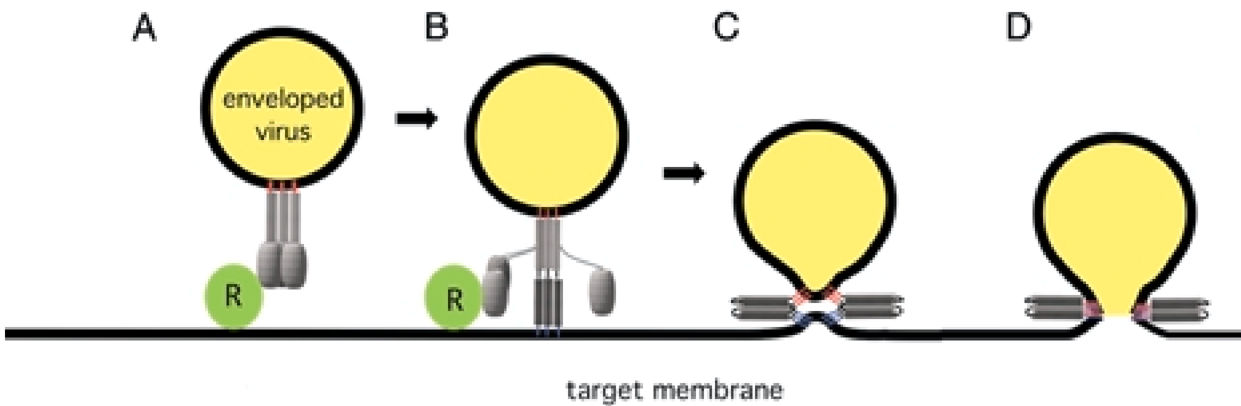

Fig. 2: Hypothetical mechanism for membrane fusion by virus glycoproteins. (*A*) The viral glycoprotein haemagglutinin binds in its conformation at neutral pH to a receptor (R) in the target membrane. (*B*) After endocytosis, the low pH environment induces major conformational rearrangements in the viral glycoprotein. In a first conformational change, the receptor-binding domain is displaced from the fusion domain (not shown in *C* and *D*) and the fusion peptide inserts into the target membrane. (*C–D*) In a second conformational change, the fusion peptide in the target membrane and the transmembrane domain in the viral membrane are brought together and the two membranes fuse. (Modified from reference 13)

(8). However, recent work shows that a change in the transmembrane domain of haemagglutinin stabilizes a hemifusion intermediate that is able to transit to fusion (9, 10). These studies strengthen the view that hemifusion is indeed a bona fide intermediate in protein-induced membrane fusion. In addition, it was shown that hemifusion occurs prior to the opening of the fusion pore (11).

A similar fusion pathway for proteinaceous and pure lipid membranes suggests that the energy barriers in both cases are similar, regardless of the class of proteins involved and regardless of whether any proteins are involved at all (12). Indeed, it was shown that the activation energy required for fusion of pure phospholipid membranes, when induced by polyethyleneglycol, is similar to that observed in the fusion of viral and intracellular membranes (7). A possible conclusion from these results is that conformational rearrangements of fusion proteins may help to overcome the energy barriers to membrane fusion. Possibly, they catalyse lipid rearrangements similar to those postulated in the fusion of protein-free membranes.

Viral fusion proteins: driving fusion by changing conformation

The small number of different proteins contained in a virus particle makes it relatively easy to identify candidates for fusion proteins. Enveloped viruses such as influenza virus, HIV and Ebola virus carry surface glycoproteins that mediate both cell attachment and fusion of viral and cellular membranes.

The viral envelope glycoproteins are synthesized as precursor molecules, which are proteolytically processed to produce a functional receptor-binding domain and a membrane-anchored fusion domain. This cleavage causes an intramolecular conformational rearrangement of a hydrophobic glycine-rich fusion peptide and is the prerequisite for the activation of the fusion potential of the fusion domain. After cleavage, the fusion domain is thought to be in a metastable conformation, in which it can undergo major structural rearrangements upon activation. It is believed that the conformational change itself and the energy released during that process drive the fusion of the viral envelope with the cellular membrane.

In many viruses, for example HIV, the fusion domain is activated after binding of the viral receptor-binding domain to its cellular receptor. In cases such as the influenza virus, the entire virus enters its host cell by endocytosis. Its fusion domain is activated by the acidic environment in the endosomes, leading to the fusion of the virus' envelope with the endosomal membrane. Upon activation, the viral fusion protein undergoes major conformational changes, which lead to a long coiled-coil structure in which the fusion peptide is exposed and probably inserted into the target membrane (*Figure 2*).

Haemagglutinin: crystal structures of two conformations

Influenza virus haemagglutinin is the most extensively characterized viral membrane fusion protein. It is the only one for which crystal structures of the two conformations are available; the metastable conformation at neutral pH (before activation) and the coiled-coil conformation (after activation). John Skehel (London, UK) described the structural features of these two conformations and their implications for membrane fusion. Interestingly, in the activated fusion structure, part of the long α-helix is folded back and brings the fusion peptide and the transmembrane domain close together (*Figure 3*).

It is believed that a third, intermediate, conformation exists, which contains one extended helix and in which the fusion peptide and the transmembrane domain are therefore juxtaposed at different ends of the rod-like structure. Such a putative intermediate conformation could serve to insert the fusion peptide into the target membrane. The conformation, seen in the crystal structure of

the activated protein, would then be reached by a second conformational change, which could serve to draw the two membranes together. However, no direct proof for the existence of an intermediate structure exists to date.

