Secretory vesicle priming by Munc18-1

SNAREpin Assembly by Munc18-1 Requires Previous Vesicle Docking by Synaptotagmin1*

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*Running title: Secretory vesicle priming by Munc18-1

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Key words: synaptic vesicle; exocytosis; fusion; reconstitution; SNARE

Background: The cascade of reactions and proteins conferring regulated exocytosis needs to be characterized.

Results: Synaptotagmin1 is a primary vesicle docking factor and Munc18-1 accelerates subsequent v-/t-SNARE assembly/zippering.

Conclusion: Synaptotagmin1, PI(4,5)P2, complexin II, and Munc18-1 function in a sequential and concerted manner to mediate vesicle docking, SNAREpin assembly, and fast Ca2+-triggered exocytosis.

Significance: Efficient Ca2+-regulated membrane fusion was reconstituted from a minimal set of components.

SUMMARY

Regulated exocytosis requires the general membrane fusion machinery – SNARE proteins – and the SM protein Munc18. Using reconstituted giant unilamellar vesicles (GUVs) containing preassembled t-SNARE proteins (syntaxin1/SNAP-25), we determined how Munc18-1 controls the docking, priming, and fusion of small unilamellar vesicles (SUVs) containing the v-SNARE VAMP2 and the Ca2+ sensor synaptotagmin1. In vitro assays allowed us to position Munc18-1 in the center of a sequential reaction cascade: Vesicle docking by synaptotagmin1 is a prerequisite for Munc18-1 to accelerate trans SNARE complex (SNAREpin) assembly and membrane fusion. Complexin II stalls SNAREpin zippering at a late stage and hence contributes to synchronize membrane fusion in a Ca2+- and synaptotagmin1-dependent manner. Thus, at the neuronal synapse, the priming factor Munc18-1 may accelerate the conversion of docked synaptic vesicles into a readily-releasable pool by activating SNAREs for efficient membrane fusion.

In the central nervous system, synaptic vesicles are morphologically docked at the active zone and this docked pool of vesicles largely coincides with the readily releasable pool, which can fuse within less than a millisecond after sensing a local increase of the Ca2+ concentration (1,2). Although numerous components and a series of reactions are involved in the initial vesicle tethering and priming steps, the number of proteins and lipids required for the final docking step and the subsequent fusion reaction are limited by the refined reaction mechanism and spatial constraints (3-5). Evidence has accumulated that a minimal set of six proteins might be functionally required at these last reaction steps. VAMP2, a v-SNARE on the synaptic vesicles forms a trans-SNARE complex - SNAREpin - with its cognate t-SNARE consisting of syntaxin1 and SNAP-25 on the pre-synaptic plasma membrane, thereby bridging the two lipid bilayers (6). SNAREpin formation starts at the N-terminal membrane-distal end and progresses towards the C-terminal membrane-proximal end of the heptad-repeat containing SNARE motifs, finally resulting in the formation of a four-helix bundle, which extends through the membrane and, thus also includes the membrane spanning regions of VAMP2 and...
syntaxin1 (7-10). Indeed, SNAREpin assembly is sufficient to drive membrane fusion in reconstituted assays (11-13). However, three late acting regulatory proteins – Munc18-1, synaptotagmin1 (Syt1), and complexin (Cpx) – control the formation of SNAREpins and the subsequent SNAREpin zipping reaction (3-5,14-17).

Synaptotagmins and complexins confer Ca\(^{2+}\) regulation to the general SNARE fusion machinery. Cpx plays a dual role in membrane fusion and arrests SNAREpins at a distinct assembly state (18). It binds via its central \(\alpha\)-helix to the N-terminal membrane distal region of the VAMP2 and syntaxin1 SNARE motif in the SNAREpin, thereby likely stabilizing the initial v-/t-SNARE interactions (19-21). Simultaneously, its N-terminal accessory helix binds in a competitive mode with VAMP2, the C-terminal membrane-proximal part of a t-SNARE located in a second SNAREpin (21,22). Thus, Cpx stabilizes partially assembled SNAREpins and blocks further SNAREpin zipping.

To release this block and to couple the reaction to a Ca\(^{2+}\) signal, Syt1, which is anchored via its N-terminal trans-membrane domain in the vesicular membrane, binds with its two C2 domains anionic phospholipids in a Ca\(^{2+}\)-dependent manner. These Ca\(^{2+}\)-dependent lipid interactions locally perturb the membrane structure and together with Ca\(^{2+}\)-dependent SNARE interactions likely release the Cpx clamp resulting in membrane fusion (23,24). In addition to the classical Ca\(^{2+}\)-binding loops in the C2 domains, the C2B domain also contains a polybasic amino acid cluster, which confers Ca\(^{2+}\)-independent interactions with the t-SNARE and PI(4,5)P\(_2\), which itself is known to be enriched at active zones and functions at several steps of the synaptic vesicle cycle (25). These Ca\(^{2+}\)-independent interactions have been implicated in vesicle docking in vivo and in vitro (26-29).

Munc18-1, a SM protein, is essential for regulated exocytosis and has several functions in SNAREpin assembly (30-35). It stabilizes syntaxin1, contributes to the transport of syntaxin1 from the ER to the plasma membrane, and keeps syntaxin1 in a closed conformation, which blocks SNAP-25 binding and t-SNARE assembly (36-45). The release of this inhibition requires regulatory proteins such as Munc13 and the specific binding of Munc18-1 to VAMP2 or VAMP3 (46-50). The resulting syntaxin1/SNAP-25 complex then provides a binding site for Syt1, conferring vesicle docking (27-29,51). Thus, the dramatic decrease of vesicle docking observed in Munc18-1-deficient cells might be the result of impaired t-SNARE complex assembly (27,32,52-54). Indeed, the overexpression of SNAP-25 can rescue the docking phenotype in Munc18 knockout cells (27). However, the cells overexpressing SNAP-25 are still exocytosis-deficient indicating a post-docking role of Munc18-1 (27,55). Interestingly, reconstituted liposome assays demonstrated that Munc18-1 stimulates specific vesicle docking and membrane fusion when preassembled t-SNAREs had been reconstituted into liposomes (50,54,56). However, in order to observe the fusion stimulation in these reconstituted assays, Munc18-1 needed to be simultaneously pre-incubated with v- and t-SNARE liposomes at low temperature for an extended time period, indicating that another factor is required to prime the SNAREs for this Munc18-1 function. Thus, the mechanism of how Munc18-1 functions at this step still remains elusive.

