BIOCHEMISTRY

Reconstitution of calcium-mediated exocytosis of dense-core vesicles

Alex J. B. Kreutzberger,1,2 Volker Kiessling,1,2 Binyong Liang,1,2 Patrick Seelheim,1,2 Shrutee Jakhanwal,3 Reinhard Jahn,3 J. David Castle,1,4 Lukas K. Tamm1,2*

Regulated exocytosis is a process by which neurotransmitters, hormones, and secretory proteins are released from the cell in response to elevated levels of calcium. In cells, secretory vesicles are targeted to the plasma membrane, where they dock, undergo priming, and then fuse with the plasma membrane in response to calcium. The specific roles of essential proteins and how calcium regulates progression through these sequential steps are currently incompletely resolved. We have used purified neuroendocrine dense-core vesicles and artificial membranes to reconstitute in vitro the serial events that mimic SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-dependent membrane docking and fusion during exocytosis. Calcium recruits these vesicles to the target membrane aided by the protein CAPS (calcium-dependent activator protein for secretion), whereas synaptotagmin catalyzes calcium-dependent fusion; both processes are dependent on phosphatidylinositol 4,5-bisphosphate. The soluble proteins Munc18 and complexin-1 are necessary to arrest vesicles in a docked state in the absence of calcium, whereas CAPS and/or Munc13 are involved in priming the system for an efficient fusion reaction.

INTRODUCTION

Regulated exocytosis is used by cells to export stored secretory products—proteins, neurotransmitters, hormones, and small molecules—in response to calcium-mediated signaling (1). In neurons and neuroendocrine cells, the final step of exocytosis is membrane fusion, which is catalyzed by the SNARE [soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor] proteins syntaxin-1 and SNAP-25 in the plasma membrane and synaptobrevin-2/VAMP-2 (vesicle-associated membrane protein 2) in the secretory vesicle membrane (1–3). Whereas SNARE proteins are sufficient to catalyze membrane fusion (4), several other proteins are involved in regulating the assembly of SNARE complexes and in coupling the calcium signal to the fusion process (2, 3). These include synaptotagmin (syt), complexin, Munc18, Munc13, and CAPS (calcium-dependent activator protein for secretion) (2, 3, 5). Numerous studies have identified prospective roles for these proteins in the vesicle docking, priming, and triggering steps that contribute to exocytosis in situ (6–11), whereas others have analyzed how their interactions affect reconstituted SNARE-mediated fusion in vitro (12–16). However, significant gaps remain in a precise molecular understanding of how and in what order the specific interactions among these proteins relate to the steps of the exocytotic process and interface with calcium’s actions in triggering release. Although several in vitro studies have reported an enhancement of fusion by calcium, complexin, and syt under specialized conditions (12, 17), it has not been possible, so far, to reconstitute a fusion-arrested docked state in vitro in which a robust fusion response is triggered by calcium under conditions containing all known in situ requirements for exocytosis.

RESULTS

To build on current knowledge concerning how the complex molecular machinery of exocytosis might function in vivo, we have sought to use a reconstitution strategy in which individual steps in the process can be resolved and analyzed and the full range of candidate molecular machinery can be evaluated through addition/subtraction approaches. Therefore, we have devised a hybrid system of reconstituted exocytosis in which native neuroendocrine dense-core vesicles (DCVs) and either liposomes or supported planar bilayers containing plasma membrane SNARE proteins serve as fusion partners. DCVs were purified from rat pheochromocytoma (PC12) cells under iso-osmotic conditions using velocity sedimentation and subsequent fractionation on a discontinuous iodoxanol gradient (Fig. 1A; see Materials and Methods). The DCVs are functionally active in an ensemble fusion assay in which lipid mixing between DCVs and liposomes was monitored by fluorescence dequenching of NBD-DOPA [1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazole-4-yI)] (Fig. 1B). Moreover, DCVs specifically labeled with a fluorescent secretory protein, neuropeptide Y (NPY) fused to Ruby (expressed in the PC12 cells before fractionation), enable observation of both docking and fusion events with reconstituted supported planar bilayers containing the plasma membrane SNARE proteins (Fig. 1C and fig. S1). DCV fusion was SNARE-dependent in all conditions tested (Fig. 1D and table S1). Lipid mixing in the ensemble fusion assay was accelerated by calcium (fig. S2), consistent with the results for other purified secretory vesicle preparations (18, 19). The amount of docking and the rate and probability of fusion of DCVs to supported bilayers were enhanced by calcium (Fig. 1, E to G, and fig. S3). Calcium-dependent docking did not require SNARE proteins (Fig. 1D), whereas both docking and fusion in the presence of calcium were enhanced by phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] and reduced in the absence of negatively charged lipids (Fig. 1H).

Both syt (a resident integral protein of synaptic vesicles and DCVs) and CAPS [a soluble but also vesicle-associated protein (Fig. 1A)] have been implicated in the regulation of exocytosis by calcium (5). Numerous studies have identified syt as a calcium sensor that triggers fusion (3, 20). Notably, syt has been shown to accelerate fusion by interacting with PI(4,5)P2 (18, 19, 21). Some of these effects could be due to increased cross-linking of vesicles by syt, although single-particle fusion assays have indicated that syt may have little effect on docking in the presence of SNARE proteins (14). CAPS, which was originally identified as a fusion trigger (22), includes a PI(4,5)P2-binding pleckstrin homology
(PH) domain, a C2 domain, and a syntaxin-1a–interacting MUN domain (5). Although it has received less attention than syt and has been questioned previously as a prospective fusion trigger (23), recent studies have shown convincing roles of CAPS in the more proximal steps of docking and priming of DCVs for fusion (5, 8, 11). The abilities to examine both docking and fusion of DCVs to supported bilayers and to manipulate the levels and functions of DCV-associated syt and CAPS have provided us a unique opportunity to examine the roles of these proteins comparatively. We have used two approaches toward this goal: (i) adding function-blocking antibodies to the in vitro assay and (ii) using short hairpin RNA (shRNA)–mediated depletion before purification of DCVs. In the case of syt, the anti-syt antibodies, which bind to the C2 domains, had no effect on the extent of calcium-enhanced docking (Fig. 2A). In addition, shRNA-mediated knockdown of syt-1 and syt-9 (the DCV-associated syts that control exocytosis (24)) to >85% for both isoforms (fig. S4) did not affect the extent of docking (Fig. 2A). However, both treatments profoundly inhibited calcium-accelerated fusion (Fig. 2B and fig. S5A). Notably, the effect on fusion was rescued by expressing RNA interference (RNAi)–resistant syt-1 during knockdown (Fig. 2, A and B).

