

i-SNAREs: inhibitory SNAREs that fine-tune the specificity of membrane fusion

Oleg Varlamov,¹ Allen Volchuk,¹ Vahid Rahimian,¹ Claudia A. Doege,¹ Fabienne Paumet,¹ William S. Eng,¹ Nancy Arango,¹ Francesco Parlati,¹ Mariella Ravazzola,² Lelio Orci,² Thomas H. Söllner,¹ and James E. Rothman¹

¹Department of Cellular Biochemistry and Biophysics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

²Department of Morphology, University of Geneva Medical School, 1211 Geneva Switzerland

A new functional class of SNAREs, designated inhibitory SNAREs (i-SNAREs), is described here. An i-SNARE inhibits fusion by substituting for or binding to a subunit of a fusogenic SNAREpin to form a nonfusogenic complex. Golgi-localized SNAREs were tested for i-SNARE activity by adding them as a fifth SNARE together with four other SNAREs that mediate Golgi fusion reactions. A striking pattern emerges in which certain subunits of the cis-Golgi

SNAREpin function as i-SNAREs that inhibit fusion mediated by the trans-Golgi SNAREpin, and vice versa. Although the opposing distributions of the cis- and trans-Golgi SNAREs themselves could provide for a countercurrent fusion pattern in the Golgi stack, the gradients involved would be strongly sharpened by the complementary countercurrent distributions of the i-SNAREs.

Introduction

Membrane fusion in the cell is triggered by SNARE proteins that assemble between lipid bilayers to form SNAREpins, transducing the energy made available from protein folding to do work on closely apposed bilayers (Weber et al., 1998; McNew et al., 2000a; Hu et al., 2003). Each SNAREpin consists of a bundle of four α -helices; three derived from the t-SNARE and the fourth from the cognate v-SNARE (Sutton et al., 1998). For fusion to occur, all of the t-SNARE subunits (three for intracellular membranes, two for plasma membranes) must reside in one bilayer, with the v-SNARE in the other (Parlati et al., 2000).

SNARE-mediated fusion is so specific for cognate v- and t-SNAREs that the pattern of protein flow in the cell can be predicted with extraordinary accuracy based solely on the intrinsic specificity of fusion by isolated SNAREs reconstituted into artificial lipid bilayers. Of 275 tetrameric combinations of SNAREs encoded in the yeast genome (all 11 potential v-SNAREs tested with the plasma membrane, early endosomes/TGN, cis- and trans-Golgi, and vacuole t-SNAREs) tested for the capacity to fuse, only nine are fusogenic, and all but one correspond to a known transport

pathway (McNew et al., 2000b; Parlati et al., 2000; Paumet et al., 2001). Therefore, the predictive power of the SNARE hypothesis for the specificity of membrane fusion (correct in at least 274/275 cases) exceeds 99% accuracy.

All of these tests of specificity have involved various combinations of isolated SNAREs allocated four at a time between two bilayers, reflecting the structural requirements of SNAREpins. Because every compartment of the secretory pathway may host a vast variety of SNAREs present in the same bilayer, we were curious as to what effect adding a fifth SNARE to the mixture of the cognate SNAREs might have. In most cases, one would anticipate no effect; however, one might imagine that in some cases the additional SNARE could effectively compete with and substitute for a fusogenic subunit, thereby inhibiting fusion. Such an inhibitory SNARE would be termed an i-SNARE. Here, we report that i-SNAREs indeed exist, and that they are likely used in the Golgi stack to fine-tune compartmental specificity.

Results

i-SNAREs from the Golgi

The Golgi houses a large set of SNARE proteins, reflecting the multiple transport pathways required for entry into,

Address correspondence to James E. Rothman, Department of Cellular Biochemistry and Biophysics, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., Box 251, New York, NY 10021. Tel.: (212) 639-8598. Fax: (212) 717-3604. email: j-rothman@ski.mskcc.org

F. Parlati's present address is Rigel Pharmaceuticals, Inc., 240 East Grand Avenue, South San Francisco, CA 94080.

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Abbreviations used in this paper: CGN, cis-Golgi network; i-SNARE, inhibitory SNARE; t_{cis} , cis-Golgi t-SNARE; t_{trans} , trans-Golgi t-SNARE; v_{cis} , cis-Golgi v-SNARE; v_{trans} , trans-Golgi v-SNARE.

recycling from, and traversal of this polarized organelle, and is therefore a suitable place to begin a search for possible i-SNAREs. A single syntaxin required for function of the Golgi in secretion in yeast, Sed5 (mammalian orthologue syntaxin 5; Hardwick and Pelham, 1992), serves as the common heavy chain for building two functionally distinct Golgi t-SNAREs. The cis-Golgi t-SNARE (t_{cis}) has Bos1 (membrin) and Sec22 (ERS-24/Sec22b) as its light chains, and Bet1 is its sole cognate cis-Golgi v-SNARE (v_{cis}) in the yeast cell (McNew et al., 2000b). The trans-Golgi t-SNARE (t_{trans}) has Gos1 (Gos-28) and Ykt6 as its light chains, and Sft1 (GS15) is its sole trans-Golgi v-SNARE (v_{trans} ; Parlati et al., 2002).

Therefore, there are two mutually exclusive v-SNARE/t-SNARE fusion systems operating in the Golgi stack, whose distribution has been analyzed by immuno-EM in the better-organized animal cell Golgi stacks (Volchuk et al., 2004). The cis-Golgi v- and t-SNAREs, as their name suggests, are most concentrated at the cis face of the stack, and gradually decline in concentration toward the trans face. Although the v_{trans} is present in a concentration gradient that increases toward the trans end of the stack, the distribution of the t_{trans} is likely equivalent across the Golgi stack (Volchuk et al., 2004). These findings naturally suggest a spatial segregation of the two distinct fusion processes.

To test for i-SNAREs, we examined the effect of increasing amounts of a fifth noncognate Golgi SNARE on fusion mediated by $v_{cis} + t_{cis}$ and by $v_{trans} + t_{trans}$. The fifth SNARE (potential i-SNARE) was added on the t-SNARE side (Fig. 1 B). To compare the relative inhibitory potentials of the i-SNAREs, we establish the K_{50} , i.e., the molar ratio of the i-SNARE to the t-SNARE needed to reduce fusion by 50% (Fig. 1 C).

Both the cis- and trans-Golgi fusion reactions were tested against the potential i-SNAREs that are known to form complexes in solution with Sed5 (Tsui et al., 2001). The cis-Golgi fusion reaction ($t_{cis} = \text{Sed5/Sec22}$, Bos1; $v_{cis} = \text{Bet1}$) is strongly inhibited by the t_{trans} light chain *Gos1* ($K_{50} = 0.8$) and the TGN/endosome-localized light chain *Tlg1* (syntaxin 6; Holthuis et al., 1998; Bock et al., 2001; Paumet et al., 2001; $K_{50} = 1.0$), and is significantly inhibited by the v_{trans} *Sft1* ($K_{50} = 2.0$; Fig. 2). Typically, a threefold molar excess of i-SNAREs inhibited 90% of the fusion activity. The t_{trans} light chain Ykt6, the Sed5-interacting SNARE Vti1 (localized both to the Golgi and to a prevacuolar compartment; Nichols and Pelham, 1998), and the yeast homologue of v-SNARE synaptobrevin Snc1 (Protopopov et al., 1993) had no significant effect (Fig. 2).

The trans-Golgi fusion reaction ($t_{trans} = \text{Sed5/Ykt6}$, Gos1; $v_{trans} = \text{Sft1}$) is strongly inhibited by the v_{cis} *Bet1* ($K_{50} = 0.4$) and *Tlg1* ($K_{50} = 2.5$), and is slightly inhibited by the t_{cis} light chains *Bos1* ($K_{50} = 7.5$) and *Sec22* ($K_{50} = 10$; Fig. 3). In contrast, Vti1 and Snc1 had no significant effect (Fig. 3). During the course of our work, we noticed that a high concentration of Tlg1 inhibits incorporation of the t-SNAREs into liposomes. However, the lower concentrations of Tlg1 used for calculation of K_{50} did not have a significant effect on t-SNARE reconstitution, yet resulted in at least 50% inhibition of fusion (Fig. 2 B and Fig. 3 B; Tlg1, lanes 2 and 3).

