The N- and C-terminal Domains of Tomosyn Play Distinct Roles in Soluble N-Ethylmaleimide-sensitive Factor Attachment Protein Receptor Binding and Fusion Regulation

Background: Tomosyn regulates vesicle fusion but its mechanism remains incompletely understood.

Results: Tomosyn uses its C-terminal domain to arrest SNARE-dependent fusion reactions, whereas its N-terminal domain is required for syntaxin interaction.

Conclusion: We have uncovered distinct roles of the N- and C-terminal domains of tomosyn in SNARE binding and fusion regulation.

Significance: Our findings shed light upon vesicle transport in the cell.

Tomosyn negatively regulates SNARE-dependent exocytic pathways including insulin secretion, GLUT4 exocytosis, and neurotransmitter release. The molecular mechanism of tomosyn, however, has not been fully elucidated. Here, we reconstituted SNARE-dependent fusion reactions in vitro to recapitulate the tomosyn-regulated exocytic pathways. We then expressed and purified active full-length tomosyn and examined how it regulates the reconstituted SNARE-dependent fusion reactions. Using these defined fusion assays, we demonstrated that tomosyn negatively regulates SNARE-mediated membrane fusion by inhibiting the assembly of the ternary SNARE complex. Tomosyn recognizes the t-SNARE complex and prevents its pairing with the v-SNARE, therefore arresting the fusion reaction at a pre-docking stage. The inhibitory function of tomosyn is mediated by its C-terminal domain (CTD) that contains an R-SNARE-like motif, confirming previous studies carried out using truncated tomosyn fragments. Interestingly, the N-terminal domain (NTD) of tomosyn is critical (but not sufficient) to the binding of tomosyn to the syntaxin monomer, indicating that full-length tomosyn possesses unique features not found in the widely studied CTD fragment. Finally, we showed that the inhibitory function of tomosyn is dominant over the stimulatory activity of the Sec1/Munc18 protein in fusion. We suggest that tomosyn uses its CTD to arrest SNARE-dependent fusion reactions, whereas its NTD is required for the recruitment of tomosyn to vesicle fusion sites through syntaxin interaction.

Exocytosis, the fusion of exocytic vesicles with the plasma membrane, allows the cell to be in constant communication with the environment (1). The core engine of intracellular vesicle fusion is the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)2 (1–4). SNAREs are membrane-associated proteins localized to both the vesicle (v-SNAREs or R-SNAREs) and the target membrane (t-SNAREs or Q-SNAREs) (5–12). Membrane fusion is initiated when the v-SNARE pairs with the t-SNAREs to form a four-helix trans-SNARE complex (or SNAREpin). N- to C-terminal assembly of the trans-SNARE complex brings the two membranes into close proximity to fuse (13–16).

Exocytosis is the basis of a wide range of fundamental biological processes including neurotransmitter release, hormone secretion, and inside-outside movement of surface transporters and receptors (1). In particular, exocytosis plays crucial roles in maintaining blood glucose homeostasis in mammals. In response to elevated blood glucose levels (e.g. after a meal), the peptide hormone insulin is released from pancreatic beta cells through exocytosis. Insulin is then transported by the bloodstream to reach its target tissues including muscles and adipocytes (17–19). Binding of insulin to its receptor on target tissues activates a cascade of signaling events that ultimately relocate GLUT4 from intracellular storage vesicles to the cell surface. The relocation is achieved by the fusion of GLUT4-containing exocytic vesicles with the plasma membrane. Once on the cell surface, GLUT4 facilitates the uptake of excess blood glucose into the cells for disposal (20, 21). Although insulin secretion and GLUT4 exocytosis are regulated by distinct trigger signals, both pathways require syntaxin-4 and SNAP-23 as the t-SNAREs, and VAMP2/synaptobrevin as the primary v-SNARE (22–26). In insulin secretion, the vesicle fusion reaction also involves another t-SNARE complex, syntaxin-1 and SNAP-25 (25–27). Another widely studied exocytic pathway is the release of neurotransmitters at chemical synapses, which serves the major form of cell-cell communication in the brain (3). The neurotransmitter release requires the t-SNAREs syntaxin-1/SNAP-25 and the v-SNARE VAMP2.

This work was supported, in whole or in part, by National Institutes of Health Grant R01DK095367 (to J. S.). 1 Pew Scholar in the Biomedical Sciences. To whom correspondence should be addressed. Tel: 303-492-6166; Fax: 303-492-7744; E-mail: jingshi.shen@colorado.edu. 2 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; v-SNARE, vesicle SNARE; t-SNARE, target-SNARE; NTD, N-terminal domain; SM, Sec1/Munc18; MBP, maltose-binding protein; CTD, C-terminal domain; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-2-hydroxy-1-propanesulfonic acid; CD, cytoplasmic domain.
Distinct Roles of Tomosyn Domains

In addition to SNAREs, exocytosis also requires a group of SNARE-binding regulatory factors that control the temporal and spatial precision of the vesicle fusion reaction. One such regulatory factor is tomosyn, a 120–130-kDa protein that negatively regulates SNARE-dependent exocytosis (28–41). Originally identified as a syntaxin-binding molecule (42), tomosyn possesses two distinct domains, a large N-terminal domain containing WD40 repeats and a small C-terminal domain harboring an R-SNARE-like motif (see Fig. 6A for a schematic diagram). Mutations in tomosyn are implicated in type 2 diabetes (43).

