The carboxy-terminal domain of complexin I stimulates liposome fusion

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Regulated exocytosis requires tight coupling of the membrane fusion machinery to a triggering signal and a fast response time. Complexins are part of this regulation and, together with synaptotagmins, control calcium-dependent exocytosis. Stimulatory and inhibitory functions have been reported for complexins. To test if complexins directly affect membrane fusion, we analyzed the 4 known mammalian complexin isoforms in a reconstituted fusion assay. In contrast to complexin III (CpxIII) and CpxIV, CpxI and CpxII stimulated soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-pin assembly and membrane fusion. This stimulatory effect required a preincubation at low temperature and was specific for neuronal t-SNAREs. Stimulation of membrane fusion was lost when the carboxy-terminal domain of CpxI was deleted or serine 115, a putative phosphorylation site, was mutated. Transfer of the carboxy-terminal domain of CpxI to CpxIII resulted in a stimulatory CpxIII-I chimera. Thus, the carboxy-terminal domains of CpxI and CpxII promote the fusion of high-curvature liposomes.

exocytosis | SNARE

Membrane fusion is initiated by the pairing of compartment-specific v-SNAREs and t-SNAREs between opposite membranes, resulting in the formation of so-called "trans-SNARE" complexes or SNAREpins (1). At the neuronal synapse, the v-SNARE VAMP2/synaptobrevin2 is located on synaptic vesicles and binds the t-SNARE, consisting of syntaxin 1 (Syx1) and SNAP-25, at the presynaptic plasma membrane (2). The assembly of the SNAREpin starts at the membrane distal end of the SNARE motifs and proceeds in a membrane proximal direction bringing the lipid bilayers in close proximity (3–5). This process occurs in a stepwise manner, and regulatory proteins likely stabilize intermediates along the assembly pathway. Indeed, synaptic vesicles docked at the active zone appear to be linked to the plasma membrane by such partially assembled SNAREpins, which ensure membrane fusion within a submillisecond response time (6). The physiological trigger for synaptic vesicle fusion is a local increase in the calcium concentration, caused by the opening of calcium channels (7, 8). In the presence of calcium, the calcium sensor synaptotagmin 1 localized on synaptic vesicles interacts with t-SNAREs and negatively charged phospholipids, leading to fusion pore opening (9).

In addition to synaptotagmin 1, complexins (also called synaphins) participate in calcium-dependent exocytosis (10–12). Complexins are small cytoplasmic proteins that contain a central α-helix, which binds distinct SNAREs. In *Drosophila*, only 1 complexin orthologue has been identified, and its inactivation results in a dramatic increase in the spontaneous fusion of synaptic vesicles at neuromuscular junctions (13). In vertebrates, 4 complexin isoforms are known (14). Complexin I (CpxI) and CpxII are highly homologous to each other and more distantly related to CpxIII and CpxIV, which contain CAAX boxes at their carboxy-termini, anchoring these isoforms to membranes. CpxI/II double- and CpxI/II/III triple-knockout mice die at birth and are impaired in synaptic vesicle exocytosis at the Ca^{2+}-dependent step (12, 15). In contrast to the complexin knockout in *Drosophila*, nonsynchronous release is slightly reduced in CpxI/II double- and CpxI/II/III triple-knockout mice (15). Other studies, including complexin overexpression, in vitro liposome fusion assays, and cell-cell fusion experiments, indicate that complexins can also function as fusion clamps (16–19). The clamp is released by synaptotagmin 1 in a calcium-dependent manner. Recent analysis revealed that distinct domains of complexins differently control neurotransmitter release and membrane fusion. The amino-terminal 26 amino acids of CpxI contain a stimulatory function followed by an inhibitory α-helix (amino acids 29–48) and the SNARE binding helix (amino acids 48–70) (20, 21). Although low-affinity binding of CpxI to neuronal t-SNAREs has been reported, high-affinity binding requires v-/t-SNARE complexes (22–25). The crystal structure of CpxI, together with the assembled cis v-/t-SNARE core complex, shows that CpxI binds in an antiparallel manner to a groove at the surface of the 4-helix SNARE bundle, which is formed by Syx1 and VAMP2 (26, 27). Thus, complexins are perfectly positioned to control SNAREpin assembly directly, and it has been suggested that complexins stabilize SNAREpin intermediates (26). In addition, clusters of conserved amino acids are present in the amino- and carboxy-terminal regions that flank the SNARE complex-binding helix of complexins. It is largely unknown to what degree and how these amino- and carboxy-terminal regions control membrane fusion. Thus, to shed light on the function of complexins in SNARE complex assembly and membrane fusion, we compared the molecular function of the 4 mammalian complexin isoforms in an in vitro liposome fusion assay. Remarkably, we observed a complexin isoform-specific stimulation of membrane fusion that requires the carboxy-terminus of complexins. The removal of the stimulatory carboxy-terminus of CpxI or point mutations in S115 abolish the stimulation. The transfer of the stimulatory CpxI carboxy-terminus to CpxIII results in a stimulatory chimera.