Strikingly, blocking CAPS function using antibodies that bind near the PH and C2 domains (of both isoforms) or shRNA-mediated CAPS depletion (>90% for both isoforms; fig. S5) gave results that significantly contrasted with those for syt. Here, the calcium-dependent increase of DCV docking was inhibited (Fig. 2C and table S5). However, for fusion,
the CAPS antibodies had no effect, whereas knockdown decreased the amount of fusion in the absence and presence of calcium as compared to CAPS-containing DCVs (Fig. 2D and table S5). CAPS deficiency also substantially increased the delay time between docking and fusion (fig. S5), likely indicating a role for CAPS in SNARE assembly. Coexpression of RNAi-resistant CAPS-1 during knockdown rescued the effects on both docking and fusion (Fig. 2, C and D, and table S5). As shown by the cumulative distribution for elapsed time between docking and fusion, synt knockdown had no other kinetic effect, although it blocked calcium-accelerated fusion (fig. S5).

The protein machinery necessary for fusion to respond to calcium appears to be present on the secretory vesicle (14, 18, 25, 26), but a large amount of SNARE-dependent fusion occurs in the absence of calcium (~40%; Fig. 1F), which is consistent with the SNAREs representing the minimal fusion machinery (4). Other proteins are well known to regulate the interaction between fusion partners in situ and affect SNARE complex assembly. Thus, we sought to modify the initial hybrid system by replacing the truncated version of syntaxin (residues 183 to 288, containing the transmembrane and SNARE domains) with the full-length protein containing, in addition, the N-terminal and Habc domains that are thought to regulate SNARE complex function. Further, we included the SNARE regulatory proteins Munc18 and complexin-1 to test the effects on docking and fusion in the absence and presence of calcium. The overall goals of this strategy were to suppress calcium-independent fusion and distinguish separate states of vesicle docking and calcium-triggered fusion analogous to those that occur in situ. The soluble proteins Munc18 and complexin-1 are known to be essential in vivo, but it is debated how they positively or negatively regulate fusion (2, 3). Munc18 interacts with the regulatory Habc domain and N-peptide of syntaxin-1a (27–29), and its deletion completely blocks exocytosis (30). Recently, it was proposed that a Munc18 homolog serves as a template for SNARE zipping (31). Complexin-1 has been reported to block spontaneous synaptic vesicle exocytosis in the absence of calcium while increasing synchronized release (32, 33), and earlier in vitro studies claimed that it binds the fully assembled synaptic SNARE complex and serves as a clamp to prevent fusion in advance of calcium signaling (34, 35). However, very recent observations show that complexin-1 may act more proximally by binding a binary complex of syntaxin-1a and SNAP-25, thereby reducing the assembly of synaptobrevin-2 into the fully zipped SNARE complex (36). As a consequence of these observations, Munc18 and complexin-1 were our first priorities for addition during reconstitution.

In our hybrid assay, docking and fusion differed depending on whether the supported bilayers were reconstituted with a truncated syntaxin-1a (residues 183 to 288) or full-length syntaxin-1a (residues 1 to 288). As compared to truncated syntaxin-1a, full-length syntaxin-1a reduced docking in the absence of calcium and reduced fusion in the absence and presence of calcium (Fig. 3, A to D, No Additions columns). When Munc18 was added to the assay, it had little effect on docking or fusion to bilayers containing truncated syntaxin-1a (Fig. 3, A and B). However, it increased docking in the absence of calcium and increased fusion in the absence and presence of calcium when the bilayers contained full-length syntaxin-1a (Fig. 3, C and D), consistent with its ability to bind the full-length protein. When complexin-1 was added to our assay (without Munc18), docking was abolished in the absence of calcium regardless of which syntaxin-1a construct was present (Fig. 3, A and C). Inclusion of calcium with complexin-1 enabled docking and fusion for both truncated and full-length syntaxin-1a, although fusion was decreased when the full-length syntaxin-1a was used (Fig. 3, B and D). When Munc18 and complexin-1 were added together in assays containing truncated syntaxin-1a, docking was blocked in the absence of calcium, but both docking and fusion occurred in the presence of calcium (Fig. 3, A and B). Notably, when full-length syntaxin-1a was used, addition of Munc18 and complexin-1 allowed DCV docking but no fusion in the absence of calcium, whereas the inclusion of calcium allowed fusion to occur readily (Fig. 3, C and D). Using these
conditions, which closely approximate those that occur physiologically, this last set of experiments achieved, for the first time, the reconstitution of the calcium dependence of docking and fusion as distinct observable events under full control of this key set of regulatory proteins.

