Synip Arrests Soluble N-Ethylmaleimide-sensitive Factor Attachment Protein Receptor (SNARE)-dependent Membrane Fusion as a Selective Target Membrane SNARE-binding Inhibitor*5

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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 zipper 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

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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-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. 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 lytically 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, (N-(7-nitro-2,1,3-benoxadiazole-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 60:17:10:1.5:1.5. 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:l 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...
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 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. 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.

**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 proteoliposomes reconstituted with syntaxin-4 monomer (Fig. 1B), 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. 1B). We found that synip binding did not prevent the pairing of syntaxin-4 with SNAP-23 to form the binary t-SNARE complex (Fig. 1B). 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. 1A). Addition of VAMP2 CD to the t-SNARE liposomes resulted in the formation of the ternary SNARE complex on the membrane (Fig. 1C). Interestingly, synip bound to the
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**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.

t-SNAREs and strongly inhibited its pairing with VAMP2 CD (Fig. 1C). 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. 2A 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 calcine-containing t-SNARE liposomes released the self-quenched calcein, leading to the massive dilution and dequenching of calcein dye. **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 calcine-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/F0) over initial fluorescence (F0). 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.

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. S2A). We found that GLUT4 exocytic v- and t-SNAREs promoted the docking of the liposomes (supplemental Fig. S2B). The SNARE-dependent liposome docking was strongly inhibited when synip was added (supplemental Fig. S2B). 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 lipidosome 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-
The N-terminal regulatory domain of syntaxin plays critical roles in the inhibitory function of synip. To determine the importance of 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.

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. 5A). Interestingly, these fusion reactions (labeled lysosomal fusion v and yeast exocytosis v) were strongly inhibited by synip (Fig. 5B). Therefore, the specificity of synip is determined by the t-SNAREs, not the v-SNARE, in agreement with the interaction of synip with the t-SNARE complex in the liposome co-flotation assays (Fig. 1C). 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
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 Sf9 insect cells using baculovirus. When added to the reconstituted SNARE-mediated fusion reaction (Fig. 6A), Munc18c strongly accelerated the fusion kinetics (Fig. 6B). The stimulation of fusion by Munc18c was abrogated when synip was added to the SNAREs (Fig. 6B), 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

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**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.
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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 zipper 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.

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.

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