Concerted Auto-regulation in Yeast Endosomal t-SNAREs*

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In yeast, the assembly of the target (t)-SNAREs [Tlg2p/Tlg1p,Vti1p] and [Pep12p/Tlg1p,Vti1p] with the vesicular (v)-SNARE Snc2p promotes endocytic fusion. Here, selected mutations and truncations of SNARE proteins were tested in an in vitro fusion assay to identify potential regulatory regions in these proteins, and two distinct regions were found. The first is represented by the combined effect of the three t-SNARE N-terminal regions and the second is located within the Tlg1p SNARE motif. These internal controls provide a potential mechanism to enable SNARE-dependent fusion to be regulated.

The core mechanism of SNARE1-NSF attachment protein (SNAP) receptor-mediated fusion is strikingly simple: a target-SNARE (t-SNARE) generates fusion only with its cognate vesicular-SNARE (v-SNARE) (1–5). The cognate t- and v-SNARE pair forms a stable helical complex composed of four helices (three from the t-SNARE and one from the v-SNARE). Each of the four helices comes from a different subfamily: the Syntaxins, the Bet1 group, the Bos1 group, and the R-SNAREs (6). Upon assembly, all membrane anchors of the helical bundle are at one end and the N-terminal domains at the other end (7, 8). The largely unstructured monomers undergo a conformational change during SNARE assembly, which releases enough free energy to overcome the repulsive forces of the opposed membranes, and leads to lipid mixing (9). After fusion, the ATPase NSF and α-SNAP disassemble SNARE complexes, and monomers are recycled (10).

The finding that SNAREs constitute the core fusion machinery and are sufficient for membrane fusion raises the question of how they are regulated. Many SNAREs, in particular the Syntaxin heavy chains, possess N-terminal extensions, which can independently fold and are able to modulate SNARE activity. The neuronal Syntaxin1, as well as Sso1p (yeast homolog of Syntaxin1) and Syntaxin7 (late endocytic mammalian Syntaxin), adopt a “closed” conformation. In this case, their N-terminal domain, structured in a three-helix bundle, is able to interact with the SNARE motif, generating the so-called closed conformation. This interaction blocks the binding of the light chains and inhibits the t-SNARE complex formation (11–15). The removal of the Syntaxin N-terminal domain results in an increase of fusion (16).

The Syntaxin Vam3p on the other hand, uses a different mechanism. In the yeast vacuole, the three-helix bundle of Vam3p-N-terminal domain does not interact intramolecularly with the SNARE motif (17). Nonetheless, the removal of this N-terminal domain influences the SNARE complex assembly (18). Despite the fact that the mechanisms differ (closed or open Syntaxins), all heavy chain N-terminal domains tested act by reducing t-SNARE assembly (11–15, 18).

The light chains also have N-terminal domains, but their role is less clear. Neither appears to adopt a closed intra-molecular conformation nor does their N-terminal domain seem to influence the rate of the t-SNARE complex formation (15, 19, 20).

Previously, we characterized two yeast endocytic complexes, the early endosome/trans-Golgi network complex [Tlg2p/Tlg1p,Vti1p] and the late endocytic complex [Pep12p/Tlg1p,Vti1p], both fusing with Snc2p v-SNARE although very slowly in vitro (4, 5). In the present study, using these SNARE complexes, we determined the influence of their N-terminal domains on fusion. Furthermore, we investigated whether the SNARE motif itself influences the kinetics of liposome fusion.

EXPERIMENTAL PROCEDURES

Pepetides—Sn2C-C-pept (GERLTSIEDKADNLAIASAGQFKRGANVRRKQR) was synthesized by the Microchemistry Core Facility of the Memorial Sloan Kettering Cancer Institute (New York) and VAMP8-C-pept (GENEHLRNKRTELATSEFHKTSKQVARKFWVVK) by SynPep Corp. (Dublin, CA). Both peptides were dissolved in 10 mM HCl and then diluted in a reconstitution buffer (25 mM Hepes-KOH pH 7.4, 100 mM KCl, 10% glycerol) to a concentration of 3 mg/ml.