Results

CpxI and CpxII Stimulate Membrane Fusion. To analyze Cpx function, we used an in vitro fusion assay that consists of liposomes containing reconstituted SNAREs. When v-SNARE liposomes with a quenched pair of *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl) (NBD)-labeled and rhodamine-labeled lipids fuse with unlabeled t-SNARE liposomes, the NBD fluorescence increases (1, 28). VAMP2 and preassembled full-length Syx1/SNAP-25 complexes were reconstituted into liposomes at a protein-to-lipid ratio of about 1:200, which corresponds approximately to the VAMP2/lipid ratio found in purified synaptic vesicles (29).

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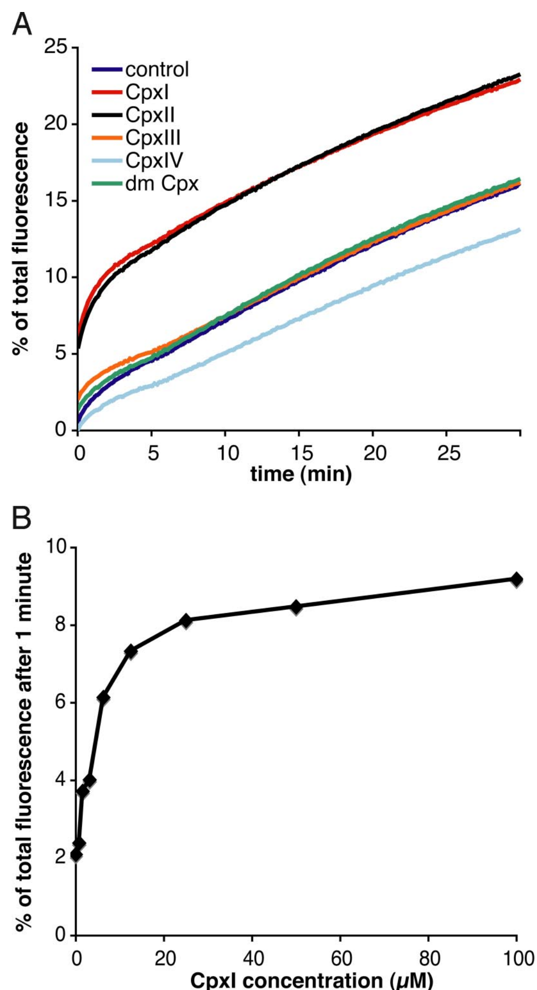


Fig. 1. Complexin isoforms differentially stimulate/inhibit membrane fusion. (*A*) Effect of complexin isoforms on liposome fusion kinetics. v-SNARE (VAMP2) liposomes labeled with rhodamine and NBD lipids were incubated in the absence or presence of the indicated complexin isoforms with unlabeled t-SNARE (Syx1/SNAP-25) liposomes for 1 h on ice. With the exception of *Drosophila* complexin (dm Cpx), all other complexin isoforms were human orthologues (CpxI–CpxIV). After warming up of the reaction to 37 °C, the increase in NBD fluorescence was monitored for 30 min at 37 °C. The results were normalized to the maximum NBD fluorescence signal after detergent lysis as described in *Material and Methods*. (*B*) Dose-dependent stimulation of fusion by CpxI. Constant amounts of v- and t-SNARE liposomes were incubated with increasing amounts of CpxI, and the fusion was monitored and analyzed as described in *A*. The gain of total NBD fluorescence 1 min after warming up to 37 °C was plotted against the CpxI amount.

VAMP2 liposomes and Syx1/SNAP-25 liposomes were mixed and preincubated in the absence or the presence of purified complexin isoforms for 1 h at 4 °C. The samples were warmed up, and fusion was immediately measured for 30 min at 37 °C. To control for unspecific fusion, v-SNARE liposomes were preincubated with botulinum neurotoxin D (BoNT/D) to remove VAMP2 from the liposome surface. These samples did not show any increase in NBD fluorescence when added to t-SNARE liposomes, confirming that fusion was VAMP2 dependent [supporting information (SI) Fig. S1]. The background signal derived from these BoNT/D-treated control reactions was subtracted from the other measurements.

