Extended synaptotagmins are Ca^{2+}-dependent lipid transfer proteins at membrane contact sites

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Organelles are in constant communication with each other through exchange of proteins (mediated by trafficking vesicles) and lipids [mediated by both trafficking vesicles and lipid transfer proteins (LTPs)]. It has long been known that vesicle trafficking can be tightly regulated by the second messenger Ca^{2+}, allowing membrane protein transport to be adjusted according to physiological demands. However, it remains unclear whether LTP-mediated lipid transport can also be regulated by Ca^{2+}. In this work, we show that extended synaptotagmins (E-Syts), poorly understood membrane proteins at endoplasmic reticulum–plasma membrane contact sites, are Ca^{2+}-dependent LTPs. Using both recombinant and endogenous mammalian proteins, we discovered that E-Syts transfer glycerophospholipids between membrane bilayers in the presence of Ca^{2+}. E-Syts use their lipid-accommodating synaptotagmin-like mitochondrial lipid binding protein (SMP) domains to transfer lipids. However, the SMP domains themselves cannot transport lipids unless the two membranes are tightly tethered by Ca^{2+}-bound C2 domains. Strikingly, the Ca^{2+}-regulated lipid transfer activity of E-Syts was fully recapitulated when the SMP domain was fused to the cytosolic domain of synaptotagmin-1, the Ca^{2+} sensor in synaptic vesicle fusion, indicating that a common mechanism of membrane tethering governs the Ca^{2+} regulation of lipid transfer and vesicle fusion. Finally, we showed that microsomal vesicles isolated from mammalian cells contained robust Ca^{2+}-dependent lipid transfer activities, which were mediated by E-Syts. These findings established E-Syts as a novel class of LTPs and showed that LTP-mediated lipid trafficking, like vesicular transport, can be subject to tight Ca^{2+} regulation.

lipid transfer | organelle | synaptotagmin | membrane contact sites

The endoplasmic reticulum (ER) is the primary site for the synthesis of proteins and lipids needed to maintain and propagate membrane-bound organelles (1). Proteins are transported from the ER to other organelles by small, sac-like trafficking vesicles, which shuttle between organelles through cycles of budding and fusion reactions (1, 2). Vesicles also carry lipids, but a substantial portion of intracellular lipid trafficking is mediated by lipid transfer proteins (LTPs) independent of vesicular transport (3). Operating at narrow membrane contact sites (MCSs) between organelles, LTPs extract lipids from one membrane and subsequently deliver them to another membrane (4, 5).

It has long been known that vesicle trafficking can be tightly regulated by the second messenger Ca^{2+} in certain pathways, allowing membrane protein flow to be adjusted according to physiological demands (6–8). One prominent example of Ca^{2+}-regulated vesicle trafficking is the release of neurotransmitters at the chemical synapses of neurons, which serves as the brain's major form of cell-to-cell communication (2). Neurotransmitters are released when synaptic vesicles fuse with the plasma membrane (PM), a process driven by the vesicle fusion engine SNAREs (soluble *N*-ethylmaleimide–sensitive factor attachment proteins) (9). The Ca^{2+} regulation of synaptic release is achieved by superimposing the Ca^{2+} sensor synaptotagmin-1 (Syt1) on the SNAREs (10, 11).

It remains unclear whether LTP-mediated lipid trafficking can also be subject to Ca^{2+} regulation. In this study, we showed that extended synaptotagmins (E-Syts), integral membrane proteins located at the contact sites between the ER and the PM (12–15), function as Ca^{2+}-regulated LTPs. E-Syts are conserved molecules that possess an N-terminal membrane anchor followed by a synaptotagmin-like mitochondrial lipid binding protein (SMP) domain and multiple C2 domains (*SI Appendix*, Fig. S1) (15–19). The SMP domain exhibits a β-barrel structure, which is common to the tubular lipid binding superfamily (20), and harbors lipids in its hydrophobic cavity (21–23). C2 domains are autonomously folded structures that bind to lipids and Ca^{2+} (12, 24, 25). E-Syts facilitate the tethering of the ER to the PM in the presence of Ca^{2+} (14, 15, 26, 27), but it remains unknown whether they perform other biological functions. Using reconstituted assays, we discovered that E-Syts nonselectively transfer glycerophospholipids between membrane bilayers in a Ca^{2+}-dependent manner. The lipid transfer function of E-Syts requires the SMP domain; however, the SMP domain alone is insufficient for lipid transport. Lipid transfer occurs only when the C2 domains of E-Syts bind to Ca^{2+}. Additional analysis indicated that Ca^{2+} promotes lipid transfer by tethering the two membrane bilayers, similar to the mechanism by which Syt1 regulates synaptic vesicle fusion. These findings establish E-Syts as a novel class of LTPs with activities that are dependent on Ca^{2+}. Thus, like vesicle transport, LTP-mediated lipid trafficking in the cell can also be subject to Ca^{2+} regulation, allowing lipid transport to be coupled to intracellular signaling.