Plasmid Constructs—The coding sequences of the mammalian SNAREs were cloned as following: hSyntaxin8 using FO61 (GGGCATATCCATATGGCCCCGACCCCGTCTTGTCGTTCTCAGTTGGT) and FO82 (GGCAATTCATAGCGAGTCCAGGACACTGCC), mVti1b using FO58 (GGGCATATCCATATGGCCCCGACCCCGTCTTGTCGTTCTCAGTTGGT) and FO62 (GCGAATTCTCAGTTGGTCGGCCAGACTGC), hSyntaxin8 using FO50 (GGGCATATCCATATGTCTTACACTCCAGGATCTCGTCGTTCTCAGTTGGT) and FO59 (GCGAATTCTCAGTGGTTCAATCCCCAAA), and hVAMP8 (hVAMP8-TMD) was amplified with FO108 and FO109 (GGGCATATCCATATGCGGCCTCGCCCGGCGTCTTGTCGTTCTCAGTTGGT) and FO59 (GGCAATTCATAGCGAGTCCAGGACACTGCC), tMemb1 using FO58 (GGGCATATCCATATGGCCCCGACCCCGTCTTGTCGTTCTCAGTTGGT) and FO62 (GCGAATTCTCAGTTGGTCGGCCAGACTGC), and hVAMP8 using FO108 (GGGCATATCCATATGCGGCCTCGCCCGGCGTCTTGTCGTTCTCAGTTGGT) and FO109 (GGCAATTCATAGCGAGTCCAGGACACTGCC), and hVAMP8 using FO108 (GGGCATATCCATATGCGGCCTCGCCCGGCGTCTTGTCGTTCTCAGTTGGT) and FO109 (GGCAATTCATAGCGAGTCCAGGACACTGCC).

All PCR products were digested with NdeI and EcoRI and ligated in myc-pGEX-2T (modified from Amersham Biosciences) resulting, respectively, in FD136 (GST-myc-hVAMP8) and FD196 (GST-hVAMP8-TMD). Thrombin sites (indicated with an asterisk) were inserted in Tlg2p with the primers F03 (GGGCATATCCATATGGCCACCCCGTCTTGTCGTTCTCAGTTGGT) and FO4 (GCTGTCTCTGCAACGTWTC) and resulting in Tlg2* and Tlg2p with the primers FO3 (GGGCATATCCATATGGCCACCCCGTCTTGTCGTTCTCAGTTGGT) and FO4 (GCTGTCTCTGCAACGTWTC) and resulting in Tlg2* and Tlg2p with the primers FO3 (GGGCATATCCATATGGCCACCCCGTCTTGTCGTTCTCAGTTGGT) and FO4 (GCTGTCTCTGCAACGTWTC) and resulting in Tlg2* and Tlg2p with the primers FO3 (GGGCATATCCATATGGCCACCCCGTCTTGTCGTTCTCAGTTGGT) and FO4 (GCTGTCTCTGCAACGTWTC) and resulting in Tlg2* and Tlg2p with the primers FO3 (GGGCATATCCATATGGCCACCCCGTCTTGTCGTTCTCAGTTGGT) and FO4 (GCTGTCTCTGCAACGTWTC).
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The N-terminal Domains of the t-SNARE Constitute a Potential Regulatory Switch—Both yeast endocytic t-SNAREs, [Tlg2p/Tlg1p,Vti1p] and [Pep12p/Tlg1p,Vti1p], must be activated to promote fusion of liposomes (4, 5). This distinguishes them from all other fusogenic SNAREs tested. In vitro, this activation is provided artificially by a peptide (Snc2-C-pept) corresponding to the C-terminal part of the v-SNARE helical motif (4). It has been demonstrated that such peptides are able to bind and restructure t-SNARE complexes (23). The heavy chain, as well as both light chains of each endocytic t-SNARE, contributes a large N-terminal domain. These N-terminal extensions are neither homologous to each other nor to other SNAREs.