The analysis of the 4 complexin isoforms revealed that CpxI and CpxII accelerated the initial fusion kinetics (Fig. 1*A*). To ensure that the fusion reactions contained similar complexin amounts, aliquots were analyzed postfusion by SDS/PAGE and Coomassie Blue staining (Fig. S2). The stimulatory effect was strictly dependent on the preincubation of CpxI with v-SNARE and t-SNARE liposomes at low temperature (Fig. S3), which usually blocks membrane fusion in the in vitro assay (1). Remarkably, in the presence of CpxI and CpxII, a significant fraction of liposomes already fused at low temperature and during the warming up phase (see elevated initial signal in Fig. 1*A* and Fig. S1). Separate preincubations of CpxI with either v-SNARE or t-SNARE liposomes did not stimulate membrane fusion, indicating that CpxI acts on SNAREpins (data not shown). Fusion stimulation by CpxI and CpxII was dose dependent (Fig. 1*B*). Plotting the NBD fluorescence signal 1 min after sample warming up against the complexin amount showed that half-maximal stimulation was reached when CpxI (5 μM) and the surface-exposed t-SNARE reached a ratio of ≈1:1 (Fig. 1*B*). CpxIII and *Drosophila* complexin showed only minor effects on the fusion signal at the indicated concentrations (Fig. 1*A*). CpxIV inhibited membrane fusion to some extent, consistent with the findings of a previous publication (16). CpxIII had no effect on liposome fusion at the indicated concentration (Fig. 1*A*) but inhibited fusion when the amounts were increased (Fig. S4), which is consistent with a lower affinity of CpxIII for SNAREs (14). We also tested *Drosophila* complexin in combination with the mammalian SNAREs and the *Drosophila* SNARE orthologues in reconstituted liposomes. Similar to the human CpxIII, the *Drosophila* complexin inhibited the fusion reaction when used at high concentrations (data not shown). Remarkably, the inhibitory effect of CpxIV occurred independent of the low temperature preincubation (Fig. S3). Thus, the fusion stimulation is specific to complexin isoform and stage.

CpxI Stimulates SNAREpin Assembly. Because CpxI stimulates the initial phase of the fusion reaction, it could accelerate SNAREpin assembly. To test this hypothesis, it is necessary to detect SNAREpins. Preincubation of v- and t-SNARE liposomes at low temperature prevents membrane fusion to a large degree but allows the formation of SNAREpins (1). VAMP2 on the liposome surface, which is not incorporated into SNAREpins, can be removed by the cleavage with tetanus toxin, whereas VAMP2 assembled into SNARE complexes is protected (6). However, VAMP2 is also trapped in the lumen of the liposomes, and thus is toxin resistant. To analyze the VAMP2 pool on the liposome surface selectively, v-SNARE liposomes were labeled with membrane-impermeable amino-reactive succinimidylester-activated biotin (Fig. 2*A*). The majority (96%) of the biotinylated VAMP2 molecules on v-SNARE liposomes was cleaved by tetanus toxin in the absence or presence of complexin (Fig. 2*B*). Further, the biotinylation of VAMP2 did not affect the fusion properties of the liposomes (data not shown). Thus, biotinylated VAMP2 liposomes were incubated with t-SNARE liposomes in the absence or the presence of complexins for distinct periods of time at 4 °C to allow SNAREpin formation. Subsequently, an excess of tetanus toxin was added, and the incubation was continued for an additional 2 h at 4 °C. The reaction was stopped by the addition of SDS-containing sample buffer, and the toxin-resistant biotinylated VAMP2 was quantitated by Western blot analysis using antibiotin antibodies. The results show that CpxI significantly increased the number of toxin-resistant VAMP2 molecules, indicating an accelerated SNAREpin assembly (Fig. 2*B*). A preincubation of 10 min already increased the amount of toxin-resistant VAMP2 (VAMP2 incorporated into SNARE complexes) by a factor of 2. During prolonged preincubations, more VAMP2 becomes toxin resistant, which, in part, might reflect postfusion complexes.

