Supporting Online Material for

Alternative Zippering as an On-Off Switch for SNARE-Mediated Fusion

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Material and Methods

Constructs, Stable Cell Lines and Peptides

Site-specific mutations were introduced on CPX-I-GPI using the Quick Change Site Directed mutagenesis kit-II (Stratagene). HeLa stable cell lines co-expressing flipped SNAREs with the fluorescent protein markers were generated using the Tet-Off gene expression system (*S1*) (Clontech) as described (*S2, S3*). Two different types of SNARE expressing HeLa cells were mainly used in this paper, cells lines stably expressing flipped-VAMP2 and DsRed2-nes (v-cells) or cells lines stably expressing

flipped Syntaxin 1, flipped SNAP-25 and CFP-nls (t-cells). Peptides coding for VAMP2 N-terminal half (VN, residue 25-57) or the C-terminal half (VC, residues 57-93) were purchased 95% purity from Biosynthesis.

Cell-cell fusion assay

$5.5x10^5$ HeLa v-cells grown for at least 3 days in complete medium in the absence of Doxycycline (to initiate flipped VAMP2 and dsRed expression) were seeded in a 6-Well-Plate. The next day the cells were used for transient transfection to express Complexin, Synaptotagmin and YFP-nls marker (4 µg CPX-I-GPI plasmid, 4 µg Flipped-Syt-I plasmid, 4 µg YFP-nls plasmid and 15 µl Lipofectamine 2000 (Invitrogen)), Complexin and the YFP-nls marker (CPX-I-GPI plasmid, 4 µg YFP-nls plasmid and 10 µl Lipofectamine 2000 (Invitrogen) or YFP-nls marker alone for control experiments (4 µg YFP-nls plasmid and 8 µl Lipofectamine 2000). The same day of the transfection, $4.5x10^4$ HeLa t-cells previously grown for at least five days in complete medium in the absence of Doxycycline (to initiate expression of the flipped-t-SNARE subunits and CFP-nls) were seeded on sterile 12 mm glass coverslips contained in 24-well plates. The following day, transfected HeLa-v cells were detached from the dishes with Sodium Citrate cell detaching solution (11 g/l KCl, 4.4 g/l Na Citrate). The detached cells were centrifuged at 200 g, and resuspended in HEPES-buffered DMEM supplemented with 10% FCS and counted in a hemacytometer.

The assay was initiated by adding $5x10^4$ v-cells to each coverslip already containing t-cells. After overnight incubation at 37°C in 5% CO_2, the coverslips were gently washed

once with HEPES-buffered DMEM supplemented with 1% BSA and 1mM DTT (recovery solution).

The recovery from the CPX-I-GPI block was performed by adding pre-warmed recovery solution containing 1 U/ml Phosphatidylinositol Specific Phospholipase-C (PI-PLC, Molecular Probes), and 20μg/ml laminin, in the presence or absence of 1.8 mM EGTA (as indicated). The addition of PI-PLC is defined as t=0. Cells were incubated for the indicated period of time at 37°C in the water bath, then the medium was aspirated, the cells were fixed with 4% paraformaldehyde for 10 min. Cells were washed three times with PBS supplemented with 0.1 g/l $CaCl_2$ and 0.1 g/l $MgCl_2$ (PBS++) and mounted with Prolong Antifade Gold mounting medium (Molecular Probes). Confocal images were collected as indicated in the Image Acquisition section.

Protein expression and purification

Recombinant soluble CPX-I wt, CPX-$I^{D27L/E34F}$ and CPX-$I^{E34F/R37A}$ were purified following the protocol described in (*S2*). The flow-through after Benzamidine treatment was incubated with 50 μl of (100%) Benzamidine Sepharose for 20 min at room temperature to bind Thrombin. Benzamidine Sepharose Sepharose beads were collected in a spin column and the flow thru was saved. The Flow-through was dialyzed at 4°C against 1 L HEPES-buffered DMEN Media with two changes of media. The dialyzed pool was then concentrated using a Centricon 20 (Amicon, Millipore) at 4,000 RPM for 10 min at 4°C. The concentration of the soluble Complexin-I was then determined using the protein determination assay (BioRad) and the purity of the protein was analyzed by SDS-PAGE gel.

Image Acquisition

Images were acquired on a Leica TCS SP2 AOBS confocal microscope, equipped with Leica LCS software and usually using a HCX PL APO 40X, 1.25 NA oil immersion objective. For higher magnification images a HCX PL APO 63X, 1.4 NA oil immersion objective was used. The images were processed with Adobe Photoshop software.

