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The Transmembrane Domain of Syntaxin 1A Is Critical for Cytoplasmic Domain Protein-Protein Interactions*

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Assembly of the plasma membrane proteins syntaxin 1A and SNAP-25 with the vesicle protein synaptobrevin is a critical step in neuronal exocytosis. Syntaxin is anchored to the inner face of presynaptic plasma membrane via a single C-terminal membrane-spanning domain. Here we report that this transmembrane domain plays a critical role in a wide range of syntaxin protein-protein interactions. Truncations or deletions of the membrane-spanning domain reduce synaptotagmin, α/β-SNAP, and synaptobrevin binding. In contrast, deletion of the transmembrane domain potentiates SNAP-25 and rbSec1A/nsec-1/munc18 binding. Normal partner protein binding activity of the isolated cytoplasmic domain could be "rescued" by fusion to the transmembrane segments of synaptobrevin and to a lesser extent, synaptotagmin. However, efficient rescue was not achieved by replacing deleted transmembrane segments with corresponding lengths of other hydrophobic amino acids. Mutations reported to diminish the dimerization of the transmembrane domain of syntaxin did not impair the interaction of full-length syntaxin with other proteins. Finally, we observed that membrane insertion and wild-type interactions with interacting proteins are not correlated. We conclude that the transmembrane domain, via a length-dependent and sequence-specific mechanism, affects the ability of the cytoplasmic domain to engage other proteins.

Syntaxin 1A was initially identified as a 35 kDa protein in the plasma membrane of amacrine cells (1), as a subunit of Ca^{2+} channels (2, 3) and as a synaptotagmin-binding protein (4). Since these initial reports, the function of syntaxin as a central component in the synaptic vesicle membrane fusion machinery has been well established (reviewed in Refs. 5–7). Syntaxin forms a putative membrane fusion apparatus by assembling into a four-helix bundle (8) with the plasma membrane protein SNAP-25[1] and the synaptic vesicle protein synaptobrevin, to form a SNARE complex (9). Assembly of this complex is necessary (10, 11) and may be sufficient to drive membrane fusion (12–14). One current view is that the zippering together of the four-helix bundle drives membrane fusion by pulling the vesicle and target membranes together (8, 12, 15). In this model, the transmembrane domains (TMDs) of synaptobrevin and syntaxin would form part of a fusion pore (16). Thus, structure-function relationships of these TMDs may reveal insights into the mechanism of membrane fusion (13, 14, 17–19).

Syntaxin functions as a key element in membrane traffic and membrane fusion by interacting with a wide range of other proteins. The many binding partners of syntaxin, in excess of twenty, include rbSec1A/nsec-1/munc18 (20–22), CSP (23), syntaphilin (24), α/β-SNAP (9, 25), sec6/8 (26), tomosyn (27), Munc-13 (28), and as mentioned above synaptotagmin (4, 29) as well as a growing assortment of channels/receptors (see, for example Refs. 2, 3, 30–32).

Biochemical studies of syntaxin, including structural determinations (8, 33, 34), have made almost exclusive use of the cytoplasmic domain of the protein. Yet, a number of reports indicate that the TMD of syntaxin is a critical determinant for protein-protein interactions; removal of the TMD inhibits synaptotagmin, synaptobrevin, and α/β-SNAP (25, 29) binding activity. In addition, insertion of the transmembrane region into membranes is required for cleavage of syntaxin by botulinum neurotoxin C1 (35, 36), and the membrane anchors of syntaxin and synaptotobrevin are required for maximal stability of the SNARE complex (37).

To better understand syntaxin protein-protein interactions, we have carried out a detailed investigation of the role of the syntaxin TMD in mediating syntaxin-target protein interactions. We provide evidence that the TMD of syntaxin affects the ability of its cytoplasmic domain to engage partner proteins. The TMD fulfills this role in a length- and sequence-specific manner that is not dependent upon TMD-mediated dimerization. Furthermore, mutagenesis experiments demonstrate that membrane insertion and wild-type partner protein binding activity can be completely uncoupled. We propose that the TMD affects target protein interactions by affecting the conformation of the cytoplasmic domain of syntaxin.

MATERIALS AND METHODS

Recombinant Proteins—Mutagenesis (truncation, deletion, and chimeric protein construction), expression, and purification of recombinant proteins were carried out as described (38, 39). cDNA to generate syx-mult and syx-A15 (19) were kindly provided by D. Langosch (Heidelberg, Germany). cDNA encoding rbSec1A (21, 40), syntaxin 1A (4), synaptobrevin 2/VAMP2 (41), and synaptotagmin I (42) were kindly provided by P. De Camilli (New Haven, CT), R. Scheller (Stanford, CA) and T. C. Sudhof (Dallas, TX).

Rat Brain Detergent Extracts—Crude synaptosomes were prepared by homogenization of 1–2 fresh rat brains in 320 mM sucrose buffer. The homogenate was centrifuged at 5000 rpm for 2 min in a Beckman JA-17 rotor; the pellet was discarded, and the crude synaptosome fraction was collected by centrifuging the supernatant at 11,000 rpm for 12 min in the same rotor. The resulting pellet was resuspended in 25–30 ml of 50 mM HEPES, pH 7.4, 100 NaCl buffer plus 1% Triton X-100 and protease

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[1] The abbreviations used are: SNAP-25, synaptosome-associated protein of 25 kDa; α/β-SNAP, soluble NSF attachment protein; SNARE, soluble NSF attachment protein receptor; TMD, transmembrane domain; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 20 μg/ml aprotinin) and solubilized for 30–45 min at 4 °C on a rotator. Insoluble material was removed by centrifugation at 17,000 rpm for 20 min in a Beckman JA-17 rotor. The final detergent extract yielded 1 mg/ml protein, and 1-mg aliquots were incubated with 30 μg of immobilized fusion protein as described below.

Binding Assays—All binding assays were carried out by immobilizing one protein on glutathione-Sepharose beads. Immobilized fusion proteins were incubated with either purified soluble binding partners in Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl) plus 0.5% Triton X-100 or rat brain detergent extracts (1 ml at 1 mg/ml, described above) with either 2 mM EGTA or 1 mM free Ca^{2+} for 1–2 h at 4 °C. Beads were washed three times in binding buffer. Bound proteins were solubilized by boiling in SDS-sample buffer, subjected to SDS-PAGE, and visualized by staining with Coomassie Blue or by immunoblotting. For blotting, mouse monoclonal antibodies directed against synaptotagmin I (604.4 and 41.1), α/β-SNAP (77.1), SNAP-25 (71.2), and synaptobrevin II (69.1) were kindly provided by R. Jahn and S. Engers (Gottingen, Germany). Immunoreactive bands were visualized using enhanced chemiluminescence. Each binding assay was carried out in at least three independent trials, and representative experiments are shown in the figures.