It remains incompletely understood how tomosyn regulates SNARE-dependent exocytosis. Previous biochemical studies of tomosyn were performed mostly in static solution binding assays using truncated proteins. These truncated proteins, however, cannot recapitulate the physiological function of full-length (FL) tomosyn in exocytosis (44, 45). In this study, we expressed and purified active FL tomosyn and examined its molecular mechanism of action in reconstituted systems that recapitulate the vesicle fusion reactions of insulin secretion, GLUT4 exocytosis, and neurotransmitter release. We demonstrated that tomosyn negatively regulates SNARE-mediated membrane fusion by inhibiting the assembly of the ternary SNARE complex. Tomosyn recognizes the t-SNARE complex and prevents its pairing with v-SNARE, therefore arresting the fusion reaction at a pre-docking stage. The inhibitory function of tomosyn is dominant over the stimulatory activity of the Sec1/Munc18 (SM) protein. The inhibitory function of tomosyn is mediated by its CTD that contains an R-SNARE-like motif, confirming previous studies carried out using truncated fragments of tomosyn. Interestingly, the NTD of tomosyn is required (although not sufficient) for the binding of tomosyn to the syntaxin monomer, indicating that FL tomosyn presents unique features not found in the widely studied CTD fragment. These findings suggest that tomosyn uses its CTD to arrest SNARE-dependent membrane fusion, whereas the NTD is required for the recruitment of tomosyn to vesicle fusion sites through syntaxin interaction.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant t- and v-SNARE proteins were expressed in *Escherichia coli* and purified by affinity chromatography. The t-SNARE complexes, comprised of untagged syntaxin-4 and His<sub>6</sub>-tagged SNAP-23 or untagged syntaxin-1 and His<sub>6</sub>-tagged SNAP-25, were expressed as previously described (46–48). SNAREs were stored in a buffer containing 25 mM HEPES (pH 7.4), 400 mM KCl, 1% n-octyl-β-D-glucoside, 10% glycerol, and 0.5 mM Tris(2-carboxyethyl)phosphine.

Human tomosyn-1 gene (ATCC) was subcloned into a baculovirus transfer vector pFastBacMBP to generate a construct encoding a His<sub>6</sub>-MBP-tomosyn fusion protein separated by a tobacco etch virus protease cleavage site. The fusion protein was expressed in SF9 cells according to the manufacturer’s instruction (Bac-to-Bac Baculovirus Expression System, Invitrogen). Briefly, recombinant Bacmid DNA was obtained by transforming the pFastBac-MBP-tomosyn construct into DH10Bac cells. The Bacmid DNA was transfected into SF9 cells grown at 27 °C in SF900-III serum-free medium (Invitrogen). Recombinant viruses were harvested 4 days after transfection and further amplified twice to obtain viral stocks with the desired titer. At the density of 2 × 10<sup>6</sup> cells/ml, SF9 cells were infected with the recombinant virus at multiplicity of infection of 1. Cells were harvested 72 h after infection and cell pellets were stored at −80 °C. Tomosyn proteins were purified by both the nickel and amylose affinity chromatography. The His<sub>6</sub>-MBP tags were removed from tomosyn proteins by tobacco etch virus protease and the proteins were subsequently dialyzed overnight against a storage buffer (25 mM HEPES, pH 7.4, 150 mM KCl, 10% glycerol and 0.5 mM Tris(2-carboxyethyl)phosphine). The NTD of tomosyn (amino acids 1–1010) was expressed and purified using a procedure we previously described for Munc18-1 (46). Recombinant Munc18c protein was produced in SF9 cells as previously described (24, 48).

Reconstitution of Proteoliposomes—All lipids were obtained from Avanti Polar Lipids Inc. For t-SNARE reconstitution, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine, and cholesterol were mixed in a molar ratio of 60:20:10:10. For v-SNARE reconstitution, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine, cholesterol, (N-[7-nitro-2,1,3-benzoazadiazole-4-yl]-1,2-dipalmitoyl phosphatidyethanolamine (NBD-DPPE), and N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidyethanolamine (Rhodamine-DPPE) were mixed at a molar ratio of 60:17:10:10:5:5. SNARE proteoliposomes were prepared by detergent dilution and isolated on a Nycodenz density gradient flotation (49–51). Complete detergent removal was achieved by overnight dialysis of the samples in Novagen dialysis tubes against the reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 10% glycerol, and 1 mM DTT). To prepare sulforhodamine-loaded liposomes, t- or v-SNARE liposomes were reconstituted in the presence of 50 mM sulforhodamine B (Sigma). Free sulforhodamine B was removed by overnight dialysis followed by liposome flotation on a Nycodenz gradient. The protein lipid ratio was 1:200 for v-SNAREs and 1:500 for t-SNARE liposomes. To ensure the consistency in SNARE liposome preparations, we routinely monitored the sizes and morphologies of reconstituted liposomes using dynamic light scattering and electron microscopy.

FRET-based Lipid and Content Mixing Assays—A standard lipid mixing reaction contained 45 μl of unlabeled t-SNARE liposomes and 5 μl of v-SNARE liposomes labeled with NBD and rhodamine, and was conducted in a 96-well Nunc plate at 37 °C. Prior to fusion, NBD emission from the v-SNARE liposomes was quenched by neighboring rhodamine molecules through FRET. After fusion, the NBD dyes were diluted such that their emission was dequenched. Increase in NBD fluorescence at 538 nm (excitation 460 nm) was measured every 2 min in a BioTek Synergy HT microplate reader. At the end of the reaction, 10 μl of 10% CHAPSO was added to the liposomes. Fusion data were presented as the percentage of maximum fluorescence change. To assess their regulatory activities, 5 μM tomosyn was added to the fusion reaction. The maximum fusion rate within the first 10 min of the reaction was used to represent the initial rate of a fusion reaction. Full accounting of
Distinct Roles of Tomosyn Domains

RESULTS

Tomosyn Inhibits the Assembly of the Ternary SNARE Complex, but Not the Formation of the t-SNARE Complex—The formation of the binary t-SNARE complex is a key regulatory step in exocytic vesicle fusion (3, 4). Tomosyn was initially isolated as a syntaxin-binding molecule (42), but it remains unclear how the tomosyn-syntaxin dimer regulates SNARE assembly on the membrane. There are two tomosyn genes in mammals, tomosyn-1 and tomosyn-2 (54). Because the proteins encoded by these two tomosyn genes appear to have similar domain structures and biological functions, tomosyn-1 was used in this study and hereafter referred to as tomosyn.

