THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 26, pp. 18885–18893, June 28, 2013

Synip Arrests Soluble *N*-Ethylmaleimide-sensitive Factor Attachment Protein Receptor (SNARE)-dependent Membrane Fusion as a Selective Target Membrane SNARE-binding Inhibitor*[S]

Received for publication, February 27, 2013, and in revised form, April 29, 2013 Published, JBC Papers in Press, May 12, 2013, DOI 10.1074/jbc.M113.465450

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Background: Synip is a SNARE-binding regulatory factor whose molecular mechanism remains unclear.
Results: Synip acts as a selective t-SNARE-binding inhibitor that arrests membrane fusion by preventing the initiation of ternary SNARE complex assembly.
Conclusion: Synip function likely represents a novel regulatory mechanism of vesicle fusion.
Significance: Studies of vesicle fusion regulation provide key insights into the mechanisms of vesicle transport.

The vesicle fusion reaction in regulated exocytosis requires the concerted action of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) core fusion engine and a group of SNARE-binding regulatory factors. The regulatory mechanisms of vesicle fusion remain poorly understood in most exocytic pathways. Here, we reconstituted the SNARE-dependent vesicle fusion reaction of GLUT4 exocytosis *in vitro* using purified components. Using this defined fusion system, we discovered that the regulatory factor synip binds to GLUT4 exocytic SNAREs and inhibits the docking, lipid mixing, and content mixing of the fusion reaction. Synip arrests fusion by binding the target membrane SNARE (t-SNARE) complex and preventing the initiation of ternary SNARE complex assembly. Although synip also interacts with the syntaxin-4 monomer, it does not inhibit the pairing of syntaxin-4 with SNAP-23. Interestingly, synip selectively arrests the fusion reactions reconstituted with its cognate SNAREs, suggesting that the defined system recapitulates the biological functions of the vesicle fusion proteins. We further showed that the inhibitory function of synip is dominant over the stimulatory activity of Sec1/Munc18 proteins. Importantly, the inhibitory function of synip is distinct from how other fusion inhibitors arrest SNARE-dependent membrane fusion and therefore likely represents a novel regulatory mechanism of vesicle fusion.

Regulated exocytosis is the basis of a wide range of fundamental biological processes, including neurotransmitter release, hormone secretion, and inside-outside distributions of surface transporters and receptors (1, 2). One prominent example of regulated exocytosis is the insulin-controlled trafficking of the glucose transporter GLUT4, which plays a central role in maintaining blood glucose homeostasis (3–5). GLUT4 is normally sequestered in intracellular vesicles in adipocytes and skeletal muscles. In response to elevated levels of blood glucose, insulin binds to cell surface receptors and activates a complex signaling cascade, ultimately leading to the exocytosis of GLUT4-containing vesicles. Once on the cell surface, GLUT4 facilitates the uptake of excess blood glucose into the cell for disposal (6–12).

GLUT4 exocytosis is mediated by the fusion of GLUT4-containing exocytic vesicles with the plasma membrane (13). The core engine of intracellular membrane fusion is the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)[2] (1, 14–16). SNAREs are membrane-associated proteins localized to both the vesicle (v-SNAREs, or R-SNAREs) and the target membrane (t-SNAREs, or Q-SNAREs) (17–23). Membrane fusion is initiated when the v-SNARE pairs with the t-SNAREs to form a four-helix *trans*-SNARE complex. N- to C-terminal zippering of the *trans*-SNARE complex brings the two membranes into close proximity to fuse (24–27). In GLUT4 exocytosis, syntaxin-4 and SNAP-23 constitute the t-SNAREs whereas VAMP2 serves as the primary v-SNARE (28–31).

In addition to SNAREs, regulated exocytosis also requires a group of regulatory factors that are superimposed upon the SNAREs to achieve the spatial and temporal regulation of vesicle fusion (7, 9, 13). One of the SNARE regulators in GLUT4 exocytosis is synip, a soluble factor expressed in insulin-responsive tissues (32, 33). It has been suggested that synip binds to the syntaxin-4 monomer and negatively regulates GLUT4 exocytosis (33). The molecular mechanism of synip in membrane fusion, however, remains unclear due to the complexity of the cellular environment.

Here, we reconstituted the SNARE-dependent vesicle fusion reaction of GLUT4 exocytosis *in vitro* using purified components. Using this defined fusion system, we demonstrated that

* This work was supported, in whole or in part, by National Institutes of Health Pathway to Independence Award DK080080 and Grant DK095367 (to J. S.).
[S] This article contains supplemental Figs. S1 and S2.
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[2] The abbreviations used are: SNARE, *N*-ethylmaleimide-sensitive factor attachment protein receptor; CD, cytoplasmic domain; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; MSP, membrane scaffold protein; NBD, *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl; SM, Sec1/Munc18; t-SNARE, target membrane SNARE; TCEP, Tris(2-carboxyethyl)phosphine; v-SNARE, vesicle membrane SNARE.

the regulatory factor synip binds to GLUT4 exocytic SNAREs and arrests fusion at an intermediate stage. Synip arrests fusion by binding the t-SNARE complex and preventing the initiation of ternary SNARE complex assembly. We showed that synip inhibits the docking, lipid mixing, and content mixing of the SNARE-dependent fusion reaction. Although synip also interacts with the syntaxin-4 monomer, it does not inhibit the pairing of syntaxin-4 with SNAP-23. Interestingly, synip selectively arrests the fusion reactions reconstituted with its cognate SNAREs, suggesting that the defined system recapitulates the physiological function of synip in exocytosis. We further showed that synip inhibit the fusion reaction in the presence of the Sec1/Munc18 (SM) protein, a positive regulator of GLUT4 exocytosis. Hence, the inhibitory function of synip is dominant over the stimulatory activity of the SM protein. The inhibitory function of synip is distinct from how other fusion inhibitors arrest SNARE-dependent membrane fusion and therefore represents a novel regulatory mechanism of vesicle fusion.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant t- and v-SNARE proteins were expressed in *Escherichia coli* and purified by affinity chromatography. GLUT4 exocytic t-SNAREs, comprising the untagged syntaxin-4 and the His_6-tagged SNAP-23, were expressed using the same procedure as described previously for synaptic t-SNAREs (34, 35). The v-SNARE proteins were expressed in a similar way as VAMP2 (36) and had no extra residues left after the tags were proteolytically removed. Lysosomal and yeast exocytic SNAREs were purified as described previously (34–37). 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 (TCEP).

