Genetic analysis of the Complexin trans-clamping model for cross-linking SNARE complexes in vivo

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Complexin (Cpx) is a SNARE-binding protein that regulates neurotransmission by clamping spontaneous synaptic vesicle fusion in the absence of Ca²⁺ influx while promoting evoked release in response to an action potential. Previous studies indicated Cpx may cross-link multiple SNARE complexes via a trans interaction to function as a fusion clamp. During Ca²⁺ influx, Cpx is predicted to undergo a conformational switch and collapse onto a single SNARE complex in a cis-binding mode to activate vesicle release. To test this model in vivo, we performed structure-function studies of the Cpx protein in Drosophila. Using genetic rescue approaches with cpx mutants that disrupt SNARE cross-linking, we find that manipulations that are predicted to block formation of the trans SNARE array disrupt the clamping function of Cpx. Unexpectedly, these same mutants rescue action potential-triggered release, indicating trans-SNARE cross-linking by Cpx is not a prerequisite for triggering evoked fusion. In contrast, mutations that impair Cpxmediated cis-SNARE interactions that are necessary for transition from an open to closed conformation fail to rescue evoked release defects in cpx mutants, although they clamp spontaneous release normally. Our in vivo genetic manipulations support several predictions made by the Cpx cross-linking model, but unexpected results suggest additional mechanisms are likely to exist that regulate Cpx's effects on SNARE-mediated fusion. Our findings also indicate that the inhibitory and activating functions of Cpx are genetically separable, and can be mapped to distinct molecular mechanisms that differentially regulate the SNARE fusion machinery.

synapse | neurotransmitter release | exocytosis

Neurotransmitter release at synapses is a tightly controlled process that is initiated presynaptically within milliseconds of an action potential. Like most cellular fusion events, synaptic vesicle exocytosis is mediated by soluble NSF attachment protein receptors (SNARE) proteins (1). v-SNAREs present on the vesicle (VAMP2/Synaptobrevin) associate with their cognate t-SNAREs on the target plasma membrane (SNAP25 and Syntaxin 1) to form a coiled-coil four-helix SNAREpin bundle that drives membrane fusion (2, 3). During vesicle fusion, the v-SNARE Synaptobrevin is predicted to zipper onto a preassembled t-SNARE dimer to form the fully assembled SNARE complex, bringing the synaptic vesicle closer to the plasma membrane (4, 5). This process is characterized by a sequential SNARE folding pathway that includes a half-zippered intermediate SNARE complex (6). It is this intermediate SNARE complex that may be the key control point for regulating fusion, with proteins that inhibit or promote zippering serving as clamps or activators of the release process, respectively. Indeed, several neuronal specific SNAREbinding proteins have emerged as key regulators of the SNARE fusion machine (7, 8). The synaptic vesicle protein Synaptotagmin (Syt) serves as a Ca^{2+} sensor that stimulates synaptic vesicle fusion in response to action potential triggered Ca^{2+} influx (9, 10). In addition to Syt, the cytosolic protein Complexin (Cpx) has emerged as an important cofactor for controlling synaptic vesicle

fusion, potentially through its ability to regulate SNARE zippering (11, 12).

Cpx is a cytosolic α -helical protein that binds to SNARE complexes (13). Initial in vitro studies using cell-cell fusion or lipid-mixing assays indicated that Cpx prevents in vitro membrane fusion events, suggesting Cpx may act as a fusion clamp to prevent premature exocytosis in the absence of Ca^{2+} (11, 14, 15). Consistent with the role of Cpx in clamping synaptic fusion, in vivo experiments in Drosophila revealed a dramatic increase in spontaneous synaptic vesicle fusion events (minis) in cpx null mutants (16). In addition to enhanced minis, cpx mutants have reduced evoked release. Together with data from other genetic systems (17–21), an emerging model is that Cpx plays a dual role in release, acting as a vesicle fusion clamp that prevents spurious fusion of vesicles at some synapses, while simultaneously promoting synchronous release in response to an action potential at all synapses (16, 17, 19-22). Structurally, these dual activities are likely to require an α -helix motif found within Cpx that is divided into an accessory and central helix region (Fig. 1A). Binding of the Cpx central helix to the SNARE complex is absolutely required for both clamping and stimulating properties (17, 23). Outside the central helix, the N terminus and the accessory helix has been postulated to contribute to the stimulatory and inhibitory properties of Cpx (22). How these distinct domains are coordinated to regulate spontaneous and evoked release, and whether these activities are required sequentially or in parallel, is poorly understood.

Studies using a C-terminal truncated v-SNARE protein to mimic partial SNARE zippering, together with "superclamp"

Significance

Synaptic vesicle fusion at synapses is the primary mechanism by which neurons communicate. A highly conserved membrane fusion machine known as the SNARE complex mediates this process. In addition, neuronal SNARE-binding regulatory proteins have evolved to control the kinetics and speed of SNARE assembly at synapses. One such SNARE-binding protein, Complexin, has been found to inhibit synaptic vesicle fusion in the absence of an action potential and activate SNARE-mediated release during stimulation. Here we examine molecular models for how Complexin can mediate these unique effects on the SNARE fusion machine using genetic rescue experiments in *Drosophila*. We find that alternate SNARE-binding mechanisms by Complexin are likely to contribute to distinct inhibitory and activating functions in vivo.

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mediated by a trans Cpx/SNARE array that forms between the accessory/ central domains of Cpx and partially zippered prefusion SNARE complexes. (A) Structure of Cpx, and the Cpx mutants used in this study. (B) The trans Cpx/SNARE array (zigzag array) that is proposed to form between Cpx and SNARE complexes. A conformational switch in Cpx from an open (trans-SNARE) to a closed (cis-SNARE) state is proposed to mediate Ca²⁺-dependent triggering of vesicle fusion. The positions of mutations used in this study are marked with colors matching Fig. 1A. (C) Hydrophobic residues that mediate the interaction of the Cpx accessory helix with t-SNAREs of a partially zippered SNARE complex are conserved in both mCpx and DmCpx. Space fill model of the accessory domain of mCpx and Dm Cpx is shown. Blue and gray residues represent the interface between the Cpx accessory domain and t-SNAREs. Conserved hydrophobic residues are illustrated in gray. (D) Both mCpx and DmCpx exhibit clamping properties in in vitro cell-cell fusion assays. (E) SB mCpx exhibits clamping properties similar to WT mCpx in cellcell fusion assays. (F) SB mCpx exhibits decreased Syt-stimulated cell-cell fusion compared with WT mCpx. Error bars are SEM, and horizontal lines indicate statistical comparisons determined using one-way ANOVA with post hoc Tukey analysis for D-F.