Previous biochemical and biophysical studies mostly used truncated fragments of tomosyn. We found that FL tomosyn could not be expressed in E. coli. Therefore, we expressed and purified FL tomosyn in insect cells using baculovirus. Insect cell-expressed FL tomosyn proteins were pure and highly soluble (Fig. 1A). In a liposome co-flotation assay, we observed that tomosyn bound to proteoliposomes reconstituted with either syntaxin-4 or syntaxin-1 monomer (Fig. 1, B and C). Tomosyn did not bind to protein-free liposomes (Fig. 1, B and C), indicating that the tomosyn-syntaxin interaction was specific. When added as a soluble factor, SNAP-23 or SNAP-25 readily assembled with syntaxin-4 or syntaxin-1, respectively, to form the binary t-SNARE complex (Fig. 1, B and C). Unexpectedly, we found that tomosyn prevented neither the pairing of syntaxin-4 with SNAP-23 nor the pairing of syntaxin-1 with SNAP-25 (Fig. 1, B and C). Thus, tomosyn-associated syntaxin monomers are fully competent for t-SNARE complex formation.

After t-SNARE complex formation, the v-SNARE assembles with the t-SNAREs to form the ternary SNARE complex that forces the two membranes into close proximity to fuse (1). Next we tested whether and how tomosyn regulates the assembly of the ternary SNARE complex. The t-SNARE complex, composed of syntaxin-4/SNAP-23 or syntaxin-1/SNAP-25, was reconstituted into proteoliposomes (Fig. 2, A and B). Addition of VAMP2 cytoplasmic domain (CD) to the t-SNARE liposomes resulted in the formation of the ternary SNARE complex on the membrane (Fig. 2, A and B). We observed that tomosyn bound to the t-SNARE complex and blocked its pairing with VAMP2 CD, therefore preventing the assembly of the ternary SNARE complex (Fig. 2, A and B). When added to pre-formed ternary SNARE complexes on the liposome membrane, tomosyn did not displace VAMP2 CD from the t-SNAREs (Fig. 2, C and D), indicating that tomosyn inhibits SNARE assembly only prior to the full formation of the ternary SNARE complex. Together, these results demonstrate that tomosyn inhibits the ternary SNARE complex and inhibits the assembly of the ternary SNARE complex.

Tomosyn Negatively Regulates the SNARE-mediated Membrane Fusion Reaction—Next we sought to determine how tomosyn regulates the dynamic SNARE-dependent membrane fusion reaction. Proteoliposomes were reconstituted using SNAREs involved in insulin secretion, GLUT4 exocytosis, and neurotransmitter release, syntaxin-1/SNAP-25/VAMP2 or syntaxin-4/SNAP-23/VAMP2 (Fig. 3A). Fusion of the v- and t-SNARE liposomes was first monitored by a fluorescence/ Förster resonance energy transfer (FRET)-based lipid mixing assay. We observed that the exocytic SNAREs drove efficient levels of lipid mixing (Fig. 3, B and C). When FL tomosyn was added, the SNARE-mediated lipid mixing reactions were reduced to background levels (Fig. 3, B and C). The inhibitory effects of tomosyn in membrane fusion were comparable with those of the dominant negative inhibitor VAMP2 CD (Fig. 3, B and C).
FIGURE 1. Tomosyn binds to the syntaxin monomer but does not inhibit assembly of the t-SNARE complex. A, Coomasie Blue-stained SDS-PAGE gels showing the input materials of liposomes and proteins. Syn4, Syntaxin-4; Syn1, Syntaxin-1. B, Coomasie Blue-stained SDS-PAGE gel showing the binding of tomosyn and SNAP-23 to protein-free or syntaxin-4 liposomes. Syntaxin-4 liposomes were incubated with or without the recombinant tomosyn protein at 4 °C for 1 h, before SNAP-23 was added. After another hour of incubation at 4 °C, the samples were floated up on a Nycodenz gradient. C, Coomasie Blue-stained SDS-PAGE gel showing the binding of tomosyn and SNAP-25 to protein-free or syntaxin-1 liposomes. Each binding reaction contained 5 μM Syntaxin. Soluble factors were added to a final concentration of 5 μM.