To complement this demonstration, we used various combinations of syntaxin-1a, SNAP-25, Munc18, and complexin-1 to reveal that syntaxin-1a and Munc18 were sufficient to dock DCVs in the absence of SNAP-25, albeit at levels that were decreased compared to when plasma membrane SNAREs were present (fig. S7). Further, we showed that the soluble synaptobrevin-2 (residues 1 to 96) inhibitor peptide blocked this docking as well as that occurring with both Munc18 and complexin-1 present, thereby indicating that reconstituted docking is synaptobrevin-2–dependent (fig. S7). This outcome supports the prospective role of Munc18 in dual binding to syntaxin-1a and synaptobrevin-2, which may be analogous to the roles of Munc18’s yeast homolog Vps33 (31).

Moreover, addition of complexin-1 in the absence of Munc18 and calcium inhibits docking under conditions with the truncated syntaxin-1a (residues 183 to 288):SNAP-25 or the full-length syntaxin-1a (residues 1 to 288):SNAP-25 complexes (Fig. 3), which agrees with recent results showing that complexin binds quite strongly to the plasma membrane SNAREs that are assembled in a strict 1:1 stoichiometry and thus blocks the interaction with synaptobrevin-2 (36). In the presence of Munc18, there is no effect of complexin-1 on docking to binary syntaxin-1a (residues 1 to 288):SNAP-25 complexes, whereas fusion in the absence of calcium is markedly inhibited by complexin-1, and this response depends strongly on increasing concentrations of complexin (Fig. 3, C and D, and fig. S8). Therefore, Munc18 likely facilitates SNARE assembly, allowing DCVs to be dockered, whereas complexin-1 acts within this complex to prevent fusion in the absence of calcium. The data in Fig. 3D also provide a good molecular explanation of the mysterious role of calcium in this complex to prevent fusion in the absence of calcium but enhances fusion when stimulated by calcium (37). In the absence of calcium, complexin (with Munc18) reduces fusion about 10-fold, and in the presence of calcium, complexin and Munc18 increase fusion by 35%, compared to fusion levels without these proteins, in our system.

Within cells, a subset of secretory vesicles exists in a readily releasable pool, where vesicles are docked at the plasma membrane and primed to undergo rapid fusion in response to elevated levels of calcium (38, 39). The condition where DCVs are docked on planar supported bilayers containing syntaxin-1a (residues 1 to 288) and lipid-anchored SNAP-25 in the presence of Munc18 and complexin-1 but in the absence of calcium likely mimics this primed releasable state. To confirm that these DCVs are fusion–competent, we first allowed them to dock under these conditions for ~30 min and then washed them extensively with a buffer containing the same amounts of Munc18 and complexin-1 to remove undocked DCVs. Then, we added a buffer containing calcium (concentrations are indicated in the figures) and a fluorescent dye (Cy5) to track calcium-containing buffer arrival, which allowed the ensuing calcium-dependent fusion kinetics to be monitored (Fig. 4A). Calcium increased the rate and probability of docked DCV fusion, thus serving as an authentic trigger (Fig. 4B and fig. S9). The observed fusion kinetics from the arrested state (Fig. 4B) was notably different from that observed between docking and fusion in the unarrested pathway (fit of data in Fig. 3D shown in table S10). The kinetics of proceeding from docking to fusion showed a delay that is characteristic of the presence of intermediate(s) (40), whereas the calcium–triggered fusion followed first-order kinetics, indicating that the final intermediate had already been populated. At 1 mole percent (mol %) PI(4,5)P$_2$ 80 to 100 μM calcium was sufficient to fully trigger a response from a ~3% spontaneous constitutive fusion to 60 to 70% fusion probability (fig. S9).

Because PI(4,5)P$_2$ is a key constituent in priming of exocytosis (41–43), we investigated its effect on our reconstituted docking and triggered fusion as well as its influence on the function of the PI(4,5)P$_2$–binding proteins syt and CAPS. The concentration of PI(4,5)P$_2$ included in the supported bilayer had no effect on spontaneous fusion in the absence of calcium but greatly enhanced triggered release (Fig. 4, C and D). The effect of PI(4,5)P$_2$ appeared near maximal from 1 to 5 mol % of total lipid in the planar bilayer, which approximates the estimated

![Fig. 3. Effects of Munc18, complexin, and the regulatory Habc domain of syntaxin-1a on docking and fusion.](Image) Docking and fusion probability for DCVs to planar supported bilayers containing truncated syntaxin-1a (residues 183 to 288):SNAP-25 (A and B) or full-length syntaxin-1a (1 to 288):SNAP-25 (C and D) in the presence of 0.5 mM Munc18, 2 mM complexin-1 (cpx1), or both. Black and red bar data were obtained in the absence and presence of Ca$^{2+}$, respectively. Summary of events and fitting results are presented in tables S9 and S10. Western blots show that Munc18 and complexin-1 were not detectable in DCV-enriched fractions prepared by centrifugation (fig. S6).
As a final issue in evaluating reconstituted two-step docking and fusion in the presence of regulatory proteins, we examined the effects of depleting syt or CAPS from the DCVs. Loss of neither syt nor CAPS appeared to affect spontaneous fusion (Fig. 4E). However, without syt, calcium-triggered fusion was completely inhibited, whereas without CAPS, triggered fusion was greatly reduced (Fig. 4, E and F). In the latter case, reduced fusion appears to be caused by a kinetic delay (evident in the cumulative distribution curve in Fig. 4F), likely indicating a role of CAPS in priming of the fusion reaction. In situ, the function of CAPS may be closely coupled to or even partially overlap with that of Munc13 (11), a protein that is essential for priming of synaptic fusion (45). Thus, we were interested whether the reduced fusion incurred upon depletion of CAPS might be rescued by adding to our reconstitution a polypeptide containing the C2 and MUN domains that are common to CAPS and Munc13. For this purpose, we used purified recombinant polypeptides containing MUN domain alone or the MUN domain preceded by the C1 and C2 domains (C1C2-MUN) of Munc13. As shown in Fig. 4 (E and F), inclusion of MUN partially rescues, whereas C1C2-MUN fully rescues, the kinetic delay in fusion caused by the CAPS knockdown. This result indicates that we have apparently repopulated the final (primed) step leading to fusion. While under these precise conditions the Munc13 C1C2-MUN domain seems to be interchangeable with CAPS in the kinetic priming of the docked DCV state, these two proteins are not redundant players in vivo and more detailed studies of the respective interactions and effects on priming will need to be addressed in the future.