To study their influence on fusion, we generated t-SNARE constructs in which one or more of these N-terminal domains could be removed by thrombin cleavage. For GST-Syn7, GST-Syn8, GST-Vti1b, GST-VAMP8, and GST-Tlg2, the first 10 min of each fusion reaction is due to the temperature equilibration of the fluorophore. For GST-Syn7, GST-Syn8, GST-Vti1b, GST-VAMP8, and GST-Tlg2, the small decrease observed during the first 10 min of each fusion reaction is due to the temperature equilibration of the fluorophore. For GST-Syn7, GST-Syn8, GST-Vti1b, GST-VAMP8, and GST-Tlg2, the small decrease observed during the first 10 min of each fusion reaction is due to the temperature equilibration of the fluorophore. For GST-Syn7, GST-Syn8, GST-Vti1b, GST-VAMP8, and GST-Tlg2, the small decrease observed during the first 10 min of each fusion reaction is due to the temperature equilibration of the fluorophore.
Both the yeast [Pep12p/Tlg1p,Vti1p] and the mammalian [Syntaxin7/Syntaxin8,Vti1b] t-SNAREs are necessary for fusion at the late endosome (25–27). In addition, Syntaxin7 can complement yeast Pep12p mutants defective in fusion to the prevacuolar compartment (28), showing their homology. We tested whether various yeast/mammalian mixed complexes

**FIG. 1.** The endocytic t-SNARE N-terminal domains regulate the kinetic of fusion. A. wild-type t-SNAREs [Tlg2p/Tlg1p,Vti1p] and [Pep12p/Tlg1p,Vti1p] were reconstituted into acceptor liposomes and Snc2p into donor liposomes. Acceptor liposomes were preincubated for 2 h at room temperature with 0.05 unit of thrombin/μl of liposomes (red curves). Control samples were incubated for 2 h at room temperature in absence of thrombin (black curves). 100 mM AEBSF was added to all samples to stop the thrombin reaction. Donor and acceptor liposomes were mixed (5:45 l), and N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine fluorescence was monitored in the presence or absence of Snc2-C-pept (3 nmol) during 2 h at 37 °C. The results were converted to rounds of fusion as described (16). B, the cleavable Syntaxins (indicated by an asterisk) were included in the following t-SNARE complex [Tlg2*/Tlg1,Vti1] and [Pep12*/Tlg1,Vti1], replacing the wild-type proteins. Both acceptor populations were treated with (red curves) and without (black curves) thrombin, and fusion was monitored as described for A. C, cleavable light chains were inserted in the following t-SNARE complexes [Tlg2/Tlg1*,Vti1] or [Tlg2/Tlg1,Vti1*] and [Pep12/Tlg1*,Vti1] or [Pep12/Tlg1,Vti1*], replacing the wild-type proteins. All acceptor populations were treated with (red curves) and without (black curves) thrombin, and fusion was monitored as described for A. In A–C, all constructs are described in the schematics, with the thrombin site labeled by the red arrow. Wild-type proteins are insensitive to thrombin cleavage. Both Syntaxin N-terminal domains as well as each light chain N-terminal domain exert a negative effect on fusion.

Both the yeast [Pep12p/Tlg1p,Vti1p] and the mammalian [Syntaxin7/Syntaxin8,Vti1b] t-SNAREs are necessary for fusion at the late endosome (25–27). In addition, Syntaxin7 can complement yeast Pep12p mutants defective in fusion to the prevacuolar compartment (28), showing their homology. We tested whether various yeast/mammalian mixed complexes
were fusogenic, the hypothesis being that if the yeast proteins carry a regulatory element in their sequence, replacing it with the mammalian homolog will release the negative regulation. We reconstituted the different t-SNARE yeast/mammal combinations into acceptor liposomes (Fig. 4) and tested their fusion capacity in the presence or absence of Snc2-C-pept.

