Organization of SNAREs within the Golgi Stack

Jörg Malsam and Thomas H. Söllner

Heidelberg University Biochemistry Center, 69120 Heidelberg, Germany

Correspondence: thomas.soellner@bzh.uni-heidelberg.de

Antero- and retrograde cargo transport through the Golgi requires a series of membrane fusion events. Fusion occurs at the *cis*- and *trans*-side and along the rims of the Golgi stack. Four functional SNARE complexes have been identified mediating lipid bilayer merger in the Golgi. Their function is tightly controlled by a series of reactions involving vesicle tethering and SM proteins. This network of protein interactions spatially and temporally determines the specificity of transport vesicle targeting and fusion within the Golgi.

At steady state, the Golgi maintains its structural and functional organization despite a massive lipid and protein flow. A balanced anterograde and retrograde membrane flow are required to constantly recycle the transport machinery and cargo containers (vesicles). In the absence of efficient recycling, directional net cargo transport would cease and the Golgi would collapse. Thus, transport vesicles constantly leave and enter at both sides of the Golgi stack and bud and fuse along the rims of the cisternae. To maintain the compartmental identity, vesicle fusion occurs in a specific and orchestrated manner. These fusion events are mediated by a cascade of reactions centered around the membrane fusion proteins SNAREs (SNAP receptors) (Söllner et al. 1993b).

SNARE COMPLEX ASSEMBLY AND DISASSEMBLY

SNAREs represent a family of tail-anchored membrane proteins, whose individual members are localized to distinct intracellular

compartments along the secretory and endocytic pathway (Jahn and Scheller 2006; Malsam et al. 2008). On a functional/topological basis, SNAREs can be classified as v- and t-SNAREs, because they are usually active on vesicles and target membranes (Söllner et al. 1993b). In the case of homotypic fusion, a clear distinction between the two compartments is not possible, however, the reaction mechanisms driving fusion are comparable to those of heterotypic fusion. On a structural basis, SNARE motifs, the hallmark signature characterizing all SNARE family members, provide the underlying principle to understand SNARE complex assembly and regulation (Fasshauer et al. 1998). Upon pairing of a v-SNARE with its cognate t-SNARE on opposing membranes, the SNARE motifs assemble to form *trans*-v/t-SNARE complexes, also termed SNAREpins, a process that occurs in a zipper-like manner starting at the amino-terminal membrane-distal ends and progressing toward the carboxy-terminal membrane-proximal regions of the SNAREs (Melia et al. 2002; Pobbati et al. 2006). This assembly

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reaction pulls the two opposing membranes together to overcome the repulsive forces of the surrounding aqueous environment, which provides an energy barrier for lipid bilayer merger. Several *in vitro* and *in vivo* studies have established that SNAREs are sufficient to drive membrane merger, and thus SNAREs have been considered to be the minimal membrane fusion machinery (Weber et al. 1998; Hu et al. 2003). The fully assembled postfusion *cis*-SNARE complex, now located in a single lipid bilayer, becomes a substrate for SNAPs (soluble NSF attachment factors), which in turn recruit the hexameric ATPase NSF (N-ethylmaleimide sensitive factor) (Block et al. 1988; Whiteheart et al. 1993). Both SNAPs and NSF are cytosolic proteins acting on all *cis*-SNARE complexes at the various transport steps. ATP hydrolysis by NSF dissociates the SNARE complexes resulting in largely unstructured SNARE proteins, which now can be recycled for another round of transport (Söllner et al. 1993a; Mayer et al. 1996). Thus, membrane fusion is driven by protein folding (v-/t-SNARE complex assembly), and a net input of energy (ATP hydrolysis) is required to unfold the SNAREs.

SNARE STRUCTURE

The SNARE motif is located in the cytosolic domain of the SNAREs and in close proximity to the *trans*-membrane domain (Fasshauer et al. 1998). An individual SNARE protein usually contains a single SNARE motif. Exceptions are members of the SNAP-25 subfamily, which contain two SNARE motifs joined in a single polypeptide chain. The SNARE motif consists of a stretch of about 60–70 amino acids, which contains heptad repeats that have a propensity to form coiled-coil structures (Fig. 1). However, in the absence of a binding partner, SNARE motifs are largely unstructured (Fasshauer et al. 1997). When a functional (fusogenic) v-/t-SNARE complex forms, four SNARE motifs assemble into a stable four-helix bundle (Sutton et al. 1998). Based on the presence of the heptad repeats, 16 stacked layers of amino acid side chains stabilize the four-helix bundle

(Fig. 1). With the exception of a central “0” layer, which usually contains either a hydrophilic glutamine or an arginine residue, all other layers are characterized predominantly by hydrophobic side chains. Based on the amino acid composition of the central layer and other structural features, the classification: Q_a-, Q_b-, Q_c-, and R-SNAREs were introduced in addition to the topological/functional v- and t-SNAREs definition (Fasshauer et al. 1998). Functional SNARE complexes always contain one member of these four SNARE subfamilies.