Results

Like the synaptic vesicle fusion regulator Syt1, E-Syts possess an N-terminal membrane anchor and multiple C2 domains (*SI Appendix*, Fig. S1) (14), leading to the postulation that E-Syts may also regulate vesicle fusion (28). To examine the function of E-Syt1, we expressed and purified the entire cytosolic domain of

Significance

Lipid transfer proteins (LTPs) operating at membrane contact sites play fundamental roles in lipid homeostasis, organelle dynamics, and cell–environment interactions. Imbalances in LTPs are associated with a range of human diseases. For example, aberrant extended synaptotagmin (E-Syt) activities are implicated in neurological disorders. Our findings establish E-Syts as a novel class of LTPs with activities that are controlled by Ca^{2+}. We also show that lipid transport, like vesicle trafficking, can be subject to tight Ca^{2+} regulation, thus expanding the role of Ca^{2+} in intracellular membrane transport. These findings broaden our knowledge of lipid transport in the cell and set the stage for understanding the pathogenesis of LTP-associated diseases.

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E-Syt1 (referred to as E-Syt1 hereinafter) that contains the SMP and C2 domains. In a liposome coflotation assay, E-Syt1 bound to protein-free liposomes in the presence of Ca^{2+} (Fig. 1*A*). We then examined E-Syt1 activity in reconstituted lipid mixing and content mixing assays. Unexpectedly, we observed that E-Syt1 itself mediated an efficient level of lipid mixing in the presence of Ca^{2+} without requiring SNAREs or any other protein (Fig. 1 *B*–*D*). Robust lipid mixing was observed with Ca^{2+} concentration at or above 50 μM (*SI Appendix*, Fig. S2).

The ability of E-Syt1 to induce lipid mixing was a surprising finding, because none of other C2 domain-containing proteins were known to mediate liposome lipid mixing. For instance, no lipid mixing was observed when the synaptic regulator Syt1 was added to protein-free liposomes (Fig. 1 *C* and *D*). Interestingly, E-Syt1 was unable to drive the content mixing of liposomes (Fig. 1 *B*–*D*), in contrast to the ability of SNAREs to mediate both lipid mixing and content mixing of liposomes (Fig. 1 *C* and *D*). Because content mixing is an indicator of membrane fusion, these results show that E-Syt1 mediates liposome lipid mixing without driving membrane fusion.

The ability of E-Syt1 to drive lipid mixing but not membrane fusion raised the intriguing possibility that E-Syt1 functions as an LTP between membrane bilayers. Indeed, E-Syts are localized primarily to the ER–PM contact sites (14, 26, 29, 30), where lipid exchange is known to occur (5, 31–33). Next, we developed a fluorescent lipid transfer assay to directly examine whether E-Syt1 can transport lipids between membranes (Fig. 2*A*). We observed that E-Syt1 efficiently transferred NBD-labeled PE [*N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine] from donor liposomes to acceptor liposomes in the presence of Ca^{2+} (Fig. 2*A*). We estimated that each E-Syt1 protein was able to transfer at least 100 lipid molecules during the 15-min reaction. The strict Ca^{2+} dependence of E-Syt1 in lipid transfer correlates well with the lipid mixing data (Fig. 1 *C* and *D*). E-Syt1 also transported lipids from the inner leaflets of membrane bilayers, albeit with lower efficiency (*SI Appendix*, Fig. S3), suggesting that E-Syt1 may promote lipid flipping between membrane leaflets.

E-Syt1 also transferred NBD-labeled phosphatidylcholine (PC) and phosphatidylserine (PS) (Fig. 2*A*), indicating that it nonselectively transports glycerophospholipids. This finding is in agreement with the observation that the diacylglycerol portion of glycerophospholipids is accommodated within the SMP domains of E-Syts (21). In support of this notion, mutations in the lipid binding residues of the SMP domain strongly reduced the lipid transfer capacity of E-Syt1 (*SI Appendix*, Fig. S4). E-Syt1 also transferred cholesterol, but the transfer rate was lower than that of glycerophospholipids (*SI Appendix*, Fig. S5). To examine the directionality of E-Syt1–mediated lipid transport, we prepared His_6-tagged E-Syt1 cytosolic domain anchored to liposomes through binding to nickel-conjugated lipids (*SI Appendix*, Fig. S6*A*). Interestingly, comparable lipid mixing rates were observed in reactions with E-Syt1 anchored to either the donor or the acceptor liposomes (*SI Appendix*, Fig. S6 *B* and *C*). The location of E-Syt1—on either the donor or the acceptor liposomes—also did not affect the lipid exchange rate in lipid transfer assays (*SI Appendix*, Fig. S7). Thus, E-Syt1–mediated lipid transfer is bidirectional.