Data analysis

At each time point the percentage of transfected cells (red cytoplasm expressing the YFP-nls construct in the nucleus) containing blue nuclei (appearing either as a separate blue nucleus, or as "white" nuclei expressing both YFP and CFP) was determined. Each data point in the manuscript represents two independent coverslips from which 40-60 separate fields were randomly chosen and imaged. The total number of cells counted per coverslip was 2000-3000. Each experiment is representative of at least three independent experiments.

In previous studies using this assay, we recorded fusion as "contacting v- and t-cells that fused" (*S4, S5*). This metric limits the data to cells which have the opportunity to fuse with a cognate-SNARE expressing cell. Unfortunately, this requires an additional color marker to define the plasma membrane of the t-cells (or to fill the cytosol of the t-SNARE such that the shape of the cell is obvious). In the experiments presented in this manuscript, no additional color was available in the t-cell, and as such the total efficiency should be considered an underestimate of the actual cell-cell fusion

efficiency. In experiments following just the stable cells and utilizing a content marker to discern t-cell shape, the actual efficiency of contacting cells fusing was comparable to previous measures.

Binding Experiments

t-cells were transiently co-transfected with flipped-Vamp2 and the indicated CPX-I-GPI mutant construct or mock transfected were incubated with or without 500 mU/ml PI-PLC for 30 minutes, washed, fixed, and stained with anti-AU1 antibody. Total fluorescent intensity from individual cells in images was determined.

Modeling of the Proposed Complexin Clamping Mechanism

The precise mechanism by which synaptic vesicle fusion is arrested prior to an action potential and subsequent calcium pulse has been a mystery for half a century. Recent studies have highlighted an essential role for both synaptotagmin and complexin in both clamping to produce the arrested state and activation from that state to release transmitter in response to calcium. (*S2, S3, S6-S11*). More particularly, recent studies both in vivo and in vitro pinpoint the accessory helix of complexin as criticial for clamping, with the adjacent central helix responsible for binding the four-helix bundle (*S3, S8*). In this way, the central helix strategically positions CPX on the assembling SNARE complex, critically positioning the accessory helix to somehow interfere with the completion of the fusion process. How does that occur?

We have proposed and tested the critical predictions of a simple model, initially inspired by analysis of the sequence of the accessory helix, in which this helix forms an

alternative four-helix bundle with the membrane-proximal regions of the three helices of the synaptic t-SNARE, one from syntaxin and the other two from SNAP-25. In this alternative arrangement (Fig. 4), fusion is clamped because zippering cannot be completed. High-affinity binding of CPX, required to position the accessory helix, relies on sequences in both the v- and t-SNARE motifs present in the central and membrane-distal portions of the SNARE bundle (*S12, S13*). This ensures that CPX can only begin to interfere with SNARE assembly *after* the SNAREpin has zippered at least half-way. The accessory helix then binds weakly and therefore reversibly to sequences in the membrane-proximal portion of the t-SNARE. Given the homologies between VAMP2 and the inverted CPX-I sequence, we imagine that binding of the accessory helix to the t-SNARE likely involves all three t-SNARE chains and results in a cis-SNARE-like 4-helix bundle, and thus occupancy of the natural VAMP2 binding site (Fig. 4).

By necessity, the membrane proximal portion of VAMP2 is displaced, as speculatively shown in Fig. 4, which assumes that it remains a helix for simplicity. The main purpose of this modeling exercise was to establish that the mechanism we propose is feasible structurally. Note that the displaced sequences of VAMP2 include both the cleavage site and the protein recognition sequence for cleavage of VAMP2 by Botulinum-B toxin, which can still act on VAMP2 in the clamped state (*S2, S14*), implying that this region is unzippered in the clamped configuration as shown in Fig 4. Note also that the recognition sequence for Tetanus Toxin is assembled into the four-helix bundle in the model, explaining why the complexin-clamped intermediate was found to be resistant to this toxin (*S2*). The central helix remain in place and all

contacts with the four-helix bundle are maintained except for one residue at the junction with the accessory helix (residue R48); however mutating this residue to Ala only slightly reduces CPX-I binding to the cis-SNARE complex (*S3*). The central helix lies on the outside of the four-helix bundle, but the accessory helix is proposed to lie in the bundle in place of Vc, necessitating that the accessory helix turn inward and then re-align. These two turns at the beginning of the accessory helix are highly constrained in the model by the docking of adjoining segments of accessory and central helices based on crystal structures. Finally, note that residues 26-47 of the accessory helix are proposed to be in a helical structure in the clamped state (as they substitute for the corresponding helical region of VAMP2 in the cis-SNARE complex structure), but much of this segment is in fact disordered in crystal structures of CPX-I cis-SNARE complexes (residues 26-31 in the mammalian complex (*S13*) and residues in the squid complex (*S12*) corresponding to human 26-44).

