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The SM protein Sly1 accelerates assembly of the ER–Golgi SNARE complex

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Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Sec1/Munc18 (SM) proteins constitute the core of an ancient vesicle fusion machine that diversified into distinct sets that now function in different trafficking steps in eukaryotic cells. Deciphering their precise mode of action has proved challenging. SM proteins are thought to act primarily through one type of SNARE protein, the syntaxins. Despite high structural similarity, however, contrasting binding modes have been found for different SM proteins and syntaxins. Whereas the secretory SM protein Munc18 binds to the “closed conformation” of syntaxin 1, the ER–Golgi SM protein Sly1 interacts only with the N-peptide of Sed5. Recent findings, however, indicate that SM proteins might interact simultaneously with both syntaxin regions. In search for a common mechanism, we now reinvestigated the Sly1/Sed5 interaction. We found that individual Sed5 adopts a tight closed conformation. Sly1 binds to both the closed conformation and the N-peptide of Sed5, suggesting that this is the original binding mode of SM proteins and syntaxins. In contrast to Munc18, however, Sly1 facilitates SNARE complex formation by loosening the closed conformation of Sed5.

Significance

Every vesicle fusion reaction is driven by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that zipper into tight complexes between membranes. Members of the Sec1/Munc18 (SM) family tightly regulate this process through interactions with a member of the syntaxin SNARE subfamily. However, seemingly different interaction modes have been found for different SM proteins and syntaxins. Here we show that the ER–Golgi SM protein Sly1 interacts not only with the N-peptide of the syntaxin Sed5, as previously thought, but also with its remainder. This corroborates the idea that SM proteins and syntaxins generally make use of two spatially separated binding sites. Remarkably, binding of Sly1 relaxes the autoinhibitory closed conformation of Sed5 and thereby accelerates SNARE complex formation.

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The authors declare no conflict of interest.

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a multisubunit tethering complex known as the homotypic fusion and protein sorting complex (25). Nevertheless, the structure of Vps33 is very similar to other SM protein types despite having low sequence similarity (23, 24). It would be surprising if such bundles were not preserved to maintain similar molecular functions. This raises the question of whether there are missing pieces to our understanding of the molecular role of SM proteins.

With the idea of a conserved molecular role of SM proteins in mind, we aimed here at a more thorough comparison of Sly1 and Munc18, which are still thought to represent two examples of SM proteins with contrasting syntaxin binding modes. So far nothing is known about a second binding site in the Sly1/Sed5 complex, but the presence of a homologous N-peptide binding site in Munc18 and Sly1 reveals a certain similarity of the two SM protein types. It is debated whether binding of Sly1 to the N-peptide of Sed5 is essential for Golgi trafficking while biochemically less is known (11, 26–28). Neither the effect of Sly1 on SNARE complex assembly has been determined rigorously, nor is it clear whether Sed5 can adopt a closed conformation that interferes with its ability to form a SNARE complex. We therefore sought to determine whether Sly1, in addition to its tight interaction with the N-peptide of Sed5, interacts with the remaining part of Sed5 and, if so, whether this interaction would affect the ability of Sed5 to form a SNARE complex. We found that Sed5 adopts a closed conformation. Comparable to Munc18-1, Sly1 binds simultaneously to both the closed conformation and the N-peptide region of Sed5, although the latter is the major contributor to its affinity to the complex. Remarkably, in contrast to Munc18-1, which blocks SNARE complex formation, whereas its SNARE motif alone is not inhibitory to SNARE assembly, Sly1 binds simultaneously to both the closed conformation of Sed5 and fragments used in this study. The short N-peptide motif and the Habc helices (11) (Fig. 1A) are shown in brown and orange colors, respectively, whereas the SNARE motif is colored in red. The transmembrane region is colored in black. (A, Lower) Sed5 variants used in this study are displayed. (B, Lower) SNARE complex formation is generally accompanied by large structural changes (e.g., refs. 29, 30). When we mixed the four SNARE motifs of the ER–Golgi SNARE complex at 2 μM each (Sed5, Bos1, Bet1, and Sec22) (31–34), a major but slow increase in fluorescence anisotropy of about 400 nM OG-labeled Sec22 126–186 (C) upon mixing with 7 μM Bet1 1–118, Bos1 151–221, and full-length Sed5 (Sed5 1–320; black) or the SNARE motif of Sed5 (Sed5 211–320) in the absence (red) or presence of 28 μM of the isolated Habc domain (Sed5 1–210; gray). (D) Size exclusion chromatographic elution profiles for the individual Habc domain (Sed5 1–210) in orange, the individual SNARE motif (Sed5 211–320) in red, or the combined Sed5 fragments in gray (35 μM each). Prior to separation on a Superdex 200 column, the proteins were incubated overnight. The Habc domain cotranslates with the SNARE motif. The respective peak fractions were analyzed by SDS/PAGE and stained with Coomassie Blue. Note that the interaction in trans can be observed by native gel electrophoreses as well (Fig. S2A). Binding of the isolated Habc domain to the SNARE motif of Sed5 measured by ITC. Sed5 1–210 at 150 μM was titrated into 10 μM of Sed5 211–310. Shown are the integrated areas normalized to the amount of the Habc domain (kcal/mole) versus the molar ratio of the Habc domain to the SNARE motif. The solid curve is the best fit to the data for a single-site binding model using nonlinear least squares fit. An endothermic interaction was observed upon titration, implying that folding of Sed5 into a closed conformation is entropy-driven. (E) Thermal CD melting curves of the entire cytoplasmic domain (Sed5 1–320, black) and the Habc domain (Sed5 1–210, orange) of Sed5. The construct including the SNARE motif is about 10 °C more stable that the one lacking this domain.