**DISCUSSION**

Our hybrid system using neuroendocrine DCVs and planar supported bilayers has enabled us to reconstruct in vitro a stepwise docking, priming, and fusion process that bears a remarkable resemblance to exocytosis in situ and to clarify the roles of key supporting proteins. Using this approach, we have made the following new findings (see also Table 1 for more details): (i) Munc18 and complexin-1 in the presence of syntaxin-1a (residues 1 to 288) and SNAP-25 are able to arrest DCVs in a docked state in the absence of calcium; (ii) at 1 mol % PI(4,5)P₂, the fusion probability is triggered by 80 to 100 μM calcium to ~3% spontaneous constitutive fusion probability to 60 to 70% fusion probability, whereas at 5 mol % PI(4,5)P₂, this dynamic range is expanded from 2% spontaneous to 80% triggered fusion; this calcium trigger for fusion is strictly dependent on synaptotagmin; (iii) CAPS appears to kinetically increase the rate of the calcium response from the Munc18/complexin trans-SNARE complex docked state while also being able to dock DCVs to PI(4,5)P₂-containing membranes in response to calcium; (iv) the C1C2-MUN domain of Munc13 can replace CAPS priming under the conditions investigated here; and (v) the depletion of PI(4,5)P₂ kinetically reduces the rate of calcium triggering from the docked state in a manner that closely resembles the effects of CAPS knockdown.

These findings have led us to propose a model (Fig. 5) where DCVs can dock to membranes in a stable nonfusing state when syntaxin-1a (residues 1 to 288), SNAP-25, Munc18, and complexin-1 are present in a process that is dependent on synaptobrevin-2. CAPS can prime these docked DCVs to fuse efficiently when triggered with calcium. The actual fusion triggering is caused by synaptotagmin-1/9 in a process that requires an anionic lipid in the target membranes with a strong preference for PI(4,5)P₂. The calcium-dependent docking of DCVs facilitated by DCV-associated CAPS (11) could then function to recruit more DCVs to PI(4,5)P₂-containing membranes, allowing more fusion events to occur (Fig. 5). This calcium-dependent recruitment of DCVs
to the target membrane agrees with the electrophysiological results showing that CAPS is necessary for sustained secretion in chromaffin cells in the presence of elevated calcium (46, 47). With respect to this last point, we are encouraged by earlier cellular studies showing calcium-dependent recruitment of synaptic vesicles after depletion of docked vesicles (48) and similar behavior noted for DCVs (49) that may depend at least in part on DCV-associated CAPS (11). The direct binding of calcium by CAPS [dissociation constant ($K_d$) ~270 μM] (6) was similar to what was observed for calcium’s $K_d$ for docking in our reconstituted system [~240 μM in the presence of 1% PI(4,5)P2]. These calcium concentrations are well above the physiological level of intracellular calcium present under stimulatory conditions, but the docking

Table 1. Summary of results on the role of soluble and DCV-associated proteins and regulatory lipids obtained by the hybrid reconstitution approach of docking and fusion of single DCVs on planar supported bilayers with reconstituted SNAREs under different target membrane, vesicle membrane, and added protein conditions. 0, no effect; +, positive effect; ++++, strong positive effect; −−−−, strong negative effect; N/E, not examined.

<table>
<thead>
<tr>
<th>Starting membrane condition</th>
<th>Vesicle condition/ additions</th>
<th>Docking probability</th>
<th>Fusion probability</th>
<th>$k_{fusion}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syntaxin-1a (183–288):SNAP-25</td>
<td>Syntaxin-1a (183–288):SNAP-25</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
</tr>
<tr>
<td>Syt</td>
<td>CASP</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Complexin-1</td>
<td>No docking</td>
<td>No docking</td>
<td>No docking</td>
<td></td>
</tr>
<tr>
<td>Munc18</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>Syntaxin-1a (1–288):SNAP-25</td>
<td>Syntaxin-1a (1–288):SNAP-25</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
</tr>
<tr>
<td>Complexin-1</td>
<td>No docking</td>
<td>No docking</td>
<td>No docking</td>
<td></td>
</tr>
<tr>
<td>Syntaxin-1a (183–288):SNAP-25, Munc18</td>
<td>Syntaxin-1a (183–288):SNAP-25, Munc18</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
</tr>
<tr>
<td>Complexin-1</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>Syntaxin-1a (183–288):SNAP-25, Munc18, complexin-1</td>
<td>Syntaxin-1a (183–288):SNAP-25, Munc18, complexin-1</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
</tr>
<tr>
<td>Syt</td>
<td>CAPS</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
</tr>
<tr>
<td>Complexin-1</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>Syntaxin-1a (183–288):SNAP-25, Munc18, complexin-1</td>
<td>Syntaxin-1a (183–288):SNAP-25, Munc18, complexin-1</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
</tr>
<tr>
<td>Complexin-1</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td>EDTA Ca$^{2+}$</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. Model of DCV docking and fusion during exocytosis. Calcium-dependent release of DCV content requires an acceptor complex consisting of syntaxin-1a, SNAP-25, Munc18, and complexin as well as PI(4,5)P2 in the plasma membrane. The complex interacts with synaptobrevin-2 (Syb) and synaptotagmin-1/9 (syt) in the vesicle membrane as well as CAPS that may or may not be vesicle-associated (I). DCVs are able to dock to the complex in the absence of calcium in a SNARE-dependent fashion (IIa). The presence of complexin “clamps” the resulting pre-fusion (trans-SNARE) complex, preventing progression to an open fusion pore, and allows priming of the fusion machinery. Priming depends on CAPS (and/or Munc13 [not shown]) and PI(4,5)P2 and might involve a spatial organization of multiple copies of trans-SNARE complexes and accessory proteins as well as the organization of a specific local nanoscale lipid environment. The primed intermediate state resembles granules in the readily releasable pool in situ. Calcium influx triggers fusion pore opening catalyzed by syt (III) and eventually the collapse of the vesicle membrane into the plasma membrane (IV). Calcium also facilitates CAPS-dependent docking of DCVs to the plasma membrane (IIb). These granules proceed through some intermediates that might resemble the priming steps in the absence of calcium to a fusion pore (III) and eventually to the complete merger of the two membranes (IV).
had a large dynamic range with increases in DCV docking to planar supported bilayers observed under 100 μM calcium. Previously, PI(4,5)P₂ was shown to be enriched around syntaxin-1a (50) and act as a recruitment site for secretory vesicle docking (51). Our results indicate that calcium-dependent DCV docking increased in the presence of higher concentrations of PI(4,5)P₂, suggesting that local enrichments of PI(4,5)P₂ around syntaxin-1a could drive calcium-dependent CAPS-mediated docking to physiologically relevant calcium concentrations. Whether this is a meaningful correlation or the relatively high Kₐ reflects a current limitation of our in vitro system will require further study. In the future, it will be important to determine whether additional proteins that have been implicated in docking [for example, granuphilin (52), rabphilin-3a, and Munc13-4 (53, 54)] as well as lipid microdomains] will, when included in our analyses, reduce the calcium Kₐ for vesicle docking to micromolar or even submicromolar levels.

