Published in final edited form as:

Adv Enzyme Regul. 2010 ; 50(1): 62–70. doi:10.1016/j.advenzreg.2009.10.012.

Phosphatidylinositol 4,5-bisphosphate regulation of SNARE function in membrane fusion mediated by CAPS

Declan J. James, Chuenchanok Khodthong, Judith A. Kowalchyk, and Thomas F.J. Martin
Department of Biochemistry, University of Wisconsin, Madison, WI 53706

Introduction

The sequential transit of cargo in the secretory pathway depends upon membrane budding and membrane fusion reactions. The final delivery of cargo to the plasma membrane involves exocytic vesicle fusion, which occurs in several steps involving the tethering/docking of vesicles at the plasma membrane, priming events that enable fusion readiness, and the final fusion step. The final fusion step for Ca^{2+} -dependent vesicle exocytosis in neuroendocrine cells is mediated by topologically ordered SNARE protein interactions with VAMP2 on the vesicle zippered into heterodimer complexes of SNAP-25 and syntaxin-1 on the plasma membrane to form a twisted four helix bundle. A tightly assembled SNARE complex in *trans* across the bilayers promotes their close apposition and membrane merger (Malsam *et al.*, 2008). The Ca^{2+} -dependent triggering of SNARE-dependent fusion is mediated by several synaptotagmin isoforms on the vesicle that engage SNARE complexes and the plasma membrane. A key issue for understanding vesicle exocytosis is elucidating the pathway for SNARE protein assembly prior to triggered fusion.

The events responsible for vesicle tethering/docking at the plasma membrane are incompletely understood but appear to involve the plasma membrane SNAREs SNAP-25 and syntaxin-1 interacting with the vesicle proteins rabphilin and Slp4a, respectively (Verhage & Sorensen, 2008). Munc-18-1 also plays an essential role in the tethering/docking process presumably by

© 2009 Elsevier Ltd. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Major topics:

Phosphoinositides

PI(4,5)P₂

Membrane fusion

Vesicle exocytosis

SNARE proteins

Syntaxin

SNAP-25

VAMP

CAPS

Munc 13

PH domain

Liposomes

Proteoliposomes

Plasma membrane

Membrane microdomains

Ca^{2+} -dependent membrane fusion

Regulated vesicle exocytosis

interacting with syntaxin-1. Not all tethered/docked vesicles are competent for Ca^{2+} -triggered fusion in neuroendocrine cells (Wojcik & Brose, 2007). The ~10% of the plasma membrane-localized vesicles that fuse in response to Ca^{2+} elevations are referred to as the release ready pool of vesicles. The conversion of vesicles into this pool is considered to correspond to priming, a set of reactions that occurs after vesicle tethering/docking. Priming reactions are also responsible for maintaining secretion during prolonged stimulation following the depletion of release ready vesicle pools. It is unclear how SNAREs re-organize into *trans* SNARE complexes between the tethered/docked state and the primed state (release ready pool).

A number of lipids and proteins are essential for priming in neuroendocrine cells. Munc13-1, a multi-domain protein with C2, C1 and MH(munc13 homology) domains, was shown to be essential for the priming of synaptic vesicles in central synapses in mouse knockout studies where the phenotype was an intact docked pool of vesicles that failed to undergo Ca^{2+} -triggered exocytosis (Wojcik & Brose, 2007). Indirect measurements suggested that a release ready pool was absent. Under conditions of strong stimulation, the DAG-binding C1 domain of Munc13-1 is required, indicating a role for DAG in priming reactions. DAG may also function to activate protein kinase C isoforms, which have been implicated in the regulation of Munc18-1 function in priming. The possible mechanisms underlying Munc13-1 function in priming have been reviewed (Wojcik & Brose, 2007).