For fusion to occur, the SNARE motif and the *trans*-membrane domain need to be directly coupled, indicating that the folding of the cytosolic SNARE motifs provides direct physical work on the lipid bilayer (Grote et al. 2000; McNew et al. 2000b). Indeed, the crystal structure analysis of the assembled neuronal v-/t-SNARE complex, containing the *trans*-membrane regions, revealed a helical extension of the SNARE motifs into the membrane (Stein et al. 2009).

Remarkably, most fusogenic SNARE complexes show a strict topological restriction. Thus, when a SNARE complex is built from its cognate Q_{abc}- and R-SNAREs, the SNAREs need to be distributed in a defined manner between the two membranes to be fused. In most cases, only one out of eight possible topological distributions appears to be functional as has been shown by *in vitro* reconstitution experiments (McNew et al. 2000a; Parlanti et al. 2000). However, in the endosomal system, mediating homotypic fusion, other topological combinations have been found to fuse reconstituted liposomes (Zwilling et al. 2007). Biochemical studies have shown that t-SNAREs form stable subcomplexes, which function as templates for v-SNARE binding (Fasshauer and Margittai 2004). A functional t-SNARE complex always contains one molecule of the syntaxin/Q_a-SNARE subfamily and usually comprises three partially assembled SNARE motifs, which in many cases match the Q_{abc}-composition. (The syntaxin/Q_a-SNARE and the nonsyntaxin t-SNARE components have also been called t-SNARE heavy and light

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Figure 1. Structural organization of the SNARE four-helix bundle and SNARE motifs of ER and Golgi SNAREs. (A) Skeleton diagram of the synaptic SNARE four-helix bundle showing interacting layers (image is modified from Fasshauer et al. [1998] and reprinted, with permission, from the National Academy of Sciences ©1998). (B) SNARE motifs and membrane anchors of human SNAREs involved in ER and Golgi transport were aligned using the MULTALIN program (Corpet 1988). Interacting layers are shown in gray; amino acids in “0” layer and *trans*-membrane regions are highlighted in color. Ykt6 contains a CAXX box, which is farnesylated and palmitoylated. The Q_a -, Q_b -, Q_c -, and R-SNARE classification is shown and SNAREs are listed according to their position in the secretory pathway: SEC20 (NP_053583.2), USE1 (NP_060937.1), BET1 (NP_005859.1), GS15 (AFF37877.1), syntaxin 6 (syx6) (CAA05177.1), syntaxin 10 (syx10) (AAC05087.1), SEC22b (NP_004883.2), YKT6 (NP_006546.1), VAMP3 (NP_004772.1), VAMP4 (NP_003753.2), syntaxin 18 (BAA95213.1), syntaxin 5 (AAC71078.1), syntaxin 16 (syx16) (AAC05647.1).

chains, respectively.) Other SNARE topologies might form transient SNARE complexes, which however are not stable enough to allow SNARE-pin formation across membranes, or provide not enough energy to drive membrane fusion. This topological restriction has important implications for the regulation of membrane fusion, because a functional t-SNARE complex will only form when all three t-SNARE components reside within the same membrane. Furthermore, even when all three cognate SNARE components are within the same membrane, regulatory components still control the assembly of the t-SNARE complex and subsequent v-t-SNARE complex formation. Syntaxins/ Q_a -SNAREs are key control sites for SNARE-complex assembly. Like many other SNAREs,

they contain regulatory domains, which are found at their amino-termini and control SNARE motif accessibility and structure. The type of regulatory domains differs between SNARE subfamilies (Malsam et al. 2008).

Based on a comparative analysis of several model organisms, the four subfamilies can be further divided into 20 distinct conserved functional subgroups, which might represent the minimal repertoire of a proto-eukaryotic cell (Klopper et al. 2007). During evolution, the SNARE sets were modified by duplication and diversification (Dacks and Field 2007; Klopper et al. 2007). In particular, the SNARE repertoire in the endosomal and exocytic transport steps has undergone a substantial expansion, most likely to adapt to the rising complexity of these

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trafficking routes in metazoa. By contrast, a very limited number of different SNAREs operates at the endoplasmic reticulum and Golgi complex, suggesting that the initial fusion steps of the secretory route are highly conserved through evolution (Kienle et al. 2009).

GOLGI SNAREs

The Golgi of mammals and plants is a highly polarized compartment, which is comprised of at least three distinct cisternae: *cis*, medial, and *trans* (Farquhar and Palade 1998). On the *cis*-side, the Golgi is preceded by the intermediate compartment (IC), (also named ER-Golgi intermediate compartment [ERGIC] or *cis*-Golgi network [CGN]), which receives membrane transport intermediates from the endoplasmic reticulum (ER) (Appenzeller-Herzog and Hauri 2006). On the *trans*-side, the Golgi is succeeded by the *trans*-Golgi Network (TGN), which obtains transport vesicles from two distinct endosomal compartments: the early and late endosomes. Thus, in the simplest model, these three membrane trafficking steps that deliver material to the Golgi require three different v-/t-SNARE complexes. Additional SNARE complexes might be required to mediate vesicular transport at the rims of the cisternae, functioning in the retrieval of Golgi resident proteins and the anterograde transport of cargo in a percolating fashion within the Golgi stack. Because of variable numbers of cisternae within different cell types, and the genetic restriction of SNARE diversity, it naturally follows that one or a few SNARE complexes mediate inter-cisterna transport. To date, a total of four functional SNARE complexes have been identified in association with the Golgi (Fig. 2). A fifth complex, which mediates retrograde transport to the ER is mentioned here, because the Golgi contains SNAREs involved in this transport step.