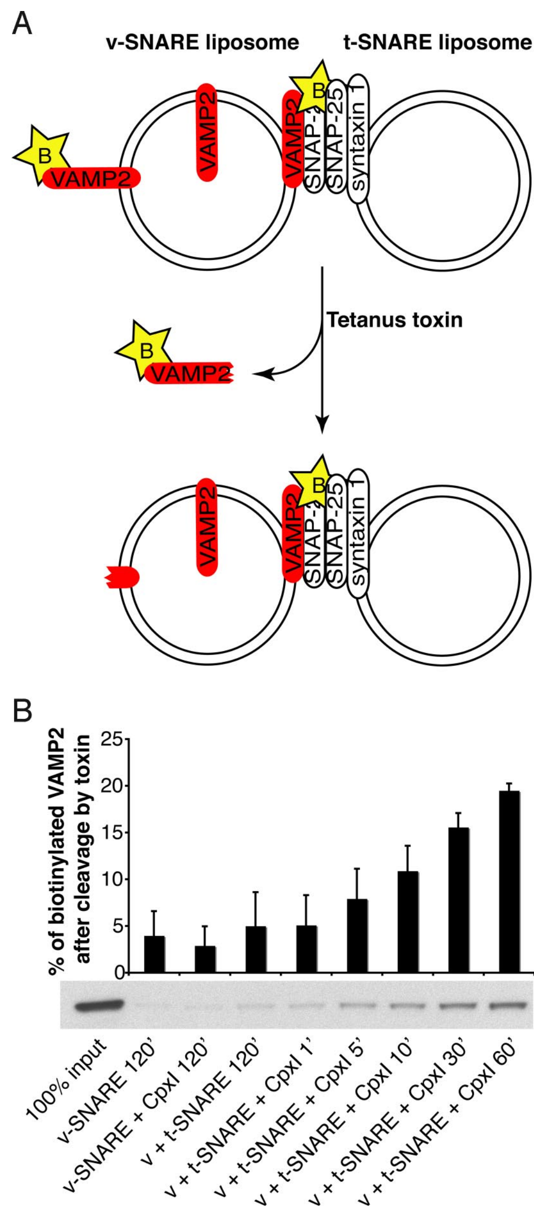


Fig. 2. CpxI stimulates SNAREpin formation/assembly. (*A*) v-SNARE liposomes were modified with membrane-impermeable amino-reactive succinimidylester-activated biotin to label the pool of surface-exposed VAMP2 molecules selectively. These liposomes were incubated with t-SNARE liposomes in the absence or the presence of CpxI to allow SNAREpin formation for distinct periods of time at 4 °C. Subsequently, an excess of tetanus toxin was added, and the incubation continued for an additional 2 h at 4 °C to remove VAMP2 molecules not incorporated into SNAREpins. The reaction was stopped by the addition of SDS-containing sample buffer. (*B*) The toxin-resistant biotinylated VAMP2 was quantitated by Western blot analysis using antibiotin antibodies (details in *Material and Methods*). Error bars = SEM (n = 2).

Stimulation Caused by CpxI Is t-SNARE Specific and Requires the Carboxy-Terminal Region of CpxI. To test if the stimulation by CpxI and CpxII shows SNARE specificity, we analyzed the fusion of VAMP2 liposomes with Syx4/SNAP-23 t-SNARE liposomes and VAMP8 v-SNARE liposomes with Syx1/SNAP-25 t-SNARE liposomes. As expected, CpxI did not affect the fusion of Syx4/SNAP-23 liposomes with VAMP2 liposomes (Fig. S5 *a* and *b*) (25). This result demonstrates that complexin has to interact with the neuronal t-SNARE to exert its stimulatory role. Unexpectedly, CpxI accelerated the fusion of VAMP8 liposomes with Syx1/SNAP-25 liposomes (Fig. S5 *c* and *d*). Consistent with this result, binding studies revealed that under the conditions used, CpxI interacts with assembled Syx1/SNAP-25/VAMP8 complexes (Fig. S5*e*), although with low affinity.

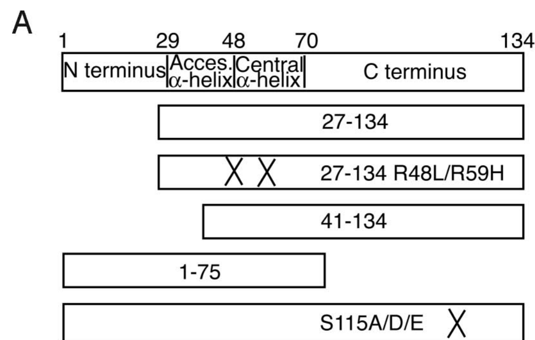
Because the stimulation by CpxI requires the neuronal t-SNARE, it might affect the amino-terminal regulatory Habc domain of Syx1. In previous experiments, we have shown that the proteolytic removal of the regulatory Habc domain stimulates membrane fusion (30). However, CpxI stimulates fusion even in the absence of the Habc domain. Thus, the amino-terminal–regulatory domain of Syx1 is not the primary target of CpxI (Fig. S6).

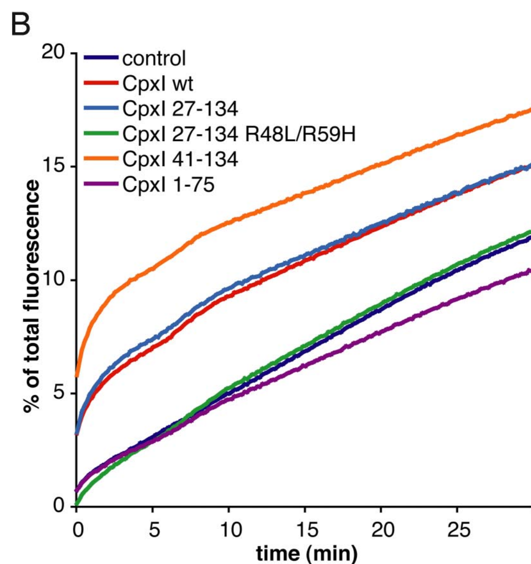
To test which part of complexin harbors the stimulatory activity, we used CpxI constructs that are either truncated at their amino-terminus (27–134, 41–134) or carboxy-terminus (1–75) (Fig. 3*A*). In our reconstituted assay, CpxI 27–134 stimulated membrane fusion to the same degree as wild-type complexin (Fig. 3*B*). A double mutation, R48L/R59H, in CpxI 27–134 revealed that stimulation requires the interaction of the central complexin helix with the SNAREs. The CpxI 41–134 construct showed increased stimulation, consistent with the disruption of the accessory inhibitory helix located in the region of amino acids 29–48 (Fig. 3*B*) (20, 21). CpxI 1–75 interacted with assembled cis v-/t-SNARE complexes (20) but completely lost its stimulatory activity (Fig. 3*B*). Moreover, CpxI 1–75 weakly but reproducibly inhibited the fusion reaction (Fig. S7 *a* and *b*).