More recently, the roles of $\text{PI}(4,5)\text{P}_2$ and CAPS in vesicle priming have become evident. $\text{PI}(4,5)\text{P}_2$ was originally identified as essential for Ca^{2+} -triggered vesicle exocytosis in permeable neuroendocrine cells by the identification of phosphatidylinositol transfer protein (Hay & Martin, 1993) and $\text{PI}(4)\text{P}$ 5-kinase (Hay *et al.*, 1995) as components required for ATP-dependent priming reactions. More recent work established that $\text{PI}(4,5)\text{P}_2$ is required for the priming of vesicles into a release ready pool (Olsen *et al.*, 2003; Milosevic *et al.*, 2005) and for ongoing rates of stimulated secretion (Aikawa & Martin, 2003). A number of $\text{PI}(4,5)\text{P}_2$ -binding proteins have been proposed for mediating the essential role of this lipid in vesicle priming reactions including synaptotagmin, CAPS, rabphilin, Munc18-1, SCAMP2 and actin-binding proteins (Martin, 1998). Our recent studies (James *et al.*, 2008) reconstituted $\text{PI}(4,5)\text{P}_2$ function in a SNARE-dependent liposome fusion assay and identified CAPS as a $\text{PI}(4,5)\text{P}_2$ -dependent regulator of SNARE protein function.

CAPS is a multi-domain protein that contains C2, PH and MH domains. It was discovered as a brain cytosolic factor that reconstituted Ca^{2+} -dependent secretion from permeable neuroendocrine cells (Walent *et al.*, 1992). Studies in permeable cells found that the process restored by CAPS exhibited the kinetics of vesicle priming and was highly dependent upon the ongoing synthesis of $\text{PI}(4,5)\text{P}_2$ (Grishanin *et al.*, 2004). Recent mouse knockout studies confirmed the essential role of CAPS in vesicle priming in adrenal chromaffin cells and in central synapses (Jockusch *et al.*, 2007; Liu *et al.*, 2008). CAPS directly binds $\text{PI}(4,5)\text{P}_2$ and undergoes conformation changes, and we proposed that CAPS is an essential effector for $\text{PI}(4,5)\text{P}_2$ in regulated vesicle exocytosis that mediates the role of this lipid in priming. The studies presented here were directed at reconstituting $\text{PI}(4,5)\text{P}_2$ and CAPS function in the SNARE-dependent liposome fusion assay. These *in vitro* studies (James *et al.*, 2008; James *et al.*, 2009) provide important clues on the mechanisms by which $\text{PI}(4,5)\text{P}_2$ and CAPS function in vesicle exocytosis to organize SNARE complexes for fusion.

Materials and Methods

Detailed materials and methods may be found in (James *et al.*, 2008; James *et al.*, 2009).

Results

Sites of vesicle docking correspond to PI(4,5)P₂- and CAPS-containing microdomains

To determine the location and size of the PI(4,5)P₂ pools potentially required for regulated vesicle exocytosis, we incubated plasma membrane sheets prepared from PC12 cells with the monovalent PI(4,5)P₂-binding PLCδ1-PH-GFP protein (Fig. 1A–D). Strong punctuate labeling on the membrane corresponded to high concentration microdomains of PI(4,5)P₂ and not to membrane invaginations. Calibration of the fluorescent signal (Fig. 1E,F) indicated that microdomains consisting of ≥ 6 mol% PI(4,5)P₂ were present on the plasma membrane of these neuroendocrine cells. Triple co-localization studies revealed that ~50% of CAPS on these membrane sheets co-localized with PI(4,5)P₂-containing microdomains and docked dense-core vesicles (Fig. 1G,H). Overall ~20% of the docked vesicles co-localized to sites containing PI(4,5)P₂ and CAPS. Because PI(4,5)P₂ and CAPS are essential for priming vesicles, these vesicles may correspond to a release ready pool. Aoyagi et al. (Aoyagi *et al.*, 2005) provided evidence for preferential vesicle exocytosis at microdomains enriched in PI(4,5)P₂ and syntaxin-1. We suggest that ~20% of the vesicles that undergo preferential release may localize to microdomains enriched for PI(4,5)P₂ and CAPS.