In summary, each of the three regulatory proteins (Munc18-1, Syt1, Cpx) has been reported to mediate vesicle docking and to contribute indirectly or directly to SNAREpin formation, stability, and zipping. To assign Munc18-1 a role in this intricate protein network and to determine the sequence of events and synergistic functions, we have employed a reconstituted assay using purified components, small unilamellar vesicles (SUVs), and giant unilamellar vesicles (GUVs) mimicking synaptic vesicles and the flat presynaptic plasma membrane, respectively. First, we directly compared the vesicle docking efficiency provided by each individual regulatory component and combinations thereof establishing a binding hierarchy. Second, we determined which binding partners (PI(4,5)P\(_2\), t-SNARE, v-SNARE) have to interact with the regulatory components to confer vesicle docking. Third, the regulatory components were analyzed individually and in combination in a reconstituted lipid-mixing assay. Based on these analyses a defined sequence of events emerges. Briefly, Syt1 appears to be the predominant vesicle-docking factor. Its vesicle docking activity generates a reaction intermediate for Munc18-1 activity, which shifts SNAREpins and docked vesicles into a highly reactive state.
CpxII together with Syt1 suppresses the Munc18-1 stimulation and renders the reaction Ca\(^{2+}\)-sensitive. Thus, in the simplest model Munc18-1, Syt1, CpxII, and the neuronal SNAREs would be the basic machinery generating a readily-releasable pool of docked vesicles, which immediately responds to a Ca\(^{2+}\) signal by membrane fusion.

**EXPERIMENTAL PROCEDURES**

**Protein purification** - Recombinant mammalian His\(_{6}\)-tagged proteins were expressed in *E. coli* BL21(DE3) bacteria (Stratagene). Protein purifications for VAMP2, Syt1, syntaxin1/SNAP-25 and CpxII were performed exactly as described previously via Ni-NTA affinity chromatography and subsequent ion exchange chromatography (13,57).

**Munc18-1 purification** - Recombinant rat Munc18-1 encoded in the pEG(KG) vector (a kind gift of Dr. Richard Scheller) was transformed into BL 21(DE3) bacteria (Stratagene). Bacterial cultures in 4 l LB medium containing 100 µg/ml ampicillin were grown at 37 °C to an optical density of 0.6 (at 600 nm). Protein expression was induced over night at 16 °C by 1 mM isopropyl-b-D-thiogalacto-pyranoside (IPTG). The following day, bacteria were collected by centrifugation and washed once with breaking buffer (25 mM HEPES-KOH pH 7.4, 150 mM KCl, 10% glycerol). After another round of centrifugation, bacteria were resuspended in a final volume of 100 ml breaking buffer and snap-frozen for storage at -80 °C. After thawing, the bacterial suspension, β-mercapto-ethanol was added to a final concentration of 3 mM, as well as a protease inhibitor cocktail (final concentrations: leupeptin (1.5 µg/ml), antipain (2.5 µg/ml), turkey trypsin inhibitor (25 µg/ml), benzamidine (12.5 µg/ml), Pefabloc SC (6.25 µg/ml), aprotinin (1.25 µg/ml), chymostatin (5 µg/ml), and pepstatin (2.5 µg/ml)). For lysis, bacteria were passed through a Microfluidizer M110L (Microfluidics) at >10,000 psi. A final concentration of 50 U/ml Benzonase (Merck), as well as 1 mM MgCl\(_2\) were added to the bacterial lysate followed by a 10 min incubation step. Subsequently, insoluble material was removed by ultracentrifugation for 1 h at 40,000 rpm at 4 °C in a 45Ti rotor (Beckman Coulter). 100 ml of the supernatant containing GST-tagged Munc18-1 were incubated with 2 ml glutathione-beads (GE Healthcare) for 1 h at 4 °C. Beads were washed 3x with 13 ml breaking buffer followed by washing 3x with 13 ml washing buffer (25 mM HEPES-KOH pH 7.4, 500 mM KCl, 5% glycerol, 3 mM β-mercapto-ethanol). Beads were resuspended in 2 ml cleavage buffer (25 mM HEPES-KOH pH 7.4, 150 mM KCl, 5% glycerol, 3 mM β-mercapto ethanol) and Munc18-1 was cleaved off the GST tag by addition of thrombin at a final concentration of 2.5 U/ml for 1 h at room temperature. Munc18-1 eluate was collected with Biospin disposable chromatography columns (BioRad). 50 µl benzanidin sepharose 6B (GE Healthcare) (washed 3x with H\(_2\)O and 3x with cleavage buffer) and 80 µl Pefabloc SC were added for 1 h at room temperature. Beads were removed by centrifugation for 3 min at 200 g and 4 °C. Protein aggregates were removed by another centrifugation step for 10 min at 55,000 rpm, and the supernatant was desalted into fusion buffer (25 mM HEPES-KOH pH 7.4, 135 mM KCl, 1 mM DTT) using a PD10 column (GE Healthcare). Finally, Munc18-1 aliquots were snap-frozen. The concentration of purified Munc18-1 was determined by SDS-PAGE and Coomassie blue-staining using BSA as standard protein and ImageJ software (NIH) for quantification.