Genes encoding mouse synip (Open Biosystems) were subcloned into a pET28a-based SUMO vector and expressed in a similar way as Munc18-1 (34, 36). After proteolysis, no tag remained on the recombinant protein. Recombinant untagged Munc18c protein was produced in *Sf9* insect cells using baculovirus infection (31). The insect cells were lysed in a lysis buffer (25 mM HEPES (pH 7.4), 400 mM KCl, 10% glycerol, 20 mM imidazole, 1% Triton X-100, and 1 mM DTT, 2 mM β-mercaptoethanol, and EDTA-free protease inhibitor mixture). The cell extract was centrifuged at 18,500 rpm for 30 min at 4 °C. Munc18c protein in the cell extract was purified by nickel affinity chromatography. The His_6 tag was removed from Munc18c by tobacco etch virus protease, and the protein was subsequently dialyzed overnight against a storage buffer (25 mM HEPES (pH 7.4), 150 mM KCl, 10% glycerol, and 0.5 mM TCEP). Mutant SNAREs and regulators were generated by site-directed mutagenesis and purified similarly to wild-type (WT) proteins.

Reconstitution of Proteoliposomes and Membrane Nanodiscs—All lipids were obtained from Avanti Polar Lipids. For t-SNARE reconstitution, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), and cholesterol were mixed in a molar ratio of 60:20:10:10. For v-SNARE reconstitution, POPC, POPE, POPS, cholesterol, (*N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-DPPE) and *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (rhodamine-DPPE) were mixed at a molar ratio of 60:17:10:10:1.5:1.5. SNARE proteoliposomes were prepared by detergent dilution and isolated on a Nycodenz density gradient flotation (36, 38). Detergents were removed 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 calcein-containing liposomes, the t-SNARE liposomes were reconstituted in the presence of 50 mM calcein. Free calcein was removed by overnight dialysis followed by liposome flotation on a Nycodenz gradient.

The v-SNARE membrane nanodiscs were prepared as described (39, 40). Briefly, lipid mixtures (of the same composition as unlabeled t-SNARE liposomes) were dried and then resuspended in a reconstitution buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1% *n*-octyl-β-D-glucoside), together with His_6-MSP and VAMP2. The molar ratio of the molecules was membrane scaffold protein (MSP):VAMP2:lipid = 2:6:120. SM-2 Bio-Beads (Bio-Rad) were subsequently added to remove the detergent. After overnight incubation, the v-SNARE nanodiscs were purified using nickel affinity chromatography and dialyzed overnight in a Novagen dialysis tube. Each v-SNARE nanodisc contained 7–8 copies of VAMP2.

Liposome 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, resulting in the dequenching of their fluorescence. 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 are presented as the percentage of maximum fluorescence change. 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 statistical significance is included for each figure based on at least three independent experiments. For content mixing assays, calcein-containing t-SNARE liposomes were directed to fuse with v-SNARE membrane nanodiscs. The fusion of liposomes with nanodiscs released the self-quenched calcein dye, leading to the massive dilution and dequenching of calcein. The increase of calcein fluorescence at 515 nm (excitation 495 nm) was measured every 2 min.

Liposome Co-flotation Assay Measuring SNARE-Regulator Interactions—Association of soluble factors with liposomes was examined using a liposome co-flotation assay (34). A soluble factor was incubated with liposomes at 4 °C with gentle agitation. After 1 h, an equal volume of 80% Nycodenz (w/v) in reconstitution buffer was added and transferred to 5 mm by 41-mm centrifuge tubes. The liposomes were overlaid with 200 μl each of 35 and 30% Nycodenz and then with 20 μl of reconstitution buffer on the top. The gradients were centrifuged for

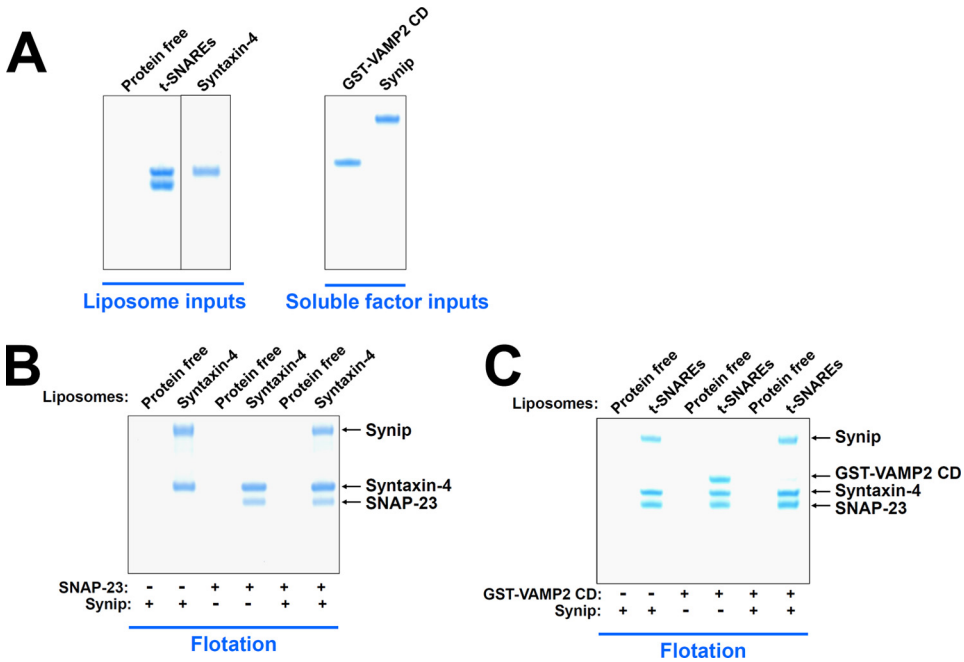

FIGURE 1. **Synip inhibits the assembly of the ternary SNARE complex, but not the formation of the t-SNARE complex.** *A*, Coomassie Blue-stained SDS-polyacrylamide gels showing the input materials of liposomes and proteins. The liposomes were prepared using the neutral lipid phosphatidylcholine. *B*, Coomassie Blue-stained SDS-polyacrylamide gel showing the binding of synip and SNAP-23 to protein-free or syntaxin-4 liposomes. Syntaxin-4 liposomes were incubated with or without synip 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*, Coomassie Blue-stained SDS-polyacrylamide gel showing the binding of synip and VAMP2 CD to protein-free or t-SNARE liposomes. 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 containing the heterodimer of syntaxin-4 and SNAP-23 were incubated with or without synip 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.

4 h at 52,000 rpm in a Beckman SW55 rotor. Samples were collected from the 0/30% Nycodenz interface (2 × 20 μl) and analyzed by SDS-PAGE.