Effect of PI(4,5)P₂ on SNARE-dependent liposome fusion

As an inverted cone-shaped lipid in the cytoplasmic leaflet, PI(4,5)P₂ would be anticipated to inhibit membrane fusion by antagonizing the high negative membrane curvature required for the formation of a hemifusion intermediate that transitions to full fusion (Chernomordik & Zimmerberg, 1995). In a SNARE-dependent liposome fusion assay, we found that concentrations of PI(4,5)P₂ in the range of 6 mol% strongly inhibited fusion (Fig. 2A,B). This appeared to correspond to the expected curvature effects of an inverted cone-shaped lipid because it was non-additive with the similar effects of lysoPC (Fig. 2C). As expected for this mechanism, similar inhibitory effects were observed with PI(3,4)P₂ but lesser inhibition was observed for PI(4)P (James *et al.*, 2008). In addition, similar inhibition was observed for inclusion of the PI(4,5)P₂ in either donor or acceptor liposomes (James *et al.*, 2008).

Syntaxin-1 and VAMP2 each contain juxtamembrane stretches of basic amino acids, which exhibit high affinity interactions with negatively charged PI(4,5)P₂. To examine the possible role of these interactions in the inhibition of fusion by PI(4,5)P₂, we generated K to A substitutions in the juxtamembrane domain of syntaxin-1. When tested in the liposome fusion assay, the K to A substitution mutants functioned normally in PC/PS liposomes (Fig. 2D). However, in liposomes containing PI(4,5)P₂, the K to A substitution mutants exhibited more strongly inhibited fusion. This suggests that syntaxin-1, via its basic membrane-proximal domain, counteracts the inhibitory effects of PI(4,5)P₂. A ring of SNARE complexes during fusion could serve to sequester PI(4,5)P₂ away from the central fusion site.

What accounts for the essential role of PI(4,5)P₂ in vesicle exocytosis?

The preceding results on the inhibition of fusion by PI(4,5)P₂ in reconstituted liposome fusion fail to account for the well-characterized positive essential role of PI(4,5)P₂ in regulated vesicle exocytosis (Hay *et al.*, 1995). We considered the possibility that additional factors missing from the minimal fusion assay may be needed to reconstitute this positive role of PI(4,5)P₂. We found that CAPS addition to the minimal system dramatically accelerated rates of fusion (Fig. 3A). CAPS stimulation of fusion was highly dependent on PI(4,5)P₂, exhibiting maximal effects at 5 mol% (Fig. 3B). In addition, the strongly synergistic effect of CAPS and PI(4,5)P₂ was only evident when PI(4,5)P₂ was present in the SNAP-25/syntaxin-1-containing acceptor liposomes (Fig. 3C). Lastly, mutations in the PH domain of CAPS that confer loss of PI(4,5)P₂ binding were found to eliminate the ability of CAPS to stimulate SNARE-dependent fusion (Fig. 3D). This modified SNARE-dependent liposome fusion assay successfully

reconstitutes many of the features that were defined by the permeable PC12 cell assay for vesicle priming (Grishanin *et al.*, 2004; James *et al.*, 2008). Both exhibit a strong dependence on PI(4,5)P₂ and CAPS. These results indicate that CAPS functions as an effector for PI(4,5)P₂ in its positive essential role for vesicle exocytosis.