mutations in the Cpx accessory helix that enhance its inhibitory activity (24), have suggested a structural basis by which Cpx might regulate these dual activities (25-27). Crystallization of the Cpx-bound truncated SNARE complex revealed that the N-terminal accessory helix domain of Cpx extends away from its central helix domain at a 45° angle to form a trans interaction with a second SNARE complex, binding within the open t-SNARE pocket of the partially zippered SNAREs (trans Cpx/SNARE or zigzag array). This finding suggested a structural basis by which Cpx clamps vesicles in a fusion capable state (26). In response to Ca²⁺, the trans Cpx/SNARE array is predicted to collapse, with the Cpx helix undergoing a conformational switch to associate with the fully zippered cis-SNARE complex, providing a switch mechanism by which Cpx might promote release (Fig. 1B) (25). However, the in vivo relevance of such a mechanism in neurotransmitter release is unknown. Here, we undertook a genetic approach to test the trans Cpx/SNARE array and switch-release model of Cpx using the *Drosophila melanogaster* (Dm) neuro-muscular junction (NMJ) as a model synapse.

Results

Drosophila has a single Cpx homolog that is enriched in presynaptic nerve terminals. Électrophysiological analysis at NMJs in cpx null mutants revealed dramatically enhanced spontaneous release and reduced evoked release compared with controls (16), consistent with Cpx's in vitro properties (14, 15, 26). Based on the crystal structure of the Cpx/SNARE array, we designed a series of mutations that were previously shown to modulate Cpx function as a vesicle clamp in in vitro cell-cell fusion assays (Fig. 1 A and B). A key molecular determinant of Cpx's ability to bridge two SNARE complexes in vitro is the presence of hydrophobic residues in the accessory helix that bind to a second partially zippered t-SNARE complex to form the trans Cpx/ SNARE array. Hydrophobic mutations in this region [termed superclamp (SC)] increased the ability of mammalian (m) Cpx to promote clamping in in vitro cell-cell "flipped" fusion assays, whereas charged residue substitutions within this region [termed nonclamp (NC)] reduced clamping (26). Furthermore, mutations that disrupt the continuity of the Cpx helix [termed helixbreaker (HB)] abolished clamping by Cpx in this assay (26).

To determine if a similar trans Cpx/SNARE array interaction might occur in *Drosophila*, we compared the accessory helix of *Drosophila* Cpx (DmCpx) to that of mCpx. The DmCpx accessory helix contains hydrophobic residues (A35, I46, and A49) that are predicted to orient and align similarly to the hydrophobic residues found in mCpx (Fig. 1*C*). We found that WT DmCpx clamps cell-cell fusion in vitro similar to WT mCpx (Fig. 1*D*) [WT DmCpx = $7.1 \pm 1.6\%$ (n = 3) vs. WT mCpx = $5.0 \pm$ 1.4% (n = 4); P > 0.05], suggesting a trans Cpx/SNARE array interface may be conserved in *Drosophila*.

In addition to clamping release to prevent spontaneous fusion, Cpx also promotes Syt-dependent vesicle fusion (16, 17, 19-22). Ca²⁺-dependent triggering of release has been suggested to require a conformational switch in Cpx from an open (Cpx extends away from the SNARE surface at a $\sim 45^{\circ}$) to a closed (Cpx lies on the surface of a postfusion SNARE complex) state (Fig. 1B) (26). This switch is mediated by hydrogen bond and salt bridge interactions of residues in VAMP2 (D64, D65, D68) with residues within the Cpx central helix (R48, Y52) (25, 28). The in vitro relevance of this interaction was tested previously using mutations within VAMP2 (25). Here, we designed an equivalent Cpx mutant [R48D/E, Y52A, termed switchbreaker (SB)] that is predicted to reduce Cpx's ability to switch from an open to closed conformation, and examined both the clamping and stimulating properties of SB mCpx using in vitro cell-cell fusion assays. Despite mutations in its central helix, SB mCpx maintained its ability to clamp in in vitro cell-cell fusion similar to WT mCpx (Fig. 1*E*) [SB mCpx = $7.6 \pm 0.8\%$ (*n* = 3) vs. WT mCpx = $5.0 \pm 0.7\%$ (*n* = 4); *P* > 0.05]. In contrast, activation of cell-cell fusion with SB mCpx was significantly reduced following addition of Ca²⁺ and Syt (Fig. 1F) [SB mCpx = $1.5 \pm 0.4\%$ fold increase (n = 3) vs. WT mCpx = 3.2 ± 0.5 fold increase (n = 4); P < 0.05]. These data indicate that interactions involving mCpx residues R48 and Y52 with zippering or fully assembled SNARES may be important to trigger release, but are not required for Cpx to clamp fusion.

To examine the in vivo relevance of the Cpx trans array clamping/switch model, we generated transgenic *Drosophila* strains expressing a series of mCpx mutants that are predicted to disrupt these activities. First, we tested the requirement of the interaction between the accessory domain of Cpx and the half-zippered t-SNARE complex using the SC and NC mCpx mutants. Using in vitro experiments, these mutants exhibited enhanced and reduced ability, respectively, to bind and clamp compared with wild-type Cpx

(26). A second aspect of the trans Cpx/SNARE array clamping model is that the Cpx helix forms a rigid bridge between two SNARE complexes (26). To test whether this rigidity is required in vivo, we expressed mCpx mutants with three glycine residues between the central helix and the accessory helix (HB) to perturb the continuous α -helical structure. In addition, we sought to express Cpx separately as a split transgenic protein, thereby directly preventing cross-linking of two SNARE complexes. One piece encoded the central SNARE binding domain (termed mCpx 51–134), whereas the second piece contained only the Cpx N-terminal fragment (1-50). However, the N-terminal fragment was unstable and not expressed properly in vivo (Fig. S1B). The Cpx C-terminal fragment was expressed, and was able to support aspects of neurotransmission (see below). Finally, we assayed whether a SB mCpx mutant designed to reduce the ability of mCpx to switch from an open to closed conformation (Fig. 1B) (25)could support synaptic transmission in vivo. A summary of these transgenic lines is shown in Fig. 1A and Table S1.