The majority of Golgi-localized SNARE complexes and their individual components have been analyzed in great detail in *in vitro* and *in vivo* studies. Reconstituted membrane fusion assays have provided evidence that among 147 tested SNARE complexes, which

could theoretically form at the Golgi, only two distinct SNARE complexes are capable of driving membrane fusion (McNew et al. 2000a; Parlanti et al. 2000; Parlanti et al. 2002). Remarkably, these studies also revealed that one particular SNARE protein can operate in two different complexes, indicating that nature uses a combinatorial code to make efficient use of a limited number of genetically encoded SNAREs (Parlanti et al. 2002; Shorter et al. 2002). Both of these Golgi SNARE complexes share the same syntaxin/ Q_a molecule: syntaxin 5 in mammals, Sed5p in yeast (Parlanti et al. 2002). One complex consists of syntaxin 5/Sed5p, membrin/Bos1p (also known as GS27) and ERS24/Sec22p, which assemble to form the t-SNARE, and rBet1/Bet1p functioning as the cognate v-SNARE (Fig. 2) (Xu et al. 2000). In the second complex, syntaxin 5/Sed5p forms a different t-SNARE with GOS28/Gos1p (also known as GS28) and Ykt6/Ykt6p to create a binding site for the cognate v-SNARE Gs15/Sft1p. Whereas the former complex is implicated in transport from the ER to the Golgi, and possibly in intra-Golgi transport between subsequent cisternae within the Golgi stack, the latter complex might exclusively function in COPI-dependent intra-Golgi transport (Newman and Ferro-Novick 1987; Newman et al. 1990; Shim et al. 1991; Hardwick and Pelham 1992; Subramaniam et al. 1996; Zhang et al. 1997; Cao and Barlowe 2000; Zhang and Hong 2001; Xu et al. 2002; Volchuk et al. 2004). The distribution of these SNAREs within the Golgi in budding profiles and COPI-coated vesicles was also analyzed by electron microscopy (Nagahama et al. 1996; Paek et al. 1997; Orci et al. 2000; Volchuk et al. 2004). By quantitative analysis, syntaxin 5 has been shown to distribute evenly throughout the stack, confirming its role as the general Golgi syntaxin/ Q_a SNARE. By contrast, a comparison of the two specific v-SNAREs revealed that they have opposite distributions within the stack. Whereas the v-SNARE GS15 is present in a gradient of increasing concentration toward the *trans*-face of the stack, its counterpart Bet1 progressively decreases in concentration from the CGN toward the *trans*-face (Fig. 2) (Volchuk et al. 2004). In fact, the distribution of both

complexes might also become substrates for SNAP and NSF, resulting in complex disassembly. Nevertheless, it has been shown in *in vitro* studies that noncognate SNARE complexes can affect membrane fusion (Varlamov et al. 2004). A reconstituted liposome fusion assay revealed that the *cis*-Golgi SNAREpin was inhibited by the addition of SNAREs that localize to the late-Golgi. Conversely, the late-Golgi SNAREpin was inhibited by the addition of *cis*-Golgi SNAREs. Because both SNARE complexes form gradients of opposite distributions within the stack, cognate SNAREs greatly outnumber SNAREs that are concentrated at the other end of the stack. Although this situation would favor the formation of cognate SNAREpins, SNAREs meant to function at the other end of the Golgi would become sequestered.

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Thus, such inhibitory i-SNAREs may be an effective tool to sharpen the continuous gradient of SNAREs within the Golgi stack. The physiological relevance of this concept remains to be established. A systematic study analyzing the presence of SNARE (sub) complexes at steady state within the Golgi stack in living cells has been difficult because of the limited spatial resolution of light microscopy. However, advances in microscopic techniques in combination with the introduction of site-specific fluorescent labels showing FRET will make such an endeavor possible in the near future.

The Q_a -SNARE syntaxin 5/Sed5p is not the only Golgi SNARE that is found in different functional complexes. As already mentioned, the R-SNARE Sec22p functions together with syntaxin 5/Sed5p and membrin/Bos1p as t-SNARE in anterograde transport from the ER to the Golgi. Sec22p is also part of a SNARE complex, which mediates retrograde transport from the Golgi to the ER. This complex contains the syntaxin 18/Ufe1p, SEC20/Sec20p, and USE1/Use1p as cognate SNARE components (Lewis et al. 1997; Hatsuzawa et al. 2000; Burri et al. 2003; Dilcher et al. 2003). Although, USE1 does not contain an R or a Q in the central layer of the SNARE motif, it has been classified as a Q-SNARE family member based on other criteria (Fasshauer et al. 1998; Dilcher et al. 2003). If Sec22p functions in this complex as a v- or t-SNARE remains to be shown. This issue could be directly resolved with liposome reconstitution experiments testing which topological SNARE combination is fusogenic. Nevertheless, an in vitro assay measuring retrograde transport from the Golgi to the ER already indicates that Sec22p and potentially Bet1 can function as retrograde-directed v-SNAREs (Spang and Schekman 1998). A study analyzing the interaction of regulatory proteins with components of the retrograde Golgi–ER SNARE complex further supports that Sec22p operates as a v-SNARE (Kraynack et al. 2005). Thus, a single SNARE protein might function as part of a t-SNARE in one direction and as v-SNARE binding to a distinct t-SNARE in the opposite transport reaction.