In Vitro Transcription and Translation—Wild-type and mutant syntaxin cDNAs cloned into pGEX-2T were used as PCR templates using a 5′ primer containing the T7 promoter plus sequence complementary to the 5′-end of pGEX-2T. The reverse primer was complementary to the 3′-end of pGEX-2T. PCR was carried out using 30 ng of plasmid DNA, 13.3 μM primers, and Pfu polymerase; samples were cycled 25 times (45 s at 95 °C, 45 s at 54 °C, and 2 min at 72 °C). PCR products (0.5 μg) were then used directly in a TnT *in vitro* transcription/translation system (Promega, Madison, WI) by incubating with 25 μl of reaction mix containing reticulocyte lysate and [^{35}S]methionine according to the manufacturer's instructions and with canine pancreatic microsomes added as indicated. To determine incorporation of syntaxin into microsomal membranes, 5 μl of the translation mix was added to 400 μl of K-Glu buffer (120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA, 20 mM HEPES, pH 7.2) and, to pellet the membranes, centrifuged at 70,000 rpm for 30 min in a Beckman TLA 100.3 rotor. As indicated, parallel samples were washed with 400 μl of 100 mM Na_2CO_3 buffer, pH 11.5, and membranes were collected by centrifugation as described above. Pellet and supernatant samples were solubilized by boiling in reducing SDS-sample buffer, and equal fractions were subjected to SDS-PAGE. Gels were processed for fluorography using Amplify (Amersham Pharmacia Biotech) and fluorographs are shown in Fig. 7.

RESULTS

Previous studies indicated that removal of the transmembrane domain (TMD) of syntaxin impaired synaptotagmin, synaptobrevin, and α/β-SNAP binding activity (25, 29). Furthermore, syntaxin must be anchored into lipid bilayers via its C-terminal membrane-spanning domain to be cleaved by botulinum neurotoxin C (35, 36). Finally, removal of the syntaxin TMD decreases the stability of fully assembled SNARE complexes (37). Whereas these reports suggested that the TMD of syntaxin is important for protein-protein interactions, it was not clear whether complete removal of the transmembrane segment of syntaxin grossly affected the structure of the protein, or whether the TMD played a more specific or direct role in mediating protein-protein interactions. Therefore, we began to investigate the role of the transmembrane domain in protein-protein interactions by constructing more subtle truncations at the C terminus of the TMD. These constructs, shown in Fig. 1*A*, were expressed as GST fusion proteins and used as affinity matrices for the binding of synaptotagmin I, α/β-SNAP, SNAP-25, and synaptobrevin II present in Triton X-100 extracts of rat brain membranes. As shown in Fig. 1*B*, truncation of the TMD resulted in the progressive loss of synaptotagmin, α/β-SNAP, and synaptobrevin binding activity. Even removal of the last two amino acids of the TMD slightly, yet reproducibly, reduced synaptotagmin interactions. Truncation to amino acid 281, which would be predicted to lie within the opposite leaflet of the lipid bilayer relative to the cytoplasmic domain, dramatically reduced binding. These data suggest that the distal region of the TMD can affect the interaction of the H3 domain of syntaxin with target proteins. We note that in these experiments, and in experiments described below, similar results were observed using purified recombinant proteins as the "ligands" in the GST pull-down assays (data not shown). Thus, the interactions reported here are direct.

In contrast to the diminished binding of synaptotagmin, α/β-SNAP, and synaptobrevin, we observed that SNAP-25 binding was enhanced by removal of the TMD, and this effect became apparent by truncating from amino acid 276 back to residue 271 (Fig. 1*B*). These data suggest that truncation of the TMD does not result in gross misfolding, but rather can differentially affect the affinity of different interacting proteins; this effect is consistent with a model in which the TMD truncations can switch syntaxin between different conformations, discussed further below. We also compared the interactions of full-length and the cytoplasmic domain of syntaxin with native (Fig. 1*C*) as well as recombinant rbSec1A/nsec-1/munc18 (Fig. 1*D*). Analogous to SNAP-25, rbSec1A bound more efficiently to the cytoplasmic domain of syntaxin than to the full-length protein. Thus, removal of the TMD inhibits synaptotagmin, α/β-SNAP, and synaptobrevin binding and enhances SNAP-25 and rbSec1A binding.

To determine whether the effects of the syntaxin TMD truncations were length- or position-sensitive, we shortened the TMD by internal deletions at the N-terminal end of the TMD (shown schematically in Fig. 2*A*). As shown in Fig. 2*B*, removal of only two amino acid residues at the N-terminal edge of the TMD reduced binding of synaptotagmin and α/β-SNAP. Binding of synaptotagmin was further reduced by progressively larger deletions of four and seven amino acids. Interestingly, synaptobrevin binding was largely unaffected by N-terminal TMD deletions, indicating that these deletions did not result in gross misfolding of syntaxin. Furthermore, SNAP-25 and rbSec1A again showed an increase in binding, and this increase required the removal of seven residues from the N-terminal side of the TMD. For comparison, increased SNAP-25 binding was not observed until more than twelve residues were removed from the C-terminal end of the syntaxin TMD. These data suggest that the role of the TMD in syntaxin binding interactions is complex and involves determinants other than simple length requirements.

We addressed this hypothesis via rescue experiments in which we tried to restore wild-type binding interactions with the 1–281 truncation and Δ265–270 deletion mutants by adding the appropriate number of amino acids onto the C-terminal tail of the TMD. To test whether the TMD must form an α-helix of a certain length, we added either a string of isoleucines, which can form an α-helix, or a string of alternating proline/phenylalanine residues (Pro/Phe), which cannot form an α-helix (shown schematically in Fig. 3*A*). As shown in Fig. 3*B*, the 1–281 mutant showed diminished synaptotagmin, α/β-SNAP, and synaptobrevin binding activity. Again, SNAP-25 binding was not impaired, providing a positive control for the folding of the mutants. Interestingly, addition of seven Ile residues to the end of the 281 mutant partially rescued α/β-SNAP and synaptobrevin binding, whereas the Pro/Phe sequence did not rescue binding of these proteins. These data indicate that α/β-SNAP and synaptobrevin binding require a full-length TMD with the ability to form an α-helix. In contrast, neither the Ile nor the Pro/Phe sequences rescued synaptotagmin binding, again indicating that the TMD fulfills different requirements for the binding of different interacting proteins. Similar experiments were conducted using the Δ265–270 deletion mutant. Consistent with the data in Fig. 2, this deletion did not affect synap-