Exchanging Tlg1p with its mammalian homolog Syntaxin8 released the blockage. In this situation, the complex [Pep12p/

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**Fig. 2. The inhibitory effect of each endocytic N-terminal domain is additive.** A, two cleavable t-SNARE proteins were inserted simultaneously in the complex in place of the wild-type proteins to generate [Tlg2*/Tlg1*,Vti1], [Tlg2*/Tlg1,Vti1*], or [Tlg2*/Tlg1*,Vti1] and [Pep12*/Tlg1*,Vti1], [Pep12*/Tlg1,Vti1*], or [Pep12*/Tlg1*,Vti1*] t-SNARE complexes. Both acceptor populations were treated with (red curves) or without (black curves) thrombin as indicated on the figure, and fusion was monitored as described in the legend to Fig 1A. B, the t-SNARE proteins were replaced with the three cleavable counter-parts to generate [Tlg2*/Tlg1*,Vti1*] and [Pep12*/Tlg1*,Vti1*] complexes. All donor populations were treated with or without thrombin as indicated on the figure, and fusion was monitored as described in the legend to Fig 1A. C, the percentage of increase is plotted, averaging at least three experiments. For each individual experiment, all results were normalized based on the fusion rate obtained after 2 h with the full-length proteins. The removal of two N-terminal domains has a stronger effect than the removal of a single one. The removal of all t-SNARE N-terminal domains dramatically enhances the fusion rate, establishing that multiple regulatory domains can act simultaneously.
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FIG. 3. The mammalian late endocytic t-SNARE complex [Syntaxin7/Syntaxin8,Vti1b] is fully fusogenic with the v-SNARE VAMP8. Syntaxin7, Syntaxin8, and Vti1b were reconstituted into acceptor liposomes and VAMP8 into donor liposomes. Both lipid populations were mixed and fusion was monitored in presence or absence of VAMP8-C-pept (3 nmol) as described. As a control, the fusion reaction was inhibited with an excess (6 nmol) of the soluble VAMP8 (VAMP8ATMD). The C-peptide increases the fusion rate but is not a requirement.

A

B

C

D

E

FIG. 4. Tlg1p mammalian homolog Syntaxin8 releases a kinetic blockage when reconstituted in the yeast t-SNARE complex. Mammalian and yeast proteins were mixed in different t-SNAREs and reconstituted into acceptor liposomes. Each t-SNARE liposomes was fused with either Snc2p (A) or VAMP8 (B) donor liposomes as indicated on the figure. The fluorescence intensity was measured during 2 h, and the final point was normalized based on [Pep12p/Tlg1p,Vti1p] fusion with Snc2p in the presence of Snc2-C-pept (corresponds to 100% of fusion (black bar in A)). This figure averages five different experiments. We observed that the replacement of Tlg1p with Syntaxin8 releases the fusion.

Syn8,Vti1p becomes fusogenic without activation with Snc2-C-pept. When replacing both yeast light chains Tlg1p and Vti1p with their mammalian counterparts Syn8 and Vti1b, the resulting t-SNARE [Pep12p/Syn8,Vti1b] is even more fusogenic, suggesting a synergistic effect of both light chains for fusion. We also observe that Snc2p (Fig. 4A) and VAMP8 (Fig. 4B) can functionally replace each other. Altogether, these results suggest that within a given cellular pathway, variations in SNARE autoregulation may be specifically encoded within the light chains of the t-SNARE.

The Autoregulation of Tlg1p Is Encoded in the N-terminal Half of Its SNARE Motif—The SNARE motif is in average 60 amino acids long and is organized in ≈16 layers based on its helical structure with the zero layer determining the center of the SNARE motif (7, 8). To identify the region responsible for the slow kinetics in yeast, we created chimeric constructs between the Syntaxin8 and the Tlg1p SNARE motif. In these constructs, the Tlg1p N-terminal part of the SNARE motif (from layers −9 to 0) was joined to the Syntaxin8 C-terminal part of the SNARE motif (from layers 0 to +9) resulting in T1-S8 protein and vice versa (resulting in S8-T1 protein). Tlg1p (T1-T1) and Syntaxin8 controls (S8-S8) were constructed using the same cloning strategy. Each of these constructs is shown in Fig. 5A. These chimeras were expressed as GST-recombinant proteins and reconstituted into donor liposomes together with the truncated forms of Pep12p and Vti1p. While both controls (T1-T1 and S8-S8) retain their fusogenic properties (Fig. 5, B and C, respectively), the chimera T1-S8 requires activation by a peptide (VAMP8-C-pept) for fusion (Fig. 5D). The chimera S8-T1, on the other hand, does not require peptide activation for fusion (Fig. 5E). We note that when C-Syn8 replaces C-Tlg1p, Snc2-C-pept does not activate the complex anymore (Fig. 5D). This effect may have different causes: either Snc2-C-pept is simply unable to bind the C-terminal part of Syn8, or Snc2-C-pept can still bind but is unable to activate the complex, suggesting in this case that an adequate cognate structure on C terminus is necessary for releasing the N-terminal switch. At this point of the study, we are unable to discriminate between both possibilities.