The remaining two SNARE complexes (syntaxin 16/Vti1a/syntaxin 6/VAMP4 and

syntaxin 16/Vti1a/syntaxin 10/VAMP3) are localized to the TGN and share two components in the corresponding t-SNARE subcomplexes (syntaxin 16/Vti1a) (Mallard et al. 2002; Ganley et al. 2008). Thus, the presence of either syntaxin 6 or syntaxin 10 in these two t-SNARE complexes apparently provides enough information to specify by itself or in connection with other factors, if either VAMP4 or VAMP3 liposomes will fuse with the TGN. The syntaxin 16/Vti1a/syntaxin 6/VAMP4 complex is involved in retrograde transport from early endosomes and the syntaxin 16/Vti1a/syntaxin 10/VAMP3 complex in retrograde transport from late endosomes (Ganley et al. 2008). In yeast, the Tlg2p/Vti1p/Tlg1p/Snc1p complex is implicated in retrograde transport to the TGN (Holthuis et al. 1998; Paumet et al. 2001).

SNARE LOCALIZATION

Because SNAREs show compartmental specificity and provide the road map for membrane trafficking, mechanisms must exist that target SNAREs to their final destinations and retain them in place. Two types of localization signals are involved in this process.

First, the membrane anchors of SNAREs, in particular the length and the degree of hydrophobicity of the *trans*-membrane domain (TMD) play a role. Distinct compartments along the secretory pathway differ in their lipid bilayer thickness, which increases toward the plasma membrane (Bretscher and Munro 1993; Munro 1995; Reggiori et al. 2000). In the case of the ER syntaxin/ Q_a -SNARE Ufe1p, it has been shown that alterations in the amino acid composition, or an increased length of the TMD result in mislocalization of this t-SNARE component (Rayner and Pelham 1997). Ykt6, functioning in intra-Golgi transport, lacks a *trans*-membrane region but it is palmitoylated and farnesylated and contains a longin domain, which together determine the intracellular distribution of Ykt6 (Fukasawa et al. 2004; Meiringer et al. 2008). In addition, membrane subdomains and lipid interactions might play a role in SNARE localization. In the case of syntaxin 1, a syntaxin/ Q_a -SNARE involved

in regulated exocytosis, cholesterol-dependent clusters have been observed in the plasma membrane (Lang et al. 2001; Sieber et al. 2007). The partitioning of Golgi SNAREs into membrane microdomains has not been determined yet.

The second type of SNARE sorting signals mediates the direct and specific binding to distinct vesicle coat components, which drive vesicle budding, cargo sorting, and determine the vesicle size (see Popoff et al. 2011). This type of interaction becomes of particular importance, when SNAREs have already reached their final destination and now function as fusion machinery, e.g., as a v-SNARE at a defined trafficking step. In this case, a budding vesicle needs to incorporate a “functional” v-SNARE to allow the fusion with the target compartment, because vesicles lacking SNAREs would become dead end transport intermediates. In the early secretory pathway, SNARE-coat interactions have been intensively studied in the anterograde ER–Golgi transport step. In this case, COPII coated vesicles bud at the ER and can fuse either with each other in a homotypic fashion generating the *cis*-Golgi network (CGN) or with the CGN (Xu and Hay 2004). Indeed, COPII vesicles contain all SNAREs required to form the functional complex (Sed5p, Sec22p, Bos1p, and Bet1p) for this fusion step. In vitro reconstituted budding assays revealed that Sec22p and Bos1p were enriched in COPII vesicles (Matsuoka et al. 1998). By contrast, the syntaxin/ Q_a -SNARE Ufe1p, which forms a functional SNARE complex with Sec20p, Sec22p, and Use1p for retrograde Golgi–ER transport, was excluded from these vesicles. Bet1p and Bos1p interact with the COPII subunits Sec23/Sec24 and in the presence of either GTP or GDP with the GTPase Sar1p, which in its GTP-bound state recruits COPII-coats onto the ER-membrane (Springer and Schekman 1998). Remarkably, structural analyses suggest that COPII coats selectively package the active v- and t-SNAREs into budding COPII vesicles. The sorting signals are short peptides, which provide low affinity interactions with the coat components, and their availability depends on the conformational state of the SNARE (Mossessova et al. 2003).

Whereas the sorting sequence of the v-SNARE Bet1p is exposed in the free v-SNARE but sequestered in the four-helix SNARE bundle, the sorting signal of Sed5p is hidden in an auto-inhibitory Sed5p conformation, but is accessible in the Sed5p/Bos1p/Sec22p t-SNARE complex (Mossessova et al. 2003). This data indicates that the vesicle coat not only selects but also sequesters cognate v- and t-SNAREs in their active conformation and thus primes these vesicles for homotypic fusion.