Dynamic Light Scattering—Dynamic light scattering was performed on a Wyatt/ProteinSolutions DynaPro 99-D instrument using a 5-s acquisition time at 25 °C. SNARE liposomes were diluted to 10 μM final lipid concentration and centrifuged at 12,000 rpm for 10 min before dynamic light scattering measurements. The sizes (in diameters) and size distributions of the liposomes were calculated using the Dynamics V6 software.

Liposome Docking Assay—The t-SNARE liposomes were prepared in a similar way as in the liposome fusion assay except that 2% biotin-conjugated DOPE lipid was included. The biotin-labeled t-SNARE liposomes were incubated with avidin-conjugated agarose beads at room temperature for 1 h. The bead-bound t-SNARE liposomes were then used to pull down rhodamine-labeled v-SNARE liposomes. The rhodamine-labeled v-SNARE liposomes were identical to the v-SNARE liposomes used in lipid mixing assays. The pulldown reactions were performed in the liposome reconstitution buffer at 4 °C in the presence or absence of 5 μM synip. After washing three times with the reconstitution buffer, CHAPSO was added to the final concentration of 1% to solubilize the bead-bound liposomes. The avidin beads were removed by centrifugation at 4,000 rpm for 2 min. Rhodamine fluorescence in the supernatant was measured in a BioTek microplate reader. In the negative control reaction, 20 μM VAMP2 cytoplasmic domain (CD) was added to prevent the assembly of the ternary SNARE complex.

RESULTS

Synip 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 (15, 16). In solution, synip binds to syntaxin-4 monomer and appears to inhibit the SNARE assembly (32, 33). However, it is unclear how synip regulates SNARE assembly and membrane fusion in the membrane environment. In a liposome co-flotation assay, we observed that synip bound to proteoliposomes reconstituted with syntaxin-4 monomer (Fig. 1, *A* and *B*). Synip did not bind to protein-free liposomes (Fig. 1*B*), indicating that the synip-syntaxin-4 interaction was specific. When added as a soluble protein, SNAP-23 readily assembled with syntaxin-4 to form the binary t-SNARE complex on the membrane (Fig. 1*B*). We found that synip binding did not prevent the pairing of syntaxin-4 with SNAP-23 to form the binary t-SNARE complex (Fig. 1*B*). These data demonstrate that the synip-associated syntaxin-4 monomer is fully competent for t-SNARE complex assembly (33).

After t-SNARE complex formation, the v-SNARE zippers with the t-SNAREs to form the ternary SNARE complex that pulls two membranes into close proximity to fuse (41). Next we examined whether synip regulates the assembly of the ternary SNARE complex. We prepared proteoliposomes using the preformed t-SNARE complex of syntaxin-4 and SNAP-23 (Fig. 1*A*). Addition of VAMP2 CD to the t-SNARE liposomes resulted in the formation of the ternary SNARE complex on the membrane (Fig. 1*C*). Interestingly, synip bound to the

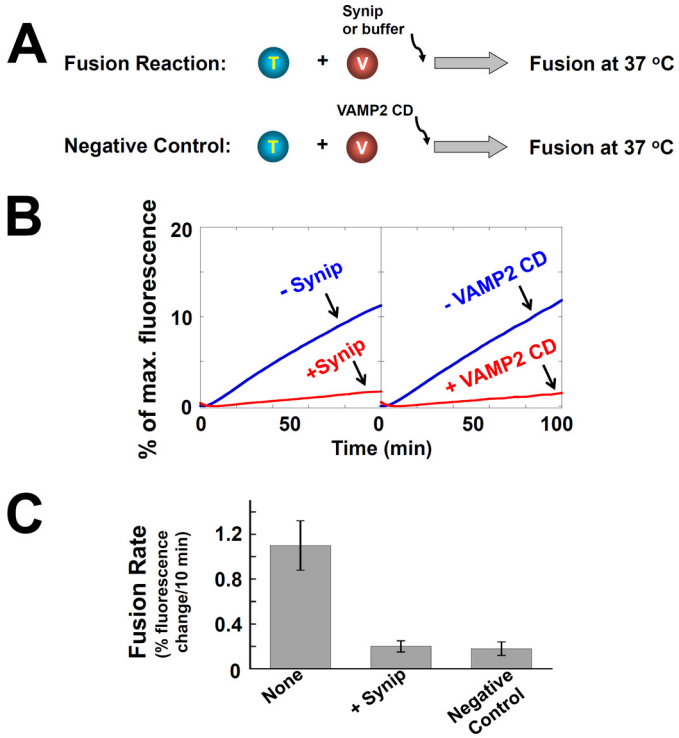

FIGURE 2. **Synip inhibits the SNARE-mediated membrane fusion reaction in a FRET-based lipid mixing assay.** *A*, reconstituted liposome fusion procedures. The t-SNARE liposomes were reconstituted with syntaxin-4 and SNAP-23, whereas the v-SNARE liposomes were prepared using VAMP2. *B*, fusion of the reconstituted proteoliposomes in the absence or presence of 5 μM synip. Negative controls: 20 μM VAMP2 CD was added at the beginning of the fusion reaction. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. The fusion reactions were measured using a FRET-based lipid mixing assay. *C*, initial rates of the fusion reactions shown in *B*. Data are presented as percentage of fluorescence change per 10 min. *Error bars* indicate S.D.

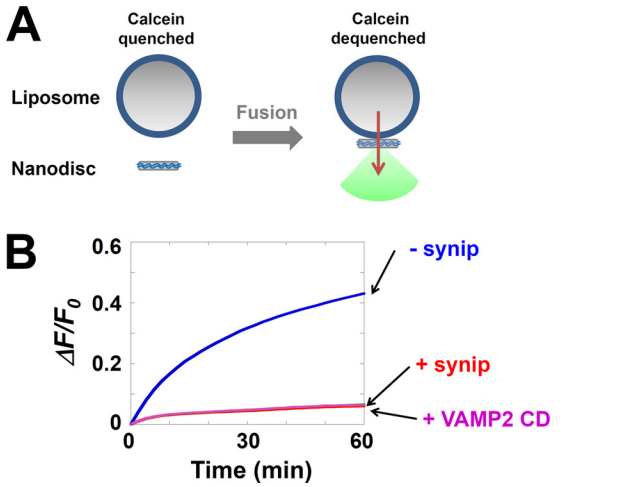

FIGURE 3. **Synip blocks the content mixing of SNARE-mediated membrane fusion.** *A*, diagram of the nanodisc-liposome content mixing assay. The fusion of membrane nanodiscs with calcein-containing proteoliposomes released the self-quenched calcein, leading to the massive dilution and dequenching of calcein dye. *B*, content mixing of the reconstituted fusion reaction. The t-SNARE liposomes were directed to fuse with VAMP2-bearing lipid bilayer nanodiscs in the absence or presence of 3.3 μM synip. Data are presented as fluorescence change (ΔF) over initial fluorescence (F_0). Each fusion reaction contained 3.3 μM t-SNAREs and 0.75 μM v-SNARE. Negative control: 20 μM VAMP2 CD was added to the fusion reaction.

t-SNAREs and strongly inhibited its pairing with VAMP2 CD (Fig. 1*C*). Together, these results indicate that synip binds to the t-SNAREs and inhibits the assembly of the ternary SNARE complex on the membrane bilayer.