**Soluble t-SNARE purification** - Mouse SNAP-25 encoded in the pET-15b vector and rat Syntaxin 1A aa1-262 in the pET-24(a) vector were co-transformed into BL21(DE3) bacteria (Stratagene). Bacterial cultures in 22 l LB medium containing 100 µg/ml ampicillin and 100 µg/ml kanamycin were grown at 37 °C to an optical density of 0.6 (at 600 nm). Protein expression was induced by 1 mM IPTG for 3 h at 37 °C. Bacteria were collected by centrifugation and washed once with PBS. Cell pellets were resuspended in a final volume of 300 ml breaking buffer (50 mM HEPES-KOH pH 7.4, 100 mM KCl, 10% glycerol) and snap-frozen for storage at -80 °C. After thawing, β-mercapto-ethanol was added to a final concentration of 3 mM, as well as a protease inhibitor cocktail described above. The further preparation of the bacterial lysate is outlined in the paragraph - Munc18-1 purification. The resulting lysate was incubated with 6 ml Ni-NTA beads (Qiagen) for 1.5 h at 4 °C on a rotating wheel. Beads were loaded into a column and washed with wash buffer (25 mM HEPES-KOH pH 7.4, 400 mM KCl, 10% glycerol, 3 mM β-mercapto-ethanol) followed by buffer A (25 mM HEPES-KOH pH 7.4, 200 mM KCl, 10% glycerol, 3 mM β-mercapto-ethanol, 50 mM imidazole). His-
tagged SNAP-25/syntaxin 1A aa1-262 complexes were eluted with buffer B (25 mM HEPES-KOH pH 7.4, 200 mM KCl, 10% glycerol, 3 mM β-mercapto-ethanol, 500 mM imidazole). Protein fractions were pooled, desalted into fusion buffer, and the protein concentration was determined as described above. Finally, SNAP-25/syntaxin 1A aa1-262 complexes were snap-frozen.

Munc18-1 binding assay - For the binding assay, 100 µl clarified bacterial lysate containing GST-Munc18-1 was incubated with 2 µl glutathione beads (GE Healthcare). After 3x washing with 13 ml breaking buffer and 13 ml washing buffer, 40 µl beads were incubated with 140 µg VAMP2 or VAMP2 fragments generated by BoNT/D cleavage (0.07 mg/ml BoNT/D, 1 h at 37 °C) in fusion buffer containing 1% OG in a final volume of 100 µl for 1.5 h at 4 °C. (Thus, GST-Munc18-1 beads were incubated with a 25-fold molar excess of His6-tagged VAMP2). Beads were washed 3x with 1 ml fusion buffer containing 1% OG. Finally, 20 µl Laemmli buffer were added and beads were boiled at 98 °C for 3 min. As a control, purified GST was bound to GSH beads and 40 µl of beads were incubated with 140 µg His-tagged VAMP2 or BoNT/D-fragmented His-tagged VAMP2. Beads were washed 3x with 1 ml fusion buffer containing 1% OG. Finally, 100 µl Laemmli buffer was added and beads were boiled at 98 °C for 3 min. SDS-PAGE was performed with 10 µl of each binding reaction.

Proteins were made visible by using the silver staining method. First, gels were fixed in 30% methanol and 10% acetic acid for 1 h followed by 2 washes in 10% ethanol for 15 min each. After treatment with 0.02% Na2S2O3 for 1 min, gels were rinsed with H2O and incubated with staining solution (2 mg/ml AgNO3, 0.037% formaldehyde) for 15 min followed by rinsing with H2O. Subsequently, developing solution (60 mg/ml Na2CO3, 0.0185% formaldehyde, 0.0002% Na2S2O3) was applied until protein bands appeared. Finally, gels were fixed in 7% acetic acid.

Protein reconstitution into liposomes - SUVs and GUVs were prepared exactly as described previously (57). All lipids were from Avanti Polar Lipids with the exception of 3H-DPPC ([1,2-dipalmitoyl phosphatidylcholine]), which was from Amersham Pharmacia Biotech. VAMP2/Syt1 lipid mix = 30 mol% POPC (1-palmitoyl-2-oleoyl-SN-glycero-3-phosphatidylcholine), 15 mol% DOPS (1,2-dioleoyl-SN-glycero-3-phosphatidylserine), 22.6 mol% POPE (1-hexadecanoyl-2-octadecenoyl-SN-glycero-3-phosphoethanolamine), 5 mol% liver PI (L-α-phosphatidylinositol), 25 mol% cholesterol (from ovine wool), 1.6 mol% Rhodamine-DPPE (N-(lissamine rhodamine B sulfonyl) 1,2-dipalmitoyl phosphatidylethanolamine) and trace amounts of 3H-DPPC (1,2-dipalmitoylphosphatidylcholine), 3 µmol total lipid. Syntaxin1/SNAP-25 lipid mix for docking assays = 34.5 mol% POPC (1-palmitoyl-2-oleoyl-SN-glycero-3-phosphatidylcholine), 15 mol% DOPS (1,2-dioleoyl-SN-glycero-3-phosphatidylserine), 20 mol% POPE (1-hexadecanoyl-2-octadecenoyl-SN-glycero-3-phosphoethanolamine), 3 mol% liver PI (L-α-phosphatidylinositol), 2 mol% brain PI(4,5)P2 (L-α-phosphatidylinositol-4,5-bisphosphate), 25 mol% cholesterol (from ovine wool) and 0.5 mol% rhodamine-DPPE (N-(lissamine rhodamine B sulfonyl), 5 µmol total lipid. The syntaxin1/SNAP-25 lipid mix for fusion assays contained no rhodamine-DPPE, but 35 mol% of POPC and trace amounts of [3H]-DPPC. Rhodamine-DPPE and 3H-DPPC were used for GUV lipid recovery calculations.