Structures of their active, but not of their inactive fusion states have also been solved for other viral fusion proteins. However, striking structural similarities between their membrane fusion structures and that of haemagglutinin strongly suggest that they too exist in at least two conformations; in a metastable conformation in complex with the receptor-binding domain and in a stable fusion conformation [for review see (13, 14)].

How do viral proteins promote fusion?

The exact pathway by which the viral fusion proteins bring about membrane fusion is still a subject for discussion. Evidence that the conformational change is indeed intimately coupled to the merger of two membranes was presented by Fred Cohen (Chicago, IL, USA) based on observations on the HIV fusion protein, which undergoes a conformational change into a six-helix-bundle structure. Membrane fusion was blocked by cone-shaped lipids, as was the conformational change of the fusion protein. This implies that both processes, conformational change of the fusion protein and lipid rearrangements, must occur simultaneously (15). However, it should be borne in mind that, alternatively, cone-shaped lipids might also affect fusion by directly interacting with fusion proteins.

It is now widely accepted that the insertion of the fusion peptide into the target membrane is a prerequisite for fusion. However, haemagglutinin – at least partially – inserts the fusion peptide into its own membrane. It was therefore suggested that the insertion of the fusion peptide into the viral membrane is one step in the fusion pathway and that the insertion of the fusion peptide into the target membrane occurs later in the fusion process.

Haemagglutinin forms trimers, and it is assumed that several haemagglutinin trimers first have to form an aggregate before fusion can be induced. It seems possible, as Joe Bentz (Philadelphia, PA, USA) suggested, that the conformational change of haemagglutinin into the extended coiled-coil structure eventually leads to the removal of the fusion peptide from the viral membrane. This would cause a hydrophobic defect in the viral membrane. Since haemagglutinin aggregates restrict the flow of lipids in the membrane plane, they would stabilize this defect. However, the defect could be relieved by the movement of lipids from the outer monolayer of the target membrane to the viral membrane, thus creating a stalk-like structure (16).

Toon Stegmann (Toulouse, France) suggested a slightly different view of the initiation of the membrane fusion process by haemagglutinin. Many researchers study viral fusion in a model system composed of two steps. In the first step, the viral fusion protein is expressed on the surface of cells. In the second step, fusion between these cells and cellular membranes lacking viral fusion proteins can be observed. Stegmann follows fusion of the intact influenza virus, either with endosomal membranes after endocytosis or with artificial membranes. He presented data suggesting that the insertion of the fusion peptide into the target membrane directly leads to the

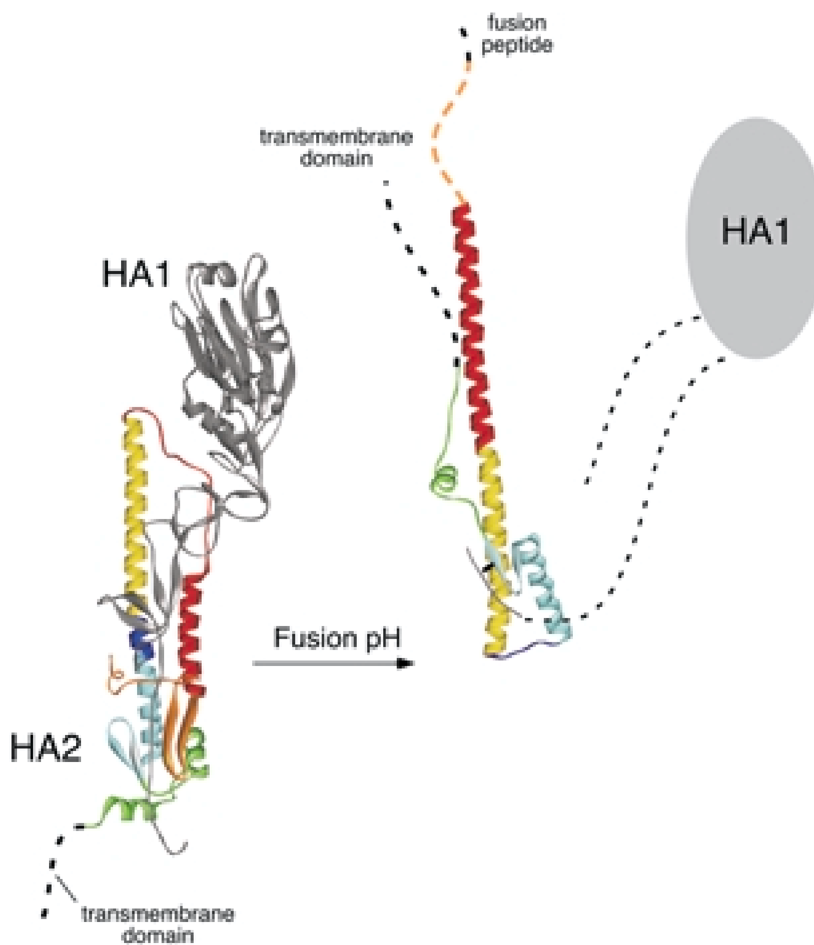

Fig. 3: Conformational changes induced in the influenza virus glycoprotein haemagglutinin upon activation by the acidic pH in the endosome. Haemagglutinin is the only viral fusion protein that has been crystallized in two different conformations so far. In both conformations, haemagglutinin forms trimers, but for simplification only one molecule for each conformation is shown. At neutral pH (*left*), the two domains of haemagglutinin HA1 (grey) and HA2 (coloured) interact. Since the exact position of HA1 at acidic pH (*right*) is not known, it is only outlined. The different parts of HA2 are coloured differently to explain the fusion pH-induced changes in its structure. The broken lines indicate parts of the structures that are unknown. (Modified from reference 13)

formation of a pore. This proteinaceous pore in the target membrane can persist when the pH is neutralized, but normally leads to the formation of a hemifusion intermediate. In his system, cooperativity of haemagglutinin trimers is not required for fusion (17).