FIGURE 2. Tomosyn binds to the binary t-SNARE complex and blocks v-SNARE entry. A, Coomasie Blue-stained SDS-PAGE gel showing the binding of tomosyn and VAMP2 CD to protein-free or t-SNARE liposomes reconstituted with syntaxin-4 and SNAP-23. To better visualize VAMP2 CD, a GST tag was included at its N terminus. The GST-tagged VAMP2 CD was fully competent for SNARE complex assembly. The t-SNARE liposomes were incubated with or without tomosyn at 4 °C for 1 h, before GST-VAMP2 CD was added. After another hour of incubation at 4 °C, the samples were floated up on a Nycodenz gradient. B, Coomasie Blue-stained SDS-PAGE gel showing binding of tomosyn and VAMP2 CD to protein-free or t-SNARE liposomes reconstituted with syntaxin-1 and SNAP-25. C, Coomasie Blue-stained SDS-PAGE gel showing the binding of tomosyn to the t-SNARE liposomes reconstituted with syntaxin-4 and SNAP-23, in the presence or absence of VAMP2 CD. The t-SNARE liposomes were incubated with or without GST-VAMP2 CD at 4 °C for 1 h before tomosyn was added. After another hour of incubation at 4 °C, the samples were floated up on a nycodenz gradient. D, Coomasie Blue-stained SDS-PAGE gel showing binding of tomosyn to t-SNARE liposomes reconstituted with syntaxin-1 and SNAP-25, in the presence or absence of VAMP2 CD. Each binding reaction contained 5 μM t-SNAREs. Soluble factors were added to a final concentration of 5 μM.

Distinct Roles of Tomosyn Domains

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 289 • NUMBER 37 • SEPTEMBER 12, 2014
We next examined how tomosyn regulates the content mixing of SNARE liposomes. The concentrated soluble dye sulforhodamine B was encapsulated in the VAMP2 liposomes in which its fluorescence was inhibited by self-quenching. Fusion of the v-SNARE liposomes with unlabeled t-SNARE liposomes led to the dilution of sulforhodamine B and dequenching of fluorescence (48). We observed that the SNAREs drove an efficient level of content mixing, and the content mixing was strongly blocked by tomosyn. The inhibitory effects of tomosyn in the content mixing reactions were comparable with those of VAMP2 CD (Fig. 4A). In leakage controls, the sulforhodamine B dye was included in both v- and t-SNARE liposomes. The sulforhodamine B emission was not increased, indicating that content leakage did not occur in the liposome fusion reactions (Fig. 4B). Therefore, tomosyn is capable of arresting both the lipid and content mixing of SNARE liposomes. These reconstitution data are consistent with liposome co-flotation results and demonstrate that tomosyn arrests the SNARE-mediated fusion at an “off” state.

**Tomosyn Arrests the Membrane Fusion Reaction at a Pre-docking Stage**—Next we sought to further dissect how tomosyn arrests SNARE-dependent membrane fusion. Since tomosyn prevents the pairing of v- and t-SNAREs, we hypothesized that it negatively regulates membrane fusion by inhibiting the initial

---

**Figure 3.** Tomosyn inhibits the SNARE-mediated lipid mixing reaction. A, illustrations of the reconstituted liposome fusion procedures. The t-SNARE liposomes were reconstituted with either syntaxin-4/SNAP-23 or syntaxin-1/ SNAP-25, whereas the v-SNARE liposomes were prepared using VAMP2. B, lipid mixing of the reconstituted proteoliposomes in the absence or presence of 5 μM tomosyn. Negative controls: 20 μM VAMP2 CD was added at the beginning of the fusion reactions. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. C, initial rates of the lipid mixing reactions shown in B. Data are presented as percentage of fluorescence change per 10 min. Error bars indicate S.D.

**Figure 4.** Tomosyn inhibits the content mixing of SNARE-mediated membrane fusion. A, content mixing of the reconstituted fusion reactions. The v-SNARE liposomes, in which sulforhodamine B was encapsulated, were directed to fuse with unlabeled t-SNARE liposomes in the absence or presence of 5 μM tomosyn. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as fluorescence increase over time. In negative controls, 20 μM VAMP2 CD was added to the fusion reactions. B, the leakage controls of the content mixing reactions. Sulforhodamine B fluorescence were not observed, indicating that no detectable content leakage occurred during the fusion reactions. C, initial content mixing rates of the fusion reactions shown in A. Data are presented as percentage of fluorescence change per 10 min. Error bars indicate S.D.
Distinct Roles of Tomosyn Domains

A

Avidin bead

\( \text{t-SNARE liposome (biotin labeled)} \)

\( \text{v-SNARE liposome (Rhodamine labeled)} \)

B

![Diagram of SNARE-liposome docking assay](image)

**FIGURE 5.** Tomosyn blocks SNARE-mediated liposome docking. A, diagram of the SNARE-liposome docking assay. B, biotin-labeled t-SNARE liposomes were anchored to avidin beads and were used to pull down rhodamine-labeled v-SNARE liposomes. The binding reactions were performed in the absence or presence of 5 \( \mu \text{g} \) tomosyn. The binding reaction containing 20 \( \mu \text{M} \) VAMP2 CD was used as a negative control to obtain the background fluorescent signal. The background fluorescence was subtracted from other binding reactions to reflect specific SNARE-dependent liposome docking. The data are presented as rhodamine fluorescence intensity. Error bars indicate S.D.

docking of SNARE liposomes. To test this possibility, avidin-immobilized t-SNARE liposomes were used to pull down rhodamine-labeled v-SNARE liposomes (Fig. 5A). We found that the v-SNARE VAMP2 interacted with the t-SNARE complex (syntaxin-4/SNAP-23 or syntaxin-1/SNAP-25) to promote the docking of SNARE liposomes (Fig. 5B). This SNARE-dependent liposome docking was strongly inhibited by tomosyn (Fig. 5B). The ability of tomosyn to block liposome docking suggests that the v- and t-SNAREs remain unpaired in the presence of tomosyn, in agreement with liposome co-flotation findings (Fig. 1). Thus, tomosyn arrests the membrane fusion reaction at a pre-docking stage.