SUVs were formed in the presence of VAMP2 (protein to lipid ratio 1/200) and Syt1 (1/800) using the lipid mix defined above and the previously described technique of dilution and dialysis followed by a Nycodenz gradient centrifugation (13). t-SNARE-GUVs (protein to lipid ratio 1/1000) were formed by electro-swelling as described previously (57).

SUV/GUV binding assay - All SUV/GUV binding studies were carried out in an ice bath, in order to suppress fusion (13,57). Potential aggregates of 3H-DPPC labeled SUVs were removed by centrifugation at 16,000 g for 1 min. Where indicated, GUVs (42 nmol lipid) were pre-incubated for 5 min with 6 µM CpxII and/or for 10 min with 0.9 µM Munc18-1 on ice in 180 µl fusion buffer (25 mM HEPES-KOH pH 7.4, 135 mM KCl, 0.1 mM EGTA, 1 mM MgCl2, 1 mM DTT). Subsequently, 20 µl SUVs (7.5 nmol lipid) were added to GUVs in a final volume of 200 µl buffer. After 5 min or 1 h of co-incubation, GUVs and associated SUVs were isolated by centrifugation for 5 min at 5000 g, 4 °C. 190 µl of the supernatant were discarded and the counts per
and/or 0.9
we have established a vesicle-docking assay using
each protein in a comparative manner and to
to determine at which step it functions. To this end,
we have incorporated into SUVs and either
SYT1/SNAP-25 or PI(4,5)P2 was present in
minutes (cpm) in the resuspended pellet were
determined. Where indicated BoNT/D- and
BoNT/C-cleavage of SNARE proteins prior to
SUV/GUV mixing was performed for 1 h at 37 °C
with toxin concentrations of 0.07 mg/ml and 0.15
mg/ml, respectively. To determine unpecific SUV
– GUV interactions and to control for the SUVs
remaining in the dead volume of the pellet, SUVs
and GUVs were pre-treated with 0.05 mg/ml
proteinase K (Sigma Aldrich) for 30 min at 37 °C.
Absolute background measurements (usually 5%
of the input) were subtracted from all samples and
the percentage of pulled-down SUVs was
calculated based on input measurements. Average
values of three independent experiments were
calculated with corresponding 95% confidence
intervals as error bars (Microsoft Excel 2008).
SUV/GUV fusion assay - The fusion assay was
performed as described previously (57). SUVs
were pre-incubated with 6 µM CpxII (5 min)
and/or 0.9 µM Munc18-1 (10 min) at room
temperature. SUVs and GUVs were mixed at room
temperature and immediately transferred into a
pre-warmed 96-well plate (37 °C). Samples, in
which VAMP2-SUVs and t-SNARE-GUVs were
preincubated in the presence of Munc18-1 for 1 h
on ice, were subsequently transferred to a 96-well
plate at room temperature to delay the onset of
fusion. Samples were measured at 37 °C in a
Synergy 4 plate reader (BioTek Instruments
GmbH) at intervals of 10 s. After 5 min, Ca²⁺ was
added to a final concentration of 100 µM. The
NBD fluorescence obtained from control
incubations containing SUVs pretreated with
BoNT/D was subtracted from individual
measurement sets. The fusion-dependent
fluorescence was normalized to the maximum
fluorescent signal obtained in the presence of 0.4%
dodecylmaltoiside (Fluka). Three independent
fusion experiments were performed for each
condition.

RESULTS
SYT1-SUVs efficiently bind PI(4,5)P2 and
t-SNARE-containing GUVs - To determine the
function of Munc18-1 in the protein/lipid network
(v-SNARE, t-SNARE, SYT1, CpxII, Munc18-1,
and PI(4,5)P2) conferring vesicle docking/fusion, it
is necessary to systematically analyze the role of
each protein in a comparative manner and to
determine at which step it functions. To this end,
we have established a vesicle-docking assay using
SUVs, which contain reconstituted VAMP2 and/or
membrane anchored Syt1, as well as GUVs
containing syntaxin1/SNAP-25 and/or PI(4,5)P2.
VAMP2 and Syt1 were reconstituted at a protein
to lipid ratio of 1/200 and 1/800, respectively,
corresponding to the physiological concentrations
found in synaptic vesicles (58). These SUVs were
radio-labeled by the incorporation of 3H-DPPE to
allow the quantification of the SUV/GUV
interaction. GUVs, containing pre-assembled
syntaxin1/SNAP-25 complexes at a protein to lipid
ratio of 1/1000 and/or 2 mol% PI(4,5)P2, were
prepared by electroselling and labeled by the
fluorescent lipid rhodamine-DPPE to determine
lipid recovery. When GUVs (filled with 250 mM
sucrose) are resuspended in an iso-osmolar
reaction buffer of lower density, GUVs will
sediment at low centrifugal force, thus allowing
their separation from free SUVs, which remain in
the supernatant. The protein pattern of
reconstituted liposomes and the purity of all
regulatory components used in this study are
shown in supplemental Figure S1.