The controversial discussions and the different concepts for the viral fusion pathway presented at this meeting demonstrate that, despite the fact that several crystal structures have been available for a long time, it has not been possible to elucidate the molecular events during viral fusion in a satisfactory manner. Not even a basic model of the process which integrates all experimental data available and which is accepted by all scientists in the field has been developed to date. This gives some indication of the difficulties associated with the analysis of intracellular membrane fusion, which involves the interaction of hundreds of molecules with different properties.

SNARE proteins – the core machinery of intracellular membrane fusion

The process of intracellular membrane fusion is highly regulated and involves many sequential protein-protein interactions. Several steps can be distinguished before a vesicle can fuse with its target membrane. First, the vesicle is transported to the location where fusion will occur. Second, the vesicle docks onto the target membrane before in a third step, the priming, the fusion machinery is activated – only then can fusion take place.

One of the first steps in the investigation of intracellular membrane fusion was the identification of the proteins involved in the process. Genetic screening of the yeast Saccharomyces cerevisiae was one of the most powerful approaches to this end. About seven years ago, the so-called SNARE protein family (SNARE: soluble NSF attachment protein receptor) was discovered (18); its members are now widely believed to constitute the core machinery for fusion of a vesicle membrane with its target membrane.

The best characterized proteins in this family are the synaptic SNAREs, the SNAREs involved in the exocytosis of neurotransmitters from synaptic vesicles. They include synaptobrevin 2 (also known as VAMP 2) and the plasma membrane proteins syntaxin 1a and SNAP-25. All three contain one or more conserved stretches of about 60 amino acids in length, the so-called SNARE motifs. Synaptobrevin 2 and syntaxin 1a each contain a single SNARE motif adjacent to their transmembrane domain, whereas SNAP-25 harbours two SNARE motifs. SNAP-25 does not contain a transmembrane domain, but is anchored to the membrane by palmitoyl modifications.

It is currently believed that the assembly of these three proteins into a stable ternary complex between the two membranes drives the fusion reaction (19) (*Figure 4*). Studies on the cytoplasmic parts of the synaptic SNARE proteins have revealed that the SNARE motifs are largely unstructured in solution and readily assemble into a stable ternary complex. It has been suggested that the assembly process provides the energy required for the membrane fusion reaction. The core of the ternary complex consists of a tightly packed parallel four-helix-bundle structure, syntaxin 1a and synaptobrevin 2, each contributing one helix and SNAP-25 two helices to the bundle (20) (*Figure 5*).

A slow fusion process was observed when synaptobrevin 2 was reconstituted into one population and syntaxin 1a together with SNAP-25 into another population of liposomes (21, 22). In this case, a liposome fusion assay based on the dequenching of a fluorescent lipid dye in one population of liposomes was used. This assay was established by Dick Hoekstra (Groningen, Netherlands) who demonstrated that a protein complex of the ATPase p97 with its cofactor p47 can induce membrane fusion efficiently in this assay and even more rapidly than the SNARE proteins (23). However, it is not yet clear to what extent this in vitro system reflects the processes which take place in real membranes.

One of the basic questions still to be solved is: how does the ternary SNARE complex assemble between two membranes? Since the simultaneous interaction of all three proteins seems unlikely, an assembly via an intermediate complex has been postulated.

Pathway of SNARE assembly

Data from the exocytotic SNARE complex of yeast suggest that a binary complex formed by the yeast syntaxins Sso1 or Sso2 and the SNAP-25 homologue Sec9 on the plasma membrane serves as a high-affinity binding site for the vesicular synaptobrevin homologues Snc1 and Snc2 (25, 26). However, as presented by Reinhard Jahn (Göttingen, Germany), a »binary« complex of the synaptic SNAREs consists of a parallel four-helix-bundle structure similar to the ternary complex. This »binary« complex contains SNAP-25 and two molecules of syntaxin 1a. The second syntaxin is at the binding site for synaptobrevin 2, and has to be replaced to enable the formation of the ternary SNARE complex. Richard Scheller (Stanford, CA, USA) suggested a different intermediate complex for the synaptic SNAREs. His laboratory uses an in vitro assay for neuronal exocytosis in permeabilized PC12 cells. Experiments in which peptides comprising SNARE motifs were introduced suggest an interaction between synaptobrevin 2 and SNAP-25 as an intermediate.