CAPS stimulates trans SNARE complex formation on liposomes

It was unlikely that CAPS stimulated SNARE-dependent liposome fusion solely through interactions with PI(4,5)P₂. A CAPS PH domain fusion protein not only failed to stimulate SNARE-dependent fusion but prevented CAPS from doing so (James *et al.*, 2008). In addition, full length CAPS did not promote aggregation of the PI(4,5)P₂, SNARE-containing liposomes, which excluded an aggregation mechanism for the increased membrane fusion stimulated by CAPS. The asymmetric requirement for PI(4,5)P₂ in the SNAP-25/syntaxin-1-containing acceptor liposomes for CAPS stimulation suggested that CAPS is preferentially recruited to these membranes and interacts directly with the SNARE proteins. Studies of CAPS binding to SNARE-containing liposomes were undertaken (James *et al.*, 2009) and revealed the ability of CAPS to directly interact with membrane-integrated syntaxin-1 (Fig. 4A–D). Previous attempts to demonstrate CAPS interactions with SNARE proteins were unsuccessful because truncated soluble SNARE proteins were utilized. Our current work indicates that CAPS exhibits high affinity interactions with membrane-integrated SNAREs because of concomitant CAPS-SNARE and CAPS-membrane binding. Extensive characterization of the SNARE-binding properties of CAPS and that of its MH domain will be described elsewhere (N. Daily *et al.*, in preparation; C. Khodthong *et al.*, in preparation).

Fusion is mediated through the formation of heterotrimeric *trans* SNARE complexes across the membrane bilayers. Heterotrimeric *cis* SNARE complexes have been characterized as highly stable and can be detected by SDS-PAGE. While it is not known whether *trans* SNARE complexes possess the same intrinsic thermostability as *cis* complexes, *trans* complexes would collapse into *cis* complexes at the time of liposome solubilization in detergent. We analyzed liposome fusion reactions by SDS-PAGE and found that CAPS strongly stimulated the formation of ~165kD complexes that contained each of the three SNAREs (shown for syntaxin-1 in Fig. 4E). These complexes likely represented the accumulation of *cis* SNARE complexes as a consequence of SNARE-dependent fusion. However, we found that blocking fusion with lysoPC (Fig. 4F) or by holding reactions at 0–4°C (not shown) where fusion fails to occur, reduced but did not eliminate CAPS-dependent formation of ~165kD SNARE complexes. The characteristics of these SNARE complexes, and independent confirmation of their formation (James *et al.*, 2009), indicated that CAPS stimulates the formation of *trans* SNARE complexes on the liposomes, which is consistent with the CAPS stimulation of fusion that ensues under permissive conditions. It is noteworthy that SNARE complex formation promoted by CAPS is dependent on PI(4,5)P₂ and syntaxin-1 binding by CAPS (James *et al.*, 2008; James *et al.*, 2009). For the latter, we demonstrated that a C-terminal portion of CAPS, which contains a Munc-13 homology domain (MHD-1), competed for CAPS binding to t-SNAREs and inhibited CAPS-stimulated SNARE-dependent fusion. In summary, we find that CAPS requires both PI(4,5)P₂ binding and SNARE interactions for efficient stimulation of SNARE-dependent membrane fusion.

Discussion

Herein we summarized a number of key experiments that motivate a model (Fig. 5) for describing the roles of PI(4,5)P₂ and CAPS in priming vesicles for exocytosis. Local high concentrations of PI(4,5)P₂ on the plasma membrane, which we measure as ≥ 6 mol% (James *et al.*, 2008), may represent microdomains for the organization of vesicle fusion sites, as also proposed by Aoyagi *et al.* (Aoyagi *et al.*, 2005). Strong interactions between PI(4,5)P₂ and the

basic linker domain of syntaxin-1 may recruit SNAP-25/syntaxin-1 heterodimers to specific sites on the plasma membrane. PI(4,5)P₂ at 1–5 mol% was recently shown to promote disassembly of syntaxin-1 homo-oligomers (Murray & Tamm, 2009), which may drive SNAP-25/syntaxin-1 associations. The high local concentrations of PI(4,5)P₂ might serve to inhibit spontaneous vesicle fusion prior to Ca²⁺-triggered exocytosis. However, neutralization of the basic linker domain of syntaxin-1 was found to enhance the inhibitory effects of PI(4,5)P₂ on fusion (James *et al.*, 2008), which suggests that syntaxin-1-mediated sequestration counteracts the inhibitory effects of PI(4,5)P₂. It is noteworthy that neutralization of the basic linker domain of syntaxin-1 was recently reported to compromise stimulated secretion in PC12 cells (Lam *et al.*, 2008).