The WT and mutant mCpx transgenes were placed in the *cpx* null background and expressed under control of the GAL4-UAS system. We used the phiC31-attP recombination system to insert all mCpx transgenes into the same integration site on the third chromosome, eliminating variability in transgene expression. The mCpx transgenes were tagged with an N-terminal myc epitope to allow detection of the transgenic proteins by Western and immunocytochemical experiments. Western analysis demonstrated that all mutants, with one exception, were expressed at similar levels to WT mCpx I (Fig. S14). The C-terminal mCpx 51–134 fragment was expressed at slightly lower levels compared with WT mCpx. All of the mutant mCpx transgenic proteins localized normally to presynaptic terminals at *Drosophila* NMJs (Fig. S1*B*), indicating these alterations do not disrupt Cpx trafficking.

We first examined the clamping properties of WT and mutant mCpxs by analyzing spontaneous release (minis) in *cpx* null and rescued animals (Fig. 2). *cpx* mutants exhibit a dramatic enhancement in spontaneous release frequency (Fig. 24). Expression of WT mCpx presynaptically in *cpx* null animals significantly reduced the elevated spontaneous release rate in *cpx* mutants [*cpx* = 73.8 ± 3.6 Hz (n = 9) vs. WT mCpx rescue = 14.6 ± 1.1 Hz (n = 18); P < 0.001], although it remained elevated compared with WT control lines [control = 2.6 ± 0.1 Hz (n = 9) vs. WT mCpx rescue = 14.6 ± 1.1 Hz (n = 18); P < 0.001]. We next tested the relevance of the accessory helix of Cpx to associate with the prefusion t-SNARE complex (26) (Fig. 1 A and B). Mutations that enhanced Cpx/t-SNARE binding (SC mCpx) have been

previously shown to increase the ability of the protein to inhibit fusion in vitro (26). We found that animals expressing SC mCpx more effectively rescued the *cpx* mini phenotype compared with WT mCpx [SC mCpx = 5.6 ± 0.6 Hz (n = 15) vs. WT mCpx rescue = 14.6 ± 1.1 Hz (n = 18); P < 0.05] (Fig. 2). In contrast, mCpx mutants that disrupted the accessory helix interaction with t-SNAREs (NC mCpx) displayed mini frequencies that were only slightly elevated compared with WT mCpx rescue = 14.6 ± 1.1 Hz (n = 18); P > 0.05] (Fig. 2); this was surprising because in vitro cell–cell fusion assays demonstrated a strong reduction in the clamping properties of NC mutants (26).

To exclude the possibility that NC mCpx does not alter association of Cpx with Drosophila t-SNAREs as expected, we performed isothermal titration calorimetry (ITC) experiments using blocked prefusion, partially assembled Drosophila SNAREs, following the strategy used in previous studies (26). The partially assembled Drosophila SNARE complex (pDmSNARE) was preformed with the SNARE motifs from Drosophila syntaxin (residues 194-265), Drosophila SNAP25 (residues 18-89 and 149-211), and the N-terminal SNARE motif from Drosophila VAMP (residues 48-79). The interaction of pDmSNARE with the Cpx central helix was blocked using truncated mCpx (residues 48-134). mCpx 48-134 binds tightly to pDmSNARE with an affinity constant 667 ± 90 nM (Fig. S2), which is similar to its binding affinity with partially assembled mammalian SNARE complex (26). This tight binding reflects saturation of the central helix binding sites of pDmSNARE with mCpx 48-134 when mCpx 48-134 is added in molar excess. Thus, with blocked pDmSNARE, only the interaction between the mCpx accessory helix and unzippered t-SNARE would be measured. WT mCpx, NC mCpx (A30E A31E L41E A44E), and a mCpx containing only two mutations in the accessory helix, NC 2× mCpx (L41E, A44E), were titrated into the blocked pDmSNARE mixture. The binding affinity, $K_{\rm D}$, of the mCpx accessory helix to blocked pDmSNARE gradually weakened with mutations in the hydrophobic residues (Fig. S3 and Table S2): $K_D = 11 \pm 1 \mu M$ for WT mCpx, $45 \pm 8 \mu M$ for NC 2x mCpx, and undetectable for the quadruple mutant, the equivalent to the NC mCpx mutant used in these studies. This finding demonstrated that NC mCpx indeed disrupted association of the Cpx accessory helix with prefusion Drosophila SNAREs in vitro. Because the disrupted association between NC mCpx with t-SNARES was confirmed in ITC experiments, the ability of NC mCpx to function only slightly less efficiently than WT mCpx



Fig. 2. Mini frequency of elav-GAL4 transgenic rescued animals using WT or mutant mCpxs in the *cpx*^{SH1} mutant background. (A) Sample traces of spontaneous release events from muscle 6 of control, *cpx*^{SH1}, and rescue lines expressing WT mCpx, SC mCpx, NC mCpx, SB mCpx, HB mCpx, or mCpx 51–134. (B) Summary of mean mini frequency (hertz ± SEM) for each line. Numbers in parentheses (*n*) represent individual NMJ recordings from indicated genotypes. Horizontal lines indicate statistical comparisons determined using ANOVA with post hoc Tukey analysis.



Fig. 3. Evoked release properties of WT mCpx and individual mutant mCpx rescues. (*A*) Averaged traces of EJPs from control, cpx^{SH7} , and rescued strains expressing indicated mutant mCpxs. Recordings were performed at various Ca²⁺ concentrations (0.15, 0.2, 0.4, and 1 mM). (*B*) Summary of mean EJP amplitude (millivolts \pm SEM) plotted at the indicated Ca²⁺ concentrations. Numbers in parentheses (*n*) represent individual muscle recordings at the indicated [Ca²⁺]; data were collected from at least three independent larvae for each genotype.

in rescuing spontaneous release in vivo suggests that the association of the Cpx accessory helix with a t-SNARE may not be absolutely required for the clamping function, or this interaction is largely maintained in vivo but not in vitro (*Discussion*).