In summary, a survey of the known Golgi SNAREs establishes a new functional classification of SNAREs (termed

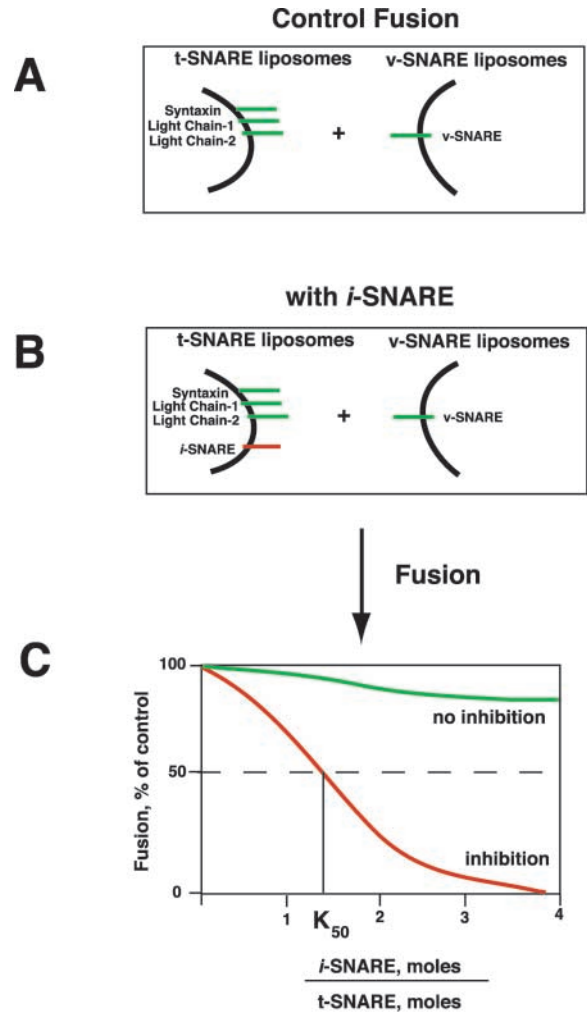


Figure 1. Design of tests for i-SNAREs. (A) The pairing of t-SNAREs composed of one syntaxin heavy chain and two nonsyntaxin light chains with cognate v-SNAREs on the opposite membranes mediates membrane fusion (control fusion). (B) To test whether the presence of additional SNAREs (candidate i-SNAREs) can modulate the activity of fusogenic SNARE complexes, we incorporated candidate i-SNAREs at increasing concentrations into t-SNARE-containing liposomes. (C) Fusion of each type of liposome is compared with control fusion, and is plotted as a function of the molar ratio of the candidate i-SNAREs to the t-SNARE. K_{50} is the molar ratio of the i-SNARE to the t-SNARE needed to reduce fusion by 50%.

i-SNAREs), and reveals an interesting pattern: subunits of the cis-Golgi SNAREpin inhibit fusion mediated by the trans-Golgi SNAREpin, and vice versa.

Mechanism of action of i-SNAREs

How do i-SNAREs inhibit fusion? One possibility (competitive inhibition) is that the i-SNARE substitutes for one of the subunits of the functional tetramer, forming a nonfunctional tetrameric complex. The other possibility (noncompetitive inhibition) would be that the i-SNARE binds to the fusogenic tetramer, forming a nonfunctional oligomeric complex. In the former mechanism, the i-SNARE could compete with and substitute for a t-SNARE subunit (to form a pseudo t-SNARE that is not functional in fusion), or alternatively, it could compete with and substitute for the cognate

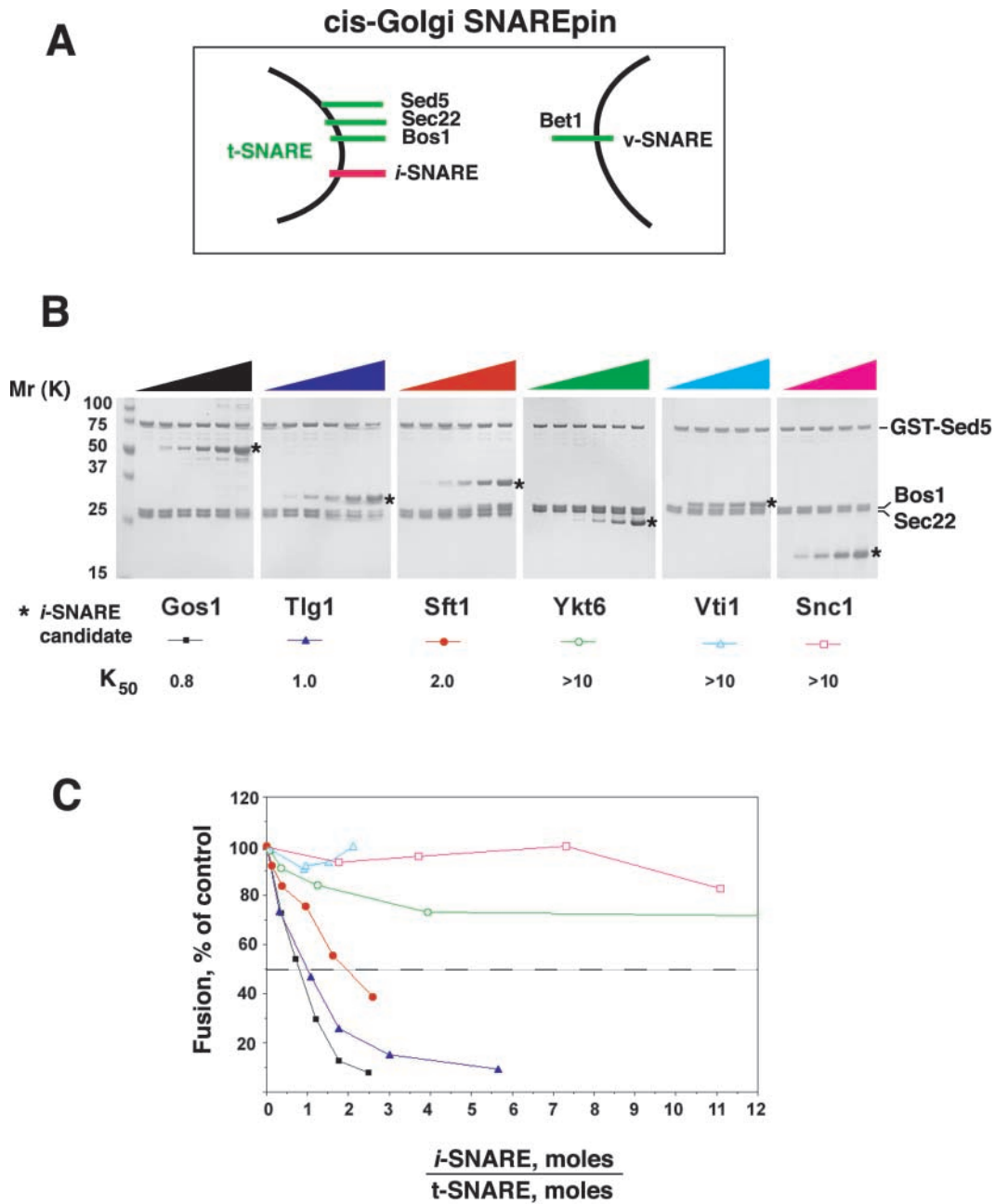


Figure 2. **i-SNAREs for the cis-Golgi fusion reaction.** (A) Increasing concentrations of candidate i-SNAREs were incorporated into acceptor liposomes containing the t_{cis} complex. (B) Proteoliposomes were analyzed by SDS-PAGE and Coomassie blue staining. The position of each candidate i-SNARE is indicated by asterisks. The stoichiometry of proteins in liposomes was determined by densitometry. (C) The resulting acceptor liposomes were incubated with donor liposomes containing the v-SNARE Bet1, and relative fusion activities are plotted as a function of the molar ratio of the candidate i-SNARE to the t-SNARE.

v-SNARE (acting as a pseudo v-SNARE), thereby precluding interaction of the t-SNARE with the authentic v-SNARE.