For the interaction studies, SUVs (7.5 nmol
lipid) and GUVs (42 nmol lipid) were incubated
for 5 min in the absence or presence of the
preferred regulatory component(s) in an ice-bath.
Free and docked SUVs were separated by
centrifugation at 5000 g for 5 min and the
percentage of SUVs docked to GUVs was
determined by measuring the radioactivity
recovered in the pellet. The inactivation of
VAMP2 and t-SNARE by botulinum neurotoxin D
(BoNT/D) and botulinum neurotoxin C (BoNT/C)
cleavage, respectively, shows that unpecific
protein-independent interaction of SUVs with
GUVs is minimal (≤ 5% for all controls) (Figure
1). Proteinase K-treated samples were used to
determine absolute background values that were
subtracted from all samples. Remarkably,
VAMP2-SUVs and t-SNARE-GUVs display very
weak binding (6.5 ± 1.8%) within the 5 min
incubation period on ice indicating that SNAREs
located in their native membrane environment
inefficiently form SNAREpins or that SNAREpins
are instable. The presence of PI(4,5)P2, which is
known to bind to the t-SNARE, did not increase
v-/t-SNARE-mediated docking (4.7 ± 3.8%). In
contrast, vesicle docking was significantly
enhanced when membrane-anchored Syt1 was
incorporated into SUVs and either
syntaxin1/SNAP-25 or PI(4,5)P2 was present in
the GUVs. This Syt1-mediated docking was largely VAMP2-independent. Syt1/PI(4,5)P$_2$-dependent docking (57 ± 4.0%) was slightly more efficient than Syt1/t-SNARE-dependent docking (50 ± 5.7%), which might be due to the 20-fold higher surface concentration of PI(4,5)P$_2$ compared to the t-SNARE. Since the interaction of PI(4,5)P$_2$ with positively charged amino acids close to the trans-membrane domain of syntaxin1, generates syntaxin1 and PI(4,5)P$_2$ clusters, we also tested if such PI(4,5)P$_2$ clusters might have an effect on Syt1-mediated vesicle docking (59). To this end, GUVs containing 2% PI(4,5)P$_2$ or GUVs containing 2% PI(4,5)P$_2$ and the membrane remnants of BoNTC-cleaved syntaxin1 (amino acids 254-288) were generated. We did not observe any significant difference in Syt1-mediated vesicle docking between these two GUV populations suggesting that PI(4,5)P$_2$ clusters are not required for Syt1/PI(4,5)P$_2$-mediated vesicle docking under the conditions employed (supplemental Figure S2). Most efficient docking was observed when all components were present and nearly 100% of SUVs added to the reaction were recovered in the GUV pellet upon the 5 min incubation, Munc 18-1 stimulated vesicle docking (51). However, a 1 h co-incubation step, Munc18-1 was already pre-loaded onto the t-SNARE-GUVs and could directly function as a docking factor via its VAMP2 interaction. A 5 min co-incubation in the presence of Munc18-1 did not significantly increase the SUV/GUV docking efficiency (7.2 ± 1.1%) (Figure 3A). However, after 1 h co-incubation, Munc18-1 stimulated vesicle docking 3-fold (22 ± 3.0%). Parallel lipid mixing experiments revealed that the prolonged co-incubation on ice does not result in membrane fusion, demonstrating that the vesicles are still at the docking stage (supplemental Figure S4). In summary, Munc18-1 is capable to increase vesicle docking, but only after prolonged incubation times, confirming the role of Syt1 as primary docking factor.

Partially assembled SNAREpins also provide high affinity binding sites for complexins, which in turn can stabilize SNAREpins and vesicle docking. To determine if CpxII enhances vesicle docking, 6 µM CpxII were added to t-SNARE-GUVs 5 min prior to mixing with VAMP2-SUVs before co-incubations of 5 min or 1 h on ice. 6 µM CpxII are saturating amounts as shown by the maximal signal obtained in a reconstituted Ca$^{2+}$-regulated lipid mixing assay (57). CpxII reproducibly stimulated vesicle docking, but only after the 1 h incubation time (Figure 3A). However, the statistical analysis showed that this stimulation was not significant (p < 0.2, independent t-test). Nevertheless, a combination of Munc18-1 and CpxII also showed a small additive effect after 1 h co-incubation (Figure 3A). Thus,
CpxII is a much weaker stimulator than Munc18-1 and Syt1. In the absence of Syt1, docking stimulation by each Munc18-1 and CpxII was strictly VAMP2-dependent. Finally, combinations of CpxII with Syt1 in the absence or presence of Munc18-1 were tested. Addition of CpxII and Munc18-1 did not significantly enhance the prominent docking effect of Syt1 under any condition chosen (supplemental Figures S5 and S6).

**Munc18-1 - SNARE and N-terminal SNARE - SNARE interactions synergize to stimulate vesicle docking** - Next, we attempted to further refine the molecular mechanism by which Munc18-1 enhances vesicle docking and SNAREpin formation. It is known that Munc18-1 shows a specific but very weak interaction with VAMP2 (49). Such weak interactions between single molecules are difficult to detect by classical binding experiments, but can be visualized after protein crosslinking or by NMR (49). However, in the membrane environment and in the presence of several Munc18-1 and VAMP2 copies, the simultaneous occurrence of such low affinity interactions could result in efficient vesicle docking by increased avidity. To further characterize the mechanism, we made use of the fact that the binding site for Munc18-1 has been mapped by crosslinking experiments to amino acids 87-91 and by NMR studies to amino acids 75-95 (49). This region is located in the C-terminal half of VAMP2 and remains membrane-anchored after BotNT/D cleavage. BotNT/D is a site-specific protease, which cleaves VAMP2 before Lys60 removing the N-terminal part of the VAMP2 SNARE motif, but leaving behind the binding site for Munc18-1 on the truncated VAMP2 remnant (61). Thus, if the single interaction of Munc18-1 with the membrane proximal binding site on VAMP2 is sufficient for efficient vesicle docking, BotNT/D cleavage should not affect docking. However, Figure 3A shows that BotNT/D cleavage efficiently abolished Munc18-1 mediated vesicle docking. Thus, the dual binding of Munc18-1 to the C-terminus of VAMP2 and the N-terminal portions of the v-/t-SNARE motifs synergize to confer SNAREpin formation/assembly. To exclude the remote possibility that the N-terminus of VAMP2 harbors a yet unidentified binding site for Munc18-1, GST-Munc18-1 was immobilized on glutathione beads and incubated with full length VAMP2 or VAMP2 cleaved by BotNT/D, generating the N- and C-terminal fragments. To detect the low affinity Munc18-1/VAMP2 interactions, bead-associated proteins were visualized by silver staining. Figure 3B shows that both full length VAMP2 and the C-terminal fragment show similar binding activities to GST-Munc18-1. An interaction of the N-terminal VAMP2 fragment with Munc18-1 was not detectable. Binding was specific because neither full length VAMP2 nor its fragments bind GST. Thus, both SNARE - SNARE interactions and Munc18-1 - SNARE interactions are required to enhance vesicle docking and to stabilize SNAREpins.