Synip Arrests the SNARE-mediated Membrane Fusion Reaction—Next we examined how synip regulates the dynamic SNARE-mediated membrane fusion reaction. GLUT4 exocytic SNAREs were reconstituted into a defined fusion system, in which the v- and t-SNAREs were anchored in separate populations of lipid bilayers (Fig. 2*A* and supplemental Fig. S1). In this defined fusion system, SNAREs and regulators can be added or altered individually in the absence of other potentially confounding factors.

The fusion of v- and t-SNARE liposomes was first monitored using a FRET-based lipid mixing assay (42). GLUT4 exocytic SNAREs alone drove an efficient level of lipid mixing (Fig. 2, *B* and *C*). In the presence of synip, the SNARE-mediated membrane fusion was reduced to a background level similar to the negative control reaction in which the dominant negative inhibitor VAMP2 CD was added (Fig. 2, *B* and *C*). We also examined the content mixing of the fusion reaction using a nanodisc-liposome fusion assay. VAMP2 was reconstituted into nanodiscs, small synthetic lipoprotein complexes that harbor a small piece of circular membrane bilayer wrapped by two molecules of MSP (40). The soluble dye calcein (50 mM) was encapsulated in the t-SNARE liposomes. At that concentration, the fluorescent emission of calcein was inhibited by self-quenching. The fusion of VAMP2-bearing nanodiscs with calcein-containing t-SNARE liposomes led to the release and massive dequenching of the calcein dye (Fig. 3*A*). Using this liposome-nanodisc fusion assay, we observed that GLUT4 exocytic SNAREs drove an efficient level of content mixing (Fig. 3*B*). This SNARE-mediated content mixing was strongly inhibited by synip (Fig. 3*B*). Therefore, synip can arrest both the lipid and content mixing of SNARE-mediated membrane fusion. Because the lipid- and content-mixing experiments yielded similar results, lipid-mixing assays were used in the rest of this study.

Next we sought to further dissect how synip arrests SNARE-dependent membrane fusion. We used a liposome docking assay to examine how synip regulates the docking of v- and t-SNARE liposomes. The t-SNARE liposomes were immobilized on avidin-agarose beads and were used to pull down rhodamine-labeled v-SNARE liposomes (supplemental Fig. S2*A*). We found that GLUT4 exocytic v- and t-SNAREs promoted the docking of the liposomes (supplemental Fig. S2*B*). The SNARE-dependent liposome docking was strongly inhibited when synip was added (supplemental Fig. S2*B*). The ability of synip to block liposome docking suggests that the v- and t-SNAREs remained unpaired in the presence of synip, in agreement with liposome co-flotation findings (Fig. 1). Thus, synip arrests membrane fusion by blocking the initiation of ternary SNARE complex assembly. Together, these results demonstrate that synip functions as a t-SNARE-binding inhibitor that arrests SNARE-mediated membrane fusion at an intermediate state.

The N-terminal Regulatory Domain of Syntaxin-4 Is Dispensable for the Inhibitory Function of Synip—The zippering of the ternary *trans*-SNARE complex is mediated by the SNARE motifs (core domains) of the v- and t-SNAREs (18, 43). In addi-

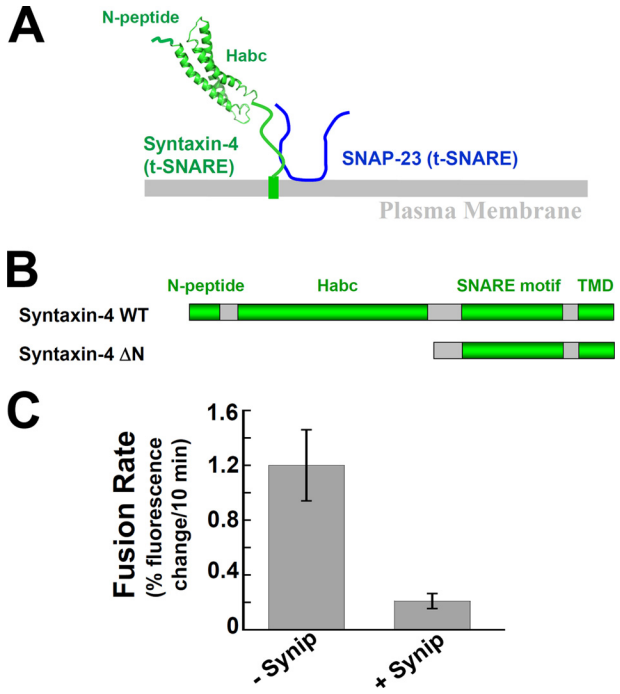

FIGURE 4. **The N-terminal regulatory domain of syntaxin is dispensable for the inhibitory function of synip.** *A*, diagram of the GLUT4 exocytic t-SNARE complex. The N-terminal regulatory domain of syntaxin-4 contains the N-peptide motif and the Habc domain. The Habc domain was modeled using the atomic structure of syntaxin-1 Habc domain (66). *B*, diagrams of WT syntaxin-4 and the syntaxin-4 ΔN mutant in which the N-terminal regulatory domain was removed. *TMD*, transmembrane domain. *C*, initial fusion rates of the indicated SNARE-dependent fusion reactions in the absence or presence of 5 μM synip. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. The fusion reactions were measured using a FRET-based lipid mixing assay. Data are presented as percentage of fluorescence change per 10 min. *Error bars* indicate S.D.