Furthermore, to test whether restraining of the carboxy-terminus of CpxI affects the stimulatory role, a lipid anchor was introduced. Therefore, a CpxI construct containing a single cysteine (CpxI C105S/C135) was covalently coupled to maleimide-activated phosphatidylethanolamine and reconstituted into v- or t-SNARE liposomes. The immobilized CpxI stimulated liposome fusion even more potently than free CpxI (Fig. S8 and data not shown), most likely because of the high local concentration at the membrane.

Point Mutations in Position 115 of CpxI Abolish the Stimulation of Liposome Fusion. To locate the stimulatory function within the carboxy-terminus of CpxI further, we introduced point mutations in position 115. A recent publication showed that S115 can be phosphorylated in vitro and in vivo by casein kinase II (31). Although the functional consequences of this phosphorylation remain unclear, such a posttranslational modification likely has a regulatory role. The following point mutations were introduced by in vitro mutagenesis: S115A, S115D, and S115E; the latter 2 mutations were intended to serve as phosphomimetics. When the recombinant CpxI constructs were added to the liposome fusion assay, each of the mutants significantly reduced the stimulatory effect of CpxI (Fig. 3*C*). The phosphomimetic mutation S115E completely abolished the stimulation. To ensure that S115E is indeed inactive, higher concentrations of this mutant were added to the fusion assay. Even high levels of CpxI S115E did not stimulate fusion (Fig. S9 *a* and *b*). Thus, a single-point mutation in the carboxy-terminus can abolish the stimulatory role of CpxI in liposome fusion. Interestingly, changes of serine 115 to hydrophobic or negatively charged amino acids reduce the stimulatory effect. Thus, serine 115 itself appears to be critical for stimulation.

The Stimulatory Carboxy-Terminus of CpxI Is Transferable to CpxIII. Because all complexin isoforms bind to SNARE complexes via their central helix but differ in their inhibitory and stimulatory activities, we speculated that it should be possible to alter their activities by switching the domains flanking the central helix. Therefore, we exchanged the carboxy-terminal domain of the inhibitory CpxIII against the stimulatory carboxy-terminal domain of CpxI. As a junction site, a conserved cluster of amino





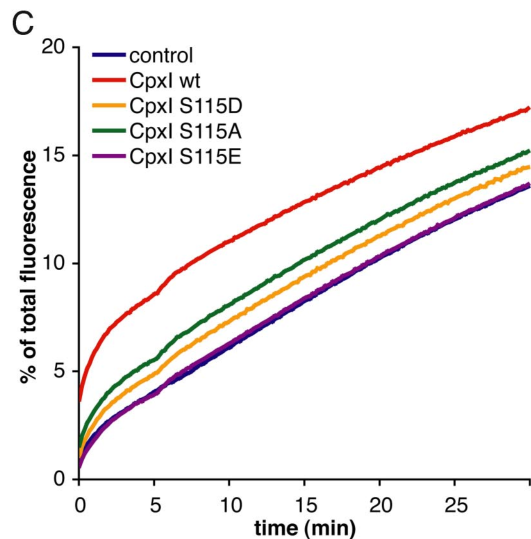
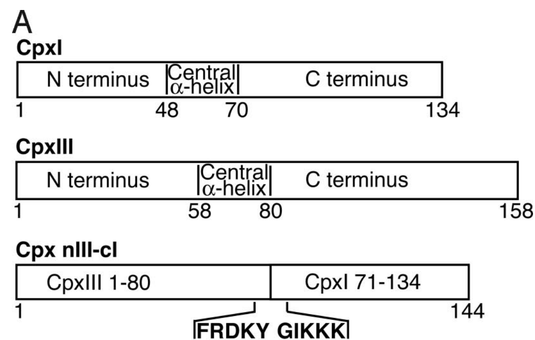


Fig. 3. The carboxy-terminal region of CpxI is required to accelerate membrane fusion. (*A*) Schematic diagram of the domain organization of CpxI and of CpxI deletion mutants. The point mutations are indicated with a cross, and the amino acid changes are listed. (*B*) t-SNARE liposomes were incubated with v-SNARE liposomes in the absence or the presence of wild-type complexin (CpxI wt) or complexin constructs, which are either truncated at their amino-terminus (amino acids 27–134, amino acids 41–134) or their carboxy-terminus (amino acids 1–75). In addition, the double mutation R48L/R59H was introduced into CpxI 27–134 to test whether SNARE binding is required for fusion stimulation. (*C*) Point mutations in position 115 of CpxI reduce the stimulatory activity. Fusion was monitored and analyzed as described.