One probable regulator of the SNARE interaction is contained in the SNARE syntaxin 1a itself. It comprises an N-terminal domain that can form an independent three-helix-bundle structure. For the yeast syntaxins Sso1 and Sso2, which are involved in the fusion of secretory vesicles with the plasma membrane, removal of the N-terminal domain was shown to speed up SNARE complex formation in vitro (26). It was therefore suggested that this N-terminal bundle provides a groove that can bind its own SNARE motif and competes for the binding of that motif with the other SNARE proteins. The three-helix-bundle conformation of syntaxin 1a was termed »closed conformation« to distinguish it from the conformation of syntaxin 1a as part of the SNARE complex (»open confor-

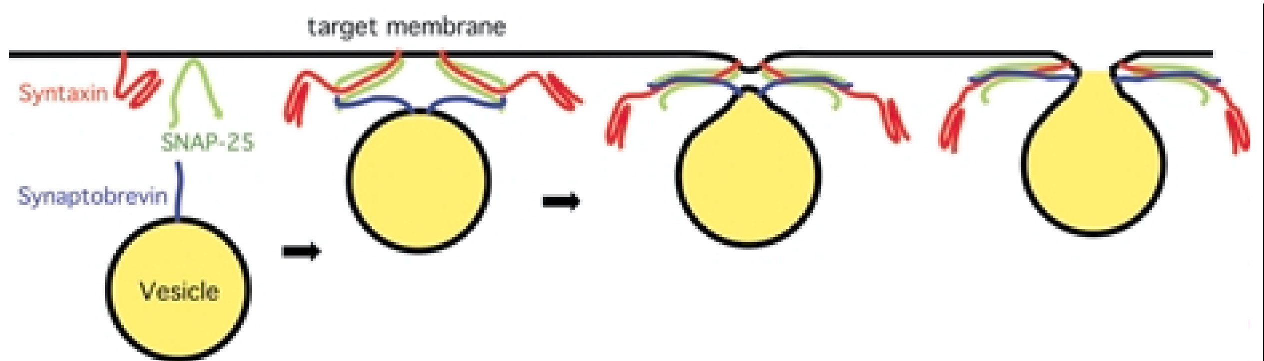

Fig. 4: Hypothetical mechanism for membrane fusion by the SNARE proteins synaptobrevin 2, syntaxin 1a and SNAP-25. SNARE proteins from opposing membranes specifically form a complex between vesicle and target membrane. Further assembly of the tight four-helix-bundle SNARE complex eventually leads to the fusion of the two membranes.

mation«). The closed conformation of syntaxin 1a is thus thought to be a target for regulation of SNARE complex assembly (*Figure 5*) (27).

The existence of the closed conformation was confirmed when the crystal structure of the protein munc-18 (also referred to as n-Sec1) in a complex with syntaxin 1a was solved, as presented by Richard Scheller (28). munc-18 forms a tight complex with syntaxin 1a and keeps it in the closed conformation. munc-18 thereby prevents the assembly of the synaptic SNARE complex in vitro. Since the complex between munc-18 and syntaxin 1a is stable, it is not yet clear as to how the transition to the SNARE complex can be achieved. It is thought that other factors must regulate the binding affinity between syntaxin 1a and munc-18.

The crystal structure of the yeast syntaxin, Sso1, presented by Fred Hughson (Princeton, NJ, USA), confirmed the existence of a closed conformation even for an uncomplexed syntaxin molecule (29). Data obtained by Hughson together with Peter Novick and coworkers (New Haven, CT, USA) suggest that Sec1, the yeast homologue of munc-18, binds to the ternary SNARE complex of the yeast plasma membrane and not the closed conformation of Sso1 (30).

Interestingly, Josep Rizo presented NMR data on several other yeast syntaxin homologues, demonstrating that some syntaxins, for example, Vamp7, do not form a closed conformation. It would therefore appear that not all syntaxins can undergo a conformational switch between an open and closed conformation. And so the general function of the conformational switch between the closed and the open conformation for the regulation of SNARE complex assembly remains unclear.

Regulation of SNARE assembly

Eukaryotic cells contain extensive membrane-enclosed compartments. Each compartment is characterized by a unique set of proteins and these proteins are presumably sorted to their correct organelle by successive steps of membrane vesicle budding, transport and fusion. To maintain the integrity of the organelles and thus the viability of the cell, these steps have to be specific and regulated.

Once the crucial role of the SNARE proteins in exocytosis had been discovered (18), it became clear that SNARE proteins constitute a family of proteins which is probably involved in all intracellular membrane fusion steps. If, as is widely believed, the SNAREs constitute the basic membrane fusion machinery, it is unlikely that they also contain the specificity required in each fusion reaction. Other proteins must hence regulate the interaction of SNARE proteins.

Moreover, before two membranes undergo SNARE protein-mediated fusion, many other regulatory proteins are thought to control the correct docking of the two membranes and the priming step. Much insight into these processes and the fusion was supplied by the study of homotypic fusion of yeast vacuoles (William Wickner, Hanover, NH, USA). Powerful yeast genetics, combined with a cell-free assay to monitor fusion of vacuoles, facilitated both the identification of many of the factors involved and a description of their complicated interplay (24).

Regulators of SNARE interactions during regulated exocytosis

In cells where secretion occurs in response to an extracellular signal, secreted molecules are stored in secretory vesicles. The membrane fusion machinery for these vesicles is not activated until a signal causes a transient increase of the cytosolic Ca^{2+} concentration. One or several Ca^{2+}-binding proteins then transmit the signal by a still unknown mechanism to the fusion machinery. To achieve rapid communication between neurons, the fusion mechanism of the synapse has evolved to achieve Ca^{2+}-stimulated exocytosis of neurotransmitters at high speed.

Nils Brose (Göttingen, Germany) presented data on the protein factors complexin 1 and 2 and munc-13. These seem to be essential proteins, since mice lacking them die at birth. munc-13, a large multidomain protein, is thought to be involved in the priming of synaptic vesicles for fusion. munc-13 can interact with the N-terminus of syntaxin 1a and it was suggested that it might establish the formation of SNARE complexes (31).