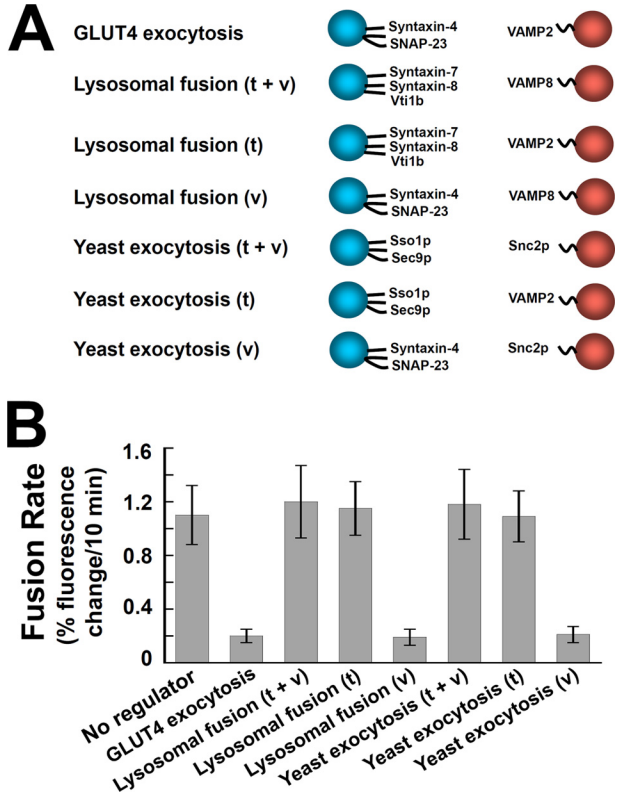

FIGURE 5. **The specificity of synip in regulating the SNARE-mediated fusion reaction.** *A*, liposome fusion pairs. The proteoliposomes were reconstituted with SNAREs isoforms involved mammalian GLUT4 exocytosis, mammalian lysosomal fusion, or yeast exocytosis. *B*, initial fusion rates of the indicated SNARE-dependent fusion reactions in the absence or presence of 5 μM synip. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. The fusion reactions were measured using a FRET-based lipid mixing assay. The fusion reaction labeled *No regulator* represents the control SNARE-mediated fusion reaction in the absence of regulatory factors. Data are presented as percentage of fluorescence change per 10 min. *Error bars* indicate S.D.

tion to the universal SNARE motif, the syntaxin subunit also possesses an N-terminal regulatory domain comprising an N-peptide motif and a Habc domain (Fig. 4, *A* and *B*). The N-terminal regulatory domain of syntaxin plays critical roles in multiple SNARE-regulator interactions (44, 45). To determine its role in the inhibitory function of synip, the N-terminal regulatory domain was removed from syntaxin-4 (Fig. 4*B*). When reconstituted into proteoliposomes, the syntaxin-4 ΔN mutant behaved similarly to WT syntaxin-4 in driving membrane fusion (Fig. 4*C*). The syntaxin-4 ΔN-containing fusion reaction was still strongly inhibited by synip, with the inhibitory efficiencies comparable with those in WT fusion reactions (Fig. 4*C*). Thus, the N-terminal regulatory domain of syntaxin is dispensable for the inhibitory function of synip.

Synip Selectively Inhibits Its Cognate SNAREs—Next we examined the intrinsic specificity of synip in regulating the SNARE-mediated fusion. The GLUT4 exocytic SNAREs in the reconstituted fusion system were substituted with SNARE isoforms involved in other fusion pathways including mammalian lysosomal fusion (syntaxin-7, syntaxin-8, Vti1b, and VAMP8), and yeast exocytosis (Sso1p, Sec9p, and Snc2p) (Fig. 5*A*). Although the v- and t-SNAREs of these fusion pathways can cross-pair to drive membrane fusion, they exhibit little sequence similarities (34, 41, 46, 47). Strikingly, synip failed to inhibit the fusion reactions driven by lysosomal SNAREs (labeled *lysosomal fusion t + v*), or yeast exocytic SNAREs (labeled *yeast exocytosis t + v*) (Fig. 5, *A* and *B*). Thus, the inhibitory function of synip is specific to GLUT4 exocytic SNAREs.

Next we fused the liposomes containing VAMP2, the GLUT4 exocytic v-SNARE, with the liposomes bearing the lysosomal t-SNAREs (syntaxin-7, syntaxin-8, and Vti1b), or yeast exocytic t-SNAREs (Sso1p and Sec9p). Again, neither of these fusion reactions (labeled *lysosomal fusion t* or *yeast exocytosis t*) was blocked by synip (Fig. 5, *A* and *B*). We also fused GLUT4 t-SNARE liposomes with v-SNARE liposomes reconstituted with either the lysosomal v-SNARE VAMP8 or the yeast exocytic v-SNARE Snc2p (Fig. 5*A*). Interestingly, these fusion reactions (labeled *lysosomal fusion v* and *yeast exocytosis v*) were strongly inhibited by synip (Fig. 5*B*). Therefore, the specificity of synip is determined by the t-SNAREs, but not the v-SNARE, in agreement with the interaction of synip with the t-SNARE complex in the liposome co-flotation assays (Fig. 1*C*). In the cell, the interactions between SNAREs and regulatory factors are exclusively specific to ensure the accuracy of vesicle transport (41). The stringent specificity of synip observed in this study supports that our reconstituted system has recapitulated the biological function of synip in exocytosis.

The Inhibitory Function of Synip Is Dominant Over the Stimulatory Activity of Munc18c—In addition to SNAREs, intracellular membrane fusion also requires the conserved SM family

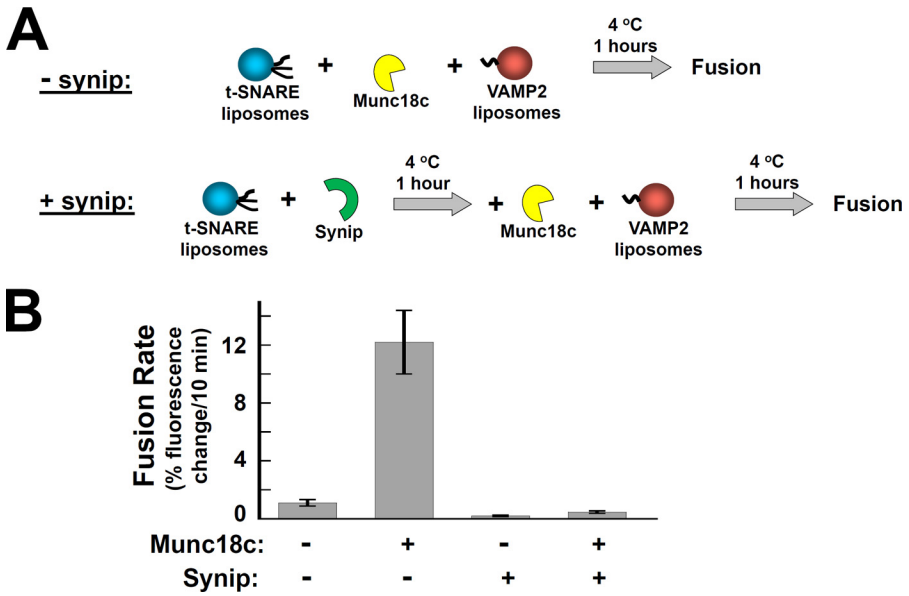

FIGURE 6. **The inhibitory function of synip is dominant over the stimulatory activity of Munc18c in fusion.** *A*, diagram illustrating the experimental procedures for the reconstituted fusion reactions. *B*, initial fusion rates of the indicated SNARE-mediated fusion reactions showing the inhibitory activity of synip in the presence or absence of Munc18c. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. The final concentration of each SNARE regulator was 5 μM. The fusion reactions were measured using a FRET-based lipid mixing assay. Data are presented as percentage of fluorescence change per 10 min. *Error bars* indicate S.D.