**Tomosyn Uses Its CTD to Arrest SNARE-mediated Membrane Fusion Whereas Its NTD Is Required for Binding to Syntaxin Monomer**—Tomosyn contains a large NTD that contains WD40 repeats, and a small R-SNARE-like motif in the CTD (Fig. 6A). Next we dissected the functional roles of the NTD and CTD in reconstituted assays. The NTD was expressed in insect cells, whereas the CTD was expressed in *E. coli*. Circular dichroism (CD) measurements showed that the overall folding of the NTD fragment was similar to that of the FL tomosyn protein (Fig. 6B). As expected, the CD spectrum of the CTD fragment was characteristic of a mainly unstructured polypeptide (Fig. 6B).

Here we focused on the t-SNAREs syntaxin-4/SNAP-23 and v-SNARE VAMP2, which are required for both insulin secretion and GLUT4 exocytosis. We found that the NTD of tomosyn had no effect on the SNARE-mediated lipid mixing reaction. By contrast, the CTD of tomosyn inhibited the lipid mixing with the same efficiency as FL tomosyn (Fig. 6C). These data indicate that the C-terminal R-SNARE-like motif mediates the inhibitory function of tomosyn in membrane fusion. Whereas this conclusion has been proposed in previous studies (42, 54–58), our findings using full-length tomosyn provided the direct evidence. In a liposome docking assay, the CTD, but not the NTD, of tomosyn inhibited the docking of the v- and t-SNARE liposomes (Fig. 6D).

We further examined the SNARE-tomosyn association in the liposome co-flotation assay. FL tomosyn bound to both syntaxin-4 and syntaxin-4/SNAP-23 liposomes (Figs. 1B and 6E). We observed that the CTD of tomosyn interacted with the t-SNARE liposomes but, unexpectedly, not with the syntaxin-4 monomer (Fig. 6E). Thus, the R-SNARE-like motif of tomosyn is insufficient for syntaxin association. The NTD of tomosyn, on the other hand, associated with neither the syntaxin-4 monomer nor the syntaxin-4/SNAP-23 complex in the liposome co-flotation assay (Fig. 6E). These results suggest that, whereas the CTD mediates the inhibitory function of tomosyn in SNARE-mediated fusion reaction, both the NTD and CTD are required for the binding of tomosyn to the syntaxin monomer. Thus, FL tomosyn presents unique features not found in the widely studied CTD fragment. Because syntaxin binding is an important mechanism for recruiting SNARE regulators, our data suggest that the NTD is critical to the recruitment of tomosyn to vesicle fusion sites. These findings likely explain the genetic observations that the biological function of tomosyn in exocytosis requires both the NTD and CTD (44, 45).

**The Inhibitory Function of Tomosyn Is Dominant over the Stimulatory Activity of the SM Protein**—SM proteins are conserved molecules required for all intracellular vesicle fusion pathways (1). We and others have shown that SM proteins promote membrane fusion through binding to their cognate SNAREs (46, 59–62). In particular, the SM protein Munc18c positively regulates insulin secretion and GLUT4 exocytosis (63–67). In reconstituted assays, Munc18c strongly accelerates the SNARE-dependent membrane fusion reaction (48). Next we examined how tomosyn and Munc18c act in concert to regulate membrane fusion. We observed that the stimulation of fusion by Munc18c was abrogated when tomosyn was added to the SNAREs (Fig. 7, A and B), indicating that tomosyn inhibits the membrane fusion reaction in the presence of Munc18c. Further analysis showed that the CTD, but not the NTD, inhibited Munc18c function. Therefore, the inhibitory function of tomosyn is dominant over the stimulatory activity of SM proteins.

**DISCUSSION**

Tomosyn negatively regulates a range of SNARE-dependent exocytic pathways and is thought to enhance the spatial and temporal precision of vesicle fusion reactions. Whereas the physiological roles of tomosyn in exocytosis are well established, its molecular mechanism remains poorly defined. In this work we have reconstituted SNARE-dependent vesicle fusion reactions using purified components to recapitulate tomosyn-regulated exocytic pathways. In these defined fusion systems,
the mechanism of a SNARE regulator can be individually dissected without the complications of other molecules naturally found in the cell.

Previous biochemical studies of tomosyn mostly used truncated mutants that cannot recapitulate the function of FL tomosyn. Here, we expressed and purified active FL tomosyn protein and examined its activity in reconstituted fusion assays. We demonstrated that the SNARE regulator tomosyn arrests the SNARE-dependent fusion reaction at a pre-docking stage. Tomosyn arrests membrane fusion by inhibiting the t-SNARE complex, thereby preventing the initiation of ternary SNARE complex assembly. As a result, tomosyn inhibits the docking, lipid mixing, and content mixing of the SNARE-dependent fusion reaction. Notably, although originally isolated as a syntaxin-binding protein (42), tomosyn does not affect the formation of the t-SNARE complex on the membrane bilayer. Interestingly, tomosyn was recently found to be in complexes with both syntaxin monomers and t-SNARE complexes on the plasma membrane. It appears that the tomosyn/t-SNARE complex, rather than the tomosyn-syntaxin complex, mediates the inhibitory activity of tomosyn in exocytosis (68), in line with our reconstitution results.

Our analysis of FL tomosyn revealed novel features not found in the tomosyn fragments. Whereas not directly involved in SNARE inhibition, the NTD is necessary for binding to syntaxin monomer. We discovered that only FL tomosyn binds directly to syntaxin monomer, whereas the NTD and CTD fragments cannot. Because syntaxin binding is a key route for the recruitment of a SNARE regulator to vesicle fusion sites (69, 70), the NTD of tomosyn likely plays a key role in regulating membrane fusion. It is conceivable that, without this recruitment step, the efficiency of tomosyn in exocytosis
regulatory molecules. We observed that the inhibitory function of tomosyn is dominant over the stimulatory activity of the SM protein Munc18c. These results suggest that tomosyn arrests SNARE assembly at an upstream stage of the fusion reaction prior to membrane docking, whereas SM proteins regulate a downstream, post-docking step of the SNARE-dependent fusion reaction (46, 48, 70).