Previous in vitro studies indicated the Cpx helix forms a rigid bridge between two SNARE complexes (26). To test whether this rigidity is required in vivo, we expressed the HB mCpx mutant containing three glycine residues between the central and accessory helixes. Expression of HB mCpx completely failed to rescue the *cpx* mini phenotype [WT mCpx rescue = 14.6 ± 1.1 Hz (n = 18) vs. HB mCpx = 80.0 ± 2.7 Hz (n = 9); P < 0.001] (Fig. 2). This result is consistent with in vitro cell-cell fusion assays, where perturbations of the *a*-helix reduced Cpx clamping function (26). We next tested the clamping functionality of the mCpx truncation mutant (mCpx 51-134) completely lacking the accessory helix. mCpx 51-134 failed to rescue the cpx mini phenotype [WT mCpx rescue = 14.6 ± 1.1 Hz (n = 18) vs. mCpx 51– 134 rescue = 85.1 ± 2.6 Hz (n = 12); P < 0.001], consistent with a requirement for the Cpx accessory helix in clamping (Fig. 2). Taken together, we conclude that (i) the N-terminal domain of Cpx containing the N-terminal unstructured region and accessory helix domain (Fig. 1A) is critical for clamping, because mCpx 51-134 failed to rescue enhanced spontaneous release in vivo; and (ii) Cpx requires a continuous helix spanning its accessory and central domains, because disruption of this bridge impairs Cpx's ability to function as a clamp.

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In addition to its role as a fusion clamp, Cpx also promotes Ca²⁺-triggered release in both *Drosophila* and mammals (16, 18, 21, 29, 30). cpx null mutants exhibited reduced evoked release, measured as evoked junctional potentials (EJPs) in both low and high Ca²⁺ concentrations (16) (Fig. 3) [control = 34.2 ± 0.6 mV (n = 4) vs. *cpx* null = 20.0 ± 0.8 mV (n = 4) at $[Ca^{2+}] = 1$ mM; P < 0.001]. To investigate whether the formation of a trans Cpx/ SNARE array is required for Cpx's activating function in fusion, we measured EJPs for the mutant mCpx rescues. In contrast to the differential abilities of trans Cpx/SNARE array mutants to rescue the clamping function of Cpx, all of the mutants showed rescue of the impaired evoked release, similar to WT mCpx [SC mCpx rescue = 36.1 ± 0.4 mV (n = 5) and NC mCpx rescue = $32.89 \pm 0.9 \text{ mV} (n = 10)$; vs. WT mCpx rescue = $34.0 \pm 0.5 \text{ mV}$ (n = 5); P > 0.05 for SC mCpx vs. WT mCpx rescues; and P >0.05 for NC mCpx vs. WT mCpx rescues] (Fig. 3). In addition, the HB mCpx and mCpx 51-134 mutants rescued evoked release despite their inability to rescue the *cpx* mini phenotype [HB mCpx rescue = 35.2 ± 1.1 mV (n = 4) and mCpx 51–134 rescue = $35.24 \pm 0.8 \text{ mV}$ (n = 6); vs. WT mCpx rescue = $34.0 \pm 0.5 \text{ mV}$ (n = 5); P > 0.05 for HB mCpx vs. WT mCpx; and P > 0.05 for mCpx 51-134 vs. WT mCpx]. These results indicate the activating function of Cpx can occur in the absence of a trans Cpx/ SNARE array with cross-linked SNAREs in vivo.

In vitro experiments supported a Cpx switch mechanism that promotes release in response to Ca^{2+} (25). Our in vitro cell-cell fusion assays demonstrated that the SB mCpx mutant reduced cell fusion similar to WT mCpx, but exhibited attenuated stimulated fusion compared with WT mCpx (Fig. 1 E and F). To test this model in vivo, we expressed the SB mCpx mutant in cpx null mutants. Expression of SB mCpx was unable to rescue evoked responses in the cpx mutant compared with WT rescues [SB mCpx = 22.3 ± 1.8 mV (n = 5) vs. WT mCpx rescue = 34.0 ± 0.5 mV (n = 5); P < 0.001] (Fig. 3). Using ITC in vitro binding assays, we measured the thermodynamic parameters of WT mCpx and SB mCpx binding to Drosophila cis SNARE complexes (Fig. 4 A and B and Table 1). The cis DmSNARE is a fully assembled complex that was formed with Drosophila Syntaxin (residues 194-265), SNAP25 (residues 18-89 and 149-211), and VAMP (residues 1–115). The $K_{\rm D}$ of binding between mCpx and cis



Fig. 4. SB mCpx exhibits impaired binding to postfusion, cis–DmSNAREs. (*A*) ~65 μ M WT mCpx was titrated into a mixture of ~5.3- μ M DmSNARE complexes. (*B*) ~140 μ M SB mCpx was titrated into ~8- μ M DmSNAREs. (*A* and *B*, *Upper*) Raw data in power vs. time during the injection after subtracting from the baseline. (*Lower*) Integrated heat of each injection normalized by the moles of injectant vs. the molar ratio between Cpx and SNARE in the sample cell. The solid lines represented the best fit to the black squares obtained from a nonlinear least-squares fit assuming a simple one-site chemical reaction, which are listed in Table 1.

Table 1. Thermodynamic parameters of various mCpx binding to postfusion, cis-DmSNARE complexes

| Interaction | Stoichiometric coefficient, N | <i>K_D</i> , nM | ΔH , kcal·mol ⁻¹ | ΔS, cal·mol ^{−1} .°C ^{−1} | ΔG , kcal·mol ⁻¹ | $\Delta G, k_{\rm B}T$ |
|----------------------------|-------------------------------|---------------------------|-------------------------------------|---|-------------------------------------|------------------------|
| WT mCpx/postfusion DmSNARE | 0.97 ± 0.03 | 33 ± 2 | -27.2 ± 0.1 | -57.6 ± 0.5 | -10.1 ± 0.1 | 17.2 ± 0.1 |
| SB mCpx/postfusion DmSNARE | 1.01 ± 0.02 | 1104 ± 95 | -7.7 ± 0.2 | 1.1 ± 0.7 | -8.1 ± 0.1 | 13.7 ± 0.1 |

DmSNAREs is 33 \pm 2 nM, similar to the affinity constant of mCpx with mammalian cis SNAREs (25). Compared with WT mCpx, the SB mCpx mutant displayed a weaker affinity ($\sim 1.1 \pm$ 0.1 µM). Additionally, the binding enthalpy of SB mCpx with cis DmSNAREs was weaker compared with WT mCpx by ~20 kcal·mol⁻¹ (SB mCpx: $\Delta H = -7.7 \pm 0.2$ kcal·mol⁻¹ vs. WT mCpx: $\Delta H = -27.2 \pm 0.1$; Table 1). These differences are similar to those previously reported using the VAMP2 SB mutant (D64A, D65A, and D68A) designed to disrupt hydrogen bond and salt bridge interactions with the Cpx central helix (R48 and Y52). Despite the impaired association of SB mCpx with fully assembled SNARE complexes and the inability to rescue evoked release, SB mCpx was able to rescue minis similarly to WT mCpx [SB mCpx rescues = 16.8 ± 1.3 Hz (n = 11) vs. WT mCpx rescue = 14.6 ± 1.1 Hz (n = 18); P > 0.05] (Fig. 2). These data indicate the central helix is sufficient to support evoked release, whereas the accessory helix is needed to maintain its clamp function. In summary, our findings suggest that Cpx promotes Ca²⁺-dependent vesicle fusion by mechanisms that are independent of accessory domain interactions, and instead require residues within the central helix of Cpx. The independent effects on spontaneous vs. evoked release in both the SB and trans interaction-abolishing mutants indicate the Cpx clamping and promoting functions are genetically separable, consistent with prior studies (17, 19, 20, 31–33).