proteins, which promote membrane fusion through binding to their cognate SNAREs (34, 48–50). SM proteins exhibit similar loss-of-function phenotypes as SNAREs (*i.e.* abrogation of fusion) and are involved in every intracellular vesicle fusion pathway (51–53). In GLUT4 exocytosis, the cognate SM protein is Munc18c (also known as Munc18-3) (54–56). We expressed and purified recombinant Munc18c protein from *Sf*9 insect cells using baculovirus. When added to the reconstituted SNARE-mediated fusion reaction (Fig. 6*A*), Munc18c strongly accelerated the fusion kinetics (Fig. 6*B*). The stimulation of fusion by Munc18c was abrogated when synip was added to the SNAREs (Fig. 6*B*), indicating that synip can arrest the fusion reaction in the presence of Munc18c. Therefore, the inhibitory function of synip is dominant over the stimulatory activity of Munc18c in vesicle fusion.

DISCUSSION

In regulated exocytosis, the SNARE-dependent membrane fusion reaction is controlled by a group of SNARE-binding regulatory factors. Whereas the regulatory mechanisms of synaptic neurotransmitter release have been extensively studied, our knowledge about other exocytic pathways such as GLUT4 exocytosis remains primitive. Although conceptually similar to synaptic neurotransmitter release, the GLUT4 pathway is distinct in fundamental ways: (i) the kinetics of the fusion reaction is markedly slower (minutes *versus* submillisecond); (ii) specialized fusion regulators are involved; and (iii) the fusion reaction of GLUT4 exocytosis is coupled to insulin signaling (13, 57). Thus, the regulatory mechanisms of GLUT4 vesicle fusion cannot be derived directly from the available knowledge of synaptic release.

Although the physiological and medical importance of the GLUT4 exocytic pathway is well established, the underlying molecular mechanisms remain largely unknown. It is challenging to delineate complex membrane trafficking systems that involve the dynamic assembly of multiple layers of functional units at membrane-cytosol interfaces. We sought to address the question by reconstituting GLUT4 vesicle fusion in a defined system using purified components. Regulatory factors can be individually added or perturbed without the complications of other molecules naturally present in the cell, allowing their kinetic effects on fusion to be causally established.

Using the defined system, we demonstrated that the fusion regulator synip arrests the SNARE-dependent fusion reaction at an intermediate stage. Synip arrests membrane fusion by binding to the t-SNAREs and preventing the initiation of ternary SNARE complex assembly. We showed that synip inhibits the docking, lipid mixing, and content mixing of the SNARE-dependent fusion reaction. Notably, although originally isolated as a syntaxin-binding protein (33), synip does not affect the formation of syntaxin-4 with SNAP-23 into the t-SNARE complex on the membrane bilayer.

SM proteins are universal fusion regulators that promote intracellular vesicle fusion by binding to their cognate SNAREs (34, 48–50). To arrest exocytosis, a fusion inhibitor is expected to block SNARE-mediated fusion in the presence of the SM protein. Indeed, we observed that the inhibitory function of synip is dominant over the stimulatory activity of Munc18c, the cognate SM protein of GLUT4 exocytosis.

In the highly synchronized synaptic release, fusion-competent vesicles are immobilized on the plasma membrane through the docking and priming processes (57). GLUT4-containing vesicles, however, are mobile and cycle continuously beneath the cell surface. Upon insulin stimulation, GLUT4 vesicles begin to dock and fuse with the plasma membrane (58–60). These observations are consistent with our biochemical findings that synip arrests the fusion at the t-SNARE stage. With the

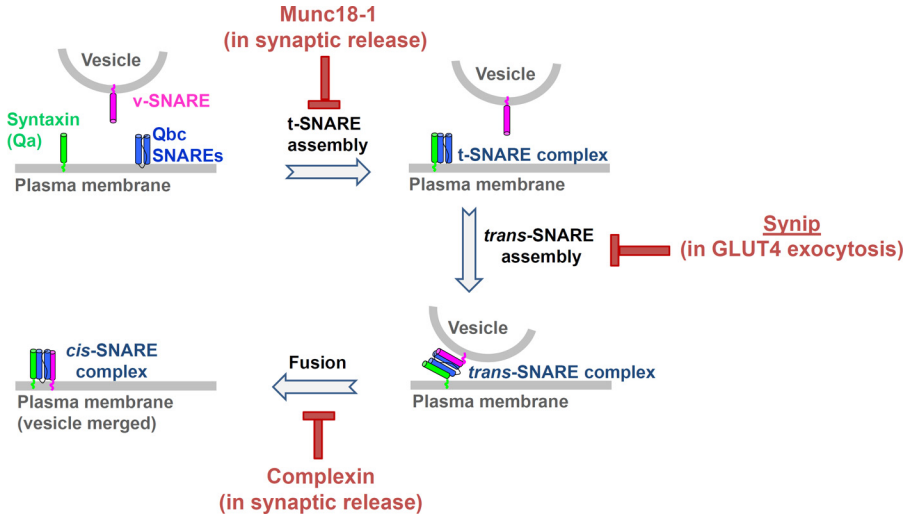

FIGURE 7. **Model showing fusion inhibitors in SNARE-mediated membrane fusion.** In synaptic neurotransmitter release, the fusion regulator Munc18-1 binds to the syntaxin monomer and blocks the formation of the t-SNARE complex on the plasma membrane. Complexin, on the other hand, recognizes the partially zippered *trans*-SNARE complex and arrests fusion at a late step of the fusion pathway. Our research established that synip negatively regulates the vesicle fusion reaction of GLUT4 exocytosis by binding the t-SNARE complex and preventing the initiation of *trans*-SNARE assembly. The regulatory mechanism of synip is distinct from how Munc18-1 and complexin arrest vesicle fusion. It should be noted that, in addition to their inhibitory functions, Munc18-1 and complexin also positively regulate membrane fusion.

t-SNARE complex blocked by synip, the vesicle-rooted v-SNARE cannot pair with the t-SNAREs to form the *trans*-SNARE complex, allowing the vesicles to be fully mobile.