Overall, we drew two major conclusions from this study. First, we confirmed that the CTD mediates the inhibitory activity of tomosyn in the context of the FL protein. On the surface, these results seem not to extend the findings previously made using tomosyn fragments. However, we believe these data are a necessary step in tomosyn studies because FL SNARE regulators are frequently found to behave differently from truncated mutants. Second, we demonstrate that the NTD is required (although not sufficient) for the association of tomosyn with the syntaxin monomer. This novel observation likely provides a molecular explanation for the critical roles of the NTD in vivo (44, 45). It remains to be shown whether the inhibitory activity of tomosyn is influenced by cellular signaling pathways (e.g. insulin signaling). Moreover, it is also likely that tomosyn regulates vesicle fusion through a SNARE-independent mechanism. Nevertheless, we expect that the molecular mechanisms of tomosyn in SNARE-mediated fusion reaction revealed in this work pave the path for comprehensive understanding of tomosyn activities in physiological transport processes and their associations with human disorders.

Acknowledgments—We thank Drs. David James and Edward Stuenkel for helpful discussions.

REFERENCES

1. Südhof, T. C., and Rothman, J. E. (2009) Membrane fusion: grappling with SNARE and SM proteins. Science 323, 474–477
2. Wickner, W., and Schekman, R. (2008) Membrane fusion. Nat. Struct. Mol. Biol. 15, 658–666
3. Rizo, J., and Südhof, T. C. (2012) The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplices—guilty as charged? Annu. Rev. Cell Dev. Biol. 28, 279–308
4. Jahn, R., and Fasshauer, D. (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490, 201–207
5. Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T. H., and Rothman, J. E. (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92, 759–772
6. Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. Nature 395, 347–353
7. Schwartz, M. L., and Merz, A. J. (2009) Capture and release of partially zippered trans-SNARE complexes on intact organelles. J. Cell Biol. 185, 535–549
8. Burgoyne, R. D., and Morgan, A. (2007) Membrane trafficking: three steps to fusion. Curr. Biol. 17, R255–R258
9. Krämer, L., and Ungermann, C. (2011) HOPS drives vacuole fusion by binding the vacuolar SNARE complex and the Vam7 PX domain via two distinct sites. Mol. Biol. Cell 22, 2601–2611
10. Martens, S., and McMahon, H. T. (2008) Mechanisms of membrane fusion: disparate players and common principles. Nat. Rev. Mol. Cell Biol. 9, 543–556
11. Ohya, T., Miaczynska, M., Coskun, U., Lommer, B., Runge, A., Drechsel, D., Kalaidzidis, Y., and Zerial, M. (2009) Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. Nature 459, 1091–1097
12. Katz, L., and Brennwald, P. (2000) Testing the 3Q:1R "rule": mutational analysis of the ionic "zero" layer in the yeast exocytic SNARE complex reveals no requirement for arginine. *Mol. Biol. Cell* **11**, 3849–3858

13. Melia, T. I., Weber, T., McMew, J. A., Fisher, L. E., Johnston, R. J., Parlati, F., Mahal, L. K., Sollner, T. H., and Rothman, J. E. (2002) Regulation of membrane fusion by the membrane-proximal coil of the t-SNARE during zippering of SNAREpins. *J. Cell Biol.* **158**, 929–940

14. Pobbari, A. V., Stein, A., and Fasshauer, D. (2006) N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. *Science* **313**, 673–676

15. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Georganos, S., Tempst, P., and Rothman, J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318–324

16. Gao, Y., Zorman, S., Gunderson, G., Xi, Z., Ma, L., Sirinakis, G., Rothman, J. E., and Zhang, Y. (2012) Single reconstituted neuronal SNARE complex zipper in three distinct stages. *Science* **337**, 1340–1343

17. Eliasson, L., Abdulkader, F., Braun, M., Galvanovskis, J., Hoppa, M. B., and Rorsman, P. (2008) Novel aspects of the molecular mechanisms controlling insulin secretion. *J. Physiol.* **586**, 3313–3324

18. Rhodes, C. J. (2005) Type 2 diabetes: a matter of β-cell life and death? *Science* **307**, 380–384

19. Sheu, L., Pasyk, E. A., Ji, J., Huang, X., Gao, X., Varoqueaux, F., Brose, N., and Pessin, J. E. (2007) Ins (endocytosis) and outs (exocytosis) of asymmetric phospholipid distribution drives in vitro asymmetric membrane fusion through tomosyn displacement by VAMP2. *Biochem. Biophys. Res. Commun.* **399**, 24–30

20. Bryant, N. J., Govers, R., and James, D. E. (2002) Regulated transport of the glucose transporter GLUT4. *Nat. Rev. Mol. Cell Biol.* **3**, 267–277

21. Hou, J. C., and Pessin, J. E. (2007) Ins (endocytosis) and outs (exocytosis) of GLUT4 trafficking. *Carr. Opin. Cell Biol.* **19**, 466–473

22. D’Andrea-Merrins, M., Chang, L., Lam, A. D., Ernst, S. A., and Stuenkel, E. L. (2007) Munc18c interaction with syntaxin 4 monomers and SNARE complex intermediates in GLUT4 vesicle trafficking. *J. Biol. Chem.* **282**, 16553–16566

23. Vicogne, J., Vollenweider, D., Smith, J. R., Huang, P., Frohmam, M. A., and Pessin, J. E. (2006) Asymmetric phospholipid distribution drives in vitro reconstituted SNARE-dependent membrane fusion. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 14761–14766