Complexins are small proteins that bind to the assembled synaptic SNARE complex with high affinity (32, 33). Electrophysiological recordings on neuronal cell cultures of mice lacking complexins show a dramatically reduced efficiency in transmitter release due to decreased Ca^{2+} sensitivity of the synaptic release process. Brose therefore suggested that complexins are probably directly involved in regulating the Ca^{2+} affinity of the Ca^{2+} sensor necessary for synaptic fusion.

Release of neurotransmitters is very fast and it is difficult to reconcile this speed with the major conformational rearrangements required during SNARE complex assembly. Therefore, it is possible that, before fusion, the fusion apparatus is brought to a point where the SNAREs are almost fully assembled. Such an incompletely assembled ternary complex would then wait for a trigger that allows fusion to be completed (34). This trigger is provided by the influx of Ca^{2+} into the synaptic terminal and mediated by a Ca^{2+}-binding protein.

The best candidate for such a Ca^{2+} sensor is the protein synaptotagmin 1. It contains two Ca^{2+}-binding domains and was shown to bind to syntaxin 1a, SNAP-25 and the ternary SNARE complex in a Ca^{2+}-dependent fashion.

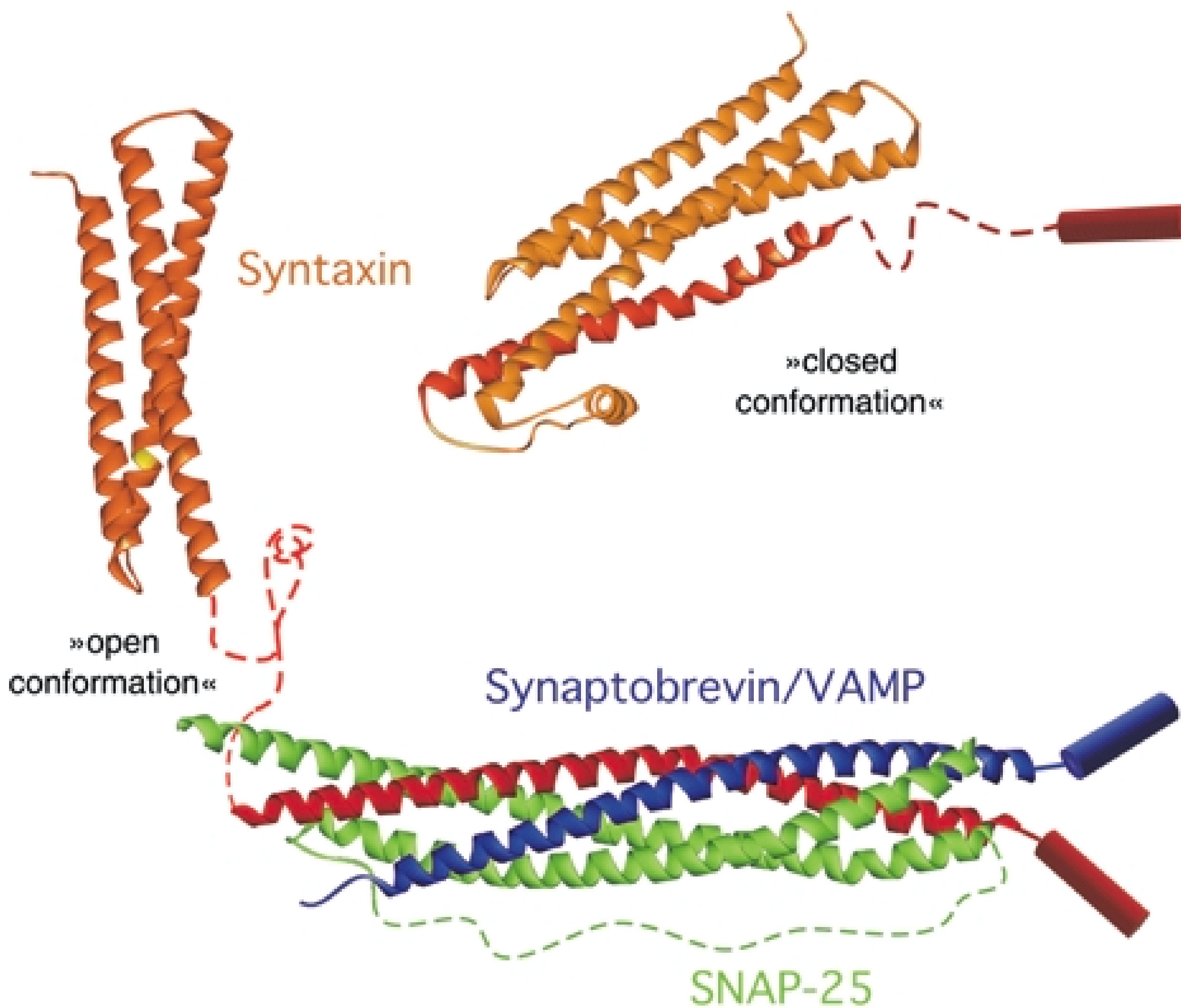

Fig. 5: Conformational changes in SNARE proteins. All three synaptic SNARE proteins undergo major conformational changes upon SNARE complex formation. Synaptobrevin 2 (blue) and SNAP-25 (green) are probably unstructured when not part of a SNARE complex. For syntaxin 1a, (red/orange) two different conformations are known. When part of a SNARE complex, syntaxin 1a consists of two domains that do not directly interact. The C-terminal SNARE motif of syntaxin 1a contributes one helix to the four-helix-bundle structure of the core SNARE complex. The structure of the N-terminal three-helix bundle was solved using an N-terminal fragment. The structure of the loop connecting the N-terminal bundle and the core SNARE complex therefore remains unknown. This structure of syntaxin 1a was termed »open conformation«. The structure of syntaxin 1a was also solved in a complex with the protein munc-18 (also known as n-Sec1). In the figure, only the structure of the syntaxin 1a molecule is shown. In this structure, the two domains of syntaxin 1a, the N-terminal three-helix bundle and the C-terminal SNARE motif, fold onto each other. Thus, this syntaxin 1a structure was termed »closed conformation«. It is probable that syntaxin 1a has to open up to be able to form a SNARE complex. The transmembrane domains are depicted as cylinders, but their structures are not determined. Further unknown parts of the structure are indicated by broken lines.