To rule out the possibility that NBD conjugation influences E-Syt1–mediated lipid transport, we next examined the transfer of unlabeled lipids between liposomes. Using high-performance TLC (HPTLC) (34, 35), we observed that unlabeled PE was transferred from the donor liposomes to the acceptor liposomes (Fig. 2*B*), similar to the results of the fluorescent lipid transfer assay (Fig. 2*A*). The identities of donor and acceptor liposomes remained intact during the lipid transfer reaction, and the liposomes were fully separated after Ca^{2+} removal (*SI Appendix*, Fig. S8). In addition, soluble contents or transmembrane proteins were not transferred from the donor to the acceptor liposomes during the reactions (*SI Appendix*, Fig. S9). These results further indicate that neither membrane fusion nor membrane leakage/lysis occurred during the reactions. The full separation of the donor and acceptor liposomes on Ca^{2+} removal suggests that no stable membrane–membrane association was formed (*SI Appendix*, Fig. S8). Nevertheless, it remains possible that the lipid exchange might result from the formation of a transient hemifusion intermediate. Together, the results of these lipid mixing, fluorescent lipid transfer, and HPTLC experiments clearly show that E-Syt1 functions as a Ca^{2+}-dependent LTP.

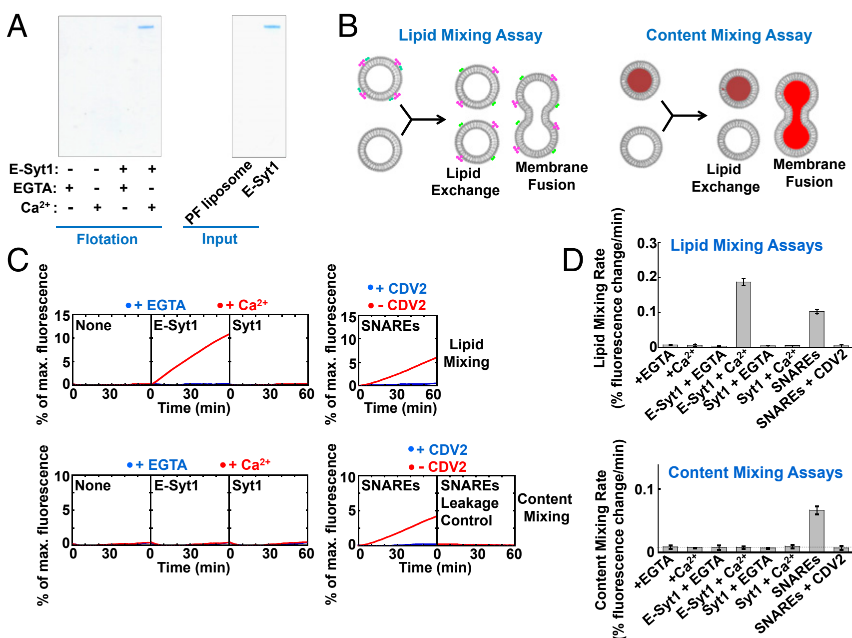

Fig. 1. E-Syt1 mediates the lipid mixing but not content mixing of liposomes in the presence of Ca^{2+}. (*A*, *Left*) Coomassie blue-stained SDS/PAGE gel showing the binding of E-Syt1 cytosolic domain (CD) to protein-free liposomes in the presence of 0.1 mM EGTA or $CaCl_2$. The liposomes contained 10% PS. (*A*, *Right*) Coomassie blue-stained gel showing the recombinant E-Syt1 CD protein. PF, protein free. (*B*) Diagrams showing the lipid mixing and content mixing assays that distinguish between lipid exchange and membrane fusion. In the lipid mixing assay, both lipid exchange and membrane fusion result in dequenching of NBD emission. In the content mixing assay, liposome fusion but not lipid exchange leads to dequenching of sulforhodamine B emission. (*C*, *Upper*) Lipid mixing and (*C*, *Lower*) content mixing of reconstituted liposomes mediated by E-Syt1 CD (1 μM), Syt1 CD (1 μM), or SNAREs. The SNARE-dependent fusion reactions were performed using t-SNARE (target membrane SNARE) liposomes containing syntaxin-1 and SNAP-25 and v-SNARE (vesicle SNARE) liposomes harboring VAMP2. The reactions mediated by E-Syt1 or Syt1 included 0.1 mM EGTA or $CaCl_2$. CDV2 indicates VAMP2 CD (amino acids 1–92) that was used as an inhibitor of SNARE assembly. In the leakage control reaction of SNARE-mediated membrane fusion, sulforhodamine B was included in both the v- and t-SNARE liposomes (48). Increases in sulforhodamine B fluorescence were not observed, indicating that no obvious content leakage occurred during these fusion reactions. (*D*) Rates of the lipid mixing and content mixing reactions shown in *C*. The dashed line indicates the background level of content mixing. Data are presented as percentages of fluorescence change per minute. Error bars indicate SD.