Results

**Sed5 Adopts a Closed Conformation That Interferes with SNARE Complex Formation.** Previous studies have shown that SNARE complex formation is generally accompanied by large structural changes (e.g., refs. 29, 30). When we mixed the four SNARE motifs of the ER–Golgi SNARE complex at 2 μM each (Sed5, Bos1, Bet1, and Sec22) (31–34), a major but slow increase in fluorescence anisotropy of about 400 nM OG-labeled Sec22 126–186 (C) upon mixing with 7 μM Bet1 1–118, Bos1 151–221, and full-length Sed5 (Sed5 1–320; black) or the SNARE motif of Sed5 (Sed5 211–320) in the absence (red) or presence of 28 μM of the isolated Habc domain (Sed5 1–210; gray). (D) Size exclusion chromatographic elution profiles for the individual Habc domain (Sed5 1–210) in orange, the individual SNARE motif (Sed5 211–320) in red, or the combined Sed5 fragments in gray (35 μM each). Prior to separation on a Superdex 200 column, the proteins were incubated overnight. The Habc domain cotranslates with the SNARE motif. The respective peak fractions were analyzed by SDS/PAGE and stained with Coomassie Blue. Note that the interaction in trans can be observed by native gel electrophoreses as well (Fig. S2A). Binding of the isolated Habc domain to the SNARE motif of Sed5 measured by ITC. Sed5 1–210 at 150 μM was titrated into 10 μM of Sed5 211–310. Shown are the integrated areas normalized to the amount of the Habc domain (kcal/mole) versus the molar ratio of the Habc domain to the SNARE motif. The solid curve is the best fit to the data for a single-site binding model using nonlinear least squares fit. An endothermic interaction was observed upon titration, implying that folding of Sed5 into a closed conformation is entropy-driven. (E) Thermal CD melting curves of the entire cytoplasmic domain (Sed5 1–320, black) and the Habc domain (Sed5 1–210, orange) of Sed5. The construct including the SNARE motif is about 10 °C more stable that the one lacking this domain.

Next, we tested more directly whether Sed5 adopts a closed conformation. Upon size exclusion chromatography, a stoichiometric mix of the Habc domain (Sed5 1–210; i.e., including the N-peptide) and the SNARE motif comigrated (Fig. 1D), supporting the idea that the individual domains of a split Sed5 can form a complex in trans. The affinity of this interaction within a split Sed5 molecule was determined to be 2.7 μM by isothermal titration calorimetry (ITC; Fig. 1E). Finally, a comparison of CD thermal melting curves demonstrated that the Habc domain is drastically stabilized in the presence of the SNARE motif in the intact Sed5 molecule (Fig. 1F), in which the effective concentration of the two domains is much higher. These results are similar to the ones obtained for the secretory yeast syntaxin Sso1 (36), which resides in a rigid closed conformation (37) that slows SNARE complex formation by three orders of magnitude (30). Altogether, our results suggest that
Sly1 also adopts a rigid closed conformation that slows SNARE complex formation drastically.