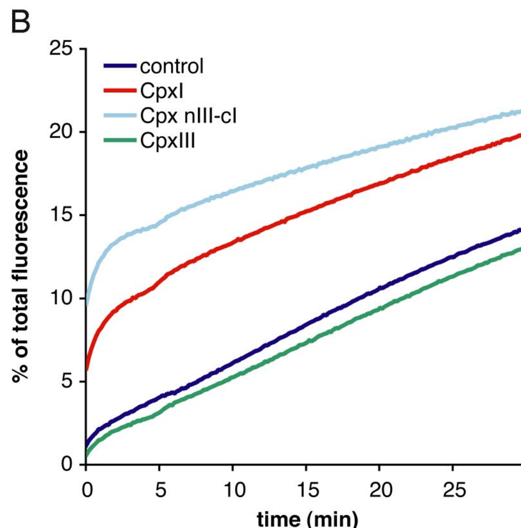


Fig. 4. A CpxIII-CpxI fusion protein, containing the amino-terminal and the α-helical regions of CpxIII and the carboxy-terminal region of CpxI accelerates fusion. (*A*) Schematic diagram of the domain organization of CpxI and CpxIII and of Cpx nIII-cI. The residues flanking the fusion site in CpxIII (amino acids 76–80) and CpxI (amino acids 71–75) are shown. (*B*) t-SNARE liposomes were incubated with v-SNARE liposomes in the absence or the presence of wild-type complexin (CpxI wt) or Cpx nIII-cI. Fusion was monitored and analyzed as described.

acids at the carboxy-terminal end of the central complexin α-helix was selected (Fig. 4*A*). As shown in Fig. 4*B*, the Cpx nIII-cI chimera stimulated fusion at least as potently as wild-type CpxI, demonstrating that the stimulatory domain of CpxI can be transferred to CpxIII.

Discussion

Here, we show that CpxI and CpxII stimulate membrane fusion in a reconstituted assay. Stimulation requires a preincubation of complexins with v- and t-SNARE liposomes at low temperature—conditions that allow the formation of SNAREpins but usually block lipid bilayer fusion (1). This result indicates that CpxI and CpxII bind and might stabilize SNAREpins. Indeed, the presence of CpxI significantly increased the amount of SNARE complexes at low temperature. The CpxI-SNAREpin complexes that accumulate at low temperature are productive intermediates, and some membrane fusion even occurs at low temperature. An increase in the temperature results in immediate vesicle fusion, restricting stimulation to the initial phase (initial 2–3 min) of the in vitro fusion reaction. When CpxI is added to v- and t-SNARE liposomes at 37 °C without preincubation, the stimulatory effect is not observed. This result suggests that CpxI does not affect the rate-limiting step in the overall fusion reaction at 37 °C. It is not clear which step is rate limiting at 37 °C: (*i*) the formation of an initial SNAREpin or (*ii*) the

binding of CpxI to such a SNAREpin. Apparently, at low temperature, these steps occur; thus, CpxI either stabilizes SNAREpins or accelerates SNAREpin zippering. Our analysis of the SNARE requirement shows that the replacement of the t-SNARE Syx1/SNAP-25 by Syx4/SNAP-23 abolishes the stimulation. Furthermore, a CpxI double mutant (R48L/R59H) impaired in VAMP2 binding abolished the stimulatory effect of CpxI. Thus, an interaction of the central helix of CpxI with both the v-SNARE and t-SNARE is a prerequisite for fusion stimulation. Unexpectedly, the stimulatory effect was also observed in the presence of VAMP8 liposomes. In previous studies, CpxI did not interact in significant amounts with cis Syx1/SNAP-25/VAMP8 complexes (25). However, under the conditions used here, CpxI binds to the Syx1/SNAP-25/VAMP8 complex, although with lower efficiency than to the Syx1/SNAP-25/VAMP2 complex.