Legends to the Figures

FIGURE S1. **Calcium/SYT-I sensitivity of CPX-I superclamp mutants.** Calcium titration experiment using v-SNARE cells co-transfected with YFP-nls, SYT-I and either CPX-I^{wt}-GPI or CPX-I-$^{D27I/E34F}$-GPI. Transfected v-cells and t-cells were incubated overnight before addition of 1 U/ml PI-PLC and 1.8 mM EGTA (t=0, where indicated) to release the complexin GPI anchor and reduce free calcium in the media to 10 nM. At t=5 min, free calcium was

raised to the indicated concentration (ranging from 0.5 μM to 1000 μM) and was keep constant for 15 min at 37° C. Cells were then fixed, data quantified as in Fig 1 and plotted as percentage of the corresponding total fusion recovery.

FIGURE S2. **Effect of site-specific mutant construct of CPX-I on the clamping effect**. Domain Structure of CPX-I-GPI deletion constructs compared with the solved crystal structure of CPX-I bound to SNARE pin (*S13*) that comprise from aa 32-72 of CPX1. B)- Sequence alignment of different CPXs, showing the identical residues in yellow, the conserved residues in light blue and the similar residues in green. The intended mutations that will be analyzed are also shown on the alignment. The SNAREpin binding region is displayed in red box. C)- The "flipped-SNARE" cell fusion assay (*S2, S3*) consist of HeLa v- cells stably expressing flipped VAMP2 and DsRed protein fused to nuclear export signals were transfected with YFP-nls (co-transfection marker) and with or without a CPX-I-GPI mutant construct. After 24 hours of expression, cells were seeded on top of HeLa t-cells attached to coverslips expressing flipped Syntaxin-1, flipped SNAP-25 and the cyan fluorescent protein fused to a nuclear localization signals. Cells were incubated overnight and the fusion efficiency was quantified as a percentage of transfected v-cells that fused. Effect of different CPX-I-GPI-mutant constructs on cell fusion (blue bars), and

efficiency of cell fusion recovery after addition of PI-PLC in the presence of SYT-I and Calcium (red bars).

FIGURE S3. **Binding of CPX-I-GPI constructs to cis-SNARE complexes.** HeLa t-cells were co-transfected with the desired CPX-I deletion/mutant construct and with a plasmid encoding for flipped-VAMP2 in order to form cis-SNARE complexes on the cell surface. Afterward, CPX-I-GPI was cleaved off from its GPI-anchor motif using PI-PLC and the remaining CPX-I bound on the cell surface was determined by IF using an anti-AU1 antibody (*S2*).

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Figure S1 -Giraudo et al. 200

A

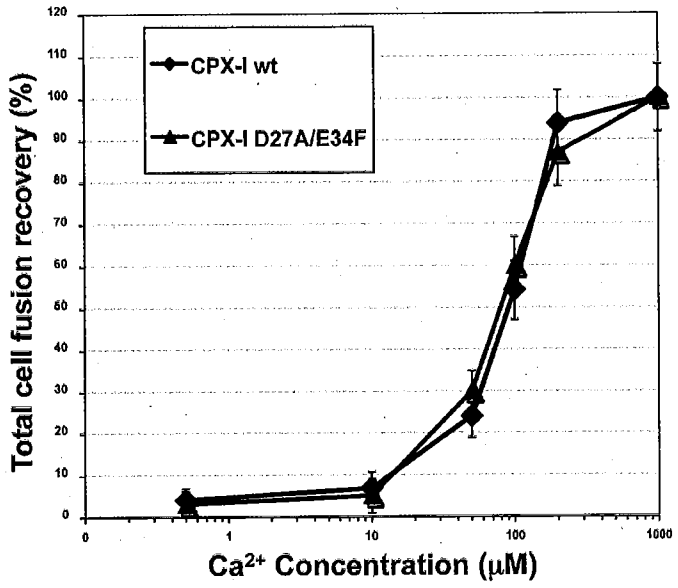
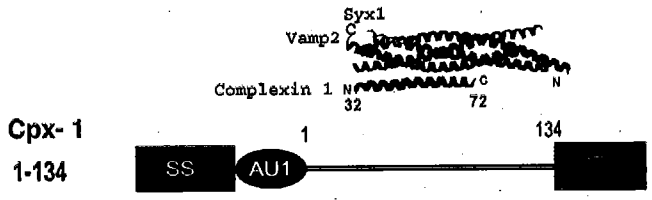