To begin to discriminate among these possibilities, we performed a functional test to check whether a high concentration of one of the authentic t-SNARE subunits can effectively compete to suppress the inhibitory effects of the i-SNAREs (this cannot meaningfully be done with the v-SNARE because excess v-SNARE in the t-SNARE vesicle simply titrates the t-SNARE and prevents fusion). If fusion is restored by a t-SNARE subunit, then the i-SNARE acts by a competitive mechanism in which it forms a pseudo

t-SNARE. If fusion is not restored by any t-SNARE subunit, then either the i-SNARE acts competitively as a pseudo v-SNARE or it forms a nonfunctional oligomer.

Fig. 4 shows the results of this analysis for the cis-Golgi fusion reaction and its i-SNAREs (*Gos1*, *Tlg1*, and *Sft1*). Inhibition was effectively reversed in all cases by an excess of the t_{cis} light chain Bos1, but was not reversed by an excess of the other light chain (Sec22) or by an excess of the heavy chain (Sed5). This result establishes that all three i-SNAREs operate in the cis-Golgi fusion reaction by the same mechanism—they compete with Bos1 to form pseudo t-SNAREs

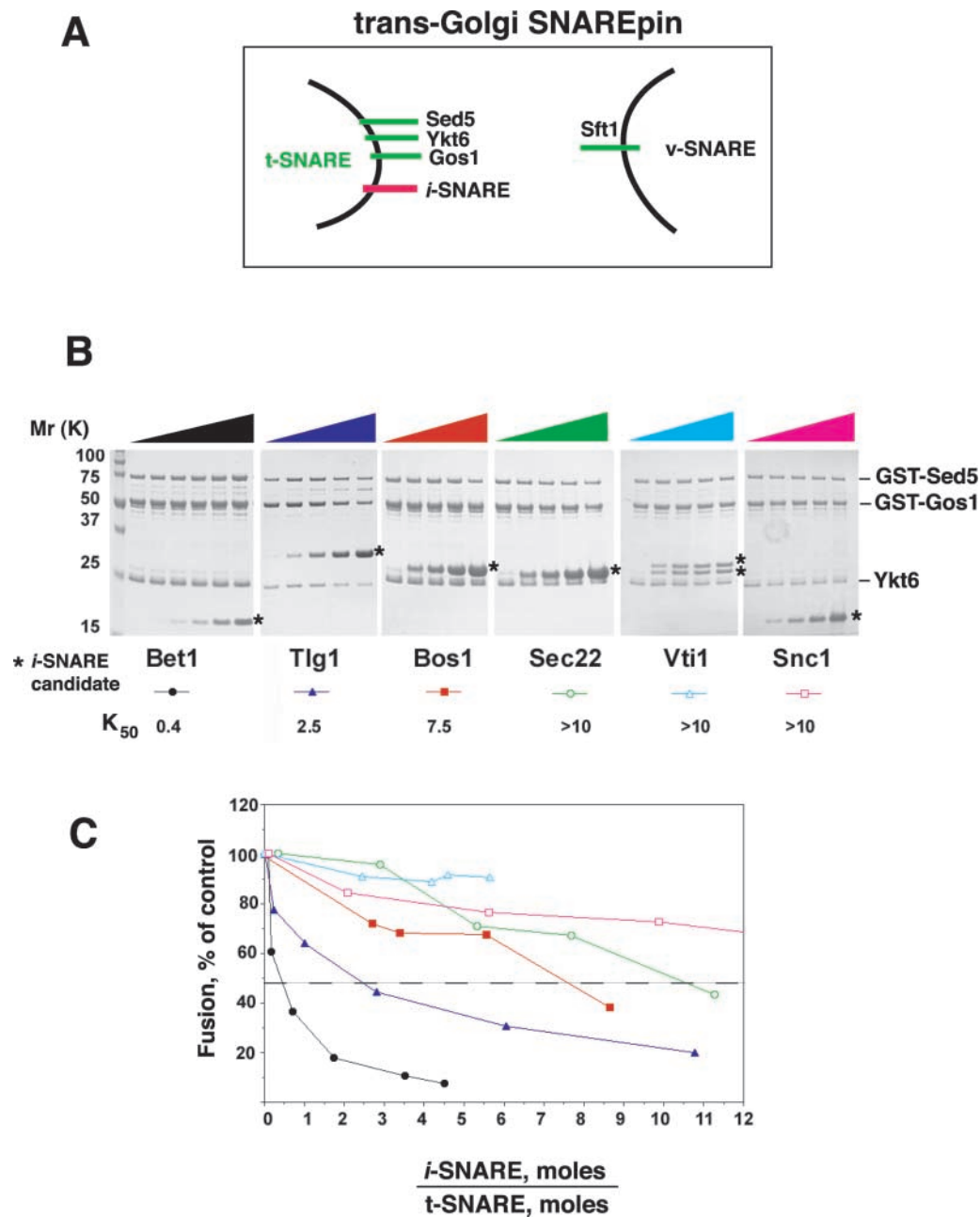


Figure 3. **i-SNAREs for the trans-Golgi fusion reaction.** (A) Increasing concentrations of candidate i-SNAREs were incorporated into acceptor liposomes containing the t_{trans} complex. (B) Proteoliposomes were analyzed by SDS-PAGE and Coomassie blue staining. The position of each candidate i-SNARE is indicated by asterisks. (C) The resulting acceptor liposomes were incubated with donor liposomes containing the v-SNARE Sft1, and relative fusion activities are plotted as a function of the molar ratio of the candidate i-SNARE to the t-SNARE.

(Sed5/Sec22, Gos1; Sed5/Sec22, Tlg1; Sed5/Sec22, Sft1). The tetrameric complex predicted by this mechanism (Sed5–Sec22–Gos1–Bet1) and the trimeric complex (Sed5–Sec22–Sft1) have been previously reported to form with cytoplasmic domains in solution by Tsui et al. (2001). All three of these pseudo t-SNAREs have been found to be non-fusogenic with v-SNAREs tested (Parlati et al., 2002). Although the trimeric i-SNARE-containing complexes Sed5/Sec22, Tlg1 and Sed5/Sec22, Sft1 form in solution, they are very labile, and thus may form only transiently in vivo (unpublished data). The quaternary complex Sed5–Sec22–Gos1–Bet1 forms in solution with high efficiency, similar

to the cognate cis-Golgi complex Sed5–Sec22–Bos1–Bet1 (Tsui et al., 2001; unpublished data). To test whether this i-SNARE-containing complex represents the “dead-end” bi-product of SNARE pairing, we examined the effect of NSF on its stability. Both Sed5–Sec22–Bos1–Bet1 and Sed5–Sec22–Gos1–Bet1 are disrupted in the presence of NSF and α -SNAP, suggesting that both the cognate and the i-SNARE quaternary complexes are the substrates for NSF (unpublished data).