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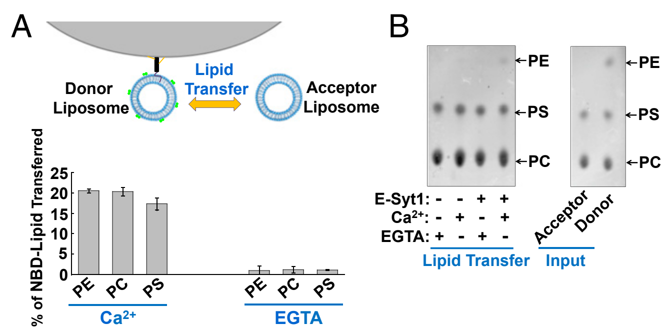

Fig. 2. E-Syt1 transfers lipids between membrane bilayers. (*A*, *Upper*) Diagram of the fluorescent lipid transfer assay. Fluorescence-labeled donor liposomes were anchored to agarose beads through the biotin–avidin conjugation. The lipid transfer reactions were carried out by incubation of bead-bound donor liposomes with unlabeled acceptor liposomes at 37 °C. The reactions were performed in the presence of 0.5 μM E-Syt1 cytosolic domain supplemented with 0.1 mM EGTA or $CaCl_2$. The donor and acceptor liposomes were subsequently separated by addition of EGTA. After centrifugation, NBD fluorescence in the resulting supernatant was measured. (*A*, *Lower*) The results of lipid transfer assays presented as the percentage of NBD-labeled phospholipids transferred from the donor liposomes to the acceptor liposomes. Error bars indicate SD. (*B*) Analysis of PE transfer using HPTLC. The lipid transfer reactions were performed as described in *A*, except that unlabeled PE was used. The acceptor liposomes contained 85% PC and 15% PS, whereas the donor liposomes contained 65% PC, 15% PS, and 20% PE. The plate was subsequently dried in vacuum and stained by 10% (wt/vol) $CuSO_4$ [in 8% (vol/vol) aqueous phosphoric acid]. After being charred at 140 °C for 3 min, the fluorescence of the plate was detected using an STORM scanner as previously described (34, 35).

The lipid transfer activity of E-Syt1 is strictly dependent on Ca^{2+}, a feature not found in previously characterized LTPs. The C2 domains of E-Syts possess Ca^{2+} binding sites and thus, likely serve as Ca^{2+} sensors in lipid transfer. The SMP domain of E-Syt1 alone was unable to mediate lipid transport, despite its capacity to accommodate lipids (Fig. 3), confirming the essential role of the C2 domains in Ca^{2+} regulation. Next, we determined which C2 domains confer the Ca^{2+} sensitivity of E-Syt1. Of five C2 domains of E-Syt1, only the C2A and C2C domains possess intact Ca^{2+} binding sites (12, 14). Previous structural and functional studies of Syt1 showed that Ca^{2+} binding is coordinated by several acidic residues of the C2 domains (36, 37). These acidic residues are conserved in the C2A and C2C domains of E-Syt1 (Fig. 3*A*). We observed that mutations of the Ca^{2+} binding sites in either the C2A or C2C domain abolished the lipid transfer activity of E-Syt1 (Fig. 3 *B*–*D*). Thus, the lipid transfer function of E-Syt1 requires Ca^{2+} binding to both the C2A and C2C domains. Deletion of the C2D and C2E domains, which are absent in E-Syt2 and E-Syt3 (*SI Appendix*, Fig. S1), had no effects on lipid transport (Fig. 3 *B*–*D*). Thus, the two C-terminal C2 domains are dispensable for the lipid transfer activity of E-Syt1. Additionally, the C2 domains of E-Syt1 must be physically linked to the SMP domain to achieve Ca^{2+} regulation. When the SMP domain and C2 domains of E-Syt1 were detached, the split fragments (ΔSMP + SMP) could no longer transfer lipids between liposomes (Fig. 3 *B*–*D*).

Next, we sought to delineate the mechanism by which Ca^{2+} regulates the lipid transfer activity of E-Syt1. In synaptic vesicle fusion, Ca^{2+} binding allows the C2 domains of Syt1 to tether the vesicle tightly to the PM, thus facilitating the zippering of the SNARE complex to drive membrane fusion (25, 38, 39). Here, membrane tethering is defined by the bridging or juxtaposition of two membrane bilayers mediated by C2 domains, distinct from the vesicle tethering in vesicular transport. We observed that E-Syt1 also promoted liposome tethering in a Ca^{2+}-dependent manner (Fig. 4*B*), and the liposomes fully dissociated after Ca^{2+} removal (*SI Appendix*, Fig. S8). Thus, the Ca^{2+} regulation of E-Syt1 may also require a membrane-tethering mechanism.

Deletion or mutations of the SMP domain did not impair the Ca^{2+}-dependent liposome-tethering activity of E-Syt1 (Fig. 4*B* and *SI Appendix*, Fig. S10), indicating that the membrane tethering is mediated solely by the C2 domains. In synaptic vesicle fusion, the C2B domain of Syt1 plays a major role in membrane tethering by binding to the PM *in trans* (38, 40). Among the five C2 domains of