It remains to be determined whether synip serves as a reversible fusion inhibitor. It has been proposed that synip is dissociated from SNAREs when its serine 99 residue is phosphorylated by Akt2, but the physiological role of this phosphorylation is being debated (32, 61). Nevertheless, it is conceivable that an insulin-induced phosphorylation may destabilize the t-SNARE-synip interaction to permit the entry of the v-SNARE. Alternatively, synip may form an irreversible inhibitor on GLUT4 exocytic t-SNAREs to help demarcate vesicle fusion sites on the plasma membrane, thereby allowing for spatial regulation of GLUT4 vesicle fusion.

Importantly, the inhibitory function of synip is distinct from how other fusion inhibitors arrest membrane fusion. For example, the synaptic factor Munc18–1 binds to syntaxin monomer and locks the latter in a "closed" configuration incompatible with t-SNARE complex assembly (44, 45). The small soluble protein complexin, on the other hand, negatively regulates synaptic vesicle fusion by arresting SNAREs at a partially zippered *trans*-SNARE configuration (62, 63). In the presence of complexin, the v- and t-SNAREs can initiate pairing but the *trans*-SNARE complex cannot complete zippering (Fig. 7) (62, 64). Therefore, SNARE-dependent membrane fusion can be arrested at each stage of the fusion pathway (Fig. 7). This versatility of fusion regulation likely allows the SNARE-mediated fusion reaction to adjust according to specific demands of a physiological response.

In summary, our studies revealed the molecular mechanism by which synip regulates the SNARE-dependent GLUT4 vesicle fusion. The inhibitory function of synip likely represents a novel regulatory mechanism of vesicle fusion. Genetic studies of GLUT4 fusion regulators such as synip often reached inconsistent and sometimes even contradictory conclusions (32, 33, 61, 65), reminiscent of the studies of synaptic fusion regulation (15, 16). In regulated exocytic pathways, multiple fusion regulators usually operate at similar or overlapping steps of the fusion reaction such that deletion of one factor might lead to unpredictable outcomes. In addition, loss of one fusion regulator might be compensated by another protein present in the cell, further complicating the analysis (57). Now, with the intrinsic regulatory mechanism of synip established, more precisely targeted *in vivo* experiments can be designed to delineate how it acts in concert with SNAREs and other fusion regulators to mediate GLUT4 exocytosis.

Acknowledgments—We thank Drs. David James (Garvan Institute, Australia) and Gustav Lienhard (Dartmouth) for helpful discussions on GLUT4 exocytosis.

REFERENCES

1. Südhof, T. C., and Rothman, J. E. (2009) Membrane fusion: grappling with SNARE and SM proteins. *Science* **323,** 474–477
2. Schekman, R., and Novick, P. (2004) 23 genes, 23 years later. *Cell* **116,** S13–15
3. Birnbaum, M. J. (1989) Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell* **57,** 305–315
4. James, D. E., Strube, M., and Mueckler, M. (1989) Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* **338,** 83–87
5. Charron, M. J., Brosius, F. C., 3rd, Alper, S. L., and Lodish, H. F. (1989) A glucose transport protein expressed predominately in insulin-responsive tissues. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 2535–2539
6. Huang, S., and Czech, M. P. (2007) The GLUT4 glucose transporter. *Cell Metab.* **5,** 237–252
7. Jewell, J. L., Oh, E., and Thurmond, D. C. (2010) Exocytosis mechanisms underlying insulin release and glucose uptake: conserved roles for Munc18c and syntaxin 4. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298,** R517–531
8. 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
9. Watson, R. T., and Pessin, J. E. (2006) Bridging the GAP between insulin signaling and GLUT4 translocation. *Trends Biochem. Sci.* **31,** 215–222
10. Vassilopoulos, S., Esk, C., Hoshino, S., Funke, B. H., Chen, C. Y., Plocik,