When the trans-Golgi fusion reaction was tested in a functional competition test, no t-SNARE subunit added in excess is capable of reversing inhibition by the i-SNAREs Bet1

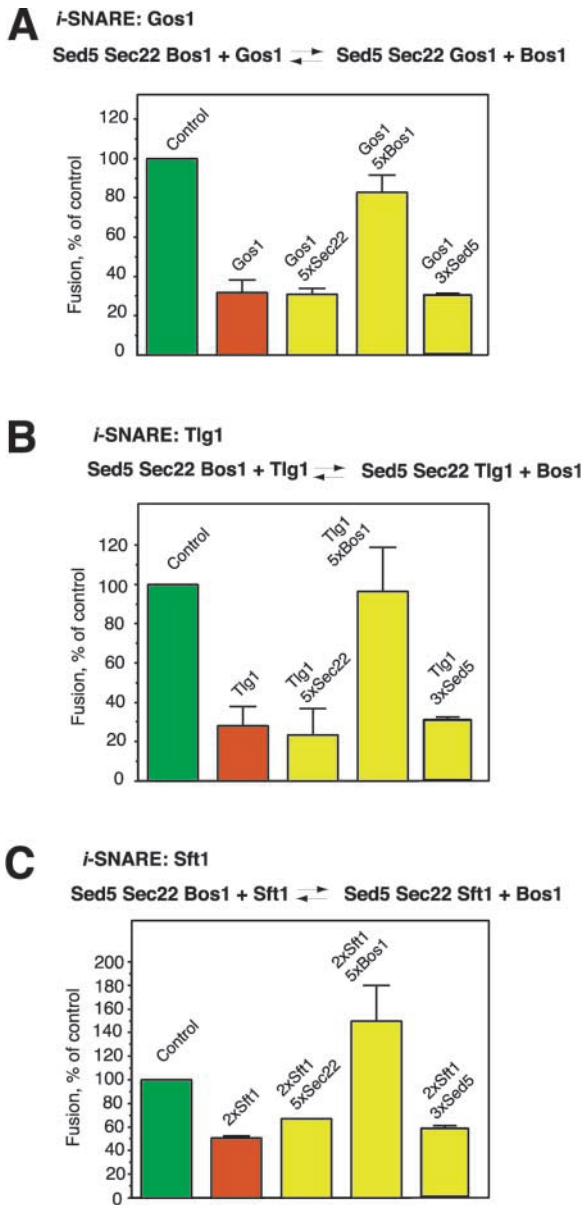


Figure 4. **i-SNAREs compete with the t_{cis} light chain Bos1, resulting in inactive pseudo t-SNARE.** (A–C) We coreconstituted stoichiometric amounts of the t_{cis} SNAREs (Sed5/Sec22, Bos1) and i-SNAREs into acceptor liposomes. Where indicated, an excess of one of the t-SNARE subunits was added. To achieve a substantial inhibition, the i-SNARE Sft1 was added at a twofold molar excess. Fusion activity of modified acceptor liposomes with donor v_{cis} Bet1 liposomes is plotted as a percentage of control fusion (control liposomes contain only t-SNAREs). The proposed mechanism of action of the i-SNARE is illustrated at the top of each graph. The inhibitors of the cis-Golgi complex may compete with the cognate light chain Bos1, generating fusion-incompetent pseudo t-SNARE.

and *Tlg1* (Fig. 5). This means either that this class of i-SNAREs acts as pseudo v-SNAREs (binding t_{trans}) or that i-SNAREs bind to the v_{trans}/t_{trans} complex, forming an inactive oligomer (the noncompetitive mechanism). The pseudo v-SNARE mechanism predicts that the i-SNAREs could compete with the cognate v-SNARE. Consistent with this, the i-SNARE Bet1 is known to form a stable complex with Sed5, Gos1, and Ykt6, which has been isolated from immu-

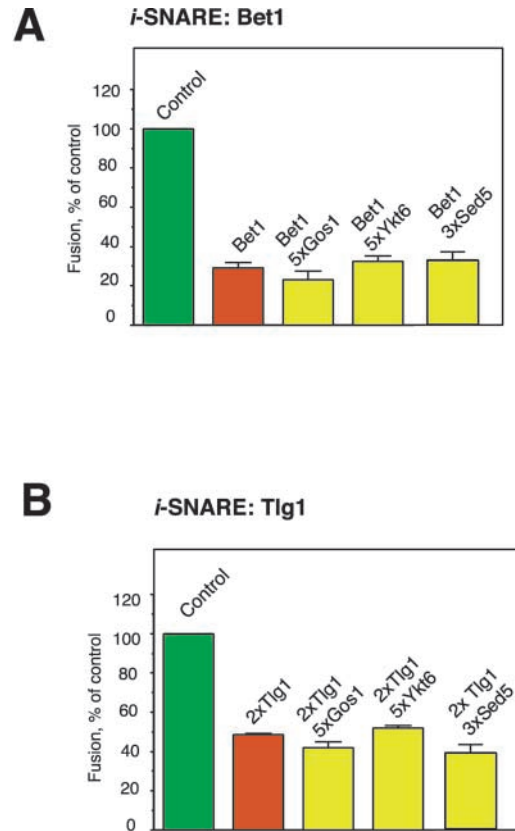


Figure 5. **i-SNAREs inhibit the trans-Golgi fusion reaction.** We coreconstituted stoichiometric amounts of the t_{trans} SNAREs (Sed5/Ykt6, Gos1) and i-SNAREs into acceptor liposomes. Where indicated, an excess of one of the t-SNARE subunits was added. To achieve a substantial inhibition, the i-SNARE Tlg1 was added at a twofold molar excess (B). Fusion activity of modified acceptor liposomes with donor v_{trans} Sft1 liposomes is plotted as a percentage of control fusion (control liposomes contain only t-SNAREs).

noprecipitates of animal cell extracts (Zhang and Hong, 2001). Out of all the i-SNAREs, Bet1 has the highest inhibitory potency toward the trans-Golgi fusion reaction (a stoichiometric amount of Bet1 inhibits fusion by 70%; Fig. 3). Binding experiments with the soluble recombinant proteins were inconclusive in further testing the possible pseudo v-SNARE mechanism. We observed no competition between the i-SNARE Bet1 and the v-SNARE Sft1 for binding to t_{trans} (unpublished data). We conclude that in the case of the cis-Golgi fusion reaction, the i-SNARE substitutes for a t-SNARE light chain Bos1 to form a nonfunctional pseudo t-SNARE. In the case of the trans-Golgi fusion reaction, the mechanism of the i-SNARE action is not established.

i-SNAREs are predicted to sharpen countercurrent fusion in the Golgi

To ascertain what effect, if any, i-SNAREs might have on the pattern of fusion mediated by the cis- and trans-Golgi SNAREpins, we used the liposome fusion assay to recreate the unique SNARE composition of the sequential compartments of the Golgi complex. We took advantage of the knowledge of the detailed cis/trans distributions and relative concentrations of the SNAREs in intermediate compartment/cis-Golgi net-