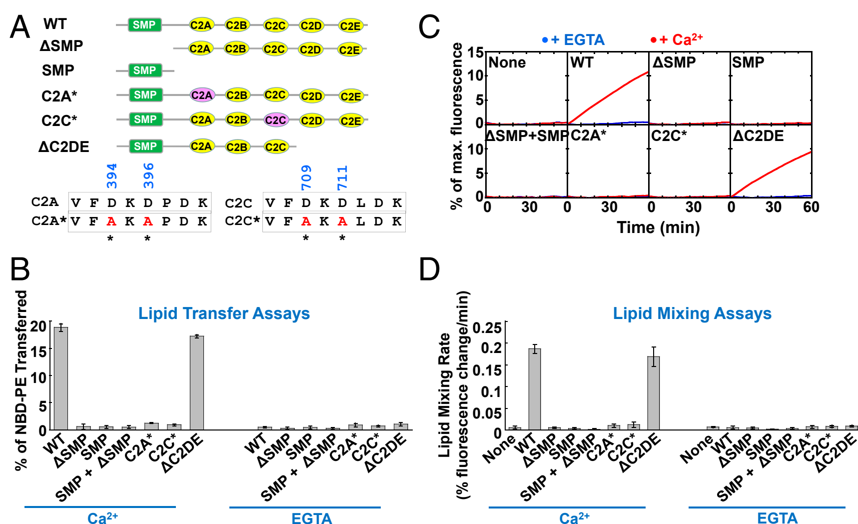

Fig. 3. The lipid transfer function of E-Syt1 requires the SMP domain and intact Ca^{2+} binding sites on both the C2A and C2C domains. (*A*, *Upper*) Diagrams of WT and mutant E-Syt1 proteins. WT C2 domains are shown in yellow, whereas mutant C2 domains are shown in pink. (*A*, *Lower*) Sequences showing the aspartic acid residues in the C2A and C2C domains of E-Syt1 that coordinate Ca^{2+} binding. Mutated residues are indicated, with residue numbers shown on the top and asterisks shown at the bottom. The SMP domain corresponds to amino acids 125–303 of E-Syt1. (*B*) Percentage of NBD-labeled PE transferred from the donor liposomes to the acceptor liposomes in the absence or presence of 0.5 μM E-Syt1 cytosolic domain (CD; WT or mutants). The lipid transfer reactions included 0.1 mM EGTA or $CaCl_2$ as described in Fig. 2*A*. Error bars indicate SD. Asterisks indicate C2 domain mutations depicted in *A*. (*C*) Lipid mixing of the protein-free liposomes in the absence or presence of 1 μM E-Syt1 CD (WT or mutants). The lipid mixing reactions included 0.1 mM EGTA or $CaCl_2$. (*D*) Rates of the lipid mixing reactions shown in *C*. Data are presented as percentages of fluorescence change per minute. Error bars indicate SD. Asterisks indicate C2 domain mutations depicted in *A*.

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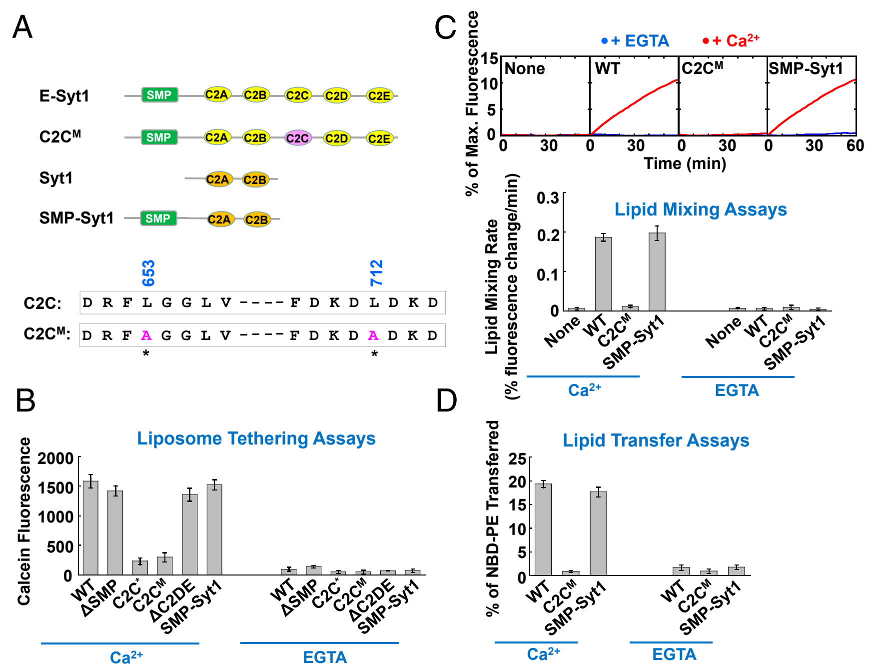

Fig. 4. Ca^{2+} stimulates the lipid transfer activity of E-Syt1 by promoting membrane tethering. (*A, Upper*) Diagrams of WT and mutant E-Syt1 proteins. WT C2 domains of E-Syt1 are shown in yellow whereas mutant C2 domains are shown in pink. The C2 domains of Syt1 are shown in orange. (*A, Lower*) Sequences of hydrophobic residues in the C2C domain of E-Syt1 that are predicted to embed in the lipid bilayer. Mutated residues are labeled with residue numbers on the top and asterisks at the bottom. (*B*) Effects of E-Syt1 mutations on liposome tethering in a tethering assay. Biotin-labeled liposomes were anchored to avidin beads and used to pull down calcein-loaded liposomes (an illustration is in *SI Appendix*, Fig. S8*A*). The tethering reactions proceeded for 15 min at room temperature in the absence or presence of 0.5 μM E-Syt1 cytosolic domain (CD; WT or mutants). The reactions included 0.1 mM EGTA or $CaCl_2$. The binding reaction without E-Syt1 CD was used to obtain the background level of fluorescence. The background fluorescence was subtracted from other binding reactions to obtain specific E-Syt1-dependent liposome tethering. The data are presented as calcein fluorescence intensity. Error bars indicate SD. Asterisks indicate C2 domain mutations depicted in Fig. 3*A*. (*C, Upper*) Lipid mixing of the acceptor and donor liposomes in the absence or presence of the indicated proteins (1 μM). The reactions included 0.1 mM EGTA or $CaCl_2$. (*C, Lower*) Rates of the lipid mixing reactions. Data are presented as percentages of fluorescence change per minute. Error bars indicate SD. (*D*) Percentage of NBD-labeled PE transferred from the donor liposomes to the acceptor liposomes. These fluorescent lipid transfer assays were performed as described in Fig. 2*A*. Error bars indicate SD.