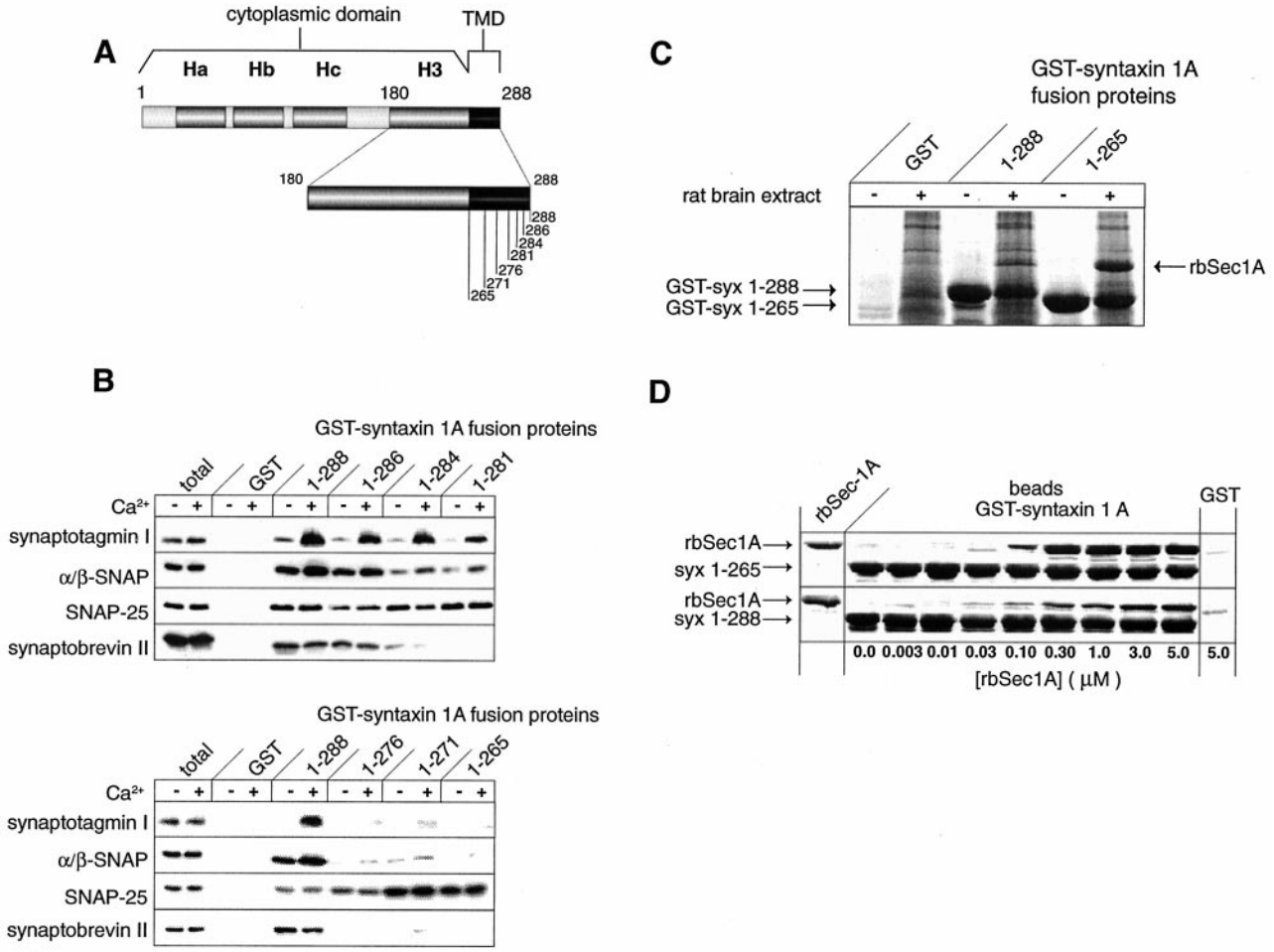

FIG. 1. **Effect of C-terminal syntaxin truncations on syntaxin protein-protein interactions.** *A,* schematic of syntaxin C-terminal TMD truncations. The Habc and H3 domains of syntaxin are *shaded*, the TMD is shown in *black*. The H3 and TMDs are enlarged to indicate the positions of the C-terminal truncations. *B,* effect of syntaxin C-terminal TMD truncations on protein-protein interactions. Thirty μg of GST or the indicated versions of GST-syntaxin was immobilized on glutathione-Sepharose beads. Beads were incubated with 1 mg of rat brain detergent extract (1 mg/ml protein) for 2 h in Tris-buffered saline with 1% Triton X-100 plus either 2 mM EGTA ($-Ca^{2+}$) or 1 mM Ca^{2+} ($+Ca^{2+}$). Beads were washed three times with binding buffer and boiled in SDS sample buffer. Thirteen percent of the bound material was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with primary antibodies directed against synaptotagmin I, α/β-SNAP, SNAP-25, or synaptobrevin. Total corresponds to 5 μg of the rat brain detergent extract. Binding of synaptotagmin, α/β-SNAP, and synaptobrevin progressively decreases with increasing TMD truncations. In contrast, SNAP-25 binding increases after truncation past residue 276. *C* and *D*, removal of the syntaxin (*syx*) TMD increases rbSec1A binding activity. In *C*, full-length syntaxin (1–288) and syntaxin lacking a TMD (1–265) were used to affinity purify rbSec1A from rat brain detergent extracts as described in *B* except that 20 mg of brain extract were used for each sample, and samples were visualized by staining with Coomassie Blue. In *D*, recombinant rbSec1A was titrated onto either full-length syntaxin or syntaxin lacking a TMD; again, bound rbSec1A was visualized by staining with Coomassie Blue. In both experiments, rbSec1A binding was enhanced by removal of the syntaxin TMD. The identity of native rbSec1 was confirmed by immunoblot analysis (data not shown).

tobrevin binding, but did affect the binding of all other proteins examined (Fig. 3*C*). In this case, the Ile sequence failed to rescue synaptotagmin or α/β-SNAP binding whereas, surprisingly, very low levels of rescue were observed with the non-helix forming Pro/Phe sequence. For these interactions, the ability of added on residues to restore wild-type binding activity depended upon whether the TMD was truncated at the N- or C-terminal end, as well as on the content of the added-on sequence. In contrast, the enhanced binding of SNAP-25 to the deletion mutant was partially abrogated by both added-on sequence stretches. In summary, the data from these rescue experiments suggest that the length, primary sequence, and the position of the primary sequence of the TMD of syntaxin are all key factors in syntaxin-target protein interactions.