Such structural information is not available for COPI-vesicles. Nevertheless, morphological and biochemical studies show that SNAREs are also recruited into COPI vesicles. Interactions of Bet1p and Sec22p with Glo3p and Arf1 have been reported (Rein et al. 2002). In addition to SNARE interactions, Glo3p also binds cargo proteins, COPI coat proteins, and functions as a GTPase-activating protein for Arf1p, which in its GTP-bound state recruits COPI coats (coat-omer) to the Golgi. These interactions provide an interesting analogy to the recruitment of SNAREs into COPII vesicles, in which Sec23 functions as GTPase-activating protein for Sar1p and together with Sec24 recruits SNAREs, cargo, and the COPII proteins (Springer and Schekman 1998; Mossessova et al. 2003). Furthermore, Arf1 itself in its activated GTP-bound state is capable of selectively binding the SNAREs GS15, Ykt6, and membrin, but not GOS28 (Honda et al. 2005; Lee et al. 2005). Nevertheless, GOS28 is enriched at Golgi rims and in COPI vesicles, but it is unclear whether GOS28 is recruited into COPI vesicles by direct coat protein interactions or by a stochastic process (Nagahama et al. 1996). In contrast, the membrane surface concentration of syntaxin 5 in COPI vesicles is twofold lower than in the Golgi stack, which is consistent with a requirement for t-SNAREs and in particular syntaxin/ Q_a -SNAREs in the cisternae (Fig. 2) (Volchuk et al. 2004). Furthermore, in COPI vesicles, the concentrations of v-SNAREs and t-SNARE light chains exceed the concentration of syntaxin 5 at least by a factor of 2. This ratio might ensure that excess free v-SNAREs are available for membrane fusion, whereas the t-SNARE components found in the same

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vesicle might cycle as cargo through the Golgi stack. In the cisternal maturation/progression model, in which cisternae form at the *cis*-Golgi, progress along the Golgi, and finally disassemble at the *trans*-Golgi, t-SNARE components need to be present in retrograde-directed COPI vesicles to ensure fusion machinery recycling (see Glick and Luini 2011).

Likewise, some of the three potential v-SNAREs (Sec22p, Bet1p, GS15p) present in a single COPI vesicle might travel as inactive cargo proteins in the process of being recycled between compartments. Alternatively, the individual v-SNAREs might be located to separate COPI vesicle populations, fusing either with the ER, the CGN/*cis*-Golgi, or at the rims of the Golgi stack. This question still remains to be resolved and will require the isolation of COPI vesicle using antibodies directed against individual v-SNAREs.

Altogether, SNAREs seem to be recruited into both COPII- and COPI-coated vesicles by a similar mechanism that requires interactions with coat components, Arfs and Arf-GAPs. Interestingly, the cold-sensitive phenotype of a Glo3 deletion mutant can be rescued by the overexpression of Bet1p, Bos1p, and Sec22p indicating that in the absence of a selective SNARE uptake mechanism, the stochastic incorporation of SNAREs into transport vesicles can suffice to ensure fusion competence and cell survival (Poon et al. 1999).

Following vesicle budding, pinching off, and uncoating, a vesicle fuses with its target compartment requiring the presence of all three t-SNARE components in a functionally active conformation. The assembly of a t-SNARE complex is mediated by regulatory proteins, which also function as transport step-specific vesicle tethering components. Thus, the location and type of tether make an important contribution, where a functional t-SNARE complex forms.

SNARE INTERACTIONS WITH TETHERS

Prior to SNAREpin formation, transport vesicles are captured by two types of membrane tethers (Sztul and Lupashin 2009). One family consists of coiled-coil proteins, and the

members associated with the Golgi are named Golgins (see Munro 2011). The other family contains heterogeneous assemblies of multisubunit protein complexes, which operate not exclusively at the Golgi, but also on other organelles. The relevant multisubunit complexes associated with the ER and the Golgi are: Dsl1, TRAPPI/II, COG, and GARP/VFT (see Table 1). Many of these proteins directly bind specific t-SNARE components, either COPI or COPII vesicle coats, and interact in a GTP-dependent manner with distinct compartment-specific small GTP binding proteins called Rabs (Stenmark 2009).

The TRAPPI and TRAPPII complexes differ significantly from the other multisubunit tethers, because they function as guanine nucleotide exchange factors (GEF) for Rabs and do not directly bind SNAREs. The TRAPPI complex is localized at the *cis*-side of the Golgi and is implicated in anterograde transport from the ER to the Golgi and COPII vesicle tethering (Sacher et al. 2001; Kim et al. 2006). It functions as a GEF for the Rab protein Ypt1 (Jones et al. 2000; Wang et al. 2000; Sacher et al. 2001). TRAPPII is implicated in intra Golgi and retrograde endosome-to-TGN transport (Cai et al. 2005). It interacts with COPI-coats and functions as a GEF for Ypt1 and Ypt31p/Ypt32p (Morozova et al. 2006). Thus, the GEF activities of TRAPPI/II complexes recruit specific Rab proteins to budding COPII and COPI vesicles, respectively.