Sly1 Accelerates SNARE Complex Assembly. The interaction of Sly1 with the N-peptide does not hinder SNARE complex assembly of Sly1-bound Sed5 (28), but the precise role of Sly1 during SNARE complex formation is still an open question. When we added Sly1 to our assay, we found that Sly1 substantially accelerates SNARE complex formation (Fig. 2A). This suggests that Sly1 must be able to control the reactivity of Sed5. As Sly1 does not accelerate SNARE assembly of the isolated SNARE motif of Sed5 (SI Appendix, Fig. S3), it is likely that Sly1 might be influencing the equilibrium between the open and closed conformation of Sed5. In whatever way Sly1 achieves this feat, our finding indicates that the interaction between Sly1 and Sed5 is not restricted to the N-peptide region of Sed5. The currently discussed modes of interaction between SM proteins and syntaxins are depicted in Fig. 2B. It should be noted that, although the crystal structure of the Sly1/Sed5 complex only contains the N-peptide region of Sed5 (12), it does not rule out that other regions interact as well. Such interaction may be weak and transient. In view of the fact that two other types of SM proteins, Munc18 and Vps45, interact with two spatially separated sites of their cognate syntaxins (15), we next probed the interaction mode of Sly1 and Sed5 in more detail.

Sly1 Interacts Not Only with the N-Peptide But Also with the Remainder of Sed5. To determine whether Sly1 also interacts with an additional site in Sed5, we first used ITC, an experimental strategy that had helped us before to shed new light on the binding mode of the SM proteins Munc18 and Vps45 (15). We observed that the isolated N-peptide (Sed5 1–21) binds to Sly1 with a favorable enthalpy (ΔH = −16 kcal/mol) and a dissociation constant of ~1.5 nM (Fig. 2C). As anticipated, no binding was observed when the N-peptide region was removed (Sed5 21–324). Binding of Sed5 21–324 could not be restored when the N-peptide was added in trans (SI Appendix, Fig. S4). We also found that the isolated SNARE motif of Sed5 (Sed5 211–320) did not bind to Sly1 (SI Appendix, Fig. S3). Together, this confirms that the N-peptide is sufficient and necessary for high-affinity binding (10, 11).

When a Sed5 construct was used that contained both the N-peptide and the adjacent Habc domain (Sed5 1–210), a small decrease in enthalpy was detected (~−18 kcal/mol) (Fig. 2C). A more pronounced decrease in enthalpy was observed when the entire cytoplasmic region of Sed5 (Sed5 1–320)—that is, a construct that also included the SNARE motif—was used (~−25.6 kcal/mole) (Fig. 2C). The difference in binding enthalpy likely reflects the contribution of additional binding surface to the interaction, suggesting that Sly1 indeed interacts with an additional part of Sed5.

We then further examined the notion that Sly1 makes use of two binding sites in Sed5 by introducing disruptive point mutations in each of the two respective regions of Sed5, the N-peptide and the SNARE motif. One substitution, F10A, was introduced into the N-peptide region of Sed5. This conserved phenylalanine binds into a hydrophobic pocket in domain I of Sly1 (12), and the mutation had been previously shown to disrupt binding to Sly1 (28). Indeed, when the point mutation was introduced into the isolated N-peptide (Sed5 1–21F10A) (Fig. 2D), the binding of Sly1 was not preserved (Fig. 2D). Interestingly, the point mutation did not prevent binding of the entire cytoplasmic region of Sed5 (Sed5 1–320F10A), but greatly reduced the affinity to ~234 nM (Fig. 2D). This indicates that the remainder of Sed5 must indeed contribute to the overall binding strength. The other substitution was introduced into the SNARE motif of Sed5 at position 290 (Sed5 1–320D290A). It is homologous to a mutation in the SNARE motif of syntaxin 1a that significantly interferes with binding of the closed conformation into the cavity of Munc18-1 (15). We found that this point mutation increased the enthalpy (SI Appendix, Table S1 and Fig. S4), suggesting that the mutated region of Sed5 is involved in binding to Sly1.