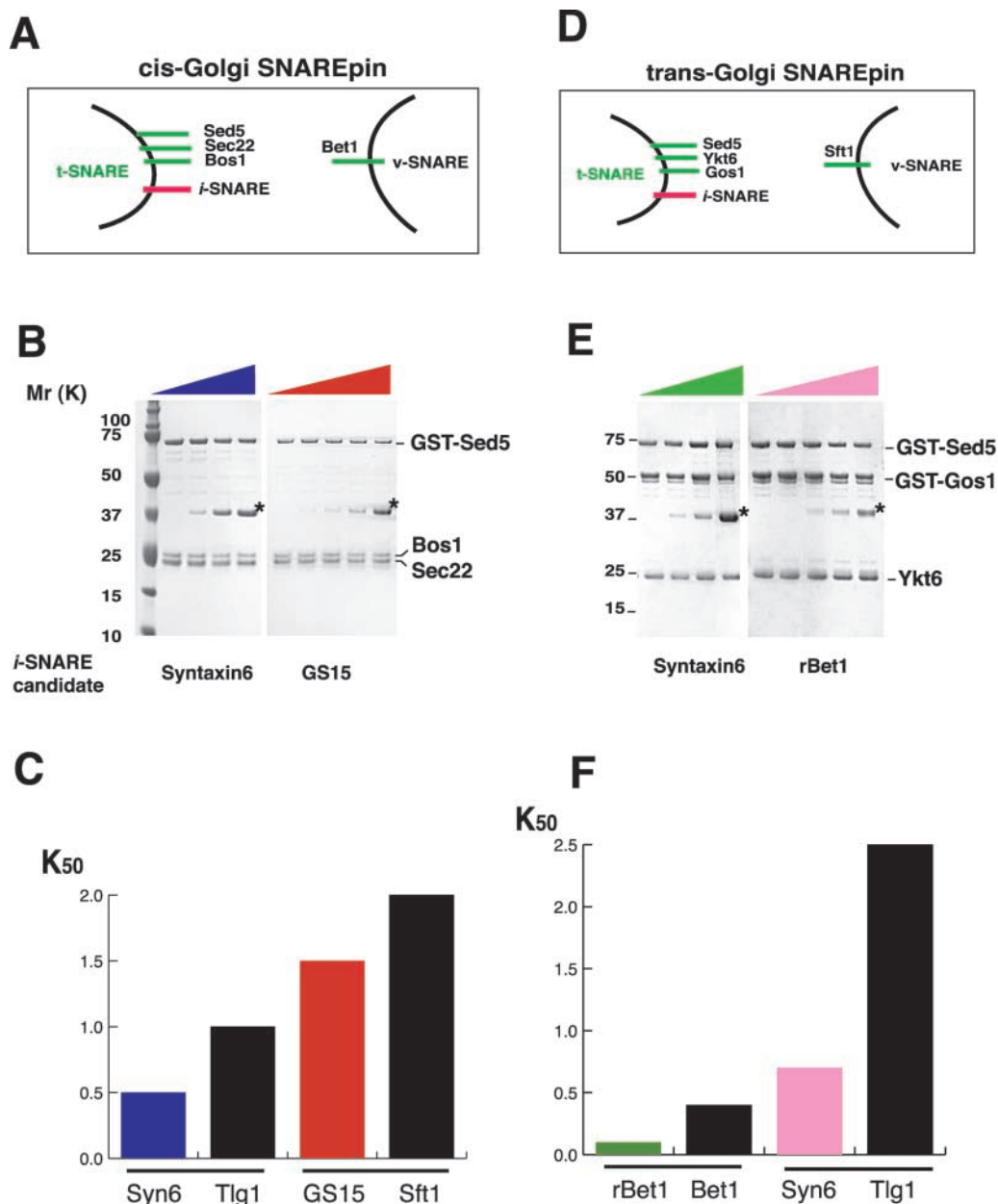


Figure 6. **Mammalian and yeast i-SNAREs are functionally conserved.** Increasing concentrations of the mammalian orthologues of yeast t_{cis} complex (A and B) or the yeast t_{trans} complex (D and E). Proteoliposomes were analyzed by SDS-PAGE and Coomassie blue staining. The position of each i-SNARE is indicated by asterisks. cis-Golgi liposomes were incubated with donor liposomes containing the v-SNARE Bet1 (C), and trans-Golgi liposomes were incubated with donor liposomes containing the v-SNARE Sft1 (F). K_{50} for each i-SNARE is calculated based on relative fusion activities plotted as a function of the molar ratio of the candidate i-SNARE to the t-SNARE (see Fig. 2 and Fig. 3 for details). Orthologues (mammalian/yeast) are as follows: syntaxin 6/Tlg1, GS15/Sft1, and rBet1/Bet1.

work (CGN), the five Golgi cisternae (C1–C5), and the TGN established in a mammalian cell line (Volchuk et al., 2004). Because the mammalian SNAREs are orthologous to the yeast SNAREs, and in some cases have been shown to substitute for the yeast equivalents *in vivo* (McNew et al., 1997; Mollard and Stevens, 1998), we thought it would be reasonable to model the successive compartments of the mammalian Golgi by creating a series of liposomes of graded SNARE composition for fusion analyses, using the yeast orthologues of the mammalian SNAREs combined in the same proportions in which the mammalian proteins are present in successive Golgi

compartments in the cell. The degree of similarity between yeast and mammalian SNAREs has been further validated by replacing yeast i-SNAREs with the mammalian orthologues (Fig. 6). Strikingly, the mammalian orthologue of Tlg1 (syntaxin 6) and the mammalian orthologue of Sft1 (GS15) inhibit fusion mediated by yeast t_{cis} + v_{cis} (Fig. 6, B and C) to a similar extent as their yeast counterparts. Similarly, the mammalian orthologue of Bet1, rBet1, and syntaxin 6 inhibit fusion mediated by yeast t_{trans} + v_{trans} (Fig. 6, E and F) even more potently than their yeast counterparts. Furthermore, both rBet1 and GS15 are functionally active and mediate fusion