The stimulatory domain is the carboxy-terminal region of CpxI, downstream of the central α-helix. Remarkably, the stimulatory domain is transferable and can convert a previously inhibitory CpxIII into a stimulatory Cpx nIII-cI chimera. Based on the crystal structure and the antiparallel orientation of the CpxI-SNARE core complex, the stimulatory domain of CpxI would emerge at the amino-terminal end of the SNAREpins (26). This region includes the membrane-distal parts of the SNARE motifs, which are involved in the initial assembly of the SNAREpin (4, 32). Thus, the carboxy-terminal domain of CpxI could target the amino-terminal regions of SNAREs, and thereby stabilize an early stage of SNAREpin formation or accelerate SNAREpin zippering. However, the removal of the Habc domain did not mimic the stimulation caused by CpxI, indicating that the amino-terminal regulatory domain of Syx1 is not the primary target of CpxI action. Although the amino-terminus of complexin emerges at the membrane distal end of the SNARE complex, the carboxy-terminal domain, which covers a stretch of about 60 aa, could fold back and reach the amino-terminal part of Cpx itself or the membrane proximal region of the SNARE complex. Indeed, single-molecule studies indicate that such a folded-back conformation, in which C105 would be near the ionic layer of the SNARE complex, can occur (33). Thus, S115, which is in the center of an amphipathic helix and critical for fusion stimulation, might interact with the accessory inhibitory helix of CpxI or directly affect SNAREpin zippering. However, without detailed structural insights, it is difficult to design appropriate complementing point mutations required to test these models in a straightforward manner. Thus, it still remains to be shown how the carboxy-terminal domain of CpxI mechanistically controls SNAREpin formation/zippering. CpxI might also modulate membrane fusion via lipid interactions. Although S115 might be engaged in protein-protein interactions, the hydrophobic side of the amphipathic α-helix could bind lipids and alter the lipid bilayer structure. Indeed, changes in the lipid composition enhance the fusogenic activity of CpxI. Consistent with a recent publication, an increase of negatively charged phospholipids resulted in a more robust fusion stimulation (34) (data not shown).

In addition to the stimulatory effect, we observed that full-length CpxI and CpxII slightly inhibit the later phase of the fusion reaction. Interestingly, on removal of the carboxy-terminal domain of CpxI (1–75), the stimulation was selectively abolished, but the clamping effect of CpxI in the late phase of the fusion reaction became more apparent. These data show that the amino-terminal domain, together with the central helix, has an inhibitory function, which is consistent with a recent electrophysiological analysis and in vitro studies that identified an inhibitory α-helix (amino acids 27–47) in the amino-terminal region of CpxI (20, 21). Indeed, when we removed a part of the accessory α-helix, the truncated construct CpxI 41–134 stimulated liposome fusion even more potently than full-length CpxI. Thus, the liposome fusion assay reproduces, at least in part, membrane fusion in vivo. However, in contrast to the electrophysiological study, which identified a stimulatory function in amino acids 1–26, we did not observe a stimulatory function in our reconstituted assay when we used the CpxI 1–75 construct. The amino-terminal region might interact with additional regulators present in intact cells, which are missing in our reconstituted assay. Apparently, the experimental conditions used in the in vivo study and in our assay differ significantly. To observe the inhibitory effects for CpxIII, CpxIV, and *Drosophila* complexin, fairly high concentrations of complexins were required, consistent with previous reports (16). It is also worth mentioning that our reconstituted assay employs high-curvature liposomes. Fusion of flat membranes requires more energy as compared with high-curvature membranes (35, 36). Thus, among other experimental differences, distinct membrane curvatures, lipid compositions, and the presence of additional regulatory proteins could influence the rate-limiting step, and thereby determine the final readout signal.

In summary, our results suggest a model in which the carboxy-terminal domains of CpxI and CpxII help to stabilize an early SNAREpin intermediate or even accelerate SNAREpin zippering. In the presence of appropriate lipids and high-curvature membranes and in the absence of additional regulators, increased membrane fusion dominates. When the conditions are less favorable for membrane fusion, an inhibitory function of CpxI, which might trap t-SNAREs and/or SNAREpin intermediates, becomes prominent. (In our minimal fusion assay containing high-curvature liposomes, it was necessary to remove the carboxy-terminal domain of CpxI to observe a clamping effect.) In addition, complexins seem not to be the only clamps present at synapses, because the inactivation of synaptotagmins in *Drosophila* and mouse increases spontaneous fusion events (37, 38). In addition, synaptotagmin I can arrest SNARE complexes and membrane fusion as shown in reconstituted liposome fusion assays (9). Thus, in the absence of calcium, complexin and synaptotagmin likely stabilize SNAREpin intermediates. On binding of synaptotagmin to calcium and anionic phospholipids, the clamp is released, allowing synchronized fusion to start. Furthermore, because CpxI and CpxII could accelerate an early stage of SNAREpin formation, the majority of newly formed SNAREpins would contain complexins, ensuring the presence of the fusion clamp. Thus, complexins have distinct functional properties, which depend on the assembly state of the SNARE complex and the local environment (e.g., presence of synaptotagmin, specific lipids, Ca^{2+}). The unique stimulatory property of CpxI and CpxII could be important for fine-tuning neurotransmitter release in the brain, allowing one to switch reversibly between inhibitory and stimulatory functions by postranslational modifications such as phosphorylation (31). Thus, by dissecting the domains of the distinct complexin isoforms in in vitro and in vivo assays, which resolve different rate-limiting steps, we shall be able to gain further insight into the complex function spectrum of complexins.