We further examined the sequence requirements of the syntaxin TMD by constructing two chimeric syntaxins that harbored TMDs from synaptobrevin II or synaptotagmin I (Fig. 4*A*). As shown in Fig. 4*B*, replacement of the syntaxin TMD with the synaptobrevin TMD resulted in a protein with wild-type, or stronger, synaptotagmin, α/β-SNAP, and synaptobrevin binding activity. In contrast, replacement with the synaptotagmin TMD resulted in only partial rescue of synaptotagmin, α/β-SNAP, and synaptobrevin binding activity. Because the TMDs of synaptobrevin and synaptotagmin are not homologous, these experiments demonstrate some degree of promiscuity in the sequence requirements within the TMD. These observations, coupled to the findings that mutations on the distal side of the membrane anchor (*i.e.* the N-terminal truncation mutants) can affect protein-protein interactions (Fig. 1*B*), prompted us to investigate the possibility that the role of the TMD is to induce oligomerization of the H3 domain of syntaxin to drive normal interactions with other proteins. Indeed, recent studies have demonstrated that the transmembrane domain of syntaxin mediates syntaxin homodimerization as well as binding to the TMD of synaptobrevin (18, 19). The same may hold true of the TMD of synaptotagmin, which has been recently shown to contain a novel clustering site within the N-terminal half of the protein (43, 44). To determine

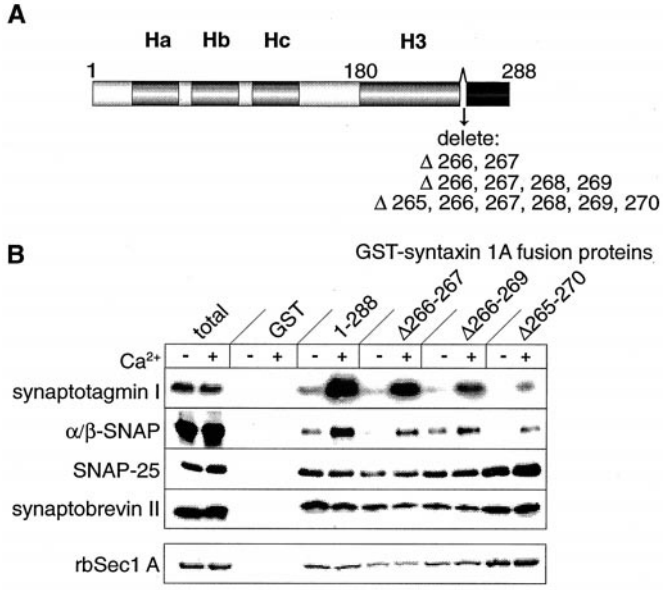

FIG. 2. **Deletions within the N-terminal end of the syntaxin-transmembrane domain affect syntaxin protein-protein interactions.** *A,* schematic of the deletions made within the N-terminal end of the syntaxin TMD. The structure of syntaxin 1A is as described in the legend to Fig. 1. *B,* effects of the deletion mutants on syntaxin protein-protein interactions. Deletion mutants were assayed for interacting protein binding activity as described in Fig. 1. Bound proteins were detected by immunoblot analysis and enhanced chemiluminescence. To assay for rbSec1A binding activity, recombinant rbSec1A (0.5 μM) was incubated with 30 μg of the indicated GST-syntaxin fusion protein for 2 h at 4 °C in 150 μl of Tris-saline pH 7.4 plus 1% Triton X-100. Samples were washed, and bound protein was visualized by staining with Coomassie Blue (note: the GST fusion proteins were cropped from the gel). 20% of the bound material was loaded onto the gel; *Total* corresponds to 1 μg of rbSec1A.

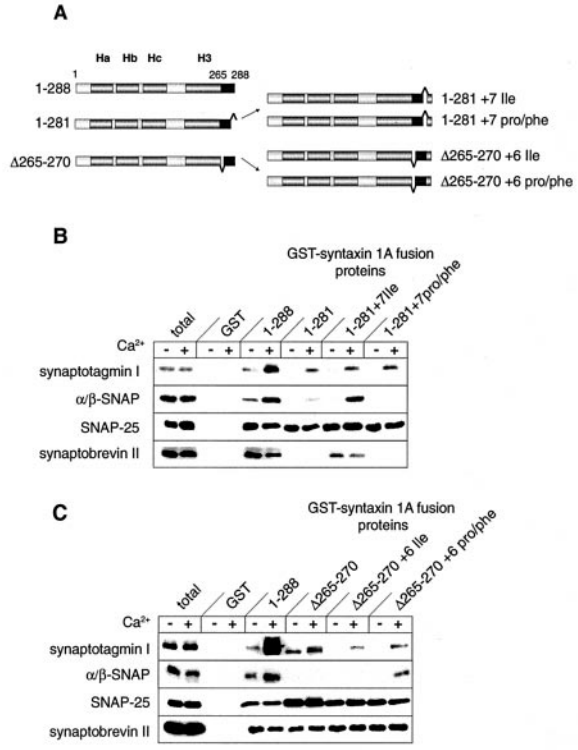

FIG. 3. **Rescue of syntaxin TMD truncation and deletion mutants by adding hydrophobic amino acids.** *A,* schematic diagram of the syntaxin TMD rescue constructs. The structure of syntaxin 1A is as described in the legend to Fig. 1. For these experiments, the 1–281 truncation mutant and the Δ265–270 deletion mutant were lengthened to the same length as the wild-type protein (288 residues) by adding either seven or six amino acids, respectively. These extensions were comprised of either Ile residues or an alternating Pro/Phe sequence. *B,* effects of adding hydrophobic residues onto the 1–281 truncation mutant. α/β-SNAP and synaptotagmin interactions were altered in the 1–281 deletion mutant, SNAP-25 binding was unaffected. Addition of seven Ile residues did not rescue synaptotagmin interactions but partially rescued α/β-SNAP and synaptobrevin interactions. Addition of the Pro/Phe sequence did not affect either interaction. *C,* effects of adding hydrophobic residues onto the Δ265–270 deletion mutant. Experiments were carried out as described above. Deletion of residues 265–270 inhibits synaptotagmin and α/β-SNAP binding, facilitates SNAP-25 binding and does not affect synaptobrevin binding. Addition of six Ile residues or the Pro/Phe sequence did not rescue synaptotagmin or α/β-SNAP binding but did decrease SNAP-25 binding to the level observed with wild-type syntaxin. The Pro/Phe sequence was able to weakly rescue α/β-SNAP binding.

whether this oligomerization activity lies within the TMD of synaptotagmin, we mapped the N-terminal clustering site. The constructs used for this analysis (shown schematically in Fig. 5*A*) were immobilized as GST fusion proteins and were assayed for their abilities to bind a His_6-tagged fragment of synaptotagmin (residues 1–265). As shown in Fig. 5*B*, the soluble synaptotagmin fragment bound to its immobilized counterpart in a Ca^{2+}-independent manner. Removal of the luminal domain did not inhibit binding, however further truncation that removed the TMD strongly reduced binding activity. These data indicate that the TMD of synaptotagmin could directly mediate oligomerization of the protein, but it is also possible that the TMD enables another region of synaptotagmin to homo-oligomerize. We confirmed these results using native synaptotagmin from brain detergent extracts as the ligand (Fig. 5*C*). However, in these experiments, Ca^{2+} facilitated binding, presumably because of Ca^{2+}-triggered oligomerization of the C2B domain of the protein (Refs. 39, 45–48).