A. M., Wright, W. E., Kucherlapati, R., and Brodsky, F. M. (2009) A role for the CHC22 clathrin heavy-chain isoform in human glucose metabolism. *Science* **324,** 1192–1196
11. Blot, V., and McGraw, T. E. (2008) Molecular mechanisms controlling GLUT4 intracellular retention. *Mol. Biol. Cell* **19,** 3477–3487
12. Lavan, B. E., and Lienhard, G. E. (1994) Insulin signalling and the stimulation of glucose transport. *Biochem. Soc. Trans.* **22,** 676–680
13. Rowland, A. F., Fazakerley, D. J., and James, D. E. (2011) Mapping insulin/GLUT4 circuitry. *Traffic* **12,** 672–681
14. Wickner, W., and Schekman, R. (2008) Membrane fusion. *Nat. Struct. Mol. Biol.* **15,** 658–664
15. 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
16. Jahn, R., and Fasshauer, D. (2012) Molecular machines governing exocytosis of synaptic vesicles. *Nature* **490,** 201–207
17. 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
18. 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
19. Schwartz, M. L., and Merz, A. J. (2009) Capture and release of partially zipped trans-SNARE complexes on intact organelles. *J. Cell Biol.* **185,** 535–549
20. Burgoyne, R. D., and Morgan, A. (2007) Membrane trafficking: three steps to fusion. *Curr. Biol.* **17,** R255–258
21. 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
22. Martens, S., and McMahon, H. T. (2008) Mechanisms of membrane fusion: disparate players and common principles. *Nat. Rev. Mol. Cell Biol.* **9,** 543–556
23. 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
24. Melia, T. J., Weber, T., McNew, 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
25. Pobbati, A. V., Stein, A., and Fasshauer, D. (2006) N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. *Science* **313,** 673–676
26. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362,** 318–324
27. Gao, Y., Zorman, S., Gundersen, G., Xi, Z., Ma, L., Sirinakis, G., Rothman, J. E., and Zhang, Y. (2012) Single reconstituted neuronal SNARE complexes zipper in three distinct stages. *Science* **337,** 1340–1343
28. Brandie, F. M., Aran, V., Verma, A., McNew, J. A., Bryant, N. J., and Gould, G. W. (2008) Negative regulation of syntaxin4/SNAP-23/VAMP2-mediated membrane fusion by Munc18c *in vitro*. *PLoS One* **3,** e4074
29. 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
30. Vicogne, J., Vollenweider, D., Smith, J. R., Huang, P., Frohman, 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
31. Latham, C. F., Lopez, J. A., Hu, S. H., Gee, C. L., Westbury, E., Blair, D. H., Armishaw, C. J., Alewood, P. F., Bryant, N. J., James, D. E., and Martin, J. L. (2006) Molecular dissection of the Munc18c/syntaxin4 interaction: implications for regulation of membrane trafficking. *Traffic* **7,** 1408–1419
32. Yamada, E., Okada, S., Saito, T., Ohshima, K., Sato, M., Tsuchiya, T., Uehara, Y., Shimizu, H., and Mori, M. (2005) Akt2 phosphorylates synip to regulate docking and fusion of GLUT4-containing vesicles. *J. Cell Biol.* **168,** 921–928
33. Min, J., Okada, S., Kanzaki, M., Elmendorf, J. S., Coker, K. J., Ceresa, B. P., Syu, L. J., Noda, Y., Saltiel, A. R., and Pessin, J. E. (1999) Synip: a novel insulin-regulated syntaxin 4-binding protein mediating GLUT4 translocation in adipocytes. *Mol. Cell* **3,** 751–760
34. 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
35. Scott, B. L., Van Komen, J. S., Liu, S., Weber, T., Melia, T. J., and McNew, J. A. (2003) Liposome fusion assay to monitor intracellular membrane fusion machines. *Methods Enzymol.* **372,** 274–300
36. Shen, J., Rathore, S. S., Khandan, L., and Rothman, J. E. (2010) SNARE bundle and syntaxin *N*-peptide constitute a minimal complement for Munc18–1 activation of membrane fusion. *J. Cell Biol.* **190,** 55–63
37. 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
38. Yu, H., Rathore, S. S., Davis, E. M., Ouyang, Y., and Shen, J. (2013) Doc2b promotes GLUT4 exocytosis by activating the SNARE-mediated fusion reaction in a calcium- and membrane bending-dependent manner. *Mol. Biol. Cell* **24,** 1176–1184
39. Shi, L., Shen, Q. T., Kiel, A., Wang, J., Wang, H. W., Melia, T. J., Rothman, J. E., and Pincet, F. (2012) SNARE proteins: one to fuse and three to keep the nascent fusion pore open. *Science* **335,** 1355–1359
40. Ritchie, T. K., Grinkova, Y. V., Bayburt, T. H., Denisov, I. G., Zolnerciks, J. K., Atkins, W. M., and Sligar, S. G. (2009) Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. *Methods Enzymol.* **464,** 211–231
41. Jahn, R., and Scheller, R. H. (2006) SNAREs: engines for membrane fusion. *Nat. Rev. Mol. Cell Biol.* **7,** 631–643
42. 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
43. Stein, A., Weber, G., Wahl, M. C., and Jahn, R. (2009) Helical extension of the neuronal SNARE complex into the membrane. *Nature* **460,** 525–528
44. Burkhardt, P., Hattendorf, D. A., Weis, W. I., and Fasshauer, D. (2008) Munc18a controls SNARE assembly through its interaction with the syntaxin *N*-peptide. *EMBO J.* **27,** 923–933
45. Misura, K. M., Scheller, R. H., and Weis, W. I. (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* **404,** 355–362
46. Brandhorst, D., Zwilling, D., Rizzoli, S. O., Lippert, U., Lang, T., and Jahn, R. (2006) Homotypic fusion of early endosomes: SNAREs do not determine fusion specificity. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 2701–2706
47. Wang, C. C., Ng, C. P., Lu, L., Atlashkin, V., Zhang, W., Seet, L. F., and Hong, W. (2004) A role of VAMP8/endobrevin in regulated exocytosis of pancreatic acinar cells. *Dev. Cell* **7,** 359–371
48. 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
49. 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
50. 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
51. Carr, C. M., and Rizo, J. (2010) At the junction of SNARE and SM protein function. *Curr. Opin. Cell Biol.* **22,** 488–495
52. Burgoyne, R. D., Barclay, J. W., Ciufo, L. F., Graham, M. E., Handley, M. T., and Morgan, A. (2009) The functions of Munc18–1 in regulated exocytosis. *Ann. N.Y. Acad. Sci.* **1152,** 76–86
53. Toonen, R. F., and Verhage, M. (2007) Munc18–1 in secretion: lonely Munc joins SNARE team and takes control. *Trends Neurosci.* **30,** 564–572
54. Hu, S. H., Latham, C. F., Gee, C. L., James, D. E., and Martin, J. L. (2007) Structure of the Munc18c/syntaxin4 *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

55. 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
56. Jewell, J. L., Oh, E., Ramalingam, L., Kalwat, M. A., Tagliabracci, V. S., Tackett, L., Elmendorf, J. 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
57. Sudhof, T. C. (2004) The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27,** 509–547
58. Lizunov, V. A., Stenkula, K. G., Lisinski, I., Gavrilova, O., Yver, D. R., Chadt, A., Al-Hasani, H., Zimmerberg, J., and Cushman, S. W. (2012) Insulin stimulates fusion, but not tethering, of GLUT4 vesicles in skeletal muscle of HA-GLUT4-GFP transgenic mice. *Am. J. Physiol. Endocrinol. Metab.* **302,** E950–960
59. Bai, L., Wang, Y., Fan, J., Chen, Y., Ji, W., Qu, A., Xu, P., James, D. E., and Xu, T. (2007) Dissecting multiple steps of GLUT4 trafficking and identifying the sites of insulin action. *Cell Metab.* **5,** 47–57
60. Xu, Y., Rubin, B. R., Orme, C. M., Karpikov, A., Yu, C., Bogan, J. S., and Toomre, D. K. (2011) Dual-mode of insulin action controls GLUT4 vesicle exocytosis. *J. Cell Biol.* **193,** 643–653
61. Sano, H., Kane, S., Sano, E., and Lienhard, G. E. (2005) Synip phosphorylation does not regulate insulin-stimulated GLUT4 translocation. *Biochem. Biophys. Res. Commun.* **332,** 880–884
62. Giraudo, C. G., Eng, W. S., Melia, T. J., and Rothman, J. E. (2006) A clamping mechanism involved in SNARE-dependent exocytosis. *Science* **313,** 676–680
63. Tang, J., Maximov, A., Shin, O. H., Dai, H., Rizo, J., and Südhof, T. C. (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. *Cell* **126,** 1175–1187
64. Kümmel, D., Krishnakumar, S. S., Radoff, D. T., Li, F., Giraudo, C. G., Pincet, F., Rothman, J. E., and Reinisch, K. M. (2011) Complexin cross-links prefusion SNAREs into a zigzag array. *Nat. Struct. Mol. Biol.* **18,** 927–933
65. Saito, T., Okada, S., Yamada, E., Ohshima, K., Shimizu, H., Shimomura, K., Sato, M., Pessin, J. E., and Mori, M. (2003) Syntaxin 4 and Synip (syntaxin 4-interacting protein) regulate insulin secretion in the pancreatic β HC-9 cell. *J. Biol. Chem.* **278,** 36718–36725
66. Fernandez, I., Ubach, J., Dulubova, I., Zhang, X., Südhof, T. C., and Rizo, J. (1998) Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. *Cell* **94,** 841–849