Using the purified native protein, synaptotagmin was shown to directly interact with phospholipid membranes, again in a Ca^{2+}-dependent manner. Its ability to bind to proteins essential for fusion as well as to membranes indeed renders it the most promising candidate for the Ca^{2+} sensor (35, 36).

To analyse the Ca^{2+}-binding properties of synaptotagmin directly, Thomas Südhof and coworkers (Dallas, TX, USA) used site-directed mutagenesis to change the putative Ca^{2+}-binding sites and amino acids close to these sites. Interestingly, he demonstrated that a direct mutation of the putative binding sites did not dramatically change the Ca^{2+}-binding properties of the protein, whereas mutations close to this site had a measurable effect. These somewhat controversial results demonstrate that the behaviour of this protein is complex, and more experiments are required to clarify its exact role in the fusion process of synaptic vesicles with the plasma membrane.

Analysing exocytotic fusion by electrophysiology

In the last decades, major progress in the characterization of the events occurring during neurotransmitter release has been made using electrophysiological methods. In his keynote lecture, Erwin Neher (Göttingen, Germany) gave a brief overview of the different methods (37). Basic parameters which can be analysed by electrophysiology include the kinetics of the fusion event, the frequency of such events and the charge released by the fusion of a single vesicle. Questions which can be addressed by these measurements range from mode and characteristics of the fusion event itself, the recycling and refilling of the vesicles to the question whether there are different pools of vesicles in the cell that can fuse with the plasma membrane.

Based on simultaneous amperometry and capacitance measurements, Manfred Lindau (Ithaca, NY, USA) and Guillermo Alvarez de Toledo (Sevilla, Spain) presented data on single fusion events (38). They showed that amperometry enables high-time resolution measurements that make it possible to analyse the characteristics of a single fusion event, its fusion pore opening and expansion kinetics.

Alvarez de Toledo presented new data for the long-lasting debate on how tightly exocytosis and endocytosis of secretory vesicles are coupled. One possibility is that the vesicles fuse completely with the plasma membrane and that the membrane material is retrieved by endocytosis in an independent process. The other possibility is that the vesicle fuses only transiently with the plasma membrane and that the same membrane patch is immediately retrieved (»kiss-and-run« mechanism). Patch amperometry recordings in rat chromaffin cells show that the kiss-and-run mechanism occurs more often when the Ca^{2+} level is raised, suggesting that the mode of membrane retrieval can be regulated by the cell (39).

Dieter Bruns (Göttingen, Germany) also used amperometry to analyse the synaptic release in serotonergic Rezius neurons of the leech Hirudo medicinalis. He could distinguish between exocytosis of small synaptic vesicles and large dense-core vesicles. By combining electrophysiology with morphological analyses using electron microscopy, he could demonstrate that the amount of transmitter released during exocytosis correlates with the volume of small synaptic vesicles. This observation suggests that the neurotransmitter is stored at a constant concentration and is completely discharged during exocytosis. The amount of transmitter released from large dense-core vesicles varied more, but the transmitter concentration is probably similar in both vesicle types (40).

The scope of the electrophysiological measurements can be considerably broadened by adding drugs to the outside medium which act on receptors and channels, by the microinjection of proteins or other compounds or by the overexpression of proteins in cell culture.

Neher and coworkers have characterized the exocytotic response of a chromaffin cell to elevated Ca^{2+} levels obtained by the photolysis of chelated Ca^{2+}. The response can be divided into an exocytotic burst event with rapid exocytosis followed by a sustained component. The exocytotic burst consists of two components, a fast and a slow component. The fast component is thought to be mediated by a vesicle pool that can be readily released upon a trigger, whereas the slow burst component represents a different pool of vesicles which react somewhat more slowly to the elevated calcium level. The sustained component probably represents vesicles that first have to be transported to the release sites (41, 42).

Evidence that the synaptic SNARE proteins synaptobrevin 2, syntaxin 1a, and SNAP-25 play key roles in the process of synaptic exocytosis was provided by the observation that proteolytic cleavage of each of the three proteins by highly specific proteases from the botulinus neurotoxin family inhibits neurotransmitter release. When these toxins were injected into chromaffin cells, they were found to act on the exocytotic burst (41). Further evidence that the assembly of the three proteins is a necessary step for synaptic membrane fusion was provided by the microinjection of a Fab fragment of a monoclonal antibody directed against SNAP-25. This Fab fragment blocked the formation of the ternary SNARE complex in vitro and showed a similar effect on the exocytosis of chromaffin cells as botulinus neurotoxins (34).

Questions raised by the conference

Several interesting question have been raised by the presentations and several areas on which further research is required have been emphasized.