Materials and Methods

Protein Reconstitution into Liposomes. All lipids were from Avanti Polar Lipids, with the exception of ^{3}H-1,2-dipalmitoyl phosphatidylcholine (^{3}H-DPPC), which was from Amersham Pharmacia Biotech. Donor lipid mix was composed of 83.3 mole% 1-palmitoyl-2-oleoyl-SN-glycero-3-phosphatidylcholine (POPC), 15.3 mole% 1,2-dioleoyl-SN-glycero-3-phosphatidylserine (DOPS), 0.7 mole% *N*-(lissamine rhodamine B sulfonyl) 1,2-dipalmitoyl phosphatidylethanolamine (rhodamine-DPPE), 0.7 mole% NBD-DPPE, and trace amounts of ^{3}H-DPPC, for a total lipid volume of 3 mM. Acceptor lipid mix was composed of 85 mol% POPC, 15 mol% DOPS or 80 mol% POPC, 15 mol% DOPS, and trace amounts of ^{3}H-DPPC, for a total lipid volume of 15 mM. In some experiments, 5 mol% maleimide-PE was included.

Liposomes were formed in the presence of VAMP2 (0.2 mg/mL) or t-SNARE complex (2 mg/mL) using the donor and acceptor lipid mixes defined previ-

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ously and a technique of dilution and dialysis followed by a Nycodenz (Axis-Shield) gradient centrifugation as described elsewhere (1). Protein expression and purification are described in detail in *SI Text*. Protein amounts in the reconstituted liposomes were determined using Coomassie blue–stained SDS/PAGE with BSA protein standards and ImageJ Quantitation Software (National Institutes of Health).

Fusion Assays. Fusion reactions and data analysis were performed as described elsewhere (1), with the following modifications:

1. In all cases, 30 μL of acceptor (unlabeled) and 5 μL of donor (labeled) liposomes were used.
2. Unless otherwise noted, acceptor and donor liposomes were preincubated on ice for 1 h in the presence or absence of complexin constructs before quickly warming up in a waterbath at 85 °C. The temperature increase of 50-μL control incubations was monitored using a microthermometer (amadigit ad 13th; Amarell), and at 30 °C, the microtiter plate was quickly transferred into a FluoroScan ascent plate reader (Thermo Scientific); samples were measured at intervals of 10 s for 30 min.
3. The NBD fluorescence obtained from control incubations containing donor liposomes pretreated with BoNT/D was subtracted from individual measurement sets.
4. The fusion-dependent fluorescence was normalized to the maximal fluorescent signal obtained in the presence of 0.4% dodecylmaltoside (Fluka).

Fusion kinetics displayed in all figures show 1 representative experiment. Experiments were repeated several times independently, yielding virtually identical results.

Biotinylation of VAMP2 Liposomes and Detection of SNAREpin Formation. VAMP2 was reconstituted into donor liposomes and dialyzed overnight against 4 L of buffer A [25 mM Hepes-KOH (pH 7.4), 100 mM KCl, 10% glycerol (wt/vol), 1 mM DTT]. Before flotation in a Nycodenz gradient, 20 μL of a 10-mM stock solution of water-soluble Biotin-NHS (Calbiochem) was added to 600 μL of dialyzed liposomes while vortexing and was incubated on ice for 1 h. Excess reactive groups were quenched by the addition of 50 mM NH_4Cl, and liposomes were purified on a Nycodenz gradient as described previously.

Over time, 2 μL of biotinylated v-SNARE liposomes alone or combinations of v-SNARE and 12 μL of t-SNARE liposomes were incubated in the presence or absence of CpxI (10 μM) in total volumes of 35 μL on ice for up to 2 h. At the time points indicated, 10-μL aliquots of each incubation were transferred to new tubes containing 2 μL of tetanus toxin (5 μg) and incubated for an additional 1 h on ice to cleave VAMP2 molecules not trapped in SNAREpins. The incubation was stopped by the addition of 5 μL 2 $\times$ SDS sample buffer while vortexing and heating the samples for 5 min at 95 °C. Samples were subjected to 16% (wt/vol) Tricine-SDS/PAGE, followed by Western blotting. Toxin-resistant VAMP2-biotin molecules were detected with an antibiotin antibody (clone BN-34 B7653; Sigma). For a detailed description of additional materials and methods, please see *SI Materials and Methods*.

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