Discussion

To characterize the mechanisms by which Cpx regulates synaptic vesicle fusion, we performed an in vivo structure–function study by expressing WT and mutant Cpx transgenes in *cpx* null mutants. Unexpectedly, Cpx mutants that disrupt the formation of a trans Cpx/SNARE array, as confirmed by in vitro binding experiments, were capable of promoting action potential-triggered release. However, these same mutants failed to rescue the clamping defect in *cpx*, indicating differential effects on Cpx's two primary roles in exocytosis. In contrast, mutants that altered Cpx interactions with the fully zippering SNARE complex through cis rather than trans interactions, failed to promote evoked vesicle release, but clamped spontaneous fusion normally. These findings demonstrate that the clamping and promoting mechanism of Cpx are genetically separable, and may be regulated by distinct mechanisms at the level of SNARE interactions.

Consistent with previous in vitro experiments (26), mutants that disrupted the ability of Cpx to form a rigid bridge between two SNARE complexes failed to support a clamping function at the synapse in vivo (see HB mCpx rescues in Fig. 2). The Cpx N terminus (1–48) was absolutely required to clamp spontaneous fusion in vivo (mCpx 51-134; Fig. 2), indicating Cpx may form a cross-linked SNARE structure in vivo. Surprisingly however, NC Cpx mutants that disrupted the interaction between Cpx accessory domain and the t-SNAREs of a second SNARE complex were able to largely rescue the elevated spontaneous fusion rate found in *cpx* null mutants (Fig. 2). This observation was not consistent with the cross-linked SNARE model given that NC mCpx exhibited reduced clamping activity in previous cell-cell fusion assays (26), and that ITC experiments demonstrated that NC mCpx does not associate with either mammalian or Drosophila SNAREs in vitro (Fig. S3 and Table S2). The NC mCpx mutant analysis raises the question of whether trans interactions are essential for clamping in vivo. It is possible that association of the Cpx N terminus with a second prefusion

SNARE complex may be more difficult to perturb in vivo than in vitro. For example, additional factors might stabilize this interaction, or secondary residues flanking the primary association site in the t-SNARE complex may contribute to the Cpx/t-SNARE association in vivo. The inability of the HB mCpx and truncated mCpx 51–134 to rescue the elevated mini rate indicates a rigid N-terminal extension away from the central SNARE-binding domain is important for Cpx-mediated vesicle clamping. However, we cannot rule out that these mutants may also disrupt a distinct in vivo Cpx–SNARE interaction beyond the predicted trans Cpx/SNARE array. Indeed, there is evidence that mutations within the N-terminal domain of Cpx may alter release by blocking cis–SNARE zippering as well (34). Unlike NC mCpx mutants, we observed a strong effect of the SC mCpx mutants, which enhanced the clamping function of Cpx in vivo, and enhanced formation of trans interactions in vitro (26).

In contrast to the effects of Cpx cross-linking mutants on their clamping properties, these same mutations were capable of rescuing action potential-triggered synaptic vesicle fusion. Indeed, even an N-terminal truncation mutant completely lacking the Cpx accessory helix was capable of restoring evoked vesicle release in vivo (Fig. 3). Although we cannot exclude the possibility that these mutants exert subtle effects on vesicle pool sizes, or vesicle release kinetics and synchronicity (19, 21, 31, 35), our findings indicate a trans Cpx/SNARE array is not absolutely required for vesicle release following an action potential.

The SB mutant that alters Cpx interactions with the fully zippering SNARE complex in cis, rather than with a second SNARE complex in trans, failed to promote evoked vesicle release efficiently (Fig. 3). Why does this SB mCpx mutant fail to rescue evoked release? The crystal structure of wild-type Cpx with the postfusion SNARE complex shows that R48 and Y52 interact with D64, D65, and D68 in the C terminus of VAMP2 (28). ITC measurements show that the SB mutations in Cpx result in a loss of $\sim -20 \text{ kCal} \cdot \text{mol}^{-1}$ in binding enthalpy to the postfusion SNARE complex (Table 1 and Fig. 4), which is similar to the enthalpy loss caused by the D64A, D65A, and D68A mutations in VAMP2 (25), indicating similar defects to those found in the SB mCpx mutants. FRET studies demonstrated that Cpx remains in the open state (Cpx extends away from postfusion SNARE surface at $\sim 45^{\circ}$) when D64, D65, and D68 are mutated (25). Hence, the R48, Y52 mutation of Cpx is likely to abolish the transition of Cpx from an open to closed state as the molecular driving force of this transition is impaired. Given the N-terminal domain of Cpx (1-50) is not required for evoked release (Fig. 3), it is also possible that a conformational transition of the Cpx central helix from an open to closed state is sufficient for evoked release. Interestingly, the SB mCpx mutant clamps spontaneous release normally (Fig. 2); this is the first Cpx mutant that selectively alters the ability of Cpx to promote evoked vesicle fusion, while leaving the clamping function intact.