E-Syt1, only the C2C domain resembles the C2B domain of Syt1 by possessing both Ca^{2+} binding sites and bulky hydrophobic residues that insert into membrane bilayers (Fig. 4*A*). Next, we sought to define the role of the C2C domain in the tethering function of E-Syt1. We observed that mutations in the Ca^{2+} binding sites of the C2C domain abrogated E-Syt1–mediated liposome tethering (Fig. 4*B*), consistent with the effects of the mutations on lipid transfer (Fig. 3). To further examine the functional role of the C2C domain in membrane tethering, we introduced mutations into its bulky hydrophobic residues to destabilize membrane association (Fig. 4*A*). Mutations in these hydrophobic residues severely impaired the ability of E-Syt1 to promote Ca^{2+}-dependent membrane tethering (Fig. 4*B*). Interestingly, these same C2C mutations also abrogated the lipid transfer function of E-Syt1 (Fig. 4 *C* and *D*). Together, these data suggest that Ca^{2+} stimulates the lipid transfer activity of E-Syt1 through C2 domain-mediated membrane tethering.

We reasoned that, if Ca^{2+} regulates E-Syt1 and Syt1 through the same membrane-tethering mechanism, the Ca^{2+}-dependent lipid transport activity of E-Syt1 should be recapitulated by replacing its C2 domains with those of Syt1. To test this possibility, we created a chimeric protein, in which the SMP domain of E-Syt1 was linked to the cytosolic domain of Syt1 (Fig. 4*A*). Strikingly, we observed that the SMP-Syt1 chimera mediated both liposome tethering and lipid transport in the presence of Ca^{2+} at levels comparable with those of E-Syt1–mediated reactions (Fig. 4 *B–D*). These results indicate that Syt1 can functionally substitute for the C2 domains of E-Syt1 in conferring the Ca^{2+} sensitivity of lipid transfer. Importantly, Syt1 is not involved in lipid trafficking and exhibits no sequence similarity with E-Syt1 beyond the C2 domains. These data strongly support that a common membrane-tethering mechanism governs the E-Syt–regulated lipid transport and Syt1-regulated vesicle fusion.

Finally, we tested whether endogenous E-Syt1 proteins can transfer lipids when anchored to native membranes. Microsomal vesicles were prepared from mouse preadipocytes and used as acceptor membranes in a cell-free lipid transfer assay (Fig. 5*A*). Microsomal vesicles, which harbor endogenous full-length E-Syt1 proteins (Fig. 5*B*), are functionally equivalent to ER membranes. Robust lipid mixing occurred between the microsomal vesicles and protein-free liposomes (Fig. 5*C*); however, no content mixing was observed (Fig. 5*D*). The lipid mixing was composed of both Ca^{2+}-dependent and -independent components (Fig. 5*C*), suggesting the existence of distinct lipid transfer activities in the microsomes. We next deleted the *Esyt1* gene from the preadipocytes using CRISPR/Cas9 genome editing and prepared microsomal vesicles from the mutant cells (Fig. 5*B*). We observed that the Ca^{2+}-dependent lipid mixing activity was abolished when the E-Syt1–deficient microsomes were used (Fig. 5*C*).

As expected, the Ca^{2+}-independent lipid mixing activity, which was likely driven by other membrane-tethered LTPs, was not affected by *Esyt1* KO (Fig. 5*C*). The Ca^{2+}-dependent lipid mixing was fully rescued by reexpression of the WT *Esyt1* gene but not the mutant gene encoding the SMP-deficient E-Syt1 (Fig. 5 *C* and *D*). The slight increase in Ca^{2+}-dependent lipid mixing observed in cells expressing the ΔSMP mutant was likely caused by the enhanced lipid transfer activities of non–E-Syt1 LTPs located at the ER–PM contact sites. Because the ΔSMP mutant mediates normal membrane tethering (Fig. 4*B*), it is expected to enhance the activities of LTPs that otherwise lack Ca^{2+} regulation.