A key feature of the model (Fig. 5) envisions a major effector role for CAPS in the essential functions of PI(4,5)P₂ in regulated exocytosis. Previous studies indicated that CAPS activity in promoting vesicle exocytosis was PI(4,5)P₂-dependent and that CAPS was recruited to the plasma membrane in part through interactions of its PH domain with PI(4,5)P₂ (Grishanin *et al.*, 2002; Grishanin *et al.*, 2004). The current work showed that CAPS was effective in promoting SNARE-dependent liposome fusion only when PI(4,5)P₂ was included in the plasma membrane-like, SNAP-25/syntaxin-1-containing acceptor liposomes. Consistent with this, mutagenesis of the CAPS PH domain was found to abrogate its stimulation of SNARE-dependent fusion (James *et al.*, 2008). Thus, a central role for PI(4,5)P₂ in vesicle fusion may be to recruit effectors like CAPS to localized fusion sites on the plasma membrane.

The effector function for CAPS in fusion resides outside of its PH domain and involves direct SNARE binding (James *et al.*, 2009). Here we describe the syntaxin-1-binding properties of CAPS and the ability of CAPS to promote *trans* SNARE complex formation on the liposomes (James *et al.*, 2009). It is noteworthy that CAPS-SNARE interactions do not absolutely require PI(4,5)P₂ but do require membrane. However, CAPS stimulation of *trans* SNARE complex formation and of SNARE-dependent fusion do require PI(4,5)P₂. While the precise details of how CAPS promotes *trans* SNARE complex assembly are under investigation, it seems clear that CAPS is an effector protein that couples membrane PI(4,5)P₂ interactions and SNARE interactions into the productive reassembly of SNARE proteins for priming vesicle exocytosis.

Summary

Ca²⁺-triggered vesicle exocytosis in neuro endocrine cells requires priming reactions that follow vesicle tethering/docking and precede triggered fusion. Priming requires PI(4,5)P₂ and priming factors, and likely involves SNARE protein complex assembly. In studies with proteoliposomes, the priming factor CAPS interacts with PI(4,5)P₂, binds the SNARE protein syntaxin-1, promotes *trans* SNARE complex formation, and stimulates PI(4,5)P₂- and SNARE-dependent liposome fusion. We propose that CAPS functions in priming vesicle exocytosis by coupling membrane binding to SNARE complex assembly.

Acknowledgments

This work was supported by an NIH grant (DK40428) to T.F.J.M. and by an AHA fellowship to D.J.J.

References

- Aikawa Y, Martin TF. ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5) bisphosphate required for regulated exocytosis. *J Cell Biol* 2003;162:647–659. [PubMed: 12925709]
- Aoyagi K, Sugaya T, Umeda M, Yamamoto S, Terakawa S, Takahashi M. The activation of exocytotic sites by the formation of phosphatidylinositol 4,5-bisphosphate microdomains at syntaxin clusters. *J Biol Chem* 2005;280:17346–17352. [PubMed: 15741173]