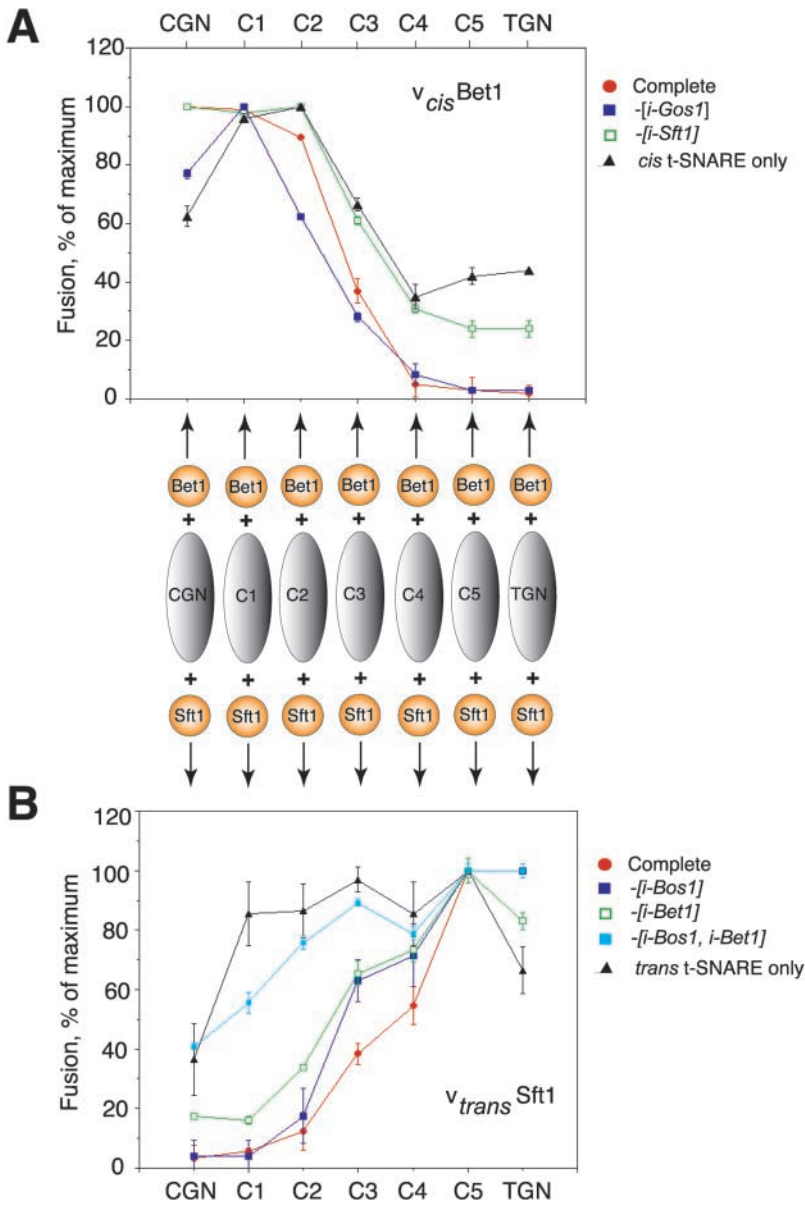


Figure 7. i-SNAREs sharpen the specificity of membrane fusion in the reconstituted Golgi-mimetic system. The Golgi-mimetic mixture of SNAREs was reconstituted into acceptor liposomes as described in the Materials and methods. Stoichiometry of SNAREs in the CGN, five Golgi cisternae (C1–C5), and the TGN was determined by quantitative immuno-EM in mammalian cells. The percentage of total immunogold particles for individual SNAREs in each cisternae (see Table I in Volchuk et al., 2004) was normalized to relative molar amounts of individual SNAREs in whole cells determined by quantitative immunoprecipitation (see Fig. 7 in Volchuk et al., 2004). Syntaxin 5 was used as a standard for normalization. (A and B) Acceptor liposomes mimicking the SNARE composition of individual cisternae, but lacking v-SNAREs Bet1 and Sft1, respectively, were generated by using the molar ratios of SNAREs in every compartment. In addition, individual i-SNAREs were omitted from the reconstitution (shown as $-[i-SNARE]$). Acceptor liposomes were mixed with either (A) Bet1- or (B) Sft1-containing donor liposomes, and fusion was monitored as described in the Materials and methods. The specific compositions of Golgi-mimetic liposomes are as follows: (A) Complete (Sed5, Bos1, Sec22, Gos1, Ykt6, Sft1); $-[i-Gos1]$ (Sed5, Bos1, Sec22, Ykt6, Sft1); $-[i-Sft1]$ (Sed5, Bos1, Sec22, Gos1, Ykt6); and cis t-SNARE only (Sed5, Sec22, Bos1). (B) Complete (Sed5, Bos1, Sec22, Bet1, Gos1, Ykt6); $-[i-Bos1]$ (Sed5, Sec22, Bet1, Gos1, Ykt6); $-[i-Bos1, i-Bet1]$ (Sed5, Sec22, Gos1, Ykt6); and trans t-SNARE only (Sed5, Ykt6, Gos1).

with yeast t_{cis} and t_{trans} , respectively (unpublished data). We conclude that yeast and mammalian SNAREs from the Golgi are functionally interchangeable in the fusion assay. Therefore, Sed5 and the six Sed5-interacting SNAREs required for Golgi function (Bos1, Sec22, Bet1, Gos1, Ykt6, and Sft1) were coreconstituted into liposomes at the molar ratios approximating those in the cis/trans Golgi compartments (see Materials and methods for details; Table I and Fig. 7 of Volchuk et al., 2004). The concentration of Sed5 was kept constant in all of the Golgi-mimetic liposomes. Each Golgi-mimetic “acceptor” liposome preparation (representing successively the CGN, C1–C5, and TGN) was then tested for the efficiency of its fusion with fluorescent probe-containing “donor” liposomes containing either the v_{cis} (Bet1) or v_{trans} (Sft1).

In this simple test, v_{cis} vesicles are markedly targeted to the cis-most cisternae in a sharp gradient of preference (Fig. 7 A, “Complete”), whereas the v_{trans} vesicles intrinsically select the trans side of the stack for fusion in an oppositely oriented preference gradient (Fig. 7 B, “Complete”). This strongly

predicts the two opposing targeting patterns for vesicles that may be enriched in one versus the other v-SNARE in the Golgi stack (Volchuk et al., 2004).

To ascertain the extent to which this predicted cis/trans preference gradient depends on the distribution of the i-SNAREs within the stack, the principal i-SNAREs were omitted singly or in combination when composing the acceptor liposomes. The cis preference gradient of v_{cis} was hardly affected by removing *Gos1*, but was affected when *Sft1* was removed, and even more so when both i-SNAREs were omitted (Fig. 7 A). Fusion of v_{cis} with the trans-most cisternae is predicted to be ~3% of that with cis-most cisternae when both i-SNAREs are present, but ~40% when they are absent. The i-SNAREs *Sft1* and *Gos1* use the same mechanism of action and compete for the same site (Fig. 4) with similarly high potency (Fig. 2); however, Sft1 is present at approximately five times the concentration of *Gos1* in the Golgi stack (Volchuk et al., 2004) so that it predominates there, explaining why removing *Gos1* had very little effect in the trans Golgi.

The trans preference gradient of v_{trans} was not much affected when either *Bos1* or *Bet1* were omitted, but when both were omitted, fusion with CGN rose from $\sim 4\%$ of fusion with trans-most cisternae to $\sim 40\%$, and fusion of C1 rose from ~ 5 to $\sim 55\%$ (Fig. 7 B). Removing both *Bos1* and *Bet1* was similar to removing all the SNAREs other than those directly required for the trans-Golgi t-SNAREs (Sed5, Gos1, Ykt6; “trans t-SNARE only”; Fig. 7 B).

In summary, although the opposing distributions of the cis- and trans-Golgi SNAREs themselves provide for opposing distributions of the distinct fusion reactions they mediate, this predicted countercurrent fusion pattern is strongly enhanced by the opposing countercurrent distributions of the i-SNAREs. This is largely the case because the primary i-SNARE for the cis-Golgi fusion reaction is itself graded toward the trans face, whereas the primary i-SNAREs for the trans-Golgi fusion reaction is graded toward the cis face. Combining the distributions of i-SNAREs with that of the pairs of v- and t-SNAREs sharpens the predicted countercurrent pattern of membrane fusion.

Discussion

The specificity of intracellular transport pathways is encoded to a remarkable degree in the intrinsic physical chemistry of its SNARE proteins such that the pattern of membrane flow in the cell is recapitulated by the pattern of fusion of artificial bilayers by isolated SNAREs. In light of this, it is of special interest that certain combinations of SNAREs that are not fusogenic between bilayers nonetheless have been found to assemble efficiently into tetrameric and trimeric complexes in solution (Fasshauer et al., 1999; Yang et al., 1999; Tsui and Banfield, 2000; Tsui et al., 2001). As one example, a complex of certain Golgi SNAREs (Sed5–Sec22–Gos1–Bet1) can efficiently form in solution (Tsui et al., 2001), but is incapable of mediating bilayer fusion (Parlati et al., 2002).

These kinds of observations were originally interpreted as indirect evidence of the promiscuity of SNARE interactions in the fusion process, an interpretation that is no longer tenable in light of the specificity of SNARE-dependent fusion established by direct testing. This left open the question of whether the nonfusogenic complexes have a biological function or whether they are artifacts. From the present work, we suggest that the nonfusogenic complexes indeed have a biological function, which can now be explained by and indeed predicted from the discovery that certain SNAREs function as i-SNAREs. Our data on i-SNAREs suggest that nonfusogenic SNARE complexes have physiological relevance in fine-tuning the specificity of fusion. Interestingly, the recently discovered non-SNARE coiled-coil inhibitor, endosome-associated hepatocyte responsive serum phosphoprotein, has been shown to inhibit the homotypic fusion of early endosomes (Sun et al., 2003), suggesting that coiled-coil-containing molecules may be the common regulators of membrane fusion.

i-SNAREs and the patterns of vesicle fusion in the Golgi

Although the distribution of v-SNAREs for both Golgi SNAREpins and the distribution of the t_{cis} is in good agreement with the predicted countercurrent pattern of membrane fusion in the Golgi, the t_{trans} subunit Gos1 has a similar

distribution throughout the Golgi and the TGN with some elevation in the CGN (Volchuk et al., 2004). The competitive nature of the i-SNAREs Gos1 and Sft1 implies that these i-SNAREs can be displaced by a sufficiently high concentration of the cognate light chain t_{cis} Bos1 (Fig. 4). Therefore, a threefold excess of Bos1 over Gos1 present in the C1 (Volchuk et al., 2004) may be sufficient to suppress the inhibitory action of the i-SNAREs in the cis-most Golgi compartments. Thus, the ratio of an i-SNARE to its competitor Bos1, rather than the absolute i-SNARE concentration, may dictate the activity of the fusogenic gradient in the cis Golgi.