Thus, the Ca^{2+}-dependent lipid transfer on the microsomal vesicles was mediated primarily by E-Syt1. The abrogation of Ca^{2+}-dependent lipid transfer in the E-Syt1 null microsomes suggests that E-Syt1 was the predominant E-Syt protein expressed in these cells. These data show that native E-Syt1 proteins function as Ca^{2+}-regulated LTPs, confirming the data obtained with recombinant proteins. These findings also indicate that microsomal vesicles isolated from mammalian cells naturally harbor robust Ca^{2+}-dependent lipid transfer activities and that these activities are mediated by E-Syts.

Discussion

The presence of lipid-accommodating SMP domains in E-Syts suggests a role in lipid trafficking; however, the SMP domain may engage in lipid binding/presenting rather than intermembrane lipid transport (3). It is also possible that E-Syts

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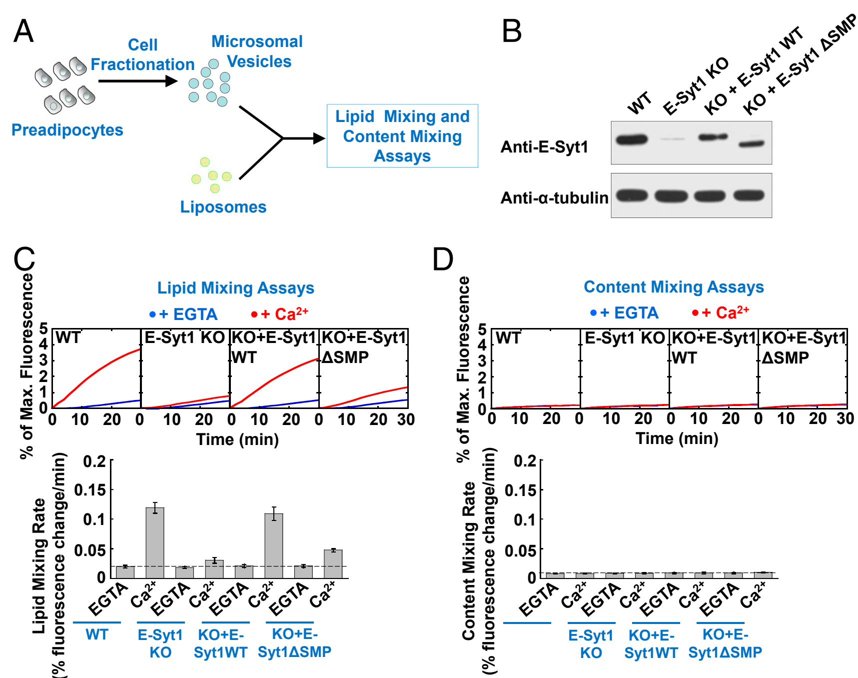

Fig. 5. Endogenous E-Syt1 proteins anchored to native membranes transfer lipids in a Ca^{2+}-dependent manner. (*A*) Diagram of the microsome–liposome lipid exchange assay using protein-free liposomes and microsomal vesicles isolated from preadipocytes. Microsomal vesicles purified from homogenized cell lysates were used as the acceptor vesicles. Protein-free liposomes labeled by NBD-PE and rhodamine-PE were used as donor liposomes for lipid mixing assays. Protein-free liposomes loaded with sulforhodamine B were used as donor liposomes for content mixing assays. (*B*) Western blots showing the expression of E-Syt1 in WT and mutant preadipocytes. The *Esyt1* gene was deleted from preadipocytes using CRISPR-Cas9 genome editing. WT E-Syt1 or a ΔSMP mutant was expressed in the E-Syt1 KO cells. (*C*, *Upper*) Lipid mixing between protein-free liposomes and microsomal vesicles isolated from the indicated preadipocytes. The reactions were carried out in the presence of 0.1 mM EGTA or $CaCl_2$. (*C*, *Lower*) Rates of the Ca^{2+}-dependent component of the lipid mixing. Data are presented as percentages of fluorescence change per minute. Error bars indicate SD. The dashed line demarcates the Ca^{2+}-independent (below) and -dependent (above) components of the lipid mixing. (*D*) Content mixing between protein-free liposomes and microsomal vesicles isolated from the indicated preadipocytes. The reactions were carried out in the presence of 0.1 mM EGTA or $CaCl_2$. Data are presented as percentages of fluorescence change per minute. Error bars indicate SD. The dashed line indicates the background level of content mixing.

constitute part of a lipid transfer machine that involves other proteins. Our findings, however, clearly showed that E-Syt1 is a minimal LTP that efficiently transports lipids between membrane bilayers. E-Syt2 and E-Syt3 exhibit similar domain structures as E-Syt1, except that they lack the C-terminal C2D and C2E domains (*SI Appendix*, Fig. S1). Our results showed that the C2D and C2E domains are dispensable for the lipid transfer function of E-Syt1, suggesting that E-Syt2 and E-Syt3 mediate lipid transport at MCS in a similar way as E-Syt1.