24. Latham, C. F., Lopez, J. A., Hu, S. H., Gee, C. L., Westbury, E., Blair, D. H., Lentz, S. I., Ashery, U., Ernst, S. A., and Stuenkel, E. L. (2007) Receptor-mediated regulation of tomosyn-syntaxin 1A interactions in bovine adrenal chromaffin cells. *J. Biol. Chem.* **282**, 22887–22899

25. Ashery, U., Bielopolski, N., Barak, B., and Yizhar, O. (2009) Friends and foes in synaptic transmission: the role of tomosyn in vesicle priming. *Trends Neurosci.* **32**, 275–282

26. Chen, K., Richltzki, A., Featherstone, D. E., Schwärzel, M., and Richmond, J. E. (2011) Tomosyn-dependent regulation of synaptic transmission is required for a late phase of associative odor memory. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 18482–18487

27. Gracheva, E. O., Burdina, A. O., Holgado, A. M., Berthelot-Grosjean, M., Ackley, B. D., Hadwiger, G., Nonet, M. L., Weimer, R. M., and Richmond, J. E. (2006) Tomosyn inhibits synaptic vesicle priming in *Caenorhabditis elegans*. *PLoS Biol.* **4**, e26185

28. Gracheva, E. O., Burdina, A. O., Touroutiane, D., Berthelot-Grosjean, M., Parekh, H., and Richmond, J. E. (2007) Tomosyn negatively regulates both synaptic transmitter and neuropeptide release at the *C. elegans* neuromuscular junction. *J. Physiol.* **380–384**

29. Hu, Z., Tong, X. J., and Kaplan, J. M. (2013) UNC-13L, UNC-13S, and Tomosyn form a protein code for fast and slow neurotransmitter release in *Caenorhabditis elegans*. *Elife* **2**, e00967

30. Gracheva, E. O., Burdina, A. O., Touroutiane, D., Berthelot-Grosjean, M., Parekh, H., and Richmond, J. E. (2007) Tomosyn negatively regulates CAPS-dependent peptide release at *Caenorhabditis elegans* synapses. *J. Neurosci.* **27**, 10176–10184

31. Dybbs, M., Ngai, J., and Kaplan, J. M. (2005) Using microarrays to facilitate positional cloning: identification of tomosyn as an inhibitor of neurosecretion. *PLoS Genet.* **1**, 6–16

32. Gracheva, E. O., Maryon, E. B., Berthelot-Grosjean, M., and Richmond, J. E. (2010) Differential regulation of synaptic vesicle tethering and docking by UNC-18 and TOM-1. *Front. Synaptic Neurosci.* **2**, 141

33. Fujita, Y., Shiratani, H., Sakisaka, T., Asakura, T., Ohya, T., Kotani, H., Yokoyama, S., Nishioka, H., Matsuura, Y., Mizoguchi, A. Scheller, R. H., and Takai, Y. (1998) Tomosyn: a syntaxin-1-binding protein that forms a novel complex in the neurotransmitter release process. *Neuron* **20**, 905–915

34. Bhatnagar, S., Oler, A. T., Rabaglia, M. E., Stapleton, D. S., Schueler, K. L., Truchan, N. A., Worzel, S. L., Stoehr, J. P., Clee, S. M., Yandell, B. S., Keller, M. P., Thurmond, D. C., and Attie, A. D. (2011) Positional cloning of a type 2 diabetes quantitative trait locus: tomosyn-2, a negative regulator of insulin secretion. *PLoS Genet* **7**, e1002323

35. Yizhar, O., Lipstein, N., Gladych,eva, S. E., Matti, U., Ernst, S. A., Retig, J., Stuenkel, E. L., and Ashery, U. (2007) Multiple functional domains are involved in tomosyn regulation of exocytosis. *J. Neurochem.* **103**, 604–616

36. Burdina, A. O., Klosterman, S. M., Shtessel, L., Ahmed, S., and Richmond, J. E. (2011) *In vivo* analysis of conserved C. elegans tomosyn domains. *PLoS One* **6**, e26185

37. Shen, J., Tareste, D. C., Paumet, F., Rothman, J. E., and Melia, T. J. (2007) Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell* **128**, 183–195

38. Scott, B. L., Van Komen, J. S., Liu, S., Weber, T., Melia, T. J., and McNee, J. A. (2003) Liposome fusion assay to monitor intracellular membrane fusion machines. *Methods Enzymol.* **372**, 274–300

39. Yu, H., Rathore, S. S., Lopez, J. A., Davis, E. M., Ouyang, Y., and Shen, J. (2013) Doc2b promotes GLUT4 exocytosis by activating the SNARE-mediated fusion mechanism.
Distinct Roles of Tomosyn Domains