Figure S2 - Giraudo et. al 2008

A



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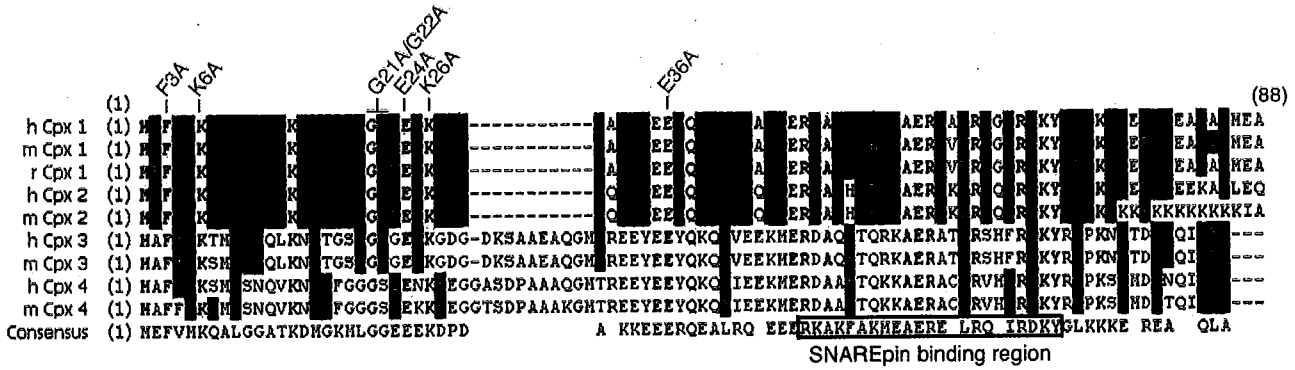

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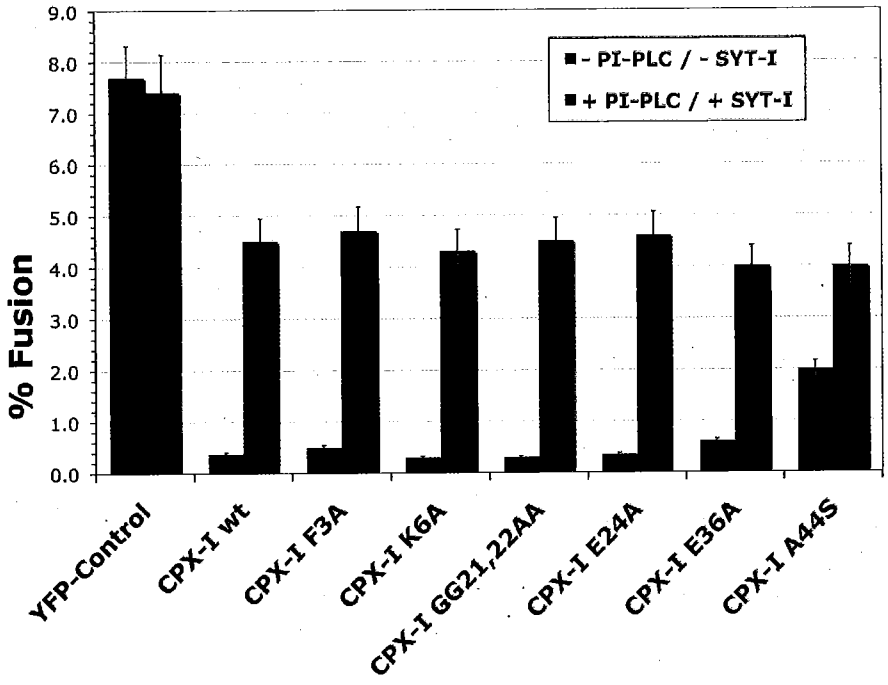

Figure S3 -Giraudo et al. 2008

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