In summary, these results suggest that the TMDs of either synaptobrevin or synaptotagmin may rescue deletion of the syntaxin TMD by conferring oligomerization activity. To test this hypothesis directly, we made use of a syntaxin TMD mutant, syx-mult, that harbors three amino acid substitutions that block TMD-mediated oligomerization (Ref. 19; Fig. 6*A*). As a control, we also analyzed a syntaxin mutant, syx-A15, which harbors a string of fifteen alanine residues from position 266–280 (Ref. 19; Fig. 6*A*). The ability of these mutant syntaxins to bind partner proteins was tested as described in Fig. 1*B*. The syx-A15 binding profile was indistinguishable from the minus TMD mutant, again demonstrating the sequence specificity of the TMDs to rescue wild-type binding activity. Surprisingly, syx-mult exhibited normal to enhanced synaptotagmin, α/β-SNAP, and synaptobrevin binding activity. This result strongly indicates that the role of the TMD is not simply to drive syntaxin into dimers for normal binding activity.

Finally, we sought to determine whether the truncation, deletion, and chimeric mutants were able to stably insert into membranes. We postulated that impaired membrane insertion would be analogous to impaired insertion into the detergent micelles used for our protein-protein interaction studies, resulting in alterations in the disposition of the cytoplasmic domain along the surface of the membrane or micelle (49). As shown in Fig. 7*A*, full-length syntaxin (residues 1–288), associated with the pellet fraction in the presence but not the absence of microsomal membranes. Furthermore, the translated protein could not be removed from the microsomal membranes upon extraction with pH 11 bicarbonate (the P2 fraction in Fig. 7*A*) but could be extracted with Triton X-100 (data not shown). These data demonstrate that syntaxin is properly inserted into membranes in this *in vitro* transcription/translation system. We then tested the ability of the C-terminal truncation mutants to stably insert into membranes. As shown in Fig. 7*A*,

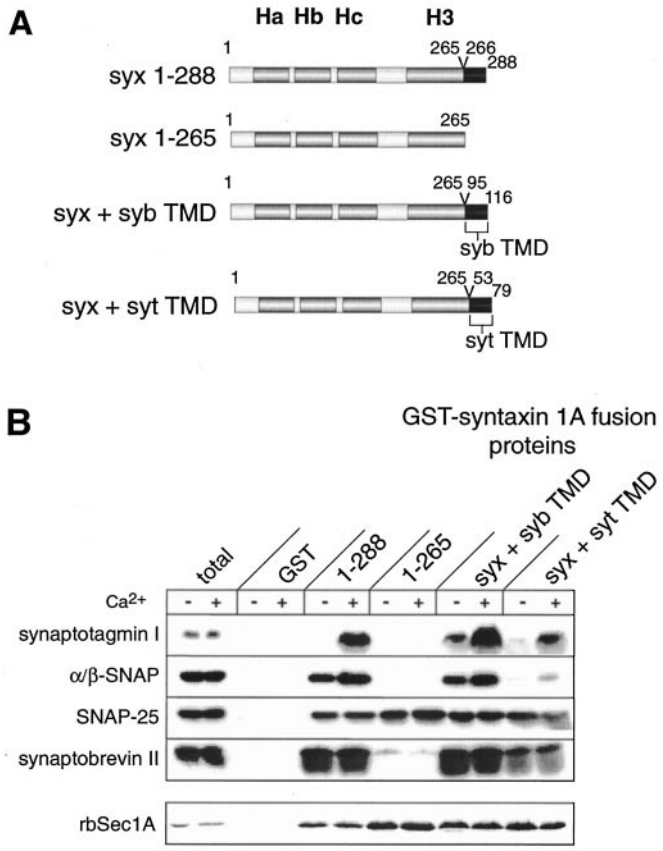

FIG. 4. **Heterologous TMDs can rescue deletion of the syntaxin TMD.** *A,* schematic diagram of syntaxin chimeras used for this analysis. Full-length syntaxin, the cytoplasmic domain, and the cytoplasmic domain fused to the twenty-two residue TMD of synaptobrevin II and the twenty-seven residue TMD of synaptotagmin I are indicated with the corresponding numbers of the amino acid residues. All TMDs are shown in *black*. Abbreviations are *syx*, syntaxin 1A; *syb*, synaptobrevin II; *syt*, synaptotagmin Ib. *B,* binding of syntaxin-interacting proteins was carried out as described in the legend to Fig. 1; binding was detected using enhanced chemiluminescence. In addition we assayed the binding of recombinant rbSec1A; binding was visualized by staining with Coomassie Blue.

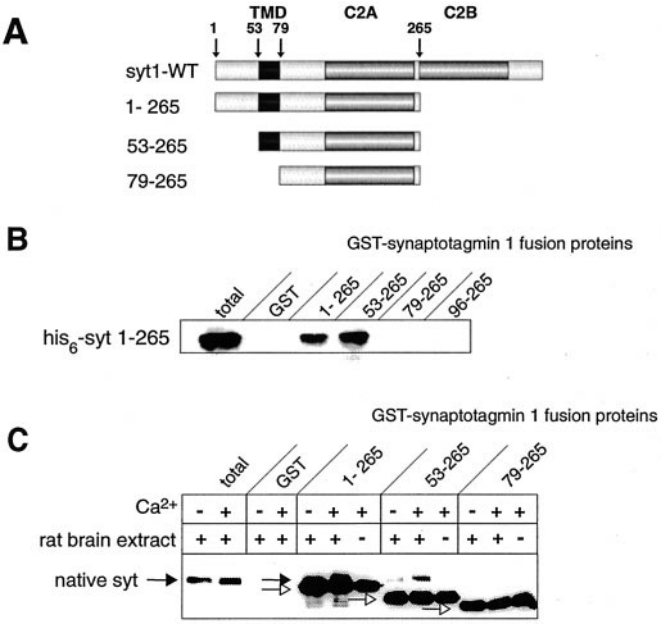

FIG. 5. **The TMD of synaptotagmin I can oligomerize.** *A,* schematic diagram of synaptotagmin (*syt*) constructs used for this analysis. *B* and *C,* the transmembrane domain of synaptotagmin I mediates oligomerization activity. In *B*, a recombinant fragment of synaptotagmin comprised of residues 1–265, His_6-syt-(1–265) (43), was incubated (0.5 μM) with the indicated immobilized GST-synaptotagmin fusion proteins for 2 h at 4 °C. Samples were washed three times in binding buffer and analyzed by SDS-PAGE and immunoblotting. Addition of Ca^{2+} or EGTA did not affect binding activity (data not shown). In *C*, these experiments were repeated using rat brain detergent extracts as a source of soluble synaptotagmin. Binding assays were carried out as described in the legend to Fig. 1, and bound native synaptotagmin was visualized by immunoblotting and enhanced chemiluminescence. Protein was visualized using an anti-C2B domain antibody that partially cross-reacts with C2A; both native synaptotagmin as well as the immobilized fusion protein are detected. *Open arrows* indicate the synaptotagmin fusion protein, the *closed arrow* indicates native synaptotagmin. The increase in binding observed with Ca^{2+} likely reflects the Ca^{2+}-triggered oligomerization of the C2B domain of the native protein (39, 45, 46, 48). Note that in the *1–265 lane*, the native protein is not well resolved from the immobilized fusion protein.

