N- to C-Terminal SNARE Complex Assembly Promotes Rapid Membrane Fusion

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glomerular basement membrane preparation [nephrotoxic serum (NTS)], thereby inducing a mouse IgG response. Serum IgG and IgM from preimmune and NTS-immunized mice were characterized for sialic acid content; total IgG sialylation was reduced on average by 40% in immunized mice as compared to the unimmunized controls (Fig. 3, A and B). The effect was specific for IgG; sialylation of IgM or transferrin was equivalent before and after immunization (Fig. 3B and fig. S9). This difference in sialylation was more pronounced when the anti-sheep–specific IgG fraction from mouse serum was analyzed, showing a 50 to 60% reduction in sialylation as compared to preimmune IgG (Fig. 3B), and these results were confirmed by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) analysis (Fig. 3C). Finally, the mouse IgG2b antibodies to sheep that were deposited in the glomeruli (*20*) displayed reduced sialic acid content as compared to the preimmunized controls (Fig. 3D).

The regulated sialylation of IgG suggests a mechanism for ensuring that steady-state serum IgG antibodies maintain an anti-inflammatory state. Upon antigenic challenge by a potential pathogen, the antigen-specific IgG antibodies can switch to a population with reduced sialic acid that is thus capable of mediating antigen clearance and a protective inflammatory response through the engagement of subclass-specific FcγRs on effector cells.

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Materials and Methods
Figs. S1 to S11
References

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N- to C-Terminal SNARE Complex Assembly Promotes Rapid Membrane Fusion

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Assembly of the soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) syntaxin 1, SNAP-25, and synaptobrevin 2 is thought to be the driving force for the exocytosis of synaptic vesicles. However, whereas exocytosis is triggered at a millisecond time scale, the SNARE-mediated fusion of liposomes requires hours for completion, which challenges the idea of a key role for SNAREs in the final steps of exocytosis. We found that liposome fusion was dramatically accelerated when a stabilized syntaxin/SNAP-25 acceptor complex was used. Thus, SNAREs do have the capacity to execute fusion at a speed required for neuronal secretion, demonstrating that the maintenance of acceptor complexes is a critical step in biological fusion reactions.

The three soluble *N*-ethylmaleimide–sensitive factor attachment protein (SNAP) receptors or SNAREs—syntaxin 1, SNAP-25, and synaptobrevin 2 (also referred to as VAMP2)—are key elements of the molecular machinery mediating the rapid exocytosis of synaptic vesicles in neurons (*1*, *2*). Syntaxin and SNAP-25 are localized within the plasma membrane, and synaptobrevin resides in synaptic vesicles. In vitro, the three proteins assemble into a stable complex with equimolar stoichiometry. It consists of a tight bundle of four α helices aligned in parallel, in which the transmembrane regions of synaptobrevin and syntaxin lie at one end (*3*). SNARE assembly is thought to be initiated at the N-terminal end and proceed toward the C-terminal membrane anchors (in a process referred to as zippering). In consequence, the membranes are pulled tightly together, overcoming the energy barrier for fusion (*4*–*6*).

Although the zipper hypothesis of SNARE function has received substantial support, the features of SNARE assembly in vitro are difficult to reconcile with the proposed role of SNAREs as catalysts of the final step in exocytotic membrane fusion. SNAREs are able to fuse liposomes, but the fusion rates are very slow, requiring hours for completion (*7*). Faster rates have been observed upon fusion of liposomes with planar membranes (*8*–*10*), but because these reactions do not require SNAP-25, the importance of these results remains questionable. On the other hand, neuronal exocytosis occurs at a submillisecond time scale (*11*). To resolve this major discrepancy, it has been proposed that, in primed fusion-ready vesicles, the SNAREs are partially assembled in trans configuration bridging the fusing membranes (*12*, *13*). However, the actual configuration of the SNARE machinery for rapid fusion has remained elusive.