Altogether, this demonstrates that a second switch region is buried in the Tlg1p SNARE motif and is restricted to its N-terminal portion (between the layers −9 and 0).

DISCUSSION

This study suggests that SNAREs possess multiple distinct autoregulatory switches situated in different regions of the t-SNARE complex. These elements can be combined in particular t-SNAREs (yeast versus mammalian endocytic complexes), providing the SNARE system with functional flexibility to answer to the specific needs of different transports pathways in different cells.

The first switch is provided by the combination of the three t-SNARE N-terminal domains. While an autoregulatory role for syntaxin N-terminal domain is well known (16) and now includes Tlg2p and Pep12p, the role of the light chain N-terminal domains has been unclear. The light chain N-terminal domains do not interact intramolecularly with the SNARE motif and have no influence on the t-SNARE complex formation in solution (15, 19). In this study, we found that the N-terminal domains of the yeast endocytic light chains can additively control the kinetics of fusion.

A second potential switch is buried in the SNARE motif of the yeast light chain Tlg1p. This regulation seems to be absent from a mammalian homolog. Possibly, the tighter autoregulation in yeast relates to the multiple roles for the endocytic v-SNARE, Snc2p. Not only do both endocytic complexes share this v-SNARE with each other, they also share it with the exocytic complex [Sso1p/Sec9p] (29). By contrast, in mammals there are several distinct VAMPs which are further functionally specialized (30).

It is interesting to note that although the C-peptide binds the C-terminal part of the SNARE motif, it releases a regulatory element located within the N-terminal half. t-SNARE complexes are partially assembled across the most N-terminal portions of the SNARE domain (23, 31). By using a peptide homologous to snc2-C-pept, our group showed previously that VAMP2-C-pept binds and structures the C-terminal part of the t-SNARE complex (called the tc-fusion switch), consequently increasing the fusion rate (23). In particular, Melia et al. (23)
observed that when the v-SNARE C-peptide is bound, t-SNARE becomes as resistant to proteolysis as when the entire soluble VAMP2 is bound, suggesting that structuring the C-terminal half is sufficient to induce complete coiled-coil formation across the SNARE bundle. Although we cannot pinpoint the site of regulation for the second potential switch in the endocytic complexes, it seems likely that the N-terminal region is refolded in response to Snc2-C-peptide binding. When the v-SNARE C-peptide is bound, the N-terminal domain of Tlg1p (32, 33). An attractive model is that the binding of Vps51p/Vps54p to Tlg1p stabilizes the N-terminal region of the SNARE motif and releases the SNARE complex to promote fusion.

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FIG. 5. The second switch is buried in the N-terminal region of Tlg1p SNARE motif. A, chimeric SNARE motifs were created between the mammalian Syntaxin8 (yellow) and the yeast Tlg1p (gray). Both SNARE motif sequences are shown. In this schematic, each layer is represented by the gray box (the red Q corresponds to the zero layer). The identity between both sequences is depicted in blue. B–E, after reconstitution of each chimera together with ΔPep12p and ΔVti1p, fusion with Snc2p liposomes was monitored in the presence or absence of Snc2-C-pept (ΔPep12/T1-T1/ΔVti1 (B), ΔPep12/S8-S8/ΔVti1 (C), ΔPep12/T1-S8/ΔVti1 (D), and ΔPep12/S8-T1/ΔVti1 (E)). As control, the fusion was inhibited with an excess (6 nmol) of the soluble Snc2p (Snc2cyt). The fusion was recorded during 2 h at 37 °C. We observed that the regulatory element is buried in the N-terminal region of Tlg1p SNARE motif.
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