**Vesicle docking by Syt1 is a prerequisite for Munc18-1 to accelerate lipid mixing** - Finally, we determined how the various regulatory components and their effects on vesicle docking influence membrane fusion, measured by lipid mixing. For this purpose similar conditions as in the docking assay were employed, but the VAMP2-SUVs now contain in addition a quenched pair of lipid-coupled fluorophores (0.8 mol% NBD-DPPE and 1.6 mol% rhodamine-DPPE). Fusion of labeled SUVs (2.5 nmol lipid) with unlabeled t-SNARE-GUVs (14 nmol lipid) results in a dramatic dilution of the fluorophores and consequently the NBD fluorescence increases due to dequenching. Thus, membrane fusion is monitored by this well-established lipid mixing assay (13,62)) (Figure 4).

Lipid mixing was measured for 5 min in the absence of Ca\(^{2+}\) and subsequently, Ca\(^{2+}\) was added (100 \( \mu \)M final concentration) to monitor the kinetics and extent of Ca\(^{2+}\)-synchronized membrane fusion for an additional 10 minutes (Figure 4). As already shown in our previous study, VAMP2-SUVs and t-SNARE-GUVs by themselves do not show any significant membrane fusion under the conditions employed (low lipid concentrations and low protein to lipid ratio) (57). The addition of CpxII shows a weak stimulatory effect consistent with its weak stimulation of docking with progressing incubation times. Membrane-anchored Syt1 (in the absence of both CpxII and Ca\(^{2+}\)) profoundly stimulates membrane fusion, consistent with its prominent stimulation of vesicle docking. Addition of Ca\(^{2+}\) results in a significant but limited burst of fast membrane fusion. The addition of CpxII suppresses the Ca\(^{2+}\)-independent stimulation by Syt1, but results in a pronounced fast Ca\(^{2+}\)-dependent burst of
membrane fusion. Furthermore, the final fusion signal significantly exceeds the signal obtained in the presence of Syt1 alone, suggesting cooperative functions of Syt1 and CpxII. The addition of Munc18-1 to fusion reactions containing only SNARE liposomes did not enhance membrane fusion during the 15 min measurement period. This result is expected because VAMP2-SUVs and t-SNARE-GUVs need to be co-incubated together with Munc18-1 on ice for 1 h (non-fusogenic condition) to observe vesicle docking and the previously reported stimulation of membrane fusion (50,56). Indeed, a prolonged co-incubation results in a pronounced stimulation of the initial fusion rate (supplemental Figure S4). Remarkably, in the presence of Syt1, Munc18-1 profoundly stimulates lipid mixing even in the absence of any co-incubation together with SUVs and GUVs. Thus, vesicle docking mediated by Syt1 has generated an intermediate that allows Munc18-1 to efficiently promote SNAREpin assembly and fusion. The addition of CpxII partially inhibits the Ca\(^{2+}\)-independent stimulation and again results in a rapid Ca\(^{2+}\)-synchronized fusion burst. Thus, following the Syt1 vesicle docking reaction, Munc18-1 efficiently stimulates SNARE complex assembly and lipid mixing, whereas CpxII functions as a fusion clamp.

**DISCUSSION**

In our approach to assign Munc18-1 to a defined late-acting reaction step in regulated membrane fusion, we have resolved how Syt1, Munc18-1 and CpxII sequentially and synergistically function to control SNAREpin formation/assembly and generate a pool of vesicles, which responds to a Ca\(^{2+}\) signal with efficient and fast synchronized lipid mixing.

1. A direct comparison of the vesicle docking capabilities of the three regulatory components reveals that only Syt1 provides fast and efficient vesicle docking, which is consistent with recent in vitro and in vivo studies of Syt1 function (27-29,57). The strong enhancement of vesicle docking by Syt1 results in a significant stimulation of membrane fusion. Correspondingly, the weak docking activity of CpxII coincides with a weak stimulation of membrane fusion. As expected, in the presence of Syt1, CpxII blocks membrane fusion, further confirming that these two proteins function synergistically as a fusion clamp (57). 2. The Syt1-mediated docking occurs VAMP2-independent, but requires either PI(4,5)P\(_2\) or syntaxin1/SNAP-25 on the opposite membrane. Under the employed conditions, vesicle docking does not require PI(4,5)P\(_2\) clusters. 3. PI(4,5)P\(_2\) and t-SNAREs compete for binding to Syt1 implying a sequential binding modus. 4. Syt1-mediated vesicle docking results in a reaction intermediate that becomes an efficient substrate for Munc18-1 action. The observation that Munc18-1 shows a more prominent liposome docking phenotype than CpxII, but does not stimulate membrane fusion (in the absence of the low temperature co-incubation) already suggests a requirement for an earlier acting factor – Syt1. In the presence of Syt1, Munc18-1 not only dramatically increases the initial fusion rate, but also the final extent of membrane fusion, consistent with its SNAREpin assembly function. Thus, the majority of the vesicles docked by Syt1 are efficiently shifted into a reactive pool, which would be consistent with studies in living cells that demonstrated that Munc18-1 can regulate the size of the readily releasable pool of vesicles (63,64). Interestingly, recent in vitro reconstitution experiments showed that Syt1 docks vesicles, but a considerable time passes before these vesicles can fuse (28). Our data now indicate that Munc18-1 actually accelerates this vesicle priming step. 5. The presence of CpxII inhibits this stimulation by Munc18-1 to a significant degree and aids in synchronizing the reaction pathway towards the Ca\(^{2+}\)-dependent reaction. However, CpxII exerts only a partial block and a distinct fraction of the vesicles still fuses in a Ca\(^{2+}\)-independent manner with progressing time. It remains to be shown if this Ca\(^{2+}\)-independent membrane fusion in the reconstituted in vitro assay, which only uses a limited number of purified components, reflects some shortcoming of the assay, or potentially reproduces spontaneous vesicle fusion in vivo. Interestingly, Munc18-1 stimulates both spontaneous and evoked release in neurons, consistent with our in vitro data (64).