SNARE assembly proceeds in an ordered fashion involving structurally defined intermediates (*14*, *15*). In vitro, syntaxin and SNAP-25 readily form a stable four-helix bundle in 2:1 stoichiometry (*16*). Because the second syntaxin occupies the position of synaptobrevin (*17*, *18*), the overall slow rate of SNARE assembly may primarily be due to competition between syntaxin and synaptobrevin for binding to a transient 1:1 syntaxin/SNAP-25 heterodimer (*15*), thus occluding the true reactivity of the final (and fusion-relevant) step in the assembly pathway.

We therefore investigated whether the formation of the 1:1 syntaxin/SNAP-25 heterodimer allowed subsequent binding of synaptobrevin at rates compatible with biological fusion reactions. We monitored the binding kinetics of fluorescently labeled synaptobrevin (*19*). To increase the concentration of the syntaxin/SNAP-25 heterodimer, we used a large excess of SNAP-25 over the SNARE motif of syntaxin [amino acids 180 to 262 (Syx180–262)]. When synaptobrevin was added, a rapid increase in fluorescence anisotropy was observed ($\sim 5 \times 10^5$ M^{-1} s^{-1}; Fig. 1A). Similarly fast binding was observed when the heterodimer was preformed with the entire cytoplasmic region of syntaxin (Syx1–262; Fig. 1B), demonstrating that syntaxin's autonomous N-terminal domain did not affect synaptobrevin binding (Fig. 1C).

Next, we attempted to stabilize the acceptor site for synaptobrevin in the 1:1 heterodimer. According to the zipper hypothesis, binding of synaptobrevin should initiate at the N-terminal end

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of the complex. Consequently, nucleation should not be affected if only the C-terminal part of the binding site is occupied, leaving the N-terminal part free. We purified stable complexes with C-terminal fragments of synaptobrevin. When the binding of synaptobrevin (Syb) was measured, rapid binding was observed to complexes containing the fragments Syb60–96, Syb49–96, or Syb42–96 (Fig. 2A), whereas no binding was observed to complexes containing N-terminally longer fragments (Syb35–96 or Syb25–96). Thus, only a short N-terminal stretch is sufficient for fast synaptobrevin binding. Syntaxin was much less efficient in competing with synaptobrevin for binding to the Syb42–96 complex (fig. S2), suggesting that the N-terminal region was largely specific for synaptobrevin. Binding of synaptobrevin to complexes containing an N-terminal fragment of synaptobrevin was rather slow (Fig. 2A), indicating that the C-terminal end was not a preferred binding site.

We used circular dichroism (CD) spectroscopy to test whether fast binding of synaptobrevin to complexes containing C-terminal fragments was facilitated by a structured N-terminal region. Purified ternary complexes containing fragments corresponding to either one of the two halves of synaptobrevin, Syb1–59 or Syb60–96, exhibited a characteristic α-helical spectrum. Upon binding of Syb60–96 to the Syb1–59 complex, a major increase in α-helical structure was observed (Fig. 2B). Thus, the Syb1–59 complex is probably structured only N-terminally, whereas the remaining portion is mostly unstructured. In contrast, no change in α-helical content was observed upon binding of Syb1–59 to the Syb60–96 complex, suggesting that its N- and C-terminal regions are well structured. Because the speed of binding of synaptobrevin to complexes containing a C-terminal fragment was comparable to that observed for the syntaxin/SNAP-25 dimer, it seems that the binding sites for synaptobrevin are structurally very similar for both complexes. Indeed, their N-termini are of similar composition, containing only the N-terminal helices of SNAP-25 and syntaxin. In contrast, the C-terminal portion of the syntaxin/SNAP-25 heterodimer, such as the complex containing an N-terminal fragment of synaptobrevin, might be less structured. For the homologous heterodimer of Sso1p (equivalent to syntaxin) and Sec9p (equivalent to SNAP-25) from yeast, a similar structural configuration was determined by nuclear magnetic resonance (*20*): The N-terminal part consists of a three-helix bundle, whereas the C-terminal part is unstructured. Binding of Snc2p (equivalent to synaptobrevin) to the Sso/Sec9 complex is 100 times slower (~6000 M^{-1} s^{-1}) (*21*) than the fast binding of synaptobrevin.