the 284, 281, and 276 truncation mutants inserted into membranes in a pH 11 extraction-resistant manner whereas the 271 mutant failed to bind membranes. The ability of the 281 and 276 truncation mutants to stably insert into membranes is notable, given that these mutations strongly affect syntaxin protein-protein interactions. Clearly, membrane insertion and target protein binding activity can be uncoupled. The C-terminal deletion mutant Δ266–269 was also inserted into membranes, but the larger Δ265–270 deletion mutant failed to become incorporated into microsomes, either because of loss of targeting, translocation, or stable insertion (Fig. 7*B*). It is notable that the Δ265–270 deletion mutant exhibited wild-type synaptobrevin II binding activity (Fig. 3*C*), further demonstrating that membrane incorporation and partner protein binding activity can be uncoupled. We also tested the membrane insertion activity of the chimeric protein that displayed different interacting protein binding avidities. The syntaxin-synaptotagmin-TMD and syntaxin/synaptobrevin-TMD were incorporated into membranes with similar efficiencies, despite their differential abilities to rescue the loss of the syntaxin TMD. Thus, at the resolution of this assay system, proper membrane insertion activity is not correlated with wild-type syntaxin-partner protein binding activity. These data argue against a model in which TMD mutations alter cytoplasmic domain interaction by affecting the disposition of the protein relative to the surface of the membrane or micelle. This argument is further supported by the findings that removal of the TMD affects syntaxin protein-protein interactions in other detergents, including detergents with small aggregation numbers such that a surface (*e.g.* CHAPS and cholate), to which the cytoplasmic domain could interact with (49), is not formed (data not shown).

In the final series of experiments we further established the specificity of the syntaxin TMD in protein-protein interactions. These experiments were carried out to rule out nonspecific direct binding interactions between TMDs and the syntaxin interacting proteins examined above. For this analysis we compared the TMDs of syntaxin and synaptobrevin in greater detail. As shown in Fig. 8*B*, neither synaptotagmin nor α/β−SNAP bound to full-length synaptobrevin, despite the presence of the synaptobrevin TMD. As a positive control, the syntaxin construct harboring the synaptobrevin TMD (also shown in Fig. 4) efficiently bound both synaptotagmin or α/β−SNAP. However, grafting the syntaxin TMD onto the cytoplasmic domain of synaptobrevin did not result in a chimeric protein with any synaptotagmin or α/β−SNAP binding activity. These data clearly demonstrate that the syntaxin TMD is not sufficient to mediate binding of target proteins. Rather, some TMDs enable conjoined cytoplasmic domains to bind other proteins with higher or lower affinities. This effect may be true for other interactions as well. For example, high affinity SNAP-25·synaptobrevin interactions also require the TMD of

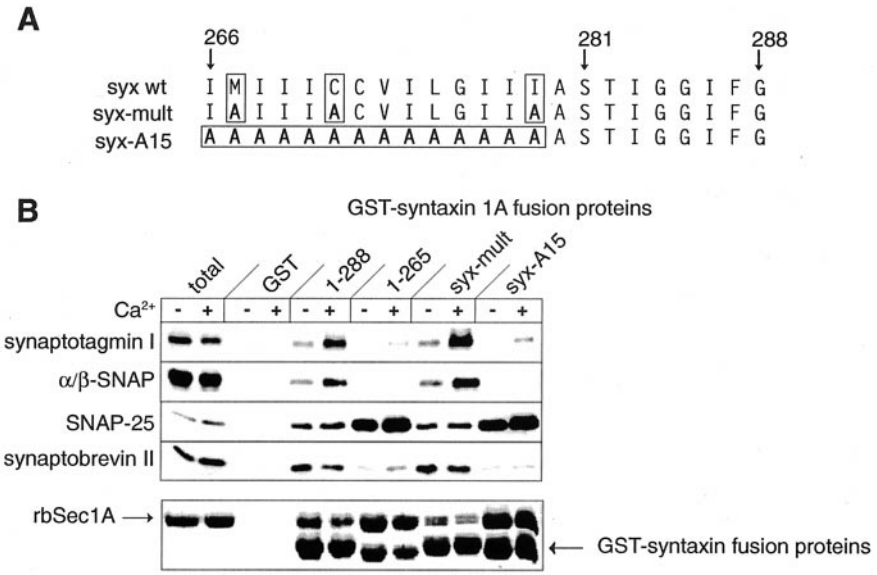

FIG. 6. **Disruption of syntaxin TMD-mediated dimerization does not disrupt TMD-dependent syntaxin protein-protein interactions.** *A,* sequence changes within the syntaxin (*syx*) TMD are indicated by *boxes*. *B,* binding assays were carried out as described in the legend to Fig. 1; rbSec1A binding assays were carried out as described in the legend to Fig. 2 except that 2 μM rbSec1A was used; bound rbSec1A was visualized by staining with Coomassie Blue (note: in the Coomassie-stained gel, the GST fusion proteins are also shown). 25% of the binding reactions were loaded onto the gel; *Total* corresponds to 3 μg of rbSec1A.

synaptobrevin (Fig. 8). Interestingly, high affinity SNAP-25 binding to the cytoplasmic domain of synaptobrevin can be partially rescued by grafting the syntaxin TMD onto synaptobrevin. Thus, the ability of TMDs to affect cytoplasmic domain interactions with other proteins may be a common phenomena among SNAREs.

DISCUSSION

Previous studies indicated that the TMD of syntaxin plays a key role in the ability of syntaxin to interact with other proteins; removal of the TMD inhibited synaptotagmin and α/β−SNAP-binding activity (25, 29). However, it was not clear from these studies whether complete deletion of the TMD had gross effects on the folding of syntaxin, whether the TMD directly participated in target protein binding interactions such that its deletion inhibited binding (18, 19), or whether the TMD contributed to protein-protein interactions via a trivial nonspecific sticky effect. Here we have addressed each of these issues and provide data to indicate that syntaxin can exist in different conformations that are influenced by its TMD.