The GARP or Vps fifty-three (VFT) complex is implicated in retrograde transport from early and late endosomes to the TGN (Siniossoglou and Pelham 2001; Conibear et al. 2003; Quenneville et al. 2006). It consists of four proteins (Vps51/52/53/54). In yeast, Vps51 directly binds the amino-terminal domain of Tlg1p and may release the putative autoinhibition of the amino-terminal domain of Tlg1p thus favoring t-SNARE complex formation (Siniossoglou and Pelham 2001; Conibear et al. 2003; Fridmann-Sirkis et al. 2006). In mammals, the GARP/VFT complex interacts with the SNARE motifs of syntaxin 6, syntaxin 16, and VAMP4 (Perez-Victoria and Bonifacino 2009). In addition, it binds the Rab protein

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Table 1. Multisubunit tethering proteins and interacting partners

Multisubunit tether	SNARE	Rab	Coat	SM	Coiled-coil tether	References
Dsl1/ZW10 (Dsl1, Tip20, Dsl3)	Ufe1/Sec20/Use1, syntaxin 18		COPI (α -COP, δ -COP)			Lewis et al. 1997; Frigerio 1998; Reilly et al. 2001; Andag and Schmitt 2003; Hirose et al. 2004; Arasaki et al. 2006
COG (Cog1–8)	Sed5/Ykt6/Gos1, Sed5, syntaxin 5, GOS28	Ypt1, Ypt6, Rab6, Rab30, Rab41	COPI (β -COP, γ -COP)	Sly1	p115, GM130	Ram et al. 2002; Suvorova et al. 2002; Zolov and Lupashin 2005; Shestakova et al. 2007; Sohda et al. 2007; Fukuda et al. 2008; Laufman et al. 2009
GARP/VFT (Vps51, Vps52, Vps53, Vps54)	Tlg1, syntaxin 10, syntaxin 16, syntaxin 6, VAMP4	Ypt6				Siniosoglou and Pelham 2001, 2002; Conibear et al. 2003; Liewen et al. 2005; Perez-Victoria and Bonifacino 2009
TRAPP I (Bet3, Bet5, Trs20, Trs23, Trs31, Trs33)		Ypt1	COPII (Sec23)			Wang et al. 2000; Cai et al. 2007
TRAPP II (TRAPPI+Trs65, Trs120, Trs130)		Ypt1, Rab1, Ypt31/32	COPI (γ -COP)			Jones et al. 2000; Yamasaki et al. 2009

Ypt6p (Siniosoglou and Pelham 2001). Thus, the GARP complex could tether vesicles containing Rab6/Ypt6p to preassembled t-SNARE complexes at the TGN.

The Dsl1 complex is implicated in the tethering of retrograde COPI vesicles directed from the Golgi to the ER (Andag et al. 2001; Reilly et al. 2001). It consists of three subunits (yeast: Dsl1, Tip20, Dsl3; mammals: ZW10, RINT-1, NAG) (Ren et al. 2009; Tripathi et al. 2009). A central acidic domain binds δ - and α -subunits of the COPI coat that function in analogy to the clathrin adaptor and cage components, respectively (Andag et al. 2001;

Reilly et al. 2001). The Dsl1 complex stabilizes a SNARE complex containing Ufe1p/syntaxin 18, Sec20p/BNIP1, and Use1p/p31 in the ER (Kraynack et al. 2005; Aoki et al. 2008). Interacting Rab proteins have not been identified. Thus, the Dsl1 complex could tether COPI-coated vesicles to preassembled t-SNARE complexes on the ER. It has been suggested that Dsl1 might also favor COPI vesicle uncoating, exposing the cognate v-SNARE (Zink et al. 2009).

The COG complex is localized to the Golgi, and implicated in retrograde trafficking from the *trans*-Golgi or an endosomal compartment to the *cis*-Golgi (Whyte and Munro 2002; Vasile

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et al. 2006; Shestakova et al. 2007). It consists of eight subunits (Cog1–8) and forms a bilobe structure (Ungar et al. 2002). Cog subunits specifically interact with COPI coat components (Suvorova et al. 2002; Zolov and Lupashin 2005). The depletion of the Cog3 subunit causes the accumulation of Golgi-derived vesicles containing Golgi residents and SNAREs (GS15 and GOS28) and blocks the retrograde traffic of Shiga toxin (Zolov and Lupashin 2005). Cog4 and Cog6 directly bind syntaxin 5 and the COG complex enhances the stability of syntaxin 5-containing SNARE complexes (Zolov and Lupashin 2005; Shestakova et al. 2007). In addition, several Rab proteins seem to bind to Cogs: Rab1/Ypt1p, Rab6/Ypt6p, Rab30, and Rab41 (Suvorova et al. 2002; Fukuda et al. 2008). Remarkably, Cog2 binds the coiled-coil tether protein p115 and Cog4 binds the SM protein Sly1 (Sohda et al. 2007; Laufman et al. 2009). Both Sly1/Sly1p and p115/Uso1p directly bind to syntaxin 5/Sed5p and regulate its activity (Sapperstein et al. 1996; Grabowski and Gallwitz 1997). Furthermore, temperature-sensitive mutants in the yeast p115 (*uso1-1*) and *cog2-ts* are synthetically lethal supporting the hypothesis that multisubunit and coiled-coil tethers work together in controlling vesicle tethering, docking, and SNARE complex assembly (Van Rhee et al. 1998). One possibility would be that one of the tethers is incorporated during budding and thus ensures that the emerging vesicle does not leave the rims of the Golgi restricting its movement. The second tether then directly affects SNARE complex assembly on the neighboring target membrane. However, the cascade of reactions and the exact functions of these two distinct types of tethers still need to be further established.