- Chernomordik LV, Zimmerberg J. Bending membranes to the task: structural intermediates in bilayer fusion. *Curr Opin Struct Biol* 1995;5:541–547. [PubMed: 8528771]
- Grishanin RN, Klenchin VA, Loyet KM, Kowalchuk JA, Ann K, Martin TF. Membrane association domains in Ca^{2+} -dependent activator protein for secretion mediate plasma membrane and dense-core vesicle binding required for Ca^{2+} -dependent exocytosis. *J Biol Chem* 2002;277:22025–22034. [PubMed: 11927595]
- Grishanin RN, Kowalchuk JA, Klenchin VA, Ann K, Earles CA, Chapman ER, Gerona RR, Martin TF. CAPS acts at a pre-fusion step in dense-core vesicle exocytosis as a PIP₂ binding protein. *Neuron* 2004;43:551–562. [PubMed: 15312653]
- Hay JC, Fiset PL, Jenkins GH, Fukami K, Takenawa T, Anderson RA, Martin TF. ATP-dependent inositide phosphorylation required for Ca^{2+} -activated secretion. *Nature* 1995;374:173–177. [PubMed: 7877690]
- Hay JC, Martin TF. Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca^{2+} -activated secretion. *Nature* 1993;366:572–575. [PubMed: 8255295]
- James DJ, Khodthong C, Kowalchuk JA, Martin TF. Phosphatidylinositol 4,5-bisphosphate regulates SNARE-dependent membrane fusion. *J Cell Biol* 2008;182:355–366. [PubMed: 18644890]
- James DJ, Kowalchuk JA, Daily N, Petrie M, Martin TFJ. CAPS drives *trans* SNARE complex formation and membrane fusion through syntaxin interactions. *Proc Natl Acad Sci U S A*. 2009 in press.
- Jockusch WJ, Speidel D, Sigler A, Sorensen JB, Varoqueaux F, Rhee JS, Brose N. CAPS-1 and CAPS-2 are essential synaptic vesicle priming proteins. *Cell* 2007;131:796–808. [PubMed: 18022372]
- Lam AD, Tryoen-Toth P, Tsai B, Vitale N, Stuenkel EL. SNARE-catalyzed fusion events are regulated by Syntaxin1A-lipid interactions. *Mol Biol Cell* 2008;19:485–497. [PubMed: 18003982]
- Liu Y, Schirra C, Stevens DR, Matti U, Speidel D, Hof D, Bruns D, Brose N, Rettig J. CAPS facilitates filling of the rapidly releasable pool of large dense-core vesicles. *J Neurosci* 2008;28:5594–5601. [PubMed: 18495893]
- Malsam J, Kreye S, Sollner TH. Membrane fusion: SNAREs and regulation. *Cell Mol Life Sci* 2008;65:2814–2832. [PubMed: 18726177]
- Martin TF. Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. *Annu Rev Cell Dev Biol* 1998;14:231–264. [PubMed: 9891784]
- Milosevic I, Sorensen JB, Lang T, Krauss M, Nagy G, Haucke V, Jahn R, Neher E. Plasmalemmal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells. *J Neurosci* 2005;25:2557–2565. [PubMed: 15758165]
- Murray DH, Tamm LK. Clustering of syntaxin-1A in model membranes is modulated by phosphatidylinositol 4,5-bisphosphate and cholesterol. *Biochemistry* 2009;48:4617–4625. [PubMed: 19364135]
- Olsen HL, Hoy M, Zhang W, Bertorello AM, Bokvist K, Capito K, Efanov AM, Meister B, Thams P, Yang SN, Rorsman P, Berggren PO, Gromada J. Phosphatidylinositol 4-kinase serves as a metabolic sensor and regulates priming of secretory granules in pancreatic beta cells. *Proc Natl Acad Sci U S A* 2003;100:5187–5192. [PubMed: 12700357]
- Verhage M, Sorensen JB. Vesicle docking in regulated exocytosis. *Traffic* 2008;9:1414–1424. [PubMed: 18445120]
- Walent JH, Porter BW, Martin TF. A novel 145 kd brain cytosolic protein reconstitutes Ca^{2+} -regulated secretion in permeable neuroendocrine cells. *Cell* 1992;70:765–775. [PubMed: 1516133]
- Wojcik SM, Brose N. Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter. *Neuron* 2007;55:11–24. [PubMed: 17610814]

Fig. 1.