The hybrid approach combining biological DCVs and reconstituted target membranes in a robust and efficient single-vesicle fusion assay enabled us to dissect and assign precise mechanistic roles to a large number of proteins (and some of their subdomains) that regulate calcium-triggered exocytosis, which has previously eluded experimental confirmation. Our results have demonstrated that complexin, Munc18, and the regulatory Habc domain of syntaxin are all necessary and sufficient to create a fusion-competent acceptor SNARE complex capable of specifically docking secretory vesicles in the absence of calcium. CAPS or Munc13 coordinates the system to be primed for subsequent efficient calcium-triggered syt-dependent fusion. This work also demonstrated that PI(4,5)P₂ in the target membrane plays critical roles in both enhancing CAPS/Munc13-dependent priming and syt-dependent fusion and that the lipid context is of critical importance in defining the protein and lipid interactions that are physiologically relevant in exocytosis. Future work using this powerful system will certainly further refine the proposed model and permit further dissection of the specific interactions and interplays between the different protein modules and lipids that lead to calcium-triggered exocytosis.

MATERIALS AND METHODS

Materials

The following materials were purchased and used without further purification: porcine brain L-α-phosphatidylcholine (bPC), porcine brain L-α-phosphatidylethanolamine (bPE), porcine brain L-α-phosphatidylserine (bPS), L-α-phosphatidylinositol (liver, bovine) (PI), phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPC), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-DOPC) were from Avanti Polar Lipids; cholesterol, sodium cholate, EDTA, calcium (Ca²⁺), Opti-MEM I, L-glutamine, 10% dialyzed fetal bovine serum, streptomycin, and penicillin were from Life Technologies; and sodium arsenate, KCl, and sucrose were from Sigma; CHAPS and ANF were from Anatrace; Hepes was from Research Products International; chloroform, ethyl acetate, and Condrad detergent, all inorganic acids and bases, and hydrogen peroxide were from Fisher Scientific. Water was purified first with deionization and organic-free filters (Virginia Water Systems) and then with a NANOpure system from Barnstead to achieve a resistivity of 18.2 megohm/cm.

Antibodies for syt-1 (mouse monoclonal), syt-5/9 (rabbit polyclonal), CAPS-1 (rabbit polyclonal), CAPS-2 (rabbit polyclonal), complexin-1/2 (mouse monoclonal), Munc18 (mouse monoclonal), and Munc13 (mouse monoclonal) were all purchased from Synaptic Systems. The calnexin antibody (rabbit polyclonal) was from Enzo Life Sciences, the secretogranin II antibody (mouse monoclonal) was from Biodesign International, and the succinate ubiquinone antibody (mouse monoclonal) was from Molecular Probes.

Protein purification

Syntaxin-1a (both constructs of residues 183 to 288 and 1 to 288), wild-type SNAP-25, soluble synaptobrevin-2 (residues 1 to 96), Munc18, and complexin-1 from Rattus norvegicus were expressed in Escherichia coli strain BL21(DE3) cells under the control of the T7 promoter in the pET28a expression vector and purified as described previously (55–57). Briefly, all proteins were purified using the nickel–nitritoltriacetic acid (Ni-NTA) affinity chromatography. After the removal of N-terminal His-tags by thrombin cleavage, proteins were further purified by subsequent ion-exchange or size-exclusion chromatography, when necessary. Wild-type SNAP-25 was quadruply dodecylated through disulfide bonding of dodecyl methaneethanesulfonate (Toronto Research Company) to its four native cysteines (58). All SNAP-25 used in this work refers to this lipid–anchored form of SNAP-25. To express Munc13 in E. coli, DNA of Munc13 (C1C2BMUN, residues 529 to 1407; EF, residues 1453 to 1531) from R. norvegicus was cloned into pET28a vector through the use of restriction enzyme sites Nde I and Hind III. Munc13 expression was carried out in BL21(DE3) cells by IPTG (isopropyl-β-D-thiogalactopyranoside) induction. Harvested cell pellets were resuspended and then lysed in the extraction buffer [20 mM Hapes (pH 7.4), 500 mM NaCl, and 8 mM imidazole]. The supernatant of lysed cells was collected and subjected to binding of Ni-NTA beads. After extensive washing of protein-bound Ni-NTA beads with the wash buffer [20 mM Hapes (pH 7.4), 500 mM NaCl, 20 mM imidazole, and 10% glycerol], Munc13 was eluted from Ni-NTA with the elution buffer [20 mM Hapes (pH 7.4) and 400 mM imidazole]. After the His-tag cleavage by bovine thrombin, Munc13 was further purified by subsequent Mono Q ion-exchange chromatography. Purities of all proteins were verified by SDS-polyacrylamide gel electrophoresis (PAGE).