Unlike other LTPs that are constitutively active, the lipid transfer activity of E-Syts can be turned on or off according to the cytosolic Ca^{2+} concentration at the ER–PM contact sites. At resting states, the cytosolic Ca^{2+} concentration is usually below 100 nM (41), which is insufficient to trigger E-Syt–mediated lipid transport (*SI Appendix*, Fig. S2). Opening of Ca^{2+} channels on either the PM or the ER membrane results in elevation of cytosolic Ca^{2+} concentration. Cytosolic buffers, such as Ca^{2+} binding proteins, can rapidly diminish Ca^{2+}. However, the local cytosolic Ca^{2+} concentration near the PM–ER membranes can rise above 100 μM (41), which is sufficient to activate the lipid transfer function of E-Syts. The lipid transport activity of E-Syts is shut down after the cytosolic Ca^{2+} concentration returns to resting levels. Thus, like vesicle trafficking, LTP-mediated lipid transport can be regulated by Ca^{2+}. Our findings also established a previously unidentified function of C2 domains—regulation of lipid trafficking at MCS.

Although lipid transport and synaptic vesicle fusion are unrelated biological processes, both are regulated by Ca^{2+} through the same mechanism of membrane tethering. Ca^{2+} binding allows C2 domains to associate with the lipid bilayer to promote tight membrane tethering. In synaptic vesicle fusion, the C2B domain of the vesicle-anchored Syt1 protein promotes membrane tethering by binding to the PM *in trans*. In E-Syt1, the C2C domain seems to be functionally equivalent to the C2B domain of Syt1 in promoting membrane tethering. Despite the similarity in their membrane-tethering activities, the C2 domains of E-Syts and Syt1 may involve distinct membrane binding interfaces and/or configurations.

The lipid-accommodating SMP domains of E-Syts are unable to transfer lipids in the absence of Ca^{2+}, suggesting that they are constructed differently from the SMP domains of constitutively active LTPs. It is possible that the SMP domains of E-Syts are intrinsically inefficient in shuttling between membrane bilayers, such that they become active only when repositioned and/or restructured by C2 domain-induced membrane tethering. To couple membrane tethering to vesicle fusion or lipid trafficking, the C2 domains and the core functional domains must be located within close proximity. In vesicle fusion, this localization is achieved by the physical interaction between Syt1 and the membrane fusion machine SNAREs. By contrast, a single E-Syt molecule containing both the Ca^{2+}-sensing C2 domains and the lipid-accommodating SMP domain accomplishes Ca^{2+}-regulated lipid trafficking.

Genetic analysis of LTPs is often impeded by isoform redundancy and their restricted roles in highly specialized processes. Nevertheless, our data clearly show that microsomal vesicles isolated from mammalian cells contain robust Ca^{2+}-dependent lipid transfer activities and that E-Syts are responsible for these activities. In support of their critical roles in lipid homeostasis, KO of E-Syts in yeasts and plants compromises the integrity of the PM and

triggers cellular stress responses (15, 19, 26, 42–44). Because they are active only at elevated cytosolic Ca^{2+} concentration, it is unlikely that E-Syts play major roles in lipid flow during membrane expansion and organelle propagation. Instead, E-Syts are expected to mediate specialized processes that involve intermembrane lipid movements associated with Ca^{2+} signaling. The identification of E-Syts as a novel class of LTPs sets the stage for understanding their physiological roles and implications in human diseases (45–47).

Methods

In fluorescent lipid transfer assays, full-length VAMP2 was biotinylated by EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) and used to anchor donor liposomes to avidin beads. Donor liposomes reconstituted with biotinylated VAMP2 were prepared in a similar way as in the liposome lipid mixing assay, except that 2% (mol/mol) NBD-labeled lipids were included. NBD-labeled PE (catalog no. 81044C), NBD-labeled PC (catalog no. 810133C), and NBD-labeled PS (catalog no. 810198C) were all obtained from Avanti Polar Lipids. The biotin-labeled donor liposomes were incubated with avidin-conjugated agarose beads at room temperature for 1 h. After washing five times with the reconstitution buffer, the bead-bound donor liposomes were incubated with acceptor liposomes at 37 °C in the presence or absence of 0.5 μM E-Syt1. The reactions included 0.1 mM EGTA or $CaCl_2$. After incubation, EGTA was added to a final concentration of 0.5 mM to remove free Ca^{2+} ions. The avidin beads were recovered by centrifugation at 4,000 rpm for 2 min. NBD fluorescence in the supernatant was measured in a BioTek Microplate Reader. The lipid transfer data were presented as the percentage of NBD fluorescence in the supernatant to the original fluorescence of donor liposomes. The number of lipid molecules transported by an E-Syt1 protein within 15 min was estimated using the following formula: $n = (60\% \times N_{\mathrm{lipids}} \times P_{\mathrm{PC}} + 20\% \times N_{\mathrm{lipids}} \times P_{\mathrm{PE}} + 10\% \times N_{\mathrm{lipids}} \times P_{\mathrm{PS}} + 10\% \times N_{\mathrm{lipids}} \times P_{\mathrm{Cholesterol}})/N_{\mathrm{E\text{-}Syt1}}$. N_{lipids} represents the total lipids in each reaction (0.03 μmol), P represents the percentage of a lipid species transferred during the 15-min reaction, and $N_{\mathrm{E\text{-}Syt1}}$ represents the total E-Syt1 proteins in each reaction (50 pmol).

Additional methods are included in *SI Appendix, SI Methods*.

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