Why do all those fusion proteins whose structures were solved contain extended helical bundle structures? Structures of all fusion proteins elucidated so far consist of long (more than 100 Å in length) and stable helical bundles, which probably represent the structure of the fusion protein after membrane merger. There are several possibilities that may account for the observed structural similarities. One explanation could be that viral fusion proteins and SNARE proteins originated from a

Fig. 6: The participants of the 82nd International Titisee Conference of the Boehringer Ingelheim Fonds on »Membrane Fusion«, held from October 25 to 29, 2000 in Titisee, Germany.

common ancestor. However, this can be dismissed, since there is neither sequence nor real structural similarity between these proteins. In addition, viral fusion proteins have evolved which induce fusion with a non-collaborating host cell, whereas intracellular fusion requires the concerted action of proteins from both membranes. Viral fusion proteins are activated by proteolytic cleavage into a metastable conformation. The protein can hence undergo the conformational change only once. In contrast, SNARE proteins are reutilized several times.

A different explanation for the structural similarities is that fusion proteins act via similar mechanisms, since the pathway to membrane fusion is dictated by the underlying physics of lipid structures. Only a particular conformational change may thus provide the energy required for membrane perturbation. Such an explanation implies that the conformational changes and provided energies of all fusion proteins are very similar, which has not yet been shown. However, it is also possible that fusion proteins use coiled-coil interactions because they provide the simplest structural motif to achieve conformational changes from less stable to highly stable protein-protein interactions.

Furthermore, it became clear during the presentations and discussions that a better understanding of the structure and behaviour of proteins in the membrane is necessary to understand membrane fusion. Although the three-dimensional structures of many proteins have been determined, structural information on membrane proteins, and in particular on their transmembrane domains, is rare. For example, the exact orientation of fusion peptide and transmembrane domain of haemagglutinin in the low energy structure is not known (*Figure 3*), since they are not part of the crystallized fragments. Similarly, the transmembrane domains of the SNARE proteins had been removed to elucidate the structure of the SNARE complex (*Figure 5*). Therefore, little is known about the structure of the transmembrane domains of syntaxin 1a and synaptobrevin 2.

Nevertheless, some information on the behaviour of transmembrane domains is emerging. The transmembrane domain of haemagglutinin has been studied in some detail using biophysical techniques (43), and Axel Brünger (Stanford,CA, USA) presented preliminary data on the structure of the transmembrane domain of synaptobrevin 2 in lipid bilayers. There are also some ideas explaining how transmembrane domains generally interact with lipid bilayers (44).

However, the goal of reaching an understanding of proteins in a lipid membrane is still a distant one. This conference demonstrated that an interaction between the different disciplines is necessary to understand membrane fusion. It became also clear that much more information on

the structure of fusion proteins in the membrane and their interactions is required before models can be developed that describe protein-lipid bilayers accurately.