In summary, the following reaction pathway emerges. During vesicle budding, distinct coat proteins recruit specific cargo proteins, SNAREs, and Rab-GEFs to the budding site. Rab-GEFs activate specific Rabs, which now bind their effectors, multisubunit and coiled-coil tethers, which in turn activate specific t-SNARE proteins on the target membrane. Then, t-SNAREs are substrates for v-SNARE binding and SNAREpin formation, which is

further controlled by another protein family called SM proteins.

SM PROTEINS OF THE GOLGI: Sly1, Vps45

SM (Sec1p/Munc18) proteins represent a family of arch-shaped cytosolic proteins, whose individual members affect SNARE complex assembly at distinct transport steps (Toonen and Verhage 2003). In addition, SM proteins seem to be involved in the proper localization and stabilization of syntaxin/Q_a-SNAREs. SM proteins are essential for membrane trafficking and their inactivation results in cell death, demonstrating the importance of their interaction with SNAREs. Consequently, SNAREs together with SM proteins have been declared as the universal fusion machinery (Südhof and Rothman 2009). A single SM member can bind several SNARE complexes and serves a subset of transport steps. The SM proteins functioning at the Golgi are Sly1 and Vps45. Sly1/Sly1p binds syntaxin 5/Sed5p in the Golgi and syntaxin 18/Ufe1p in the ER and controls anterograde and retrograde transport between the ER and Golgi and within the Golgi stack (Lupashin et al. 1996; Li et al. 2005). Vps45 binds syntaxin 16/Tlg2p at the TGN and directs transport from early/recycling endosomes and late endosomes to the TGN (Simonsen et al. 1998; Tang et al. 1998; Struthers et al. 2009). In addition, it binds Pep12 in early endosomes controlling endosomal trafficking (Tellam et al. 1997). SM protein-Q_a-SNARE interactions can stabilize the Q_a-SNARE and vice versa. Vps45p stabilizes Tlg2p, and Sly1p protects Ufe1p from degradation (Bryant and James 2001; Braun and Jentsch 2007). With the exception of the Sly1p-Sed5p interaction, which seems not to affect Sed5p stability, similar cross stabilizations have been observed for SM proteins and Q_a-SNAREs functioning in regulated exocytosis (Toonen and Verhage 2003; Braun and Jentsch 2007). Thus, it appears that SM proteins can control the amount of different Q_a-SNAREs. Remarkably, the overexpression of SM proteins can boost protein secretion by increasing the size of secretory organelles in certain cells (Peng and Fussenegger 2009).

In addition to the syntaxin/ Q_a -SNARE interaction, SM proteins bind partially assembled SNARE complexes and stimulate membrane fusion. One of the best-studied systems to understand how SM proteins direct SNARE complex assembly has been regulated exocytosis (Toonen and Verhage 2007). In this case, the neuronal SM protein Munc18-1 has both an inhibitory and stimulatory function (Hata et al. 1993; Misura et al. 2000; Verhage et al. 2000; Shen et al. 2007). Munc18-1 binds the Q_a -SNARE syntaxin 1 in a closed conformation and inhibits t-SNARE (syntaxin 1/SNAP-25) and v-/t-SNARE (syntaxin1/SNAP-25/VAMP2) complex assembly (Dulubova et al. 1999; Misura et al. 2000). Remarkably, the cognate v-SNARE VAMP2 directly binds Munc18-1 and relieves the inhibition (Xu et al. 2010). In addition, the specific binding of the SM protein to both the v- and t-SNARE stimulates membrane fusion (Shen et al. 2007). The existence of an inhibitory and a stimulatory binding mode requires distinct binding sites between the SM protein and the syntaxin/ Q_a -SNARE (Südhof and Rothman 2009). Recent data indicate that the stimulatory SM function in the neuronal system depends on the binding of a helical amino-terminal peptide of the Q_a -SNARE into a hydrophobic pocket in domain 1 of the SM protein (Hu et al. 2007; Khvotchev et al. 2007; Shen et al. 2007; Shen et al. 2010). This binding mode seems to be conserved and has been shown for the Sly1p-Ufe1p, Sly1p-Sed5p, and the Vps45p-Tlg2p interactions (Bracher and Weissenhorn 2002; Dulubova et al. 2002; Yamaguchi et al. 2002). It also has been established that the presence of the Tlg2p N-peptide can change the Vps45p-Tlg2p interaction modus (Carpp et al. 2006; Furgason et al. 2009). However, it is still debated whether all SM protein- Q_a -SNARE pairs in the Golgi show the same properties as their neuronal counterpart (MacDonald et al. 2010). Nevertheless, it is reasonable to assume that SM protein-SNARE interactions are functionally conserved and that modest changes represent transport step-specific adaptations. In any case, SM proteins bind cognate SNARE complexes. Sly1p binds the non Q_a -SNAREs Use1p/pSLT1p and