Microdomains of PI 4,5-P₂ on the plasma membrane. (A–D) PC12 cell membrane sheets were incubated with 2 μ M PLC δ 1-PH-GFP fusion protein and 100 μ M FM4-64 to image PI 4,5-P₂ (A and B) or membrane area (C and D), respectively. (E,F) Quantification of membrane PI 4,5-P₂. Supported planar bilayers containing PI 4,5-P₂ and 0.1 mol% rhodamine-PE were incubated with 2 μ M PLC δ 1-PH-GFP fusion protein and imaged. Fluorescence intensity per pixel was plotted for each supported bilayer to generate a calibration curve (E). From calibration curves prepared in parallel, pixel intensity on PC12 cell membrane sheets (incubated with or without MgATP) was used to infer PI 4,5-P₂ concentrations in microdomains, between microdomains, and averaged across the membrane (F). Mean $\pm$ SE

values are shown for +MgATP (n=13) and -MgATP (n=5). (G,H) PI 4,5-P₂ microdomains overlap with clusters of CAPS and docked vesicles. PI 4,5-P₂ microdomains in PC12 cell membrane sheets were localized by incubation with PLCδ1-PH-GFP and fixed for immunolocalization with CAPS polyclonal or chromogranin B monoclonal antibodies (DCVs) (G). Triple channel co-localizations were quantified (H). Mean ± SE (n=9) values are shown. Adapted from (James *et al.*, 2008).

Fig. 2.

PI 4,5-P₂ inhibits SNARE-dependent liposome fusion. (A) Acceptor liposomes with 40 copies of syntaxin-1/SNAP-25 (t-SNARE) in 90:10 mol% PC:PI 4,5-P₂ (open squares) or 85:15 mol % PC:PS (closed circles) were mixed with fluorescent PC:PS donor liposomes with 100 copies of VAMP-2. Parallel reactions with protein-free acceptor liposomes at 90:10 mol% PC:PI 4,5-P₂ (open circles) or 85:15 mol% PC:PS (stars) were conducted. The kinetics of fusion are shown as a percentage of total NBD fluorescence following correction for protein-free liposomes. Points represent mean values $\pm$ SE for five independent experiments. (B) Acceptor liposomes reconstituted with syntaxin-1/SNAP-25 and the indicated mol% PI 4,5-P₂ were mixed with donor liposomes at a 9:1 ratio ($\sim$ 2 mM lipid) and NBD fluorescence was monitored.

The extent of fusion at 120 min is expressed as % of fusion relative to that observed using acceptor liposomes with 85:15 mol% PC:PS. Bars indicate mean values $\pm$ SE for three independent experiments (* corresponds to $p < 0.01$). (C) Inhibition of fusion by inverted cone lipid LPC (lysophosphatidylcholine). Acceptor liposomes with 40 copies of syntaxin-1/ SNAP-25 in 85:15 mol% PC:PS or 90:5 mol% PC:PI 4,5- P_2 as indicated were incubated 5 min at 30° without (open bars) or with (closed bars) 0.2 mM LPC and mixed with donor liposomes to monitor fusion kinetics. Values (mean $\pm$ SE; n=6) of % maximal NBD fluorescence at 120 min incubation times are shown (** corresponds to $p < 0.001$ compared to untreated PC/PS liposomes). (D) The extent of liposome fusion at 120 min is shown for reactions containing donor liposomes mixed with acceptor liposomes containing wild-type or mutant (K252,253A, K264,265A and K252,253,264,265A) syntaxins in 85:15 mol% PC:PS or 90:10 mol% PC:PI 4,5- P_2 as indicated. Extent of fusion is shown as % relative to wild-type syntaxin in PC/PS liposomes (** corresponds to $p < 0.001$ compared to WT syntaxin containing PC/PS liposomes). Adapted from (James *et al.*, 2008).

Fig. 3.