In summary, these results indicate that the clamping and activating functions of Cpx are genetically separable, and are regulated through distinct SNARE interaction mechanisms. This conclusion is consistent with additional studies examining the contribution of the Cpx C terminus in regulating synaptic vesicle release (17, 31–33, 36). What mechanism is ultimately used to separate Cpx's role in spontaneous vs. evoked release is still unclear, and may involve a complex interplay with the SNARE complex and other SNARE-associated proteins, including Syt (37, 38). These distinct roles may also be manifest through effects of Cpx on molecularly distinct pools of vesicles (39), or on specific active zones dedicated to evoked vs. spontaneous release (40). Given that Cpx mutants that interrupt the trans Cpx/SNARE array support evoked release, it is unlikely that a Cpx-mediated cross-linking of SNARE complexes is required during Ca²⁺-triggered release. As such, the SB Cpx mutant may impede the transition of the Cpx central helix from an open to closed conformation with a single zippering SNARE complex. Within Cpx, therefore, the central α -helix is the primary domain that associates with SNARE complexes (28), and is absolutely required for all Cpx function (17, 20, 23). The N-terminal accessory domain is necessary for clamping functions, as demonstrated in this study and others (23, 30, 41). The nonhelical N-terminal domain of Cpx is required for promotion of evoked release at some synapses (18, 30), whereas the C terminus functions to regulate both clamping and priming through its ability to target Cpx to membranes (17, 32, 33, 36, 42). How these distinct domains work in concert to regulate the overall mode of synaptic release is an important question, and likely to be critical for distinct plasticity mechanisms that alter release through changes in Cpx function (17, 18, 30, 33). It is also possible that clamping and promotion of vesicle release may be mechanistically coupled through currently unknown pathways.

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Materials and Methods

Protein Constructs, Expression, and Purification. Recombinant proteins were expressed and purified using standard bacterial expression systems. See *SI Materials and Methods* for details.

Isothermal Titration Calorimetry Analysis. ITC experiments were performed as previously described (26). See *SI Materials and Methods* for details.

Cell-Cell Fusion Assays. Cell-cell fusion assays were performed as previously described (14, 24). See *SI Materials and Methods* for details.

Drosophila Genetics. Drosophila melanogaster were cultured on standard medium at 25 °C. Drosophila transgenic lines were generated using standard methods. Briefly, WT and mutant mCpx were transgenes were recombined into cpx^{SH1} null mutant (16) and expressed using neuronal C155 elav-Gal4 driver. See SI Materials and Methods for details.

Electrophysiology. Muscle fiber 6 (segment A3) of dissected third instar larvae was used for all sharp electrode potential recordings as previously described (16). See *SI Materials and Methods* for details.

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Supporting Information

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SI Materials and Methods

Immunostaining and Western Analysis. Immunostaining was performed on third instar larvae at wandering stage after rearing at 25 °C as described previously (1). Anti-myc (Clontech), anti-*Drosophila melanogaster* Complexin (DmCpx; 1:5,000), and anti-HRP (1:10,000, Jackson ImmunoResearch) were used for immunostaining (1). Immunoreactive proteins were visualized on a Zeiss Pascal Confocal with PASCAL software (Carl Zeiss MicroImaging, Inc.) using fluorescent secondary antibodies (Molecular Probes). Western blotting of whole adult head lysates was performed using standard laboratory procedures with anti-myc (1:1,000, 1:500; GeneTex) and anti-DmCpx (1:5,000 (1). The equivalent of one head was loaded per lane. Equal loading was assayed using anti-tubulin clone B-5-1-2 at 1:60,000 (T5168; Sigma-Aldrich). Western blots were visualized on a Li-Cor Odyssey infrared imaging system.

Protein Constructs, Expression, and Purification. SNARE motif of Drosophila Syntaxin and SNAP25. The SNARE domains of Drosophila Syntaxin and SNAP25 were cloned into a pCDFDuet-1 vector. The resulting plasmids were GST-PreScission-DmSyntaxin (containing Drosophila Syntaxin residues 194-265), GST-PreScission-dmSNAP25N (containing Drosophila SNAP25 residues 18-89), and GST-PreScissiondmSNAP25C (containing Drosophila SNAP25 residues 149-211). The details of these plasmids, and protein expression and purification were similar to that was previously described (2, 3). Briefly, these plasmids were expressed in Rosetta2 (DE3; Novagen) Escherichia coli bacterial strain. Cells were pelleted, resuspended, and passed through a cell disruptor. The lysate was centrifuged, and the supernatant was incubated with glutathione agarose (Pierce, Thermo Fisher Scientific Inc). The glutathione beads were collected and washed. The GST tag was cleaved by incubating the protein (attached to glutathione beads) with PreScission protease. Each individual protein was eluted and further purified by gel filtration chromatography (Superdex 75; GE Healthcare) with a buffer containing 25 mM Hepes (pH 7.4), 400 mM KCl, 10% (vol/vol) glycerol, and 1 mM DTT.

N-terminal domain and entire cytosolic domain of Drosophila neuronal VAMP. The N-terminal domain (residues 48-79) and entire cytosolic domain (residues 1-115) of Drosophila neuronal VAMP (n-syb) were cloned into a pET28a vector that contains N-terminal His₆-SUMO tag. The details of these plasmids, and protein expression and purification were similar to that was previously described (3-5). Briefly, these plasmids were expressed in Rosetta2 (DE3) E. coli bacterial strain. Cells were pelleted, resuspended, and passed through a cell disruptor. The lysate was centrifuged, and the supernatant was incubated with Nickel-NTA agarose (Thermo Fisher Scientific Inc). The Nickel-NTA beads were collected and washed. The His₆-SUMO tag was cleaved by incubating the protein (attached to Nickel-NTA beads) with SUMO protease. Each individual protein was eluted and further purified by gel filtration chromatography (Superdex 75; GE Healthcare) with a buffer containing 25 mM Hepes (pH 7.4), 400 mM KCl, 10% glycerol, and 1 mM DTT.

mCpx variants. Full-length WT human Complexin 1 (mCpx) and its mutant forms were cloned into a pET15b vector containing an N-terminal His₆ tag and a Thrombin cleavable site. These mCpx variants contained wild-type mCpx (residues 1–134), switchbreaker (SB) mCpx mutant (residues 1–134, R48D Y52A), nonclamp (NC) $2 \times$ mCpx mutant (residues 1–134, L41E A44E), and NC $4 \times$ mCpx mutant (residues 1–134, A30E A31E L41E A44E). The

C-terminal domain mCpx was created by cloned residues 48-134 of mCpx into a pET28a vector that contains N-terminal His₆-SUMO tag. The details of these plasmids, and protein expression and purification were similar to that described above and previously (3, 5). The N-terminal His₆ or His₆-SUMO tag was cleaved by incubating the protein (attached to Nickel-NTA beads) with thrombin (from bovine plasma; Sigma-Aldrich) or SUMO protease, respectively.