Sec20p, which are part of the Ufe1p complex (Li et al. 2005). It also shows low affinity interactions with the Sed5p t-SNARE components Bos1p and Gos1p and with the v-SNAREs Bet1p and Sft1p, and enhances SNAREpin formation (Dascher et al. 1991; Dascher and Balch 1996; Kosodo et al. 2002; Peng and Gallwitz 2002; Peng and Gallwitz 2004). Remarkably, Sly1p restricts the interactions of Sed5p with noncognate SNAREs (Peng and Gallwitz 2002). Thus, Sly1 could also act as a molecular shield to prevent the formation of unproductive (nonfusogenic) SNARE complexes. This might be of particular importance in the early secretory pathway, because most SNAREs are tail-anchored proteins, which as newly synthesized proteins are initially inserted into the ER membrane (Kutay et al. 1995; Rabu et al. 2009). Thus, the presence of various SNAREs, which travel through the ER and Golgi to their final destinations, would increase the possibility that noncognate SNARE complexes form. Sly1 could block such adverse interactions.

In summary, SM proteins contribute a layer of specificity to SNARE complex formation and favor membrane fusion, likely by specifically stabilizing cognate SNAREpins. In this context, it is also of note that SM proteins are functionally coupled to tethering proteins. Sly1 directly interacts with the COG tethering complex and both bind syntaxin 5 with distinct binding sites (Laufman et al. 2009). Obliteration of Sly1-Cog binding impairs SNARE pairing and attenuates retrograde Golgi-to-ER transport (Laufman et al. 2009). Thus, the concerted action of SM and tethering proteins controls SNAREpin assembly.

CONCLUDING REMARKS

For productive fusion to occur, several requirements need to be fulfilled on opposite membranes (Fig. 3). First, during vesicle budding, transport step-specific coat proteins select cargo proteins, specific SNAREs, and a particular Rab-GEE, which activates and recruits a specific Rab protein onto the budding vesicle. Second, the Rab protein binds a specific tether,

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Figure 3. Basic machinery controlling membrane fusion and SNARE recycling. SNAREs and tethers are recruited by vesicle coat components for uptake into a budding vesicle. The initial interaction of a vesicle with its target membrane is mediated by tethering proteins and a small Rab GTPase. Components of the tethering machinery trigger the formation of t-SNARE complexes, which assemble with their cognate v-SNAREs, resulting in the formation of SNAREpins. These events involve SM proteins and finally culminate in membrane fusion, which results in cis-v/t-SNARE complexes. In the presence of SNAPs, ATP-hydrolysis by NSF segregates v- and t-SNAREs for another round of fusion.

which together with the specific SM protein controls t-SNARE complex assembly on the opposite membrane. Third, if a cognate v-SNARE is present, it will interact with the t-SNARE while the SM protein provides an additional control level and stimulates SNAREpin assembly, resulting in membrane merger. In the absence of a cognate v-SNARE, stable SNAREpins will not be established and GTP hydrolysis by Rab will eventually release the tether, likely allowing another round of tethering to be initiated. Depending on the length, number, and type of tethers, which link a given vesicle to the Golgi, the vesicle could probe different areas of the Golgi stack, without ever leaving the Golgi, until productive SNAREpins have been established and fusion proceeds.

Thus, various proteins and a potential cascade of reactions controlling SNARE complex assembly have been identified, accompanied by a morphological map showing the distribution of these components within the Golgi stack. However, a functional map at high spatial resolution, resolving transport steps between individual cisternae, is still missing. Because, by its very nature, the transport machinery cycles between individual compartments, it will be necessary to distinguish between inactive and active components on distinct vesicle subpopulation. The identification of various COPI coats and distinct vesicle tethers clearly supports the existence of distinct vesicle populations within the Golgi (Moelleken et al. 2007). Such subpopulations can be isolated

based on their coat, SNARE, and tether composition (Malsam et al. 2005). Proteomic and lipidomics could then reveal their molecular composition. More importantly, reconstituted fusion assays employing liposomes with distinct t-SNAREs, in the absence or presence of appropriate tethers and SM proteins will show the fusion competence and specificity of the isolated vesicle subpopulations. Concomitantly, the functional/assembly status of t-SNARE and v-/t-SNARE complexes needs to be probed in living cells at the level of individual cisternae. Therefore, t-SNARE components and v-SNAREs could be labeled by fluorescent tags that have the capability to show fluorescence resonance energy transfer. In combination with advanced imaging techniques such as stimulated emission depletion microscopy, far field optical nanoscopy, fluorescence cross correlation microscopy, fluorescence life time imaging, and fluorescence recovery after photobleaching, it will be possible to show where and when t-SNARE complexes form and to which degree assembled v-/t-SNARE complexes exist at steady state in cells. Such studies should also detect potential SNARE clusters and their intra-Golgi distribution. With this information in hand, it should be possible to gain detailed insights into where and how distinct vesicle subpopulations fuse, resolving the membrane flow patterns within the Golgi stack and distinguishing between different transport models.

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