Homotypic fusion in the Golgi and the i-SNAREs

Immuno-EM data in mammalian cells indicate that the t- and v-SNAREs (as well as the potential i-SNAREs) are present both in the Golgi cisternae and vesicle membranes, raising a possibility that fusion in the Golgi has a homotypic mechanism (Volchuk et al., 2004). Therefore, complete Golgi modeling would require integration of the entire set of SNAREs both into the target and vesicle membranes. Importantly, the SNARE composition of vesicles determined in Volchuk et al. (2004) may represent an average SNARE ratio determined over many populations of vesicles, each derived from the different level of the Golgi stack. Without having the detailed information of the SNARE stoichiometry in these vesicle subpopulations, it would be very difficult to model both the target and vesicle membrane in vitro. We did test individual i-SNAREs in v-SNARE-containing donor liposomes, and observed a very modest inhibitory effect on membrane fusion (unpublished data). Thus, the present paper should be considered as the first approximation of introducing i-SNAREs as the potential regulators of membrane fusion.

Predicted buffering capacity of the Golgi

Our previous results suggest that the two most abundant i-SNAREs in the Golgi, *Sft1* for the t_{cis} and *Bet1* for the t_{trans} , are present at much greater molar excess than the syntaxin Sed5 in the trans Golgi and the cis Golgi, respectively (Volchuk et al., 2004). Because a fourfold molar excess of *Sft1* and a twofold molar excess of *Bet1* are sufficient for inhibiting 80% of the fusion activity of the t_{cis} and t_{trans} , respectively (Fig. 2 and Fig. 3), these i-SNAREs exist at greater concentrations than are necessary for simple fine-tuning of the Golgi. This suggests that by maintaining high local concentrations of i-SNAREs, the Golgi may accommodate significant fluctuations in the distribution and concentration of the t-SNAREs within the stack without alteration of the countercurrent pattern of membrane fusion, thereby acting as a buffered system. As a result, the trans-Golgi fusion system may be well buffered against any fluctuations of the t_{trans} in the CGN. Similarly, the trans-Golgi compartments may have a high buffering capacity for the cis-Golgi fusion system. The predicted buffering capacity of the Golgi may help explain the robustness of the Golgi in vivo toward cyclic physiological and pharmacological perturbations (Lippincott-Schwartz et al., 1989; Takizawa et al., 1993; Acharya et al., 1995; Rabouille et al., 1995).

i-SNAREs outside of the Golgi

Could i-SNAREs be used outside the Golgi? The existence of certain nonfusogenic SNARE complexes involving non-

Golgi SNAREs suggests this. For example, complexes of the Golgi SNAREs with endocytic SNAREs Sed5–Ykt6–Tlg1–Vti1 and Sed5–Snc2–Tlg1–Vti1 may represent nonfusogenic i-SNARE-containing complexes in the TGN, which together with a fusogenic complex v-Snc/t-Tlg2/Tlg1, Vti1 may be involved in transport at the interface of endosomes and TGN (Brickner et al., 2001; Paumet et al., 2001; Tsui et al., 2001; Parlati et al., 2002). Certainly, genetic and biochemical experiments suggest that Vti1 and Tlg1 are essential both for Golgi and endocytic functions (Lupashin et al., 1997; Coe et al., 1999). We speculate that Tlg1 could function as the negative regulator of the cis- and trans-Golgi SNAREpins in the TGN, leading to inactivation of the Sed5-based SNARE complexes and simultaneous activation of the Tlg2-based SNAREpin (Paumet et al., 2001). This simultaneous switching off the Golgi SNARE machinery (required exclusively in the Golgi) and engaging endocytic SNAREs could maintain a spatial segregation of the two adjacent compartments. In addition to the TGN, a nonfusogenic complex of a vacuolar syntaxin Vam3 with the Golgi/endocytic SNAREs Vam3–Vti1–Tlg1–Snc1 raises the possibility that i-SNARE-mediated fine-tuning of membrane fusion exists throughout the secretory and endocytic pathways (Tsui et al., 2001).

Materials and methods

Protein expression and purification

Recombinant SNARE proteins used in this work were as follows: GST-Sed5, GST-Bos1, Sec22-His₆, GST-Bet1, GST-Gos1, GST-Ykt6, GST-Sft1, Vti1-His₆, Tlg1-His₆, and Snc1-His₆. Protein expression and purification was described previously (McNew et al., 1998, 2000b; Fukuda et al., 2000; Parlati et al., 2000).

SNARE reconstitution

The SNARE proteins were reconstituted into synthetic liposomes as described previously (Parlati et al., 2000). In brief, the recombinant SNAREs were mixed in 25 mM Hepes, pH 7.4, 0.4 M KCl, 1% *n*-octyl- β -D-glucopyranoside, 10% glycerol, and 1 mM DTT. Before reconstitution, a soluble SNARE Ykt6 was lipid anchored with geranylgeranyl lipid as described previously (Parlati et al., 2002). 7 nmol of each SNARE protein was used for the formation of the t-SNARE complexes Sed5/Sec22, Bos1 and Sed5/Ykt6, Gos1, and v-SNAREs Bet1 and Sft1.

Modifications were made in the titration experiments as follows (Fig. 2 and Fig. 3): 7 nmol of the t-SNARE Sed5/Sec22, Bos1 was coreconstituted with increasing concentrations of the following SNAREs: GST-Gos1, Tlg1, GST-Sft1, Ykt6 (0–28 nmol), Vti1, and Snc1 (0–56 nmol). 7 nmol of the t-SNARE Sed5/Ykt6, Gos1 was coreconstituted with increasing concentrations of Bet1, Tlg1, Bos1, Sec22 (0–28 nmol), Vti1, and Snc1 (0–56 nmol). The proteoliposomes were analyzed by SDS-PAGE and Coomassie blue staining. The efficiency of protein incorporation was monitored by densitometry of individual bands with Quantity One[®] software (Bio-Rad Laboratories). The optical densities corrected for molecular mass were used for plotting inhibitory curves (Fig. 2 and Fig. 3).

In competition experiments (Fig. 4 and Fig. 5), the t-SNAREs were reconstituted with equimolar amounts of the i-SNAREs (7 nmol). In some cases, 35 nmol of the competing light chain or 21 nmol of a heavy chain Sed5 was added to the reconstitution reaction.

Reconstitution of the Golgi-mimetic liposomes

The Golgi-mimetic mixture of SNAREs was reconstituted into acceptor liposomes according to the standard reconstitution protocol (Weber et al., 1998). For the reconstitution experiments, we used yeast orthologues of the mammalian SNAREs at the ratios corresponding to those in the mammalian Golgi. A quantitative distribution of SNAREs in individual cisternae of the Golgi stack was previously determined with immuno-EM (Volchuk et al., 2004). The percentage of total immunogold particles for individual SNAREs in each cisternae (see Table I in Volchuk et al., 2004) was normalized to relative molar amounts of individual SNAREs in whole

cells determined by quantitative immunoprecipitation (see Fig. 7 in Volchuk et al., 2004). The resulting numbers were normalized to syntaxin 5 content in every cisternae to produce the molar ratios of SNAREs in each cisternae. Because the concentration of Ykt6 in the mammalian cell was not determined, we used the average Golgi SNARE concentration in the cell (Volchuk et al., 2004). This should not pose a problem, as Ykt6 does not have i-SNARE activity. Although the distribution of YKT6 in the mammalian Golgi was not determined by immuno-EM, immunofluorescence data indicated that Ykt6 and Gos-28 may have a similar distribution in the mammalian Golgi (Volchuk et al., 2004). Thus, we thought it would be reasonable to assume that Ykt6 and Gos-28 are present at the same ratios in the Golgi stack.