Isothermal Titration Calorimetry Analysis. To study the interactions between partially zippered SNARE and NC mCpx mutants, DmSyntaxin (residues 194-265), DmSNAP25N (residues 18-89), DmSNAP25C (residues 149-211) and the N-terminal SNARE motif from Drosophila VAMP (residues 48-79) were mixed together at a 1:1.2:1.2:1.2 molar ratio and incubated at 4 °C overnight to form the pDmSNARE79 partial complex. Before isothermal titration calorimetry (ITC) experiments, pDmSNARE79 and mCpx variants (mCpx 48–134, WT mCpx, NC 2× mCpx, and NC 4× Cpx) were purified by gel filtration using a regular or HiLoad Superdex 75 column (GE Healthcare Life Sciences) and PBS (pH 7.4; 137 mM NaCl, 3 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic) with 0.25 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Thermo Scientific Pierce) as the running buffer, respectively. Peak fractions were pooled and concentrated. To ensure that all of the central helix binding sites of the partial SNARE complex were occupied, excess mCpx 48-134 was added into pDmSNARE79 with a molar ratio of at least 2:1, and incubated overnight at 4 °C or 1 h at room temperature to form blocked pDmSNARE79. mCpx variants and blocked pDmSNARE79 were then dialyzed in the same flask against 4 L of PBS buffer with 0.25 mM TCEP for 4 h at 4 °C and then dialyzed against another 4 L of fresh PBS buffer with 0.25 mM TCEP overnight at 4 °C. The concentrations of dialyzed proteins were determined using the Thermo Scientific Pierce bicinchoninic acid protein assay kit with BSA as the standard and/or Bradford assay.

To study the interactions between postfusion, full-zippered DmSNARE complex and SB mCpx mutant (R48D, Y52A), DmSyntaxin (residues 194–265), DmSNAP25N (residues 18–89), DmSNAP25C (residues 149–211), and the complete cytosolic domain from *Drosophila* VAMP (residues 1–115) were mixed together at a 1:1.2:1.2:1.2 molar ratio and incubated at 4 °C overnight to form the full-zippered DmSNARE complex. Before ITC experiments, full DmSNARE complex and mCpx variants (WT mCpx and SB mCpx) were purified by gel filtration using a regular or HiLoad Superdex 75 column and PBS buffer, respectively. mCpx variants and full DmSNARE complex were then dialyzed in the same flask against 4 L of PBS buffer with 0.25 mM TCEP for 4 h at 4 °C and then dialyzed against another 4 L of fresh PBS buffer with 0.25 mM TCEP overnight at 4 °C.

ITC experiments were performed with a MicroCal ITC200 instrument similarly to that described previously (3, 6). Typically, ~200 μ L of SNARE solution was loaded into the sample cell, and ~60 μ L of mCpx solution was loaded into the syringe. An initial 0.1- μ L injection was followed by several injections of constant volume. A 180-s equilibration time was used after each injection to ensure complete binding. The heat change from each injection was integrated and then normalized by the moles of mCpx in the injection. All ITC experiments were carried out at 23 °C to match the temperature (*T*) at which all of the physiological experiments with *Drosophila* were performed. MicroCal Origin ITC200 software package was used to analyze the titration calorimetric data and obtain the stoichiometric

number (N), the molar binding enthalpy (ΔH), and the association constant (K_a) . A nonlinear least-squares fit assuming a simple one site chemical reaction was used. The equilibrium dissociation constant ($K_{\rm D}$), the binding free energy (ΔG), and the binding entropy (ΔS) were calculated using the thermodynamic equations

$$K_{\rm D} = 1/K_{\rm a}$$
$$\Delta G = \Delta H - T\Delta S = -RT \ln(K_{\rm a})$$

Cell-Cell Fusion Assays. Cell-cell fusion assays were performed as previously described (7-9). Stable cell lines expressing flipped WT VAMP2 and DsRed2-nuclear export signal (v-cells) were transfected with GPI-anchored Cpx [WT mCpx, DmCpx, or SB mCpx (R48D, Y52A)], YFP-nuclear localization signal (NLS), and flipped GPI-anchored Synaptotagmin I where indicated. Afterward, v-cells were seeded on top of stable cell lines expressing flipped Syntaxin 1, flipped SNAP 25, and CFP-NLS (tcells). Cells were incubated overnight and then treated with 0.5 U/mL phosphatidylinositol-specific phospholipase C (MP Biomedicals) for 30 min at 37 °C to cleave the GPI anchor. Cells were fixed with 4% (vol/vol) paraformaldehyde and mounted using Prolong Gold Antifade (Molecular Probes). Images were acquired using a Zeiss 510 confocal microscope and analyzed with Zeiss LSM imaging software. The n indicates independent experiments analyzed separately, where ~ 30 fields were imaged for each. Statistical significance was determined using one-way ANOVA with post hoc Tukey analysis (n.s., P > 0.05, *P < 0.05, **P < 0.01, **P < 0.001).

Molecular Cloning for Drosophila Genetics. WT and mutant mCpx genes subcloned into a modified pValum construct (N-terminal myc tag), which uses of the Gal4/UAS expression system (10). The constructs were injected into yv; attP third chromosome docking strains by Genetic Services Inc. Homozygous third chromosome UAS lines were then recombined into the cpx^{SH1} null mutant (1). The C155 elav-GAL4 driver was used for neuronal expression of transgenes.

Superclamp (SC) mCpx and mCpx (L41E, A44E) mutant genes were synthesized using custom gene synthesis services (IDT). For the remaining mutants, QuikChange (Stratagene) was used for site-directed mutagenesis on the existing cloned mouse mCpx I (11). The following primers (all $5' \rightarrow 3'$) were used to generate point mutations.

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- 3. Kümmel D, et al. (2011) Complexin cross-links prefusion SNAREs into a zigzag array. Nat Struct Mol Biol 18(8):927-933.
- 4. Li F, et al. (2007) Energetics and dynamics of SNAREpin folding across lipid bilayers. Nat Struct Mol Biol 14(10):890-896.
- 5. Li F, et al. (2011) Complexin activates and clamps SNAREpins by a common mechanism involving an intermediate energetic state. Nat Struct Mol Biol 18(8):941-946.
- 6. Krishnakumar SS, et al. (2011) A conformational switch in complexin is required for synaptotagmin to trigger synaptic fusion. Nat Struct Mol Biol 18(8):934-940.