References

1. Gingell, D., Ginsberg, L. (1978) Problems in the physical interpretation of membrane interaction and fusion. Cell Surf. Rev. *5*, 791–833
2. Monck, J.R., Fernandez, J.M. (1992) The exocytotic fusion pore. J. Cell Biol. *119*, 1395–1404
3. Lindau, M., Almers, W. (1995) Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. Curr. Opin. Cell Biol. *7*, 509–517
4. Chanturiya, A., Chernomordik, L.V., Zimmerberg, J. (1997) Flickering fusion pores comparable with initial exocytotic pores occur in protein-free phospholipid bilayers. Proc. Natl. Acad. Sci. USA *94*, 14423–14428
5. Zimmerberg, J., Chernomordik, L.V. (1999) Membrane fusion. Adv. Drug Deliv. Rev. *38*, 197–205
6. Heimburg, T., Biltonen, R.L. (1996) A Monte Carlo simulation study of protein-induced heat capacity changes and lipid-induced protein clustering. Biophys. J. *70*, 84–96
7. Lentz, B.R., Malinin, V., Haque, M.E., Evans, K. (2000) Protein machines and lipid assemblies: current views of cell membrane fusion. Curr. Opin. Struct. Biol. *10*, 607–615
8. Kemble, G.W., Danieli, T., White, J.M. (1994) Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. Cell *76*, 383–391
9. Melikyan, G.B., Markosyan, R.M., Roth, M.G., Cohen, F.S. (2000) A point mutation in the transmembrane domain of the hemagglutinin of Influenza virus stabilizes a hemifusion intermediate that can transit to fusion. Mol. Biol. Cell *11*, 3765–3775
10. Armstrong, R.T., Kushnir, A.S., White, J.M. (2000) The transmembrane domain of influenza hemagglutinin exhibits a stringent length requirement to support the hemifusion to fusion transition. J. Cell Biol. *151*, 425–438
11. Razinkov, V.I., Melikyan, G.B., Cohen, F.S. (1999) Hemifusion between cells expressing hemagglutinin of influenza virus and planar membranes can precede the formation of fusion pores that subsequently fully enlarge. Biophys. J. *77*, 3144–3151
12. Lee, J., Lentz, B.R. (1998) Secretory and viral fusion may share mechanistic events with fusion between curved lipid bilayers. Proc. Natl. Acad. Sci. USA *95*, 9274–9279
13. Skehel, J.J., Wiley, D.C. (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. *69*, 531–569
14. Weissenhorn, W., Dessen, A., Calder, L.J., Harrison, S.C., Skehel, J.J., Wiley, D.C. (1999) Structural basis for membrane fusion by enveloped viruses. Mol. Membr. Biol. *16*, 3–9
15. Melikyan, G.B., Markosyan, R.M., Hemmati, H., Delmedica, M.K., Lambert, D.M., Cohen, F.S. (2000) Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. J. Cell Biol. *151*, 413–424
16. Bentz, J. (2000) Membrane fusion mediated by coiled coils: a hypothesis. Biophys. J. *78*, 886–900
17. Stegmann, T. (2000) Membrane fusion mechanisms: the influenza hemagglutinin paradigm and its implication for intracellular fusion. Traffic *1*, 598–604
18. Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., Rothman, J.E. (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature *362*, 318–324
19. Hanson, P.I., Heuser, J.E., Jahn, R. (1997) Neurotransmitter release – four years of SNARE complexes. Curr. Opin. Neurobiol. *7*, 310–315
20. Sutton, R.B., Fasshauer, D., Jahn, R., Brunger, A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. Nature *395*, 347–353
21. Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., Rothman, J.E. (1998) SNAREpins: minimal machinery for membrane fusion. Cell *92*, 759–772
22. Bock, J.B., Scheller, R.H. (1999) SNARE proteins mediate lipid bilayer fusion. Proc. Natl. Acad. Sci. USA *96*, 12227–12229
23. Otter-Nilsson, M., Hendriks, R., Pecheur-Huet, E.I., Hoekstra, D., Nilsson, T. (1999) Cytosolic ATPases, p97 and NSF, are sufficient to mediate rapid membrane fusion. EMBO J. *18*, 2074–2083
24. Wickner, W., Haas, A. (2000) Yeast homotypic vacuole fusion: a window on organelle trafficking mechanisms. Annu. Rev. Biochem. *69*, 247–275
25. Fiebig, K.M., Rice, L.M., Pollock, E., Brunger, A.T. (1999) Folding intermediates of SNARE complex assembly. Nat. Struct. Biol. *6*, 117–123
26. Nicholson, K.L., Munson, M., Miller, R.B., Filip, T.J., Fairman, R., Hughson, F.M. (1998) Regulation of SNARE complex assembly by an N-terminal domain of the t- SNARE Sso1p. Nat. Struct. Biol. *5*, 793–802
27. Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T.C., Rizo, J. (1999) A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J. *18*, 4372–4382
28. Misura, K.M., Scheller, R.H., Weis, W.I. (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature *404*, 355–362
29. Munson, M., Munson, M., Chen, X., Cocina, A.E., Schultz, S.M., Hughson, F.M. (2000) Interactions within the yeast t-SNARE Sso1p that control SNARE complex assembly. Nat. Struct. Biol. *7*, 894–902
30. Carr, C.M., Grote, E., Munson, M., Hughson, F.M., Novick, P.J. (1999) Sec1p binds to SNARE complexes and concentrates at sites of secretion. J. Cell Biol. *146*, 333–344
31. Brose, N., Rosenmund, C., Rettig, J. (2000) Regulation of transmitter release by Unc-13 and its homologues. Curr. Opin. Neurobiol. *10*, 303–311
32. McMahon, H.T., Missler, M., Li, C., Sudhof, T.C. (1995) Complexins: cytosolic proteins that regulate SNAP receptor function. Cell *83*, 111–119
33. Pabst, S., Hazzard, J.W., Antonin, W., Sudhof, T.C., Jahn, R., Rizo, J., Fasshauer, D. (2000) Selective interaction of complexin with the neuronal SNARE complex: determination of the binding regions. J. Biol. Chem. *275*, 19808–19818
34. Xu, T., Rammner, B., Margittai, M., Artalejo, A.R., Neher, E., Jahn, R. (1999) Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. Cell *99*, 713–722
35. Lin, R.C., Scheller, R.H. (2000) Mechanisms of synaptic vesicle exocytosis. Annu. Rev. Cell Dev. Biol. *16*, 19–49
36. Jahn, R., Südhof, T.C. (1999) Membrane fusion and exocytosis. Annu. Rev. Biochem. *68*, 863–911
37. Neher, E. (1998) Vesicle pools and Ca^{2+} microdomains: new tools for understanding their roles in neurotransmitter release. Neuron *20*, 389–399
38. Albillos, A., Dernick, G., Horstmann, H., Almers, W., Alvarez de Toledo, G., Lindau, M. (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. Nature *389*, 509–512
39. Ales, E., Tabares, L., Poyato, J.M., Valero, V., Lindau, M., Alvarez de Toledo, G. (1999) High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. Nat. Cell Biol. *1*, 40–44
40. Bruns, D., Riedel, D., Klingauf, J., Jahn, R. (2000) Quantal release of serotonin. Neuron *28*, 205–220
41. Xu, T., Binz, T., Niemann, H., Neher, E. (1998) Multiple kinetic components

of exocytosis distinguished by neurotoxin sensitivity. Nat. Neurosci. *1*, 192–200

42. Voets, T., Neher, E., Moser, T. (1999) Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices. Neuron *23*, 607–615
43. Tatulian, S.A., Tamm, L.K. (2000) Secondary structure, orientation, oligomerization, and lipid interactions of the transmembrane domain of influenza hemagglutinin. Biochemistry *39*, 496–507
44. Killian, J.A., von Heijne, G. (2000) How proteins adapt to a membrane-water interface. Trends Biochem. Sci. *25*, 429–434.

Key words

amperometry, electrophysiology (methods), exocytosis, fusion models, haemagglutinin, membrane fusion, munc-18, SNAP-25, SNARE, synaptobrevin, synaptotagmin, syntaxin

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