We then investigated whether the presence of the C-terminal synaptobrevin fragment would prevent complete zippering or whether the binding of intact synaptobrevin would displace the fragments. The peptide dissociated from the complex (Fig. 2C and fig. S3). To measure the rate of displacement, we formed a complex containing labeled Syb49–96. The addition of Syb1–96 displaced this Syb49–96 fragment within 1 min (Fig. 2D). Thus, complexes containing a C-terminal fragment served as acceptors that allowed for SNARE complex formation at a much faster rate than hitherto observed.

Next we tested whether the Syb49−96 complex would accelerate liposome fusion. A moderate acceleration of liposome fusion occurs in the presence of a C-terminal peptide of synaptobrevin (Syb57–92) (*22*). We incorporated a Syb49−96 complex that contained the SNARE motif of syntaxin with a transmembrane region (TMR) into liposomes and monitored fusion with synaptobrevin liposomes. Lipid mixing was profoundly accelerated with liposomes containing the Syb49−96 complex [half-time ($t_{1/2}$) ≈ 1 min] when compared to liposomes containing the syntaxin/SNAP-25 2:1 complex ($t_{1/2}$ ≈ 20 min) (Fig. 3). Because the presence of the C-terminal fragment of synaptobrevin will certainly retard fusion, we predict that the fusion kinetics with an unobstructed 1:1 complex as an acceptor would be even faster.

When we determined the stability of complexes containing synaptobrevin fragments, the Syb1–59 complex unfolded in a single transition at about 66°C, whereas the Syb60–96 complex was clearly less stable (melting temperature ≈ 44°C; Fig. 2B). This agrees with deuterium exchange experiments that had inferred a lower stability of the C-terminal region for the complex (*23*). A complex that contained both fragments (a Syb1–59:Syb60–96 complex) unfolded in two steps (Fig. 2B), demonstrating that both halves of the four-