Reconstitution of SNAREs into proteoliposomes

Proteoliposomes with a lipid composition of bPC:bPE:bPS:Chol:PI:PI(4,5)P₂ (25:25:15:30:4:1) were prepared. All t-SNARE proteins were reconstituted using sodium cholate, as previously described (40, 59). The desired lipids were mixed, and organic solvents were evaporated under a stream of N₂ gas followed by vacuum desiccation for at least 1 hour. The dried lipid films were dissolved in 25 mM sodium cholate in a buffer [20 mM Hapes (pH 7.4) and 150 mM KCl] followed by the addition of an appropriate volume of syntaxin-1a and SNAP-25 in their respective detergents to reach a final lipid/protein ratio of 3000 for each protein. After 1 hour of equilibration at room temperature, the mixture was diluted below the critical micellar concentration by adding more buffer to the desired final volume. The sample was then dialyzed overnight against 1 liter of buffer, with one buffer change after ~4 hours.

Preparation of planar supported bilayers containing SNARE acceptor complexes

Planar supported bilayers with reconstituted plasma membrane SNAREs were prepared by the Langmuir–Blodgett/vesicle fusion technique as described in previous studies (60–62). Quartz slides were cleaned by dipping in sulfuric acid/hydrogen peroxide (3:1) for 15 min using a Teflon holder. Slides were then rinsed thoroughly in water. The first leaflet of the bilayer was prepared by Langmuir–Blodgett transfer directly onto the quartz slide...
using a Nima 611 Langmuir-Blodgett trough by applying the lipid mixture of bPC:Chol:DPS (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-PEG3400-triethoxysilane) (70:30:3) from a chloroform solution. After allowing the solvent to evaporate for 10 min, the monolayer was compressed at a rate of 10 cm²/min to reach a surface pressure of 31 mN/m. After equilibration for 5 to 10 min, a clean quartz slide was rapidly (200 mm/min) dipped into the trough and slowly (5 mm/min) withdrawn while a computer maintained a constant surface pressure and monitored the transfer of lipids with head groups down onto the hydrophilic substrate. Proteoliposomes reconstituted with 1:1 syntaxin-1a (residues 183 to 288 or 1 to 288);SNAP-25 at a lipid/protein ratio of 10% calf serum (Fe+) (HyClone), and 1% penicillin/streptomycin mix. Medium was changed every 2 to 3 days, and cells were passed after reaching 90% confluency by incubating 5 min in Hank’s balanced salt solution and replating in fresh medium. Cells were transfected by electroporation using ECM 830 Electro Square Porator (BTX). After harvesting and sedimentation, cells were suspended in a small volume of sterile cytomix electroporation buffer [120 mM KCl, 10 mM KH₂PO₄, 0.15 mM CaCl₂, 2 mM EGTA, 25 mM Hepes-KOH, 5 mM MgCl₂, 2 mM adenosine triphosphate, and 5 mM glutathione (pH 7.6)] and then counted and diluted to ~14 × 10⁶ cells/ml. Cell suspension (~10 × 10⁶ cells) (700 µl) and 30 µg of DNA were placed in an electroporation cuvette with a 4-mm gap, and two 255-V, 8-ms electroporations pulses were applied. Cells were then transfected to a 10-cm cell culture dish with 10 ml of normal growth medium. NPY-Ruby–transfected cells were cultured under normal conditions for 3 days after transfection and then used for fractionation. Transient knockdown cell lines were generated by transfecting shRNA as described above and maintaining cells under puromycin (2 µg/ml) selection beginning 24 hours after transfection. Cells were later transfected with NPY-Ruby and, when relevant, shRNA-resistant plasmids 3 days before fractionation.

DCV purification
DCVs were purified using an iso-osmotic medium as follows. PC12 cells (15 to 30 10-cm plates depending on experiments) were scraped into phosphate-buffered saline (PBS), pelleted by centrifugation, resuspended, and washed once in the homogenization medium (0.26 M sucrose, 5 mM Mops, and 0.2 mM EDTA). Following resuspension in 3 ml of medium containing protease inhibitor (Roche Diagnostics), the cells were cracked open using a ball bearing homogenizer with a 0.6368 cm bore and 0.6340 cm diameter ball. The homogenate was then spun at 4000 rpm (1000g) for 10 min at 4°C in fixed-angle microcentrifuge to pellet nuclei and larger debris. The postnuclear supernatant was collected and spun at 11,000 rpm (8000g) for 15 min at 4°C to pellet mitochondria. The PMS was then collected, adjusted to 5 mM EDTA, and incubated for 10 min on ice. A working solution of 50% OptiPrep (iodixanol) [five volumes of 60% OptiPrep (one volume of 0.26 M sucrose, 30 mM Mops, and 1 mM EDTA)] and homogenization medium were mixed to prepare solutions for discontinuous gradients in Beckman SW 55 tubes: 0.5 ml of 30% iodixanol on the bottom and 3.8 ml of 14.5% iodixanol, above which 1.2 ml of EDTA-adjusted PMS was layered. Samples were spun at 45,000 rpm (190,000gav) for 5 hours. A clear white band at the interface between the 30% iodixanol and the 14.5% iodixanol was collected as the DCV sample (fraction 9 of Western blot shown in Fig. 1A). The DCV sample was then extensively dialyzed in a cassette with 10,000-kDa molecular weight cutoff (24 to 48 hours, 3 × 5 liters) into the fusion assay buffer [120 mM potassium glutamate, 20 mM potassium acetate, and 20 mM Hepes (pH 7.4)].