In summary, the ITC experiments confirm that Sly1 interacts tightly with Sed5. They also reveal that the N-peptide region is the major contributor to the high-affinity interaction, whereas the remainder of Sed5, possibly in a closed conformation, contributes an additional binding surface that only moderately increases the overall affinity of the Sly1/Sed5 complex.

Sly1 Rearranges the Closed Conformation of Sed5. If we are correct and Sly1 does indeed bind to the remainder of Sed5 in a closed conformation, where the SNARE motif is tightly bound to the Habc domain, why does Sly1 then not inhibit SNARE complex formation in a similar fashion as Munc18? To address this question, we made use of the fact that the two domains of Sed5, the Habc domain (Sed5 1–210) and the SNARE motif (Sed5 211–320), form a stable complex that comigrates upon gel filtration (Fig. 1D). We also observed that a complex between Sly1 and Sed5 1–210 comigrates. However, when Sly1 was combined with both regions of a split Sed5, the Habc domain and the SNARE motif, only Sly1 and the Habc domain comigrated, whereas the SNARE motif eluted later (Fig. 3A). This suggests that in the presence of Sly1, the SNARE motif of Sed5 is less tightly bound to the Habc domain.

To more directly test whether the SNARE motif is less tightly bound in the presence of Sly1, we first compared the dissociation rates of different Sed5 fragments from Sly1. For this, we labeled different Sed5 fragments with a fluorescent dye. Upon addition of Sly1, the fluorescence anisotropy of each of the labeled Sed5 molecules rapidly increased. We then determined the off-rates of the different Sly1/Sed5 complexes by competitive dissociation

Fig. 2. Sly1 interacts with the N-peptide motif and with the remaining portion of Sed5. (A) Addition of Sly1 (11 μM) accelerates SNARE complex formation in the presence of Sed5 (Sed5 1–320, 7 μM, blue). SNARE complex formation was monitored by the increase in fluorescence anisotropy of fluorescent Sec22 126–186CTLDG as described in Fig. 1C. Note that the experiment in the absence of Sly1 (black) is the same as in Fig. 1C. Sly1 had no effect on the assembly rate when only the SNARE motif of Sed5 was used (SI Appendix, Fig. S3). Note that the acceleration observed agrees with the finding that the ER-Golgi SNAREs fuse liposomes only in the presence of Sly1 (18). (B) Representation of three different SM-syntaxin binding modes: involving solely the N-peptide (I), solely the closed conformation of the syntaxin (II), or the N-peptide and the closed conformation together (III). The SM protein is depicted in blue, whereas syntaxin is colored as in Fig. 1B. (C) Calorimetric titrations of truncated Sed5 variants Sed5 1–21 (15 μM), Sed5 1–210 (15 μM), Sed5 1–320 (15 μM), and Sed5 21–324 (30 μM) into Sly1 (12 μM). (D) Calorimetric titrations of the Sed5 mutants Sed5 1–211T10A (25 μM) and Sed5 1–320T10A (25 μM) into Sly1 (1.5 μM). Note that all ITC experiments are shown in SI Appendix, Fig. S4 and that the corresponding data are given in SI Appendix, Table S1.
To observe the influence of Sly1 on the state of the SNARE motif more directly, we next labeled the SNARE motif with a fluorescent dye (Sed5 1–210, C278-TR). The fluorescence anisotropy of the labeled SNARE domain increased upon complex formation with the Habc domain. We then determined the off-rate of the Habc domain/SNARE motif complex by rapid dilution. When the experiment was carried out in the presence of Sly1, the SNARE motif dissociated more rapidly (Fig. 3C). Correspondingly, a titration approach confirmed that the SNARE motif is less tightly bound in the presence of Sly1 (Fig. 3D).

In summary, the results substantiate the hypothesis that binding of Sly1 to Sed5 alters the configuration of the SNARE motif, maybe indirectly through a conformational change in the Habc domain, so that it can participate in SNARE complex formation.