Helixbreaker (HB) mCpx:

Forward: ggaggagcgcaaagcaggcggcggcaagtacgccaagatgg

Reverse: ccatcttggcgtacttgccgccgcctgctttgcgctcctcc

mCpx 51-134:

Forward: atgaagtacgccaagatggaggc

Reverse: tctagaagcttacttcttgaacatgtcct

NC mCpx [using mCpx (L41E, A44E) as template]:

Reverse: ccgctcttcctccttcttctcctcatcggggtccttttcctc

SB mCpx (R48E, Y52A) was made sequentially using the following primers in the order presented: mCpx Y52A

Forward: gaagaggatctcaaggccgccaagatggagg

Reverse: cctccatcttggcggccttgagatcctcttc

mCpx R48E

Forward: cagaggaggaggaggagaaagcaaaggc

Reverse: gcctttgctttctcctcctctg

WT and mutant mCpx cDNA were subcloned into a modified pValum construct with incorporated N-terminal myc tag. The constructs were injected into yv; attP third chromosome docking strains by Genetic Services Inc.

Electrophysiology. Recordings of dissected wandering third instar larva were performed in HL3.1 saline containing (in mM): 70 NaCl, 5 KCl, 4 MgCl2, 10 NaHCO3, 5 Trehalose, 115 sucrose, 5 Hepes-NaOH, 0.2 Ca²⁺Cl, pH 7.2. Evoked junctional potentials were recorded intracellularly from muscle fiber 6 of segment A3 using an AxoClamp 2B amplifier (Axon Instruments). Data acquisition and analysis was performed using Clampfit 9.0 software (Axon Instruments) and Mini Analysis (Synaptosoft Inc.) as previously described (1). Each indicated n value represents a single muscle recording, with data generated from at least three individual larvae of each genotype. All error bars are SEM. Statistical significance was determined using one-way ANOVA with post hoc Tukey analysis (n.s., P > 0.05, *P < 0.05, **P <0.01, ***P < 0.001.

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Fig. S1. Transgenically expressed mutant mCpxs are expressed and localized similarly to WT mCpx at *Drosophila* synapses. (A) Overall protein expression of transgenically expressed myc-tagged WT or mutant mCpxs driven by the neuronal C155^{elav-Gal4} driver in the indicated WT or *cpx*^{SH7} backgrounds was analyzed by Western blot analysis using adult transgenic head lysates. Blots were probed using anti-myc, anti-DmCpx, and anti-tubulin. Expression of individual mutant mCpxs was similar to WT mCpx expression with the exception of reduced expression of mCpx 51–134. Similar results were obtained in at least two independent experiments. (*B*) Third instar wandering larvae of the indicated genotypes were dissected, fixed, and costained with anti-HRP and anti-myc antisera. Representative images demonstrate that transgenically expressed myc-tagged WT and mutant mCpxs, with the exception of mCpx 1-50, are localized and enriched similarly at *Drosophila* muscle 6/7 neuromuscular junctions. (Scale bar: 20 μm.)



Fig. 52. Interaction of mCpx central helix with prefusion, partially assembled pDmSNARE79. ~330 μ M mCpx 48–134 was titrated into ~27 μ M pDmSNARE79. (*Upper*) Raw data in power vs. time during the injection after subtracting the baseline. (*Lower*) Integrated heat of each injection normalized by the moles of injectant vs. the molar ratio between mCpx and SNARE in the sample cell. The solid lines represented the best fit to the black squares obtained from a nonlinear least-squares fit assuming a simple one-site chemical reaction. The result gave the thermodynamic parameters for the binding reaction: affinity constant $K_D = 667 \pm 90$ nM, $\Delta H = -13.3 \pm 0.2$ kcal·mol⁻¹, and $\Delta S = -16.6 \pm 0.8$ kcal·mol⁻¹.°C⁻¹.



Fig. S3. Interaction of mCpx accessory helix with prefusion, partially assembled pDmSNARE79. (*A*) ~310 μ M WT mCpx was titrated into ~15.5 μ M blocked pDmSNARE79, (*B*) ~360 μ M double-mutant mCpx L41E A44E was titrated into ~25 μ M blocked pDmSNARE79, and (*C*) ~603 μ M quadruple-mutant mCpx A30E A31E L41E A44E was titrated into ~30 μ M blocked pDmSNARE79. (*Upper*) In each reaction, raw data in power vs. time during the injection after subtracting the baseline. (*Lower*) Integrated heat of each injection normalized by the moles of injectant vs. the molar ratio between mCpx and SNARE in the sample cell. The solid lines represented the best fit to the black squares obtained from a nonlinear least-squares fit assuming a simple one-site chemical reaction. The results gave the thermodynamic parameters for each binding reaction, which are listed in Table S1.

Table S1. Summary of mCpx mutants expressed in cpx null mutants

| mCpx mutant (N-terminal myc tag) | Mutations | | | |
|----------------------------------|---|--|--|--|
| Superclamp (SC) | D27L, E34F, R37A | | | |
| Nonclamp (NC) | A30E, A31E, L41E, A44E | | | |
| Helixbreaker (HB) | GGG insertion between residues A50 and K51 | | | |
| mCpx 51–134 | Deletion of N terminus 1–50 up to the central helix | | | |
| Switchbreaker (SB) | R48D/E, Y52A | | | |

Table S2. Thermodynamic parameters of various mCpx accessory helix (WT mCpx, mCpx L41E A44E, and mCpx A30E A31E L41E A44E) binding to blocked pDmSNARE79

| Interaction | Stoichiometric coefficient, <i>N</i> | <i>Κ</i> _D , μΜ | ∆ <i>H</i> , kcal·mol ⁻¹ | ∆ S , cal·mol ⁻¹ .°C ⁻¹ | ∆G, kcal·mol ⁻¹ | $\Delta G, k_{\rm B}T$ |
|---|--------------------------------------|----------------------------|-------------------------------------|--|--------------------------------|--------------------------------|
| WT mCpx/prefusion dmSNARE79 | 1.00 ± 0.05 | 11 ± 1 | -5.7 ± 0.4 | 3.6 ± 1.5 | -6.7 ± 0.1 | 11.5 ± 0.1 |
| Double mutant/prefusion dmSNARE79 Quadruple mutant/prefusion dmSNARE79 | 1.04 ± 0.15 Not detected | 45 ± 8 Not detected | -3.9 ± 0.7 Not detected | 6.9 ± 2.8 Not detected | -5.9 ± 0.1 Not detected | 10.0 ± 0.1 Not detected |

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