Overall, our results together with previous work suggest the following cascade of reactions. SNAREs at their physiological membrane concentrations do not show efficient vesicle docking and membrane fusion. Syt1 acts as an initial vesicle-docking factor. Thus, the presence of Syt1 is a prerequisite for efficient SNAREpin formation. This order of events ensures that the Ca\(^{2+}\) sensor will inevitably be incorporated into the
fusion machinery, rendering the machinery Ca\textsuperscript{2+}-responsive. Initially, Syt1 binds with low affinity to PI(4,5)P\textsubscript{2} on the pre-synaptic plasma membrane requiring its polybasic motif. This interaction may already occur in the immediate vicinity of the t-SNAREs, because syntaxin1 interacts with PI(4,5)P\textsubscript{2} and forms PI(4,5)P\textsubscript{2}-dependent clusters (59,65). Since the t-SNARE and PI(4,5)P\textsubscript{2} compete for the binding to the polybasic motif, and Syt1 has a higher affinity for the t-SNARE than for PI(4,5)P\textsubscript{2} (approx. $K_D = 250$ µM), the initial low affinity Syt1-PI(4,5)P\textsubscript{2} interaction will be replaced by the higher affinity Syt1-t-SNARE interaction (66,67). This Syt1-t-SNARE interaction will bring v-SNAREs and t-SNAREs on the opposite membranes in close proximity and SNAREpin assembly can start. Henceforth, SNAREpin assembly is controlled by CpxII and Munc18-1. Munc18-1 binds partially assembled t-SNARE complexes, the C-terminal part of the VAMP2 SNARE motif, and the subsequent linker sequence (36,49,56,68). Since Munc18-1 specifically interacts with VAMP2 and VAMP3, only vesicles containing these v-SNAREs will be efficiently primed by accelerating SNAREpin assembly (50,56). In addition, it is of note, that Munc18-1 is also a high affinity partner of syntaxin1 and keeps syntaxin 1 in a closed conformation. To release this inhibition and to allow subsequent SNARE complex assembly, a Munc13 - t-SNARE and a Munc18-1 - VAMP2 interaction are required (48,50). However, these earlier regulatory steps are bypassed in our assay, because preassembled t-SNARE complexes were used to focus on late steps of SNAREpin assembly. While Munc18-1 targets the C-terminal part of VAMP2, CpxII is directed towards the assembled N-terminal part of the partially assembled SNAREpin. Indeed, it has been shown that both complexin and Munc18-1 can simultaneously bind SNARE complexes (64). An already available structure of a complexin-SNAREpin mimetic demonstrates that complexin binds via its central helix in an anti-parallel manner to the N-terminal end of partially assembled SNAREpins (21). The N-terminal accessory helix of complexin interacts in trans with a second SNAREpin and blocks SNAREpin assembly by competing with VAMP2 for binding to the membrane proximal part of the t-SNARE complex (21). These bridging functions of complexin also result in SNAREpin oligomerization. Thus, the SNAREpins are now in an arrested state containing the complexin clamp, Syt1, the Ca\textsuperscript{2+} sensor, and presumably Munc18-1. How this reaction intermediate is organized in structural terms remains to be shown. Ca\textsuperscript{2+}-binding to Syt1 then mediates local perturbations in the lipid bilayer and the release of the complexin clamp likely via Ca\textsuperscript{2+}-dependent SNAREpin interactions, results in membrane fusion (23,24). Post fusion, complexin does not any longer bridge SNARE complexes, SNARE complex oligomers are resolved, and the accessory/inhibitory helix is solvent-exposed (19).

Having resolved basic vesicle docking and subsequent priming steps in a reconstituted assay and having assigned the corresponding machinery to distinct reactions, future work still needs to address the biophysics of the fusion reaction.
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FOOTNOTES

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The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Munc18-1, mammalian unc18-1; Syt1, synaptotagmin1; CpxII, complexin II; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; GUV, giant unilamellar vesicle; SUV, small unilamellar vesicle; wt, wild type.
FIGURE LEGENDS

FIGURE 1. Syt1, but not the v-SNARE VAMP2, mediates efficient vesicle docking by specific interactions with PI(4,5)P$_2$- and/or t-SNARE (syntaxin1/SNAP-25)-GUVs in the absence of Ca$^{2+}$. VAMP2- or VAMP2/Syt1-SUVs (7.5 nmol lipid, 38 pmol VAMP2, 9.4 pmol Syt) labeled with $^3$H-DPPC were mixed with GUVs (42 nmol lipid, 42 pmol syntaxin1/SNAP-25) lacking or containing PI(4,5)P$_2$ (0.84 nmol) in a final volume of 200 µl and incubated on ice for 5 min. Where indicated (-), VAMP2 and syntaxin1/SNAP-25 were specifically cleaved by treatment with BoNT/D and BoNT/C, respectively. SUVs bound to GUVs were isolated by centrifugation and the radioactivity associated with the pellet was measured as described in Experimental Procedures. Percentages were calculated based on input radioactivity. Error bars are 95% confidence intervals (n=3).