TIRF microscopy
Experiments examining single-vesicle docking and fusion events were performed on a Zeiss Axiovert 35 fluorescence microscope (Carl Zeiss), equipped with a 63× water immersion objective (Zeiss; numerical aperture, 0.95) and a prism-based TIRF illumination. The light source was an OBIS 532 LS laser from Coherent Inc. Fluorescence was observed through a 610-nm band pass filter (D610/60, Chroma) by an electron-multiplying charge-coupled device (CCD) (DU-860E, Andor Technology). The electron-multiplying CCD (EMCCD) was cooled to −70°C.
and the gain was set between 200 and 220. The prism-quartz interface was lubricated with glycerol to allow easy translocation of the sample cell on the microscope stage. The beam was totally internally reflected at an angle of 72° from the surface normal, resulting in an evanescent wave that decays exponentially with a characteristic penetration depth of ~100 nm. An elliptical area of 250 μm × 65 μm was illuminated. The laser intensity, shutter, and camera were controlled by a homemade program written in LabVIEW (National Instruments).

Experiments triggering DCV fusion with calcium were done on a Zeiss Axiocvert 200 fluorescence microscope (Carl Zeiss), with objective and TIRF setups as described above. The light source was a 514-nm beamline of an argon ion laser (Innova 90C, Coherent), controlled through an acousto-optic modulator (Isomet), and a diode laser (Cube 640, Coherent) emitting light at 640 nm. The characteristic penetration depth was ~102 and ~130 nm for the 514- and 640-nm lasers, respectively. An OptoSplit (Andor Technology) was used to separate the fluorescence from the NPY-Ruby and Cy5 fluorescence. Fluorescence signals were recorded by an EMCCD (iXon DV887ESC-BV, Andor Technology). The EMCCD camera was cooled to ~70°C, and the electron gain factor was set between 200 and 240.

Single DCV docking and fusion assay
Acceptor t-SNARE proteins containing planar supported bilayers were washed with fusion buffer containing EDTA or divalent metal (Ca^{2+} or Mg^{2+}), as indicated in the text. They were then perfused with DCV (50 to 100 μl depending on preparation) diluted into 2 ml of fusion buffer [120 mM potassium glutamate, 20 mM potassium acetate, and 20 mM Hepes (pH 7.4)] with additions to buffer as indicated in the text. The fluorescence from DCVs was recorded with a 532-nm laser using an EMCCD camera gain of 200 to 220. After injection of the DCV sample, the microscope was focused within no more than 30 s and then 5000 images were taken with 200-ms exposure times and spaced directly to the hard drive. One spooling set was taken for each bilayer.

Single-vesicle fusion data were analyzed using a homemade program written in LabVIEW (National Instruments). Stacks of images were filtered by a moving average filter. The maximum intensity for each pixel over the whole stack was projected on a single image. Vesicles were located in this image by a single-particle detection algorithm described in by Kessling et al. (68). The peak (central pixel) and mean fluorescence intensities of a 5-pixel × 5-pixel area around each identified center of mass were plotted as a function of time for all particles in the image series. The exact time points of docking and fusion were determined from the central pixel similar to previous work (40). Cumulative distributions were determined from the time of docking to the time of fusion for individual fusion events, and the fusion efficiency was determined from the number of vesicles that underwent fusion compared with the total number of vesicles that docked within 15 s of DCV docking.

DCV docking was normalized from each DCV preparation by running two bilayers in 100 μM EDTA with syntaxin-1a (residues 183 to 288):SNAP-25 and using the average number of docked events in these experiments to normalize all other data collected for that preparation. Docking values from preparations of DCVs from wild-type, knockdown, and RNAi rescue cell lines cannot be directly compared to each other because normalizations were done within each preparation. However, trends of calcium acceleration are comparable. Experiments to inhibit proteins by antibody binding showed no major effect on docking in the absence of calcium, but an effect of syt or CAPS knockdown on docking in the absence of calcium cannot be completely ruled out.

Single DCV Ca^{2+}-triggered assay
Acceptor t-SNAREs containing planar supported bilayers were washed with fusion buffer containing 100 μM EDTA and then incubated for 30 min with 0.5 μM Munc18, 2 μM complexin-1, and 100 μM EDTA. The DCV sample was then perfused in while keeping the concentrations of Munc18 and complexin-1 constant. After 30 min, fusion buffer containing the indicated [Ca^{2+}] and 0.5 μM soluble Cy5 dye was injected, and fusion was monitored. The presence or absence of Munc18 and complexin-1 in the fusion buffer containing Ca^{2+} had no effect on the triggered fusion results.

Western blotting
Samples were separated by SDS-PAGE and transferred to nitrocellulose filters (Invitrogen); membranes were washed with PBS and blocked with Odyssey Blocking Buffer (PBS). Primary antibodies were incubated in Odyssey Blocking Buffer with 0.1% (v/v) Tween 20. The appropriate secondary antibody coupled with IRDye 800 was incubated in a dilution of 1 to 10,000 with the nitrocellulose filter. After extensive washing with PBS, 0.1% Tween 20 blots were imaged on an Odyssey Imaging System (LI-COR).