Discussion

SNARE and the SM protein families are key for the docking and fusion of transport vesicles with their respective target membrane. Numerous genetic studies have provided compelling evidence that SM proteins act primarily through one subgroup of SNARE proteins, the syntaxins (6, 7). For instance, the secretory SM protein Munc18 forms a tight complex with syntaxin 1, whereas Sly1 interacts tightly with the syntaxin Sed5. Naturally, it was assumed that these homologous factors share a common molecular mechanism. However, this view received less support when the structures of their high-affinity complexes were revealed. Thus, a portion of the Sed5 Habc domain accounts for a slower SNARE assembly, because in its absence the assembly proceeds faster. However, whereas syntaxin 1a is slowed only by a factor of approximately 10 (39), the assembly speed of Sed5 is reduced by three orders of magnitude. Possibly, Sed5 requires more assistance than syntaxin 1a to be released from its autoinhibitory state into a state that is required for the interaction with its SNARE partners.

Our results corroborate the idea that a binding site is reversed: Whereas the N-peptide of syntaxin 1a binds, the N-peptide of Sed5 binds more directly, we next labeled the SNARE motif with a split Sed5. (A) Size exclusion chromatographic elution profiles for Sly1 (blue), the combination of Sly1 and the Habc domain of Sed5 (Sed5 1–210, dark green), and the combination of Sly1, Habc domain, and SNARE motif of Sed5 (Sed5 211–320, light green). Arrows indicate the peak maxima of the individual SNARE motif of Sed5 (red) and of the combination of the Habc domain and SNARE motif of Sed5 (gray) as shown in Fig. 1D. In the presence of Sly1, the SNARE motif of Sed5 dissociates from the Habc domain. Note that a portion of the Habc domain not bound to Sly1 still interacts with the SNARE motif. The peak fractions were analyzed by SDS/PAGE and stained with Coomassie Blue. Results of the analogous experiment with combinations of Sly1 and Sed5 1–320 or Sed5 211–320 are shown in SI Appendix, Fig. S5. (B) Determination of the off-rates of different Sly1/Sed5 complexes by competitive dissociation. The anisotropy signal is reported as a fraction of bound fluorescent molecules. The dissociation was fit by a single exponential: Sed5 1–210, dark red curve; Sed5 1–320, orange curve; Sed5 1–320, black curve. The dissociation rate of the N-peptide did not change when Sed5 211–324 was added in trans (SI Appendix, Fig. S6). (C) The dissociation rate of the Habc/SNARE motif complex was determined by rapid dilution. In the absence of Sly1, the rate was -0.006 s⁻¹. In the presence of 32 μM Sly1, the rate was -0.014 s⁻¹. Thus, Sly1 accelerates the off-rate of a split Sed5. (D) Complex formation between the individual domains of Sed5 leads to an increase of fluorescence anisotropy of a labeled SNARE motif (Sed5 211–320) when mixed with the Habc domain. To determine the effect of Sly1 on the affinity between both Sed5 segments, ~100 nM of labeled SNARE motif was incubated with increasing amounts of the Habc domain in the presence or absence of about 30 μM Sly1. The anisotropy signal is reported as a fraction of bound fluorescent molecules. Binding curves were fitted using nonlinear regression to determine the affinities between the Habc domain and the SNARE motif of Sed5. The Kd was about threefold lower in the presence of Sly1 (Kd ~ 7.3 μM) than in its absence (Kd ~ 2.3 μM). Note that the affinity determined by fluorescence anisotropy agrees well with the one obtained by ITC (Kd ~ 2.7 μM, SI Appendix, Table S1).
were still able to form a complex, albeit with less affinity. Apparently this affinity is sufficient to maintain the overall function of the protein complex. Analogous experiments have been performed to explore the role of the N-peptide binding site of Munc18-1. Point mutations in Munc18-1 were shown to strongly interfere with binding of the N-peptide; however, the mutants were still able to rescue synaptic secretion in Munc18-1 knockout neurons (43). Conversely, a syntaxin 1a, in which the N-peptide had been removed, was not able to rescue synaptic secretion in syntaxin-deficient neurons (44). The stark difference between the two studies may be explained by the fact that both binding sites are crucial for the function of the complex. The point mutations in Munc18-1 used in one study may have weakened only one interaction site. It seems feasible that this local defect could be overcome, as both binding sites act together. By contrast, in the study by Zhou et al. (44), a syntaxin variant was used in which one binding region had been deleted altogether, a deficiency that might not be able to be overcome by the secrerion apparatus. Note that comparable results were obtained for Caenorhabditis elegans (42, 45, 46).