reaction in a calcium- and membrane bending-dependent manner. Mol. Biol. Cell 24, 1176–1184
51. Rathore, S. S., Ghosh, N., Ouyang, Y., and Shen, J. (2011) Topological arrangement of the intracellular membrane fusion machinery. Mol. Biol. Cell 22, 2612–2619
52. Yu, H., Rathore, S. S., and Shen, J. (2013) Synip arrests soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent membrane fusion as a selective target membrane SNARE-binding inhibitor. J. Biol. Chem. 288, 18885–18893
53. Groffen, A. J., Jacobsen, L., Schut, D., and Verhage, M. (2005) Two distinct genes drive expression of seven tomosyn isoforms in the mammalian brain, sharing a conserved structure with a unique variable domain. J. Neurochem. 92, 554–568
54. Ishiki, M., and Klip, A. (2005) Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. Endocrinology 146, 5071–5078
55. Yizhar, O., Matti, U., Melamed, R., Hagalili, Y., Bruns, D., Rettig, J., and Ashery, U. (2004) Tomosyn inhibits priming of large dense-core vesicles in a calcium-dependent manner. Proc. Natl. Acad. Sci. U.S.A. 101, 2578–2583
56. Masuda, E. S., Huang, B. C., Fisher, J. M., Luo, Y., and Scheller, R. H. (1998) Tomosyn binds t-SNARE proteins via a VAMP-like coiled coil. Neuron 21, 479–480
57. Yamamoto, Y., Mochida, S., Miyazaki, N., Kawai, K., Fujikura, K., Kurooka, T., Iwasaki, K., and Sakisaka, T. (2010) Tomosyn inhibits synaptotagmin-1-mediated step of Ca2+-dependent neurotransmitter release through its N-terminal WD40 repeats. J. Biol. Chem. 285, 40943–40955
58. Pobbati, A. V., Razeto, A., Bödener, M., Becker, S., and Fasshauer, D. (2004) Structural basis for the inhibitory role of tomosyn in exocytosis. J. Biol. Chem. 279, 47192–47200
59. Novick, P., and Schekman, R. (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 76, 1858–1862
60. Hata, Y., Slaughter, C. A., and Südhof, T. C. (1993) Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. Nature 366, 347–351
61. Dulubova, I., Khvotchev, M., Liu, S., Huryeva, I., Südhof, T. C., and Rizo, J. (2007) Munc18-1 binds directly to the neuronal SNARE complex. Proc. Natl. Acad. Sci. U.S.A. 104, 2697–2702.
62. Schollmeier, Y., Krause, J. M., Kreye, S., Malsam, J., and Söllner, T. H. (2011) Resolving the function of distinct Munc18-1/SNARE protein interaction modes in a reconstituted membrane fusion assay. J. Biol. Chem. 286, 30582–30590
63. Hu, S. H., Latham, C. F., Gee, C. L., James, D. E., and Martin, J. L. (2007) Structure of the Munc18c/Syntaxin1 N-peptide complex defines universal features of the N-peptide binding mode of Sec1/Munc18 proteins. Proc. Natl. Acad. Sci. U.S.A. 104, 8773–8778
64. Tellam, J. T., McIntosh, S., and James, D. E. (1995) Molecular identification of two novel Munc-18 isoforms expressed in non-neuronal tissues. J. Biol. Chem. 270, 5857–5863
65. Jewell, J. L., Oh, E., Ramalingam, L., Kalwat, M. A., Tagliabracci, V. S., Tackett, L., Elmendorf, I. S., and Thurmond, D. C. (2011) Munc18c phosphorylation by the insulin receptor links cell signaling directly to SNARE exocytosis. J. Cell Biol. 193, 185–199
66. Oh, E., Spurlin, B. A., Pessin, J. E., and Thurmond, D. C. (2005) Munc18c heterozygous knockout mice display increased susceptibility for severe glucose intolerance. Diabetes 54, 638–647
67. Oh, E., and Thurmond, D. C. (2009) Munc18c depletion selectively impairs the sustained phase of insulin release. Diabetes 58, 1165–1174
68. Bielopolski, N., Lam, A. D., Bar-On, D., Sauer, M., Stuenkel, E. L., and Ashery, U. (2014) Differential interaction of Tomosyn with syntaxin and SNAP25 depends on domains in the WD40 β-propeller core and determines its inhibitory activity. J. Biol. Chem. 289, 17087–17099
69. Ma, C., Su, L., Seven, A. B., Xu, Y., and Rizo, J. (2013) Reconstitution of the vital functions of Munc18 and Munc13 in neurotransmitter release. Science 339, 421–425
70. Rathore, S. S., Bend, E. G., Yu, H., Hammarlund, M., Jorgensen, E. M., and Shen, J. (2010) Syntaxin N-terminal peptide motif is an initiation factor for the assembly of the SNARE-Sec1/Munc18 membrane fusion complex. Proc. Natl. Acad. Sci. U.S.A. 107, 22399–22406
71. Giraudo, C. G., Garcia-Diaz, A., Eng, W. S., Chen, Y., Hendrickson, W. A., Melia, T. I., and Rothman, J. E. (2009) Alternative zipperning as an on-off switch for SNARE-mediated fusion. Science 323, 512–516
72. Giraudo, C. G., Eng, W. S., Melia, T. I., and Rothman, J. E. (2006) A clamping mechanism involved in SNARE-dependent exocytosis. Science 313, 676–680
73. Sakisaka, T., Yamamoto, Y., Mochida, S., Nakamura, M., Nishikawa, K., Ishizaki, H., Okamoto-Tanaka, M., Miyoshi, J., Fujiyoshi, Y., Manabe, T., and Takai, Y. (2008) Dual inhibition of SNARE complex formation by tomosyn ensures controlled neurotransmitter release. J. Cell Biol. 183, 323–337
74. Humphrey, S. I., Yang, G., Yang, P., Fazakerley, D. J., Stöckli, J., Yang, J. Y., and James, D. E. (2013) Dynamic adipocyte phosphoproteome reveals that Akt directly regulates mTORC2. Cell Metab. 17, 1009–1020
75. Lehman, K., Rossi, G., Adamo, J. E., and Brenwald, P. (1999) Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J. Cell Biol. 146, 125–140
76. Hattendorf, D. A., Andreeva, A., Gangar, A., Brenwald, P. J., and Weis, W. I. (2007) Structure of the yeast polarity protein Sor7 reveals a SNARE regulatory mechanism. Nature 446, 567–571