Ensemble DCV lipid mixing assay
Proteoliposomes containing t-SNAREs (syntaxin-1 and SNAP-25) with fluorescence resonance energy transfer–paired lipid probes (1.5% each of Rh-DOPE and NBD-DOPE) were incubated at 37°C in fusion buffer with either EDTA or divalent metal (Ca^{2+} or Mg^{2+}) at an indicated concentration and with any additional protein, as indicated in the text. After 10 min of incubation, fluorometer recording was started, DCVs (25 to 50 μl) were added, and NBD dequenching was monitored. At the end of each experiment, 0.1% Triton X-100 was added, and the maximum NBD dequenching was recorded and used to normalize each experiment.

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/7/e1603208/DC1

**Supplementary Text**

fig. S1. Line shape of intensity traces from single DCV fusion events.

fig. S2. Ensemble lipid mixing of DCVs with reconstituted proteoliposomes containing syntaxin-1a (residues 183 to 288) (lipid/protein = 3000), dodecylated SNAP-25 (lipid/protein = 3000), no protein, or syntaxin-1a (residues 183 to 288) and SNAP-25 and distinguish which of these conditions are present in the triggered fusion results.

fig. S3. Single DCV fusion response.

fig. S4. Quantification of shRNA-mediated knockdowns.

fig. S5. DCV fusion kinetics of synt and CAPS knockdowns.

fig. S6. Western blots of complexin-1/2, Munc18, and Munc13 in fractions generated during DCV purification.

fig. S7. Controls to determine the effects of Munc18 and complexin-1 on docking to full-length syntaxin-1a (residues 1 to 288) and SNAP-25 and distinguish which of these conditions are inhibited by the soluble domain of syntaxin-1a (residues 1 to 288) and SNAP-25 in the presence of 0.5 μM Munc18 in the absence of calcium, whereas there is no effect on DCV docking.

fig. S8. Control that Munc18 interacts with syntaxin-1a (residues 1 to 288) and SNAP-25 in the presence of 0.5 μM Munc18 with the inhibitor peptide.

fig. S9. Fusion probability as a function of calcium for fusion of DCVs that were docked to full-length syntaxin-1a (residues 1 to 288) and SNAP-25 in the presence of Munc18 and complexin-1 upon triggering with calcium.

table S1. Event statistics for DCV docking and fusion to planar supported bilayers with syntaxin-1a (residues 183 to 288):SNAP-25 (lipid/protein = 3000), syntaxin-1a (residues 183 to 288) (lipid/protein = 3000), dodecylated SNAP-25 (lipid/protein = 3000), no protein, or syntaxin-1a (residues 183 to 288):SNAP-25 (lipid/protein = 3000) incubated with 2 μM syntaxobrevin-2 (residues 1 to 96) inhibitor peptide.

table S2. Event statistics for DCV docking and fusion to planar supported bilayers with syntaxin-1a (residues 183 to 288):SNAP-25 (lipid/protein = 3000) with increasing concentration of divalent metals (Ca^{2+} and Mg^{2+}).

table S3. Fit values of a parallel reaction model [N(t) = N(t) − e^{−kt}m], where N is the fusion probability, k is the rate, and m is the number of parallel reactions occurring for the
cumulative distribution function of delay times between docking and fusion for single-particle DCV events with different concentrations of divalent metals. table S5. Event statistics for docking and fusion of DCVs to planar supported bilayers with 5% DCV events with different concentrations of divalent metals. table S18. Event statistics for DCV docking and fusion to planar supported bilayers containing DCV events for conditions described in Fig. 3. table S19. Event statistics for DCV docking and fusion to planar supported bilayers containing DCV events for conditions described in Fig. 2. table S5. Event statistics for docking and fusion of DCVs to planar supported bilayers containing DCV events for conditions described in Fig. 2. table S15. Event statistics for DCV docking and fusion to planar supported bilayers containing DCV events for conditions described in Fig. 3.

REFERENCES AND NOTES


Acknowledgments: We would like to thank C. Stoupe, D. Cafiso, P. Kasson, and S.-T. Yang for their suggestions and comments; C. Doyle and R. D’Sousa for their assistance with cell culture and creating shRNA-mediated knockdown cell lines; and A. Anantharam, T. Martin, and W. Almers for plasmid gifts that are acknowledged in Materials and Methods. Funding: This work was supported by NIH (grant P01 GM072694) to L.K.T. and R.J. Author contributions: A.J.B.K. designed and performed the research, analyzed the data, and wrote the article. V.K. designed the research, contributed analytical tools, and analyzed the data. B.L. performed the research and analyzed the data. P.S. performed the research and analyzed the data. S.I. developed a protein purification method, performed the research, and analyzed the data. R.J. designed a protein purification method and analyzed the data. J.D.C. designed the research, analyzed the data, and wrote the article. L.K.T. designed the research, analyzed the data, and wrote the article. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper was requested from the authors.

Submitted 16 December 2016
Accepted 15 June 2017
Published 19 July 2017
10.1126/sciadv.1603208

Reconstitution of calcium-mediated exocytosis of dense-core vesicles
Alex J. B. Kreutzberger, Volker Kiessling, Binyong Liang, Patrick Seelheim, Shrutee Jakhanwal, Reinhard Jahn, J. David Castle and Lukas K. Tamm

Sci Adv 3 (7), e1603208.
DOI: 10.1126/sciadv.1603208

ARTICLE TOOLS http://advances.sciencemag.org/content/3/7/e1603208
SUPPLEMENTARY MATERIALS http://advances.sciencemag.org/content/suppl/2017/07/17/3.7.e1603208.DC1
PERMISSIONS http://www.sciencemag.org/help/reprints-and-permissions

Use of this article is subject to the Terms of Service

Science Advances (ISSN 2375-2548) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. 2017 © The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. The title Science Advances is a registered trademark of AAAS.