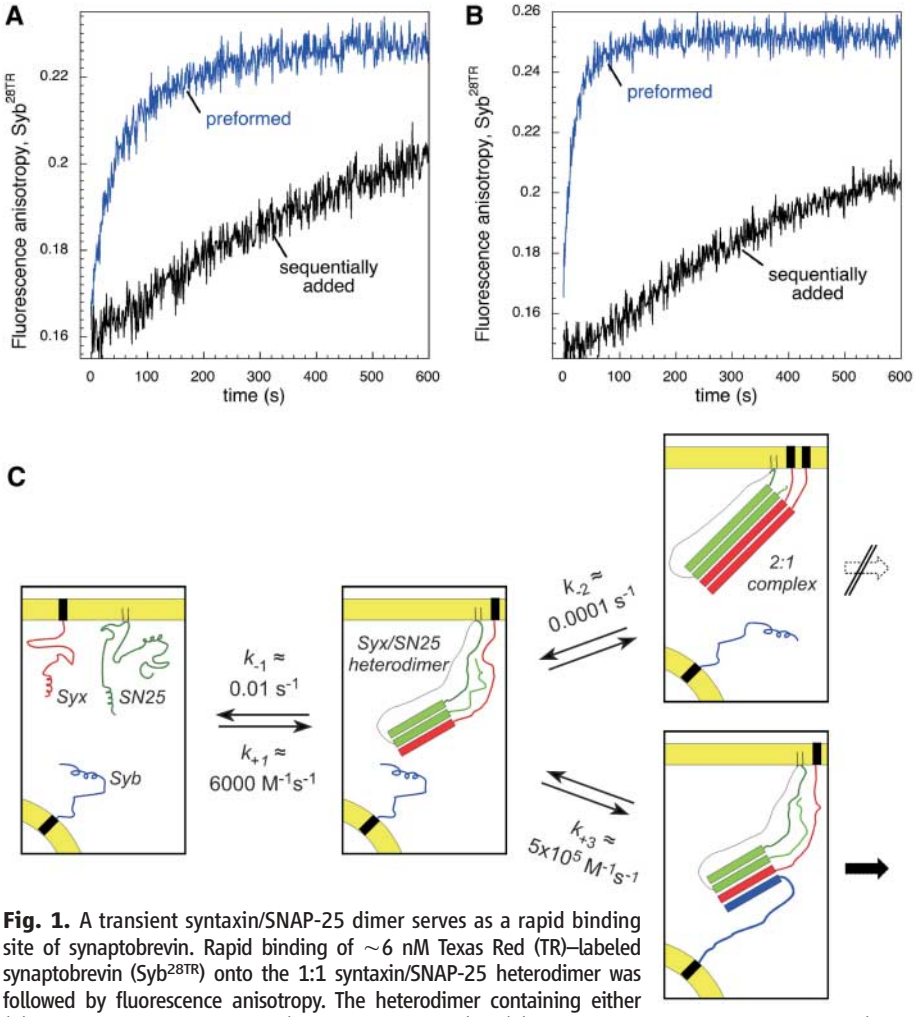

Fig. 1. A transient syntaxin/SNAP-25 dimer serves as a rapid binding site of synaptobrevin. Rapid binding of ~6 nM Texas Red (TR)–labeled synaptobrevin (Syb^{28TR}) onto the 1:1 syntaxin/SNAP-25 heterodimer was followed by fluorescence anisotropy. The heterodimer containing either (**A**) the SNARE motif of syntaxin (Syx180–262; 26 nM) or (**B**) the entire soluble domain of syntaxin (Syx1–262; 83 nM) was preformed by means of an excess of SNAP-25 (240 nM or 720 nM, respectively). When the proteins were added sequentially, a much slower binding of Syb^{28TR} was observed. (**C**) Kinetic model of the SNARE assembly pathway. The proteins are depicted between two fusing membranes; however, the rates were obtained with the use of the soluble portions of the SNAREs. First, syntaxin (Syx) and SNAP-25 (SN25) slowly assemble into a transient 1:1 heterodimer [~6000 M^{-1} s^{-1} (*15*)] that provides a rapid binding site for synaptobrevin (Syb) or a second syntaxin molecule. Synaptobrevin does not actively replace the second syntaxin (*15*), indicating that the 2:1 complex is off-pathway. The off-rate of the second syntaxin from the 2:1 complex was ~0.01 s^{-1} [(*15*), fig. S1A], and dissociation of the entire 2:1 complex occurred at ~0.0001 s^{-1} (fig. S1B). Assumed α-helical regions are boxed.

helix bundle were able to fold independently. A marked hysteresis in SNARE folding and unfolding (*14*) probably results from a kinetic barrier for the dissociation of synaptobrevin. It is conceivable that either fragment can dissociate and rebind as long as the remaining fragment holds the complex together. Thus, the first unfolding step of the Syb1–59:Syb60–96 complex may be reversible, whereas the second is not. Together, these data suggest that SNARE complex formation can be arrested halfway and that, in this configuration, the C-terminal part of an N-terminally anchored synaptobrevin can assemble reversibly.

A partially assembled trans-SNARE configuration might be reached when the force generated by SNARE assembly and the repulsive force of the charged lipid headgroups balance each other. Such an equilibrium might be exploited by another protein to block further assembly, perhaps until its grip is released upon binding of Ca^{2+}. Still, this factor would need to be able to sustain a strong SNARE assembly force. However, even a tightly fitting C-terminal synaptobrevin fragment was not able to arrest zippering between liposome membranes (Fig. 2D). Because the energetic barrier for membrane fusion is largely unknown, it remains unclear whether SNAREs can be maintained in trans configuration. An energetically more favorable way to regulate SNARE assembly might be through the control of the nucleation process, in particular by restricting the first contact of synaptobrevin with the syntaxin/SNAP-25 acceptor.