We used 7 nmol Sed5 and proportional amounts of the other SNAREs for the corresponding sets of the Golgi-mimetic liposomes. For acceptor liposomes, the estimated number of Sed5 (yeast syntaxin 5) molecules ranges from 70–110 per liposome (Parlati et al., 2000, 2002). Assuming an average 45-nm diameter of acceptor liposome (surface area $5 \times 10^{-3} \mu\text{m}^2$), the surface density of Sed5 in liposome is $\sim 18,000$ molecules/ μm^2 . The protease sensitivity test shows that typically 50–70% of the reconstituted SNARE proteins are externally oriented.

Fusion assay

We performed a standard fusion assay as described previously (Weber et al., 1998). NBD fluorescence was converted to rounds of fusion as described previously (Parlati et al., 1999). 5 μg of the COOH-terminal peptides of Sft1 was added to the fusion reaction. The presence of the COOH-terminal peptides accelerates fusion reaction, but has no effect on the relative fusion values presented in this paper.

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References

- Acharya, U., R. Jacobs, J.M. Peters, N. Watson, M.G. Farquhar, and V. Malhotra. 1995. The formation of Golgi stacks from vesiculated Golgi membranes requires two distinct fusion events. *Cell*. 82:895–904.
- Bock, J.B., H.T. Matern, A.A. Peden, and R.H. Scheller. 2001. A genomic perspective on membrane compartment organization. *Nature*. 409:839–841.
- Brickner, J.H., J.M. Blanchette, G. Sipos, and R.S. Fuller. 2001. The Tlg SNARE complex is required for TGN homotypic fusion. *J. Cell Biol.* 155:969–978.
- Coe, J.G., A.C. Lim, J. Xu, and W. Hong. 1999. A role for Tlg1p in the transport of proteins within the Golgi apparatus of *Saccharomyces cerevisiae*. *Mol. Biol. Cell*. 10:2407–2423.
- Fasshauer, D., W. Antonin, M. Margittai, S. Pabst, and R. Jahn. 1999. Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. *J. Biol. Chem.* 274:15440–15446.
- Fukuda, R., J.A. McNew, T. Weber, F. Parlati, T. Engel, W. Nickel, J.E. Rothman, and T.H. Söllner. 2000. Functional architecture of an intracellular membrane t-SNARE. *Nature*. 407:198–202.
- Hardwick, K.G., and H.R. Pelham. 1992. Sed5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.* 119:513–521.
- Holthuis, J.C., B.J. Nichols, S. Dhruvakumar, and H.R. Pelham. 1998. Two syntaxin homologues in the TGN/endosomal system of yeast. *EMBO J.* 17: 113–126.
- Hu, C., M. Ahmed, T.J. Melia, T.H. Söllner, T. Mayer, and J.E. Rothman. 2003. Fusion of cells by flipped SNAREs. *Science*. 300:1745–1749.
- Lippincott-Schwartz, J., L.C. Yuan, J.S. Bonifacino, and R.D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell*. 56:801–813.
- Lupashin, V.V., I.D. Pokrovskaya, J.A. McNew, and M.G. Waters. 1997. Characterization of a novel yeast SNARE protein implicated in Golgi retrograde traffic. *Mol. Biol. Cell*. 8:2659–2676.
- McNew, J.A., M. Søgaard, N.M. Lampen, S. Machida, R.R. Ye, L. Lacomis, P.

- Tempst, J.E. Rothman, and T.H. Söllner. 1997. Ykt6p, a prenylated SNARE essential for endoplasmic reticulum-Golgi transport. *J. Biol. Chem.* 272:17776–17783.
- McNew, J.A., J.G. Coe, M. Sogaard, B.V. Zemelman, C. Wimmer, W. Hong, and T.H. Söllner. 1998. Gos1p, a *Saccharomyces cerevisiae* SNARE protein involved in Golgi transport. *FEBS Lett.* 435:89–95.
- McNew, J.A., T. Weber, F. Parlati, R.J. Johnston, T.J. Melia, T.H. Söllner, and J.E. Rothman. 2000a. Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. *J. Cell Biol.* 150:105–117.
- McNew, J.A., R. Fukuda, F. Parlati, R.J. Johnston, K. Paz, F. Paumet, T.H. Söllner, and J.E. Rothman. 2000b. Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature.* 407:153–159.
- Mollard, G.F., and T.H. Stevens. 1998. A human homolog can functionally replace the yeast vesicle-associated SNARE Vti1 in two vesicle transport pathways. *J. Biol. Chem.* 273:2624–2630.
- Nichols, B.J., and H.R. Pelham. 1998. SNAREs and membrane fusion in the Golgi apparatus. *Biochim. Biophys. Acta.* 1404:9–31.
- Parlati, F., T. Weber, J.A. McNew, B. Westermann, T.H. Söllner, and J.E. Rothman. 1999. Rapid and efficient fusion of phospholipid vesicles by the alpha-helical core of a SNARE complex in the absence of an N-terminal regulatory domain. *Proc. Natl. Acad. Sci. USA.* 96:12565–12570.
- Parlati, F., J.A. McNew, R. Fukuda, R. Miller, T.H. Söllner, and J.E. Rothman. 2000. Topological restriction of SNARE-dependent membrane fusion. *Nature.* 407:194–198.
- Parlati, F., O. Varlamov, K. Paz, J.A. McNew, D. Hurtado, T.H. Söllner, and J.E. Rothman. 2002. Distinct SNARE complexes mediating membrane fusion in Golgi transport based on combinatorial specificity. *Proc. Natl. Acad. Sci. USA.* 99:5424–5429.
- Paumet, F., B. Brugger, F. Parlati, J.A. McNew, T.H. Söllner, and J.E. Rothman. 2001. A τ -SNARE of the endocytic pathway must be activated for fusion. *J. Cell Biol.* 155:961–968.
- Protopopov, V., B. Govindan, P. Novick, and J.E. Gerst. 1993. Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell.* 74:855–861.
- Rabouille, C., T.P. Levine, J.M. Peters, and G. Warren. 1995. An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell.* 82:905–914.
- Sun, W., Q. Yan, T.A. Vida, and A.J. Bean. 2003. Hrs regulates early endosome fusion by inhibiting formation of an endosomal SNARE complex. *J. Cell Biol.* 162:125–137.
- Sutton, R.B., D. Fasshauer, R. Jahn, and A.T. Brunger. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature.* 395:347–353.
- Takizawa, P.A., J.K. Yucel, B. Veit, D.J. Faulkner, T. Deerinck, G. Soto, M. Ellisman, and V. Malhotra. 1993. Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, ilimaquinone. *Cell.* 73:1079–1090.
- Tsui, M.M., and D.K. Banfield. 2000. Yeast Golgi SNARE interactions are promiscuous. *J. Cell Sci.* 113:145–152.
- Tsui, M.M., W.C. Tai, and D.K. Banfield. 2001. Selective formation of Sed5p-containing SNARE complexes is mediated by combinatorial binding interactions. *Mol. Biol. Cell.* 12:521–538.
- Volchuk, A., M. Ravazzola, A. Perrelet, W. Eng, M. Di Liberto, O. Varlamov, M. Fukasawa, T. Engel, T.H. Söllner, J.E. Rothman, and L. Orci. 2004. Countercurrent distribution of two distinct SNARE complexes mediating transport within the Golgi stack. *Mol. Biol. Cell.* In press.
- Weber, T., B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Söllner, and J.E. Rothman. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell.* 92:759–772.
- Yang, B., L. Gonzalez, Jr., R. Prekeris, M. Steegmaier, R.J. Advani, and R.H. Scheller. 1999. SNARE interactions are not selective. Implications for membrane fusion specificity. *J. Biol. Chem.* 274:5649–5653.
- Zhang, T., and W. Hong. 2001. Ykt6 forms a SNARE complex with syntaxin 5, GS28, and Bet1 and participates in a late stage in endoplasmic reticulum-Golgi transport. *J. Biol. Chem.* 276:27480–27487.