Although the N-peptide interaction mode of the two SM protein/syntaxin complexes exhibits nuanced differences, the results of the interaction with the remainder of syntaxin appear to be diametrically opposed: Whereas Munc18-1 interferes with the ability of the bound syntaxin to form a SNARE complex, Sly1 assists Sed5 in binding to its partner SNAREs. In fact, if one ignored the inhibitory activity of Munc18-1, Sly1’s stimulating effect would almost perfectly fit the job description of SM proteins derived from various in vivo studies. However, how can the different outcomes be explained? This question cannot yet be answered sufficiently, because many of the details appear to be missing. However, one can obtain an initial impression by taking a closer look at the fundamental role of SM proteins. It is generally thought that SM proteins orchestrate the zippering of the SNARE proteins of fusing membranes, primarily by shaping the conformation of the bound syntaxin. To alter the activity of the bound syntaxin, an SM protein must be able to directly or indirectly act on the SNARE motif that is attached to the Habc domain. In the Munc18-1/syntaxin complex, the SNARE motif is clamped securely in the cavity of Munc18-1, a conformation that impedes syntaxin’s ability to form a SNARE complex (15). The structure of the interaction of Sly1 with the remainder of Sed5 is not currently known, but because Sly1 does not interfere with but strongly supports Sed5’s activity, we can safely assume that the complex resides in a somewhat different conformation, a scenario depicted in Fig. 4. In fact, our data using the isolated domains of Sed5 suggest that the SNARE motif of Sed5 is less tightly coupled to its Habc domain in complex with Sly1 than as in the individual Sed5. By contrast, Munc18-1 stabilizes the interaction between the domains of a split syntaxin 1a (15). It is possible that during the reaction, a transition state complex is formed between the SM protein and syntaxin. In the case of Sly1 and Sed5, the lower activation energy leads, as expected, to a faster reaction. In the case of Munc18-1 and syntaxin 1a, it is possible that the complex formed does not represent the transition state complex but rather a stable configuration from which syntaxin cannot escape. The stable configuration might allow Munc18 to control syntaxin’s accessibility, a property that is well suited for tight regulation of exocytosis. Possibly, the Munc18/syntaxin complex might subsequently, with the help of other factors like the protein Munc13 (47), switch to the transition state configuration. In this regard, it should be noted that mutations in the linker region—that is, the “LE mutations” (15)—of syntaxin can overcome this block without the aid of other factors (15, 47) and without interfering strongly with the global conformation of the Munc18-1/syntaxin complex (41).

If the above outlined scenario is correct, these findings indicate that the energy barrier between the putative stable intermediate and transition state configuration might be relatively small.

In summary, our findings suggest that Sly1 makes use of two binding sites to prepare Sed5 for SNARE complex assembly. Most likely, the N-peptide binding site serves to keep Sly1 at the relevant position, whereas the other interaction site serves to act on the conformation of syntaxin. On a structural level, however, it remains to be clarified how exactly Sly1 renders Sed5 more open for its SNARE partners. Moreover, it remains unclear whether Sly1’s role is only to present an open Sed5 to the partner SNAREs or whether it also subsequently facilitates the assembly of the SNARE complex. Our data corroborate the idea that SM proteins and syntaxins originally shared a common motif—maintenance mechanism that involved two spatially separated binding sites. Because then, different homologous pairs have diverged, although to different degrees, so that their commonalities are barely discernible.

**Experimental Procedures**

**Protein Constructs.** Apart from the expression constructs for yeast Syl1 1–666 in pGEX-T7 vector and Bet1 1–118 in pET19b vector, each of the recombinant proteins was cloned into a pET28a vector, which contains an N-terminal, thrombin-cleavable His-tag. The constructs for Syl1 1–666, Sed5 21–324, Bet1 1–118, and Sec22 126–186 (13, 28) were kindly provided by R. Peng (University of Bern, Bern, Switzerland). The following truncated variants of Sed5 were generated in this study—Sed5 1–210, Sed5 1–320, and Sed5 211–320—as were the Sed5 1–320 constructs containing the point mutations F10A and I290A. The following single-cysteine variants—Sed5 1–210C(26), Sed5 1–320C(26), Sed5 211–320C(26), and Sec22 126–186C(61) were generated for fluorescence measurements. The construct encoding for the SNARE motif of Bos1 (Bos1 151–221) was also newly generated in this study. The peptides corresponding to the N-peptide regions of Sed5 (Sed5 1–21) and Uef1 (Uef1 1–21) were synthesized by Biosyntan GmbH, as were the variants containing the single-point mutation Sed5 1–21T166 and Sed5 1–21 with a C-terminal cysteine.