Here we have shown that SNARE nucleation is restricted to the N-terminal portion and that zippering proceeds in an N- to C-terminal direction. In addition, synaptobrevin binds rapidly to a syntaxin/SNAP-25 acceptor. Stabilizing the syntaxin/SNAP-25 acceptor by a peptide allowed for fast liposome fusion. Thus, SNAREs have the capacity to execute

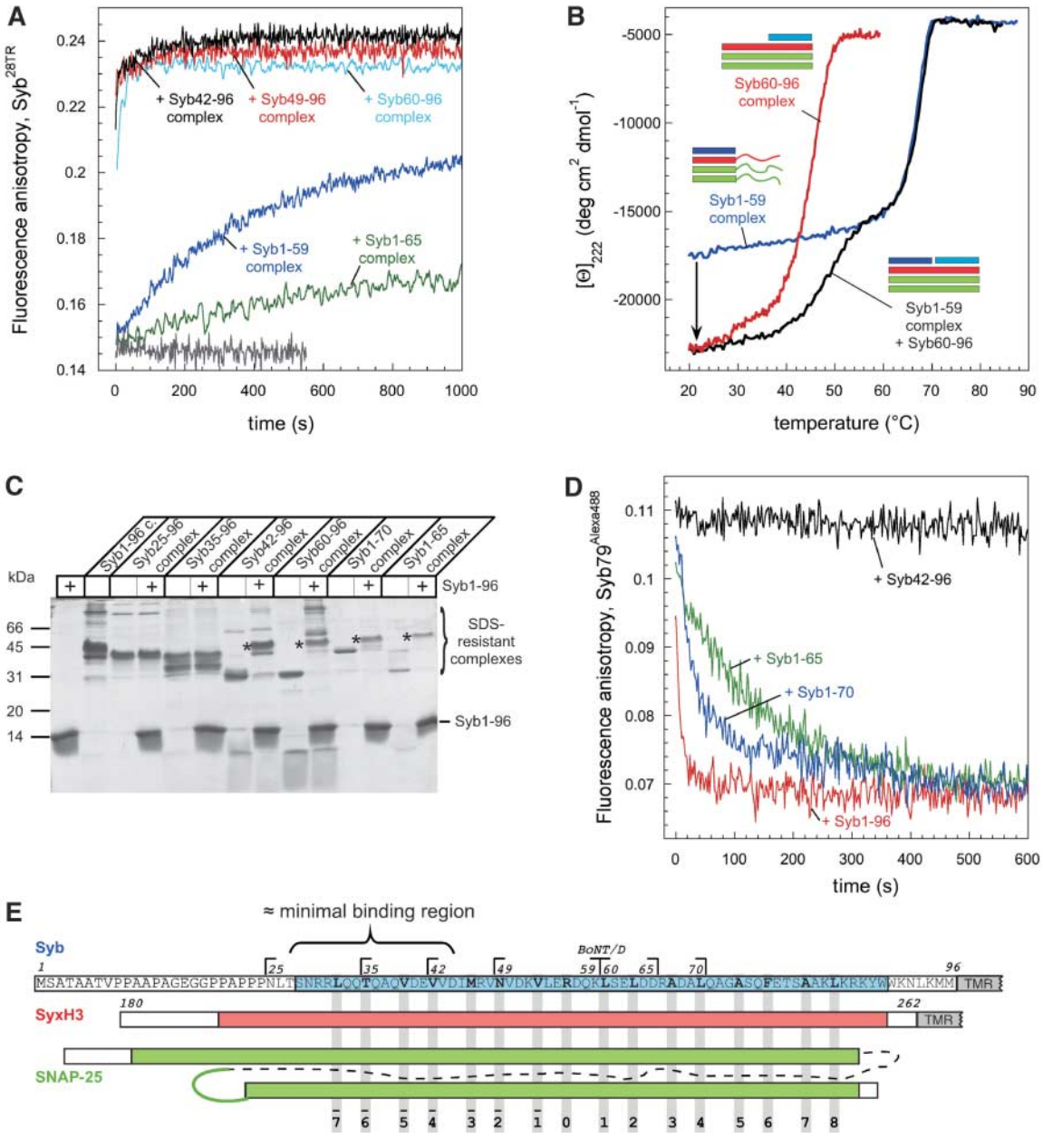

Fig. 2. Synaptobrevin fragments can be actively replaced from a ternary complex by the intact coil of synaptobrevin. (**A**) Binding of synaptobrevin to SNARE complexes containing different fragments of synaptobrevin monitored by fluorescence anisotropy. Complexes (1 μM) were mixed with Syb^{28TR} (~200 nM). Binding was inhibited by preincubation with Syb1–96 (shown for the Syb49–96 complex as the gray curve). (**B**) Thermal unfolding of SNARE complexes containing synaptobrevin fragments monitored by CD spectroscopy. The Syb60–96 complex and the Syb1–59 complex unfolded in a single transition at ~45° and ~66°C, respectively, whereas a complex containing both fragments unfolded in two steps. The large increase in molar ellipticity upon binding of Syb60–96 to the Syb1–59 complex is indicated by an arrow. [Θ], molar ellipticity. (**C**) Replacement of synaptobrevin fragments visualized by SDS–polyacrylamide gel electrophoresis and Coomassie Blue staining. SNARE complexes containing different synaptobrevin fragments were incubated with Syb1–96. Upon replacement, a SDS-resistant complex containing Syb1–96 appeared (marked by asterisks). In the first two lanes, Syb1–96 and the SNARE complex containing Syb1–96 were loaded. (**D**) Fast displacement of Syb49–96 monitored by fluorescence anisotropy. A ternary complex containing Alexa488-labeled (~200 nM) Syb49–96 was incubated with different Syb fragments (1 μM). Displacement led to a decrease in anisotropy. (**E**) Schematic depiction of synaptobrevin fragments used in the framework of the four-helix–bundle SNARE complex. The positions of the heptad repeat layers (–7 to +8) are indicated by gray vertical bars. The structured regions of the SNAREs are boxed (*3*).