We began by showing that subtle deletions/truncations of the syntaxin TMD inhibited binding of synaptotagmin, α/β−SNAP, and synaptobrevin in a graded manner. Strikingly, removal of only two residues at the C terminus of the TMD reproducibly inhibited synaptotagmin binding. It is possible that the loss of synaptotagmin binding activity could be caused by loss of direct interactions between the TMDs of these proteins (Fig. 1*B*). For example, it has been reported that the TMD of syntaxin interacts directly with the TMD of synaptobrevin (18, 19). However, this does not appear to be the case. For example, grafting the syntaxin TMD onto the cytoplasmic domain of synaptobrevin did not result in a protein that bound to either synaptotagmin or α/β−SNAP (Fig. 8). In addition, we have observed that removal of the syntaxin TMD largely abolishes the co-immunoprecipitation of syntaxin with the purified cytoplasmic domain of synaptotagmin (data not shown). Clearly, neither the cytoplasmic domain of synaptotagmin, nor α/β−SNAP, have access to the outer leaflet of the plasma membrane that contains the distal region of the syntaxin TMD or to the inside of a detergent micelle as in our experimental conditions, so it is unlikely that the TMD of syntaxin plays a direct role in binding these proteins. Rather, these data indicate that the TMD of syntaxin somehow influences the ability of its cytoplasmic domain, and in particular the H3 domain to bind synaptotagmin (29, 50, 51) and α/β−SNAP (25, 52, 53).

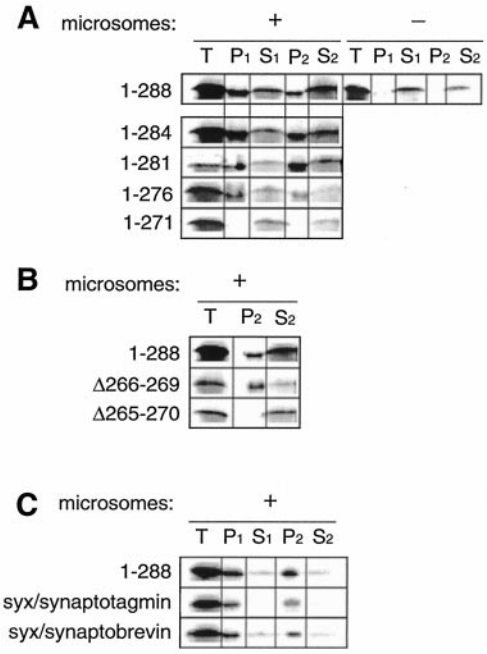

FIG. 7. **Insertion of syntaxin TMD mutants into microsomal membranes.** *A,* effect of syntaxin C-terminal TMD truncations on post-translational insertion into microsomal membranes. Wild-type and mutant syntaxin cDNA were used as templates for *in vitro* transcription/translation in the presence of ^{35}[S]methionine plus (+) or minus (−) canine pancreatic microsomes. Five-μl aliquots of the translation product were diluted into potassium-glutamate buffer or pH 11.5 bicarbonate buffer and centrifuged at 70,000 rpm in a Beckman TLA100.3 rotor for 30 min. The supernatant and pellet from samples separated in potassium-glutamate buffer are designated S1 and P1; the supernatant and pellet from the pH 11.5 wash are designated *S2* and *P2*. Total corresponds to 5 μl of translation mixture. Samples were separated by SDS-PAGE and prepared for fluorography as described under "Experimental Procedures." Full-length syntaxin was inserted into microsomal membranes as shown by partitioning, in an alkali-resistant manner, into the pellet fraction only in the presence of microsomes. Stable insertion was observed in the 284, 281, and 276 truncation mutants; 271 failed to associate with the microsomal membranes. *B,* effect of syntaxin N-terminal TMD deletions on post-translational insertion into membranes. Assays were carried out as described in *A*. The Δ266–269 mutant was stably inserted into microsomal membranes. In contrast, the Δ265–270 failed to associate with microsomal membranes. *C,* insertion of syntaxin-TMD-chimeras into membranes. Assays were carried out as described in *A*. Syntaxin harboring its own TMD, or the TMD of either synaptobrevin or synaptotagmin I, stably associated with membranes.

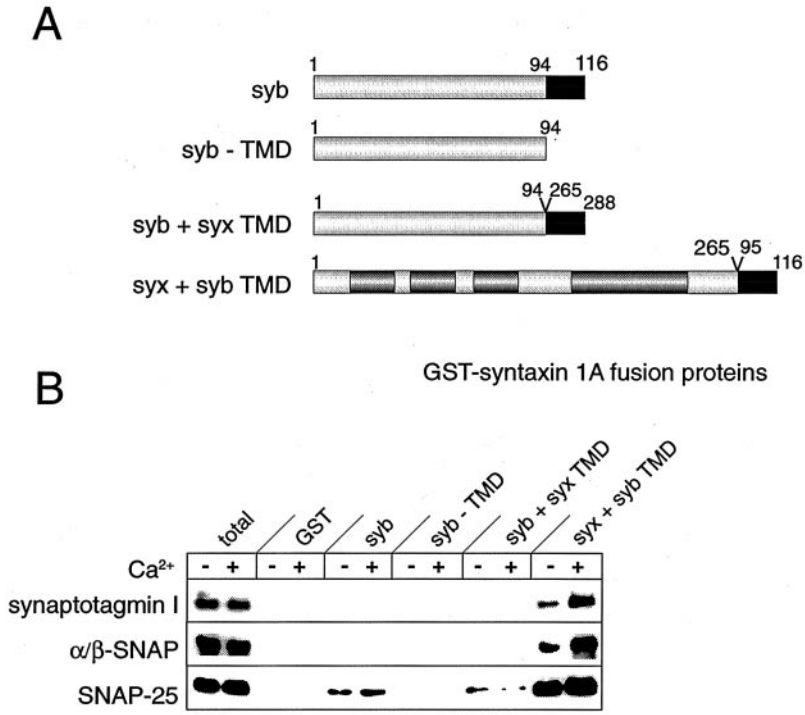

FIG. 8. **TMD grafts further establish the specificity of the TMD requirement for syntaxin-target protein interactions.** *A,* schematic of chimeric and truncated proteins analyzed in *B*. *B,* GST pull-down assays were carried out as described in the legend to Fig. 1. Synaptotagmin I and α/β-SNAP failed to bind either full-length or the cytoplasmic domain of synaptobrevin. Grafting the syntaxin TMD onto the cytoplasmic domain of synaptobrevin failed to confer synaptotagmin or α/β-SNAP binding activity. These data demonstrate that there is not a nonspecific absorption of synaptotagmin or α/β-SNAP onto *bona fide* TMDs; neither the syntaxin TMD nor the synaptobrevin TMD is sufficient to bind these proteins. As a control, and in agreement with Fig. 4, replacement of the syntaxin TMD with the synaptobrevin TMD results in a chimera with strong synaptotagmin and α/β-SNAP binding activity. Syntaxin 1A and synaptobrevin II are abbreviated *syx* and *syb*, respectively.