CAPS accelerates liposome fusion in a PI 4,5-P₂- and SNARE-dependent manner. (A) Acceptor liposomes with 40 copies of syntaxin-1/SNAP-25 (indicated by t for t-SNAREs) in 90:10 mol% PC:PI 4,5-P₂ were incubated with donor liposomes with 100 copies of VAMP-2 in 85:15 mol% PC:PS in the absence (open circles) or presence (closed circles) of 1 μ M CAPS. Similar incubations with donor and acceptor liposomes in 85:15 mol% PC:PS in the absence (open squares) or presence (closed squares) of 1 μ M CAPS were conducted. NBD fluorescence in parallel reactions with protein-free liposomes was used to correct all data. Mean values $\pm$ SE for five independent experiments are shown. (B) Rate constants were determined for fusion reactions without (open circles) or with (closed circles) CAPS (1 μ M) in incubations that contained syntaxin-1/SNAP-25 acceptor liposomes with the indicated mol% PI 4,5-P₂. Mean $\pm$ SE values are shown for four independent experiments. (C) Extent of fusion in 120 min was determined in the absence (open bars) or presence (closed bars) of 1 μ M CAPS with donor VAMP-2 or acceptor syntaxin-1/SNAP-25 liposomes that contained 85:15 mol% PC:PS or

90:10 mol% PC:PI 4,5-P₂ as indicated. Mean $\pm$ SE values are shown for three independent experiments (* corresponds to $p < 0.01$ for PC/PIP₂ compared to PC/PS; † corresponds to $p = 0.05$ compared to parallel incubations without CAPS). (D) The CAPS PH domain mutant R558D/K560E/K561E with impaired phosphoinositide binding fails to accelerate fusion. Fusion reactions with 10 mol% PI 4,5-P₂-containing acceptor liposomes were conducted in the absence (open circles) or presence of 1 μ M wild-type CAPS (closed circles) or mutant CAPS (CAPS PH^{DEE}, closed squares). Adapted from (James *et al.*, 2008).

Fig. 4.

CAPS binds syntaxin and stimulates SNARE complex formation. 1 μ M CAPS was incubated with PC/PS liposomes containing t-SNAREs (A), syntaxin-1 (B), or VAMP2 (C) or with protein-free liposomes (D) and bound CAPS (fractions 1–3) was separated from free CAPS (fractions 7,8) by Accudenz gradient centrifugation. Fractions were analyzed by immunoblotting for CAPS or syntaxin (Stx). Results representative of three experiments. (E) CAPS stimulates formation of a ~165 kDa SNARE complex. Incubations were assembled with v- or t-SNARE(5% PIP2) liposomes or with both in the absence or presence of 1 μ M CAPS and incubated for 30 min at 32°. One incubation (*) was boiled before SDS-PAGE. ~165 kDa complexes (arrowhead) were detected with syntaxin antibody. (F) Incubations were conducted

with VAMP2- and t-SNARE(5% PIP₂) liposomes for zero time (lanes 1, 4) or for 30 min at 32° (lanes 2, 3) or 4° (lanes 5, 6). 1 μ M CAPS was present as indicated (lanes 3, 6). ~165 kDa complexes (arrowhead) were detected with syntaxin antibody. Adapted from (James *et al.*, 2009).

Fig. 5. Model for CAPS function. Potential steps in priming reactions for vesicle exocytosis are shown involving assembly of *trans* SNARE complexes with SNAP-25, syntaxin-1 and VAMP2. CAPS is recruited to the plasma membrane by binding to PI(4,5)P₂ via its PH domain. Microdomains of PI(4,5)P₂ also contain syntaxin-1 (either alone or bound to SNAP-25). In its membrane-associated state, CAPS interacts with syntaxin-1 or with syntaxin-1/SNAP-25 heterodimers. CAPS is then capable of promoting *trans* SNARE complex assembly for fusion.