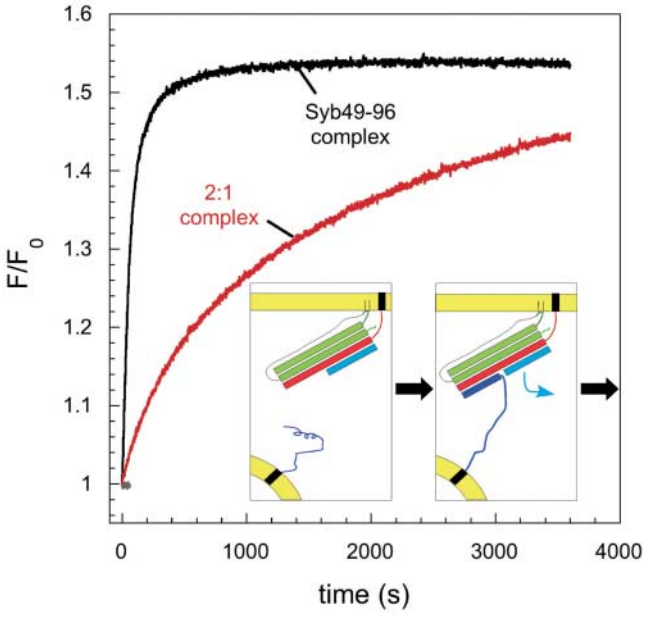

Fig. 3. A complex containing a C-terminal synaptobrevin fragment greatly accelerates liposome fusion. A SNARE complex containing SNAP-25, the SNARE motif of syntaxin with TMR (Syx183–288), and Syb49–96 and a binary 2:1 complex containing Syx183–288 and SNAP-25 were purified and inserted into liposome membranes. Fusion with liposomes containing full-length synaptobrevin (Syb1–116) was monitored by lipid dequenching. At a final protein concentration of ~200 nM for both liposome populations, the liposomes containing the Syb49–96 complex fused very rapidly ($t_{1/2} \approx 1$ min) as compared to liposomes containing the 2:1 complex ($t_{1/2} \approx 20$ min). The obtained rates are difficult to compare to published fusion rates, because other laboratories routinely use 5 to 10 μM final protein concentrations, and their reactions usually do not reach saturation even after several hours of incubation. Additionally, graphical representations of their data are often expressed as rounds of fusion (*7*, *22*, *25*). F/F_0, relative fluorescence. (**Insets**) The N-terminal region of the Syb49–96 complex offers a free binding site for synaptobrevin. Upon complete zippering, the Syb49–96 fragment is displaced and lipid mixing can occur (drawing as in Fig. 1C).

fusion at a very high rate as required for neuronal exocytosis. It remains to be established at which state the SNAREs are arrested before fusion: at the level of a free 1:1 syntaxin/SNAP-25 acceptor or at a later stage when the N-terminal tip of synaptobrevin is already in contact with the acceptor complex, with further zippering being prevented by unknown mechanisms. The latter view is supported by the fact that mutations in C-terminal coiled-coil residues of SNAP-25 selectively affect fusion triggering in vivo and also compromise the integrity of the C-terminal portion of the SNARE bundle (*24*).

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Figs. S1 to S3
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A Clamping Mechanism Involved in SNARE-Dependent Exocytosis

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During neurotransmitter release at the synapse, influx of calcium ions stimulates the release of neurotransmitter. However, the mechanism by which synaptic vesicle fusion is coupled to calcium has been unclear, despite the identification of both the core fusion machinery [soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)] and the principal calcium sensor (synaptotagmin). Here, we describe what may represent a basic principle of the coupling mechanism: a reversible clamping protein (complexin) that can freeze the SNAREpin, an assembled fusion-competent intermediate en route to fusion. When calcium binds to the calcium sensor synaptotagmin, the clamp would then be released. SNARE proteins, and key regulators like synaptotagmin and complexin, can be ectopically expressed on the cell surface. Cells expressing such "flipped" synaptic SNAREs fuse constitutively, but when we coexpressed complexin, fusion was blocked. Adding back calcium triggered fusion from this intermediate in the presence of synaptotagmin.

In the cell membrane, trafficking occurs constitutively. However, exocytosis—the fusion of vesicles containing stored secretory product—is tightly regulated by external signals and is often highly synchronized. Nevertheless, regulated exocytosis and constitutive vesicle trafficking rely on the same machinery—cognate vesicle (v-) and target membrane (t-) SNARE proteins. How, then, can fusion by a common mechanism be tightly triggered in one instance but occur spontaneously in another (*1*)?

It has been suggested that the fusion machinery is constitutively "on," so that exocytosis—in which fusion is "off" in the absence of a signal for secretion—would require a protein "clamp" to block fusion in the basal state. Then, an additional protein "trigger" would be needed to reverse the clamp when a signal for secretion appears. Accumulation of fusion intermediates arrested at a discrete stage would create a synchronized and robust burst of secretion in response to urgent but transient physiological need. Isolated SNAREs can spontaneously fuse bilayers (*2*) on a time scale shorter than secretions can be stored—hours to days—but direct evidence for a clamp mechanism has been lacking.

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