As noted above, the TMD of synaptobrevin has been reported to interact directly with the TMD of syntaxin (18, 19). In this case, removal of the TMD of syntaxin would be expected to inhibit binding of synaptobrevin. However, this does not appear to be the reason why removal of syntaxin TMD segments inhibits synaptobrevin binding in our experiments (Figs. 1*B* and 2*B*). This conclusion stems from our use of point mutations that inhibit the interaction between the TMD of syntaxin and synaptobrevin (19). As shown in Fig. 6*B*, these loss-of-function mutants actually facilitated association of these proteins under our assay conditions (Fig. 6*B*). We conclude that the TMD of syntaxin indirectly influences the ability of syntaxin to bind not only synaptotagmin and α/β−SNAP, but also synaptobrevin. Whereas C-terminal truncations had graded effects on the binding of all three of these proteins, N-terminal deletions did not diminish synaptobrevin binding but did inhibit synaptotagmin and α/β−SNAP binding (*e.g.* Δ265–270; Fig. 2*B*). These findings demonstrate that there are distinct requirements within the syntaxin TMD that are important for the binding of different proteins. Because synaptotagmin and α/β−SNAP can bind and partially penetrate into membranes (43, 51, 54, 55), it is possible that their binding sites extend a short distance into the plane of the bilayer, lending increased sensitivity to TMD N-terminal deletions.

In contrast to the diminished binding of synaptotagmin, synaptobrevin, and α/β−SNAP upon removal of portions of the syntaxin TMD, binding of SNAP-25 and rbSec1A is enhanced. These data argue against simple misfolding of the syntaxin TMD mutants. Because neither SNAP-25 nor rbSec1A have direct access to the TMD of syntaxin, these observations provide further support for a model in which the TMD affects cytoplasmic domain protein-protein interactions by influencing the conformation of the H3 domain.

It is critical to note the effects of the syntaxin TMD on protein-protein interactions is sequence specific and is not because of nonspecific binding of a hydrophobic TMD to other proteins. As shown in Figs. 3, 4, 6, and 8, a variety of full-length hydrophobic TMDs, grafted onto either syntaxin or onto synaptobrevin, fail to bind synaptotagmin, α/β−SNAP, and synaptobrevin. Furthermore, the Δ265–270 deletion inhibits binding of synaptotagmin and α/β−SNAP, but does not inhibit synaptobrevin binding. Thus, shortening the TMD does not simply allow proteins to "fall off." Our results demonstrate that the cytoplasmic domain of syntaxin must be connected to the proper length and sequence TMD to exhibit the correct binding interaction profile.

We suggest two possibilities for how the TMD of syntaxin can influence the interaction of its cytoplasmic domain with other proteins, either positively (synaptotagmin, synaptobrevin, α/β−SNAP) or negatively (rbSec1A, SNAP-25). In one model, homo-oligomerization of the TMD (19) results in multimerization of the cytoplasmic domain of the protein, and this oligomerization facilitates some interactions (synaptotagmin, synaptobrevin, and α/β-SNAP) and inhibits others (SNAP-25 and rbSec1A). In this model, removal of the TMD would inhibit binding of the former set of proteins and facilitate binding of the latter set. This model was prompted by recent reports establishing that the TMD of syntaxin directly mediates oligomerization of the protein (19). We tested this model by examining the ability of a mutant syntaxin, that fails to oligomerize via the TMD, to bind other proteins. We found that these mutations did not inhibit synaptotagmin, synaptobrevin or α/β−SNAP binding and did not enhance SNAP-25 or rbSec1A binding (Fig. 6*B*). Whereas we cannot rule out the possibility that the syntaxin oligomerization mutant exhibits some residual oligomerization activity, these data strongly argue that the effects of TMD mutations on syntaxin interactions are not simply secondary to effects on syntaxin self-association activity.

In the second model, syntaxin exists in multiple conformations that can be influenced by the presence or absence of the TMD. In this model, removal of the TMD favors a conformation in which the H3 domain has impaired interactions with synaptotagmin, synaptobrevin, and α/β−SNAP and has more favorable interactions with SNAP-25 and rbSec1A. Indeed, syntaxin has been shown to exist in at least two states; an open and a closed conformation (8, 33, 56). In the closed conformation, part of the H3 domain forms a four-helix bundle with the N-terminal Habc domain (33). Because the closed conformation forms a high affinity complex with rbSec1A (34), it is tempting

to speculate that removal of the TMD favors this conformation, whereas the full-length protein favors the open conformation. This model would also account for the ability of the full-length open conformation to bind efficiently to synaptobrevin, synaptotagmin, and α/β−SNAP, because all three proteins bind directly to the H3 domain that is exposed in this conformation (33, 56). However, this two-conformation model does not account for our SNAP-25 binding data, because SNAP-25 also assembles onto the exposed H3 domain (57). This finding suggests that syntaxin can adopt additional conformations, and the presence or absence of a TMD favors one conformation over another to influence the affinity of target protein interactions.

In binary complexes with SNAP-25 or rbSec1A, the C-terminal region of the cytoplasmic domain of syntaxin is disordered and does not form direct contacts with these interacting proteins (33, 34). This region becomes more ordered only upon assembly into SNARE complexes (8). However, the studies indicating that there is a discontinuity in the ordering of the structure between the TMD and the H3 domain, made use of the cytoplasmic domain of syntaxin. It is possible that this segment becomes ordered upon inclusion of the TMD and is involved in transmitting structural information from the TMD to the H3 domain in the context of the full-length protein. Understanding how the TMD affects the structure of the cytoplasmic domain will require high resolution structural studies focused on full-length syntaxin. In summary, we propose that the TMD can inhibit binding of SNAP-25 and rbSec1A, and can facilitate binding of synaptotagmin, α/β−SNAP, and synaptobrevin by influencing the structure of the cytoplasmic domain of the protein.

Our studies demonstrate that the length and sequence of the syntaxin TMD are critical determinants for the specific interaction of syntaxin with other proteins. In this light it is interesting to note that new isoforms and splice variants of syntaxin have been reported that lack the C-terminal transmembrane domain (58, 59). In some cases, truncated forms of syntaxin protein have been detected and shown to exhibit differential interactions with target proteins (59). Thus, removal of the TMD via alternative splicing may strongly influence the function of syntaxin in different cell types or different trafficking pathways. Furthermore, reconstitution experiments indicate that SNARE-catalyzed membrane fusion requires that only one v-SNARE and one t-SNARE need to have transmembrane anchors; the other two strands of the SNARE complex four-helix bundle do not require membrane anchors (13, 14, 60). These findings suggest that in some trafficking pathways, the SNARE complex could contain syntaxins that are not anchored to the membrane via a TMD as long as they are paired with another t-SNARE that has a transmembrane domain. Thus, splicing TMDs in and of t-SNAREs may result in SNARE complexes with unique properties.

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