FIGURE 2. The polybasic motif in the C2B domain of Syt1 is required for vesicle docking via redundant PI(4,5)P$_2$ and t-SNARE interactions. (A) Mutations in the polybasic motif of Syt1C2B impair both PI(4,5)P$_2$- and t-SNARE-dependent vesicle docking. v-SNARE-SUVs containing the Syt1C2B* triple mutation K326,327,331Q or Syt1 wt were incubated with t-SNARE-GUVs as described in the legend to Figure 1. Where indicated (-), VAMP2 and syntaxin1/SNAP-25 were cleaved by the specific BoNTs. (Last bar reproduced from Figure 1.) (B) t-SNARE and PI(4,5)P$_2$ compete for binding to Syt1. Syt1-SUVs were preincubated with a 6-fold molar excess of soluble t-SNARE and subsequently incubated with PI(4,5)P$_2$-GUVs on ice for 5 min and analyzed as described in Experimental Procedures. Error bars are 95% confidence intervals (n=3).

FIGURE 3. Munc18-1 stimulates docking of v-SNARE-SUVs to t-SNARE-GUVs, which requires both SNARE-SNARE and Munc18-1-SNARE interactions and a prolonged incubation on ice. (A) Munc18-1 shows more pronounced vesicle docking than CpxII. The weak docking activity of CpxII is statistically not considered to be significant (p < 0.2, independent t-test). t-SNARE-GUVs were pre-incubated with 0.9 µM Munc18-1 for 10 min, and/or 6 µM CpxII for 5 min on ice. v-SNARE-SUVs were added, and the incubation was continued for the indicated time periods on ice. Where indicated (VAMP2 -), VAMP2 was cleaved by BoNT/D. (The first bar was reproduced from Figure 1.) Error bars are 95% confidence intervals (n=3). (B) Munc18-1 binds the C-terminal, but not the N-terminal fragment of BoNT/D-cleaved VAMP2. GST-Munc18-1 was immobilized on glutathione beads, followed by a 1 h incubation with full-length VAMP2 or BoNT/D-cleaved VAMP2. VAMP2 was present in a 25-fold molar excess over Munc18-1. Bound proteins were separated by SDS-PAGE and visualized by silver staining; a representative experiment is shown.

FIGURE 4. In the presence of Syt1, Munc18-1 stimulates lipid mixing without a SUV/GUV pre-incubation step. VAMP2- or VAMP2/Syt1-SUVs (2.5 nmol lipid, 12.5 pmol VAMP2, 3.1 pmol Syt1), labeled with rhodamine-DPPE and NBD-DPPE were mixed with unlabeled syntaxin1/SNAP-25-GUVs (14 nmol lipid, 14 pmol t-SNARE) in the absence or presence of 90 pmol Munc18-1 and/or 600 pmol CpxII in a final volume of 100 µl and the increase in NBD fluorescence was monitored. After 5 min at 37 °C, Ca$^{2+}$ was added to a final concentration of 100 µM and the measurement continued for another 10 min. The results were normalized to the maximum NBD fluorescence signal after detergent lysis of the liposomes. Error bars are SEM (n=3).
### Figure 1

|         | VAMP2 | Syt1 | t-SNARE | PI(4,5)P_2 |
|---------|-------|------|---------|------------|
| docked SUVs (% of input) | +     | -    | -       | -          |

- VAMP2: + + + + - + - - - + + + +
- Syt1: - - - + + + + + + + + +
- t-SNARE: - + - - + - + + - +
- PI(4,5)P_2: - - + + - - + + - + +
Figure 2

A

| VAMP2 | - | + | - | + | + |
|-------|---|---|---|---|---|
| Syt1 C2B* | + | + | + | + | - |
| Syt1 wt | - | - | - | - | + |
| t-SNARE | - | - | + | + | + |
| PI(4,5)P_2 | + | + | + | + | + |

B

| VAMP2 | - | - |
|-------|---|---|
| Syt1 | + | + |
| t-SNARE | - | - |
| PI(4,5)P_2 | + | + |
| soluble t-SNARE | - | + |
Figure 3

A

![Graph showing docked SUVs (% of input) vs. conditions]

| Condition       | VAMP2 | Syt1 | t-SNARE | PI(4,5)P2 | Munc18-1 | CpxII | Co-incubation |
|-----------------|-------|------|---------|-----------|----------|-------|---------------|
|                 | +     | -    | +       | +         | -        | -     | 5'            |
|                 | +     | -    | +       | +         | +        | +     | 5'            |
|                 | +     | -    | +       | +         | -        | -     | 5'            |
|                 | +     | -    | +       | +         | +        | +     | 1 h           |
|                 | +     | -    | +       | +         | +        | +     | 1 h           |
|                 | +     | -    | +       | +         | +        | +     | 1 h           |
|                 | +     | -    | +       | +         | +        | +     | 1 h           |

B

![Image of VAMP2 fragments]

| Condition       | VAMP2 | BoNT/D |
|-----------------|-------|--------|
| Input 2 µg      | +     | +      |
| GST             | +     | -      |
| GST-Munc18-1    | +     | -      |
Figure 4

% of total fluorescence

+Ca^{2+}

VAMP2/Syt1 SUVs

control

CpxII

Munc18-1

CpxII + Munc18-1

VAMP2 SUVs

time (min)