**Protein Purification.** All proteins were expressed in the Escherichia coli strain BL21 (DE3) and, except Syl1, purified by Ni2+-nitritotriacetic acid chromatography followed by ion exchange chromatography on an Äkta system (GE Healthcare). The proteins were eluted with a linear gradient of NaCl in 20 mM Tris, pH 7.4, 1 mM EDTA (standard buffer) as previously described (15). We added 1 mM DTT for proteins containing cysteine residues. His-tags were removed using thrombin. For the entire cytosolic portion of Sed5 (Sed5 1–320), ion exchange chromatography was performed in the presence of 2 M urea to prevent aggregation. Afterward, urea was removed by dialysis against standard buffer containing 200 mM NaCl, in which purified Sed5 was stable. Syl1 was first purified by GST affinity chromatography. The GST moiety was cleaved by thrombin on the beads. Syl1 was further purified by size exclusion chromatography on a Superdex-200 column in standard buffer containing 200 mM NaCl and 1 mM EDT. Quaternary SNARE complexes consisting of Sed5 (Sed5 211–320 or Sed5 1–320), Bos1 151–221, Bet1 118–118, and Sec22 126–186 were assembled from purified components and purified by ion exchange chromatography. Generally, the purity of the proteins was greater than 95% as determined by SDS/PAGE. Protein concentrations were determined by absorption at 280 nm.

**ITC.** ITC was performed on a VP-ITC instrument (GE Healthcare) at 25 °C as previously described (15). Proteins were dialyzed twice against PBS buffer (20 mM sodium phosphate, pH 7.4, 200 mM NaCl, 1 mM DTT) and degassed before experiments. Typically, titrations were carried out by 15-μL injections.
The baseline-corrected raw data were analyzed with MicroCal Origin 7.0 to obtain the binding enthalpy (∆H), the stoichiometry (n), and the equilibrium association constant (K). Binding curves were generated by using a non-linear least squares fit for a single-site binding model.

Fluorescence Spectroscopy. Fluorescence measurements were carried out in a Fluorolog 3 spectrometer in a T configuration equipped for polarization (model FL322; Horiba Jobin Yvon). Single-cysteine variants were labeled with Texas Red maleimide or Oregon Green (OG) 488 iodoacetamide according to the manufacturer’s instructions. The concentration of labeled proteins was determined by a Bradford assay. Experiments were performed at 25 °C in 1-cm, 0.4-cm, or ultramicro quartz cuvettes (Hellma) in a PBS buffer. Local flexibility of labeled residues, which increases upon complex formation and decreases upon dissociation, was reported in terms of fluorescence anisotropy (r) essentially as described (15). For affinity titrations, the signal was averaged for 200–300 s. For competitive dissociation experiments, fluorescent molecules. Binding constants were obtained from least squares fittings to the equation: 

\[
\text{r}_{\text{min}} + \frac{(r_{\text{max}} - r_{\text{min}})}{1 + [	ext{Sed5}]^{n_{\text{eq}}}} 
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For the competitive dissociation experiments, ~50 nM of OG-labeled Sed5 variants (Sed5 1–21) was first mixed with saturating amounts of Sly1 (~50 nM) was added, and the decrease in fluorescence anisotropy was followed. In the rapid dilution experiments, ~60 nM of the labeled SNARE motif was mixed with 22 μM of Habc domain in a total volume of 100 μL. Then, the mixture was rapidly diluted to 3 mL, and the dissociation was monitored by the decrease in fluorescence anisotropy.

CD Spectroscopy. Measurements were carried out in PBS buffer as previously described (29) using a Chirascan instrument (Applied Photophysics). Spectra were recorded in quartz cuvettes with a path length of 0.1 cm. For thermal denaturations, the ellipticity at 222 nm was recorded between 20 and 95 °C at a heating rate increment of 30 °C/min. Kinetic measurements were carried out using 1-cm quartz cuvettes at 25 °C. Proteins were mixed, and the change in the CD signal was followed at 222 nm.

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