The Dsl1 Protein Tethering Complex Is a Resident Endoplasmic Reticulum Complex, Which Interacts with Five Soluble NSF (N-Ethylmaleimide-sensitive Factor) Attachment Protein Receptors (SNAREs)

**IMPLICATIONS FOR FUSION AND FUSION REGULATION**

Christoph T. A. Meiringer, Ralf Rethmeier, Kathrin Auffarth, Joshua Wilson, Angela Perz, Charles Barlowe, Hans Dieter Schmitt, and Christian Ungermann

The Dsl1 tethering complex functions in fusion of Golgi-derived vesicles at the ER4 membrane and consists of the three subunits Dsl1, Dsl3, and Tip20. It forms a stable complex with the SNAREs Ufe1, Use1, and Sec20 to mediate fusion of COPI vesicles with the endoplasmic reticulum. Here, we analyze molecular interactions between five SNAREs of the ER (Ufe1, Use1, Sec20, Sec22, and Ykt6) and the Dsl1 complex in vitro and in vivo. Of the two R-SNAREs, Sec22 is preferred over Ykt6 in the Dsl-SNARE complex. The NSF homolog Sec18 can displace Ykt6 but not Sec22, suggesting a regulatory function for Ykt6. In addition, our data also reveal that subunits of the Dsl1 complex (Dsl1, Dsl3, and Tip20), as well as the SNAREs Ufe1 and Sec20, are ER-resident proteins that do not seem to move into COPII vesicles. Our data support a model, in which a tethering complex is stabilized at the organelle membrane by binding to SNAREs, recognizes the incoming vesicle via its coat and then promotes its SNARE-mediated fusion.

Retrograde vesicular transport from the Golgi to the ER requires the Dsl1 tethering complex, which consists of the three subunits Dsl1, Dsl3, and Tip20. It forms a stable complex with the SNAREs Ufe1, Use1, and Sec20 to mediate fusion of COPI vesicles with the endoplasmic reticulum. Here, we analyze molecular interactions between five SNAREs of the ER (Ufe1, Use1, Sec20, Sec22, and Ykt6) and the Dsl1 complex in vitro and in vivo. Of the two R-SNAREs, Sec22 is preferred over Ykt6 in the Dsl-SNARE complex. The NSF homolog Sec18 can displace Ykt6 but not Sec22, suggesting a regulatory function for Ykt6. In addition, our data also reveal that subunits of the Dsl1 complex (Dsl1, Dsl3, and Tip20), as well as the SNAREs Ufe1 and Sec20, are ER-resident proteins that do not seem to move into COPII vesicles. Our data support a model, in which a tethering complex is stabilized at the organelle membrane by binding to SNAREs, recognizes the incoming vesicle via its coat and then promotes its SNARE-mediated fusion.

Vesicles transport biosynthetic cargo and lipids between different compartments of the endomembrane system. Formation of the transport vesicles requires adaptors, coat proteins, and regulatory GTPases of the Arf1/Sar1 family. The initial contact between a vesicle and its target membrane requires Rab GTPases and tethers, which are in most cases multisubunit complexes. Rab GTPases, which cycle between an inactive GDP- and active GTP-bound state, and tethers coordinate the assembly of SNARE proteins on vesicle and target membrane into a four-helix bundle, which ultimately drives bilayer fusion (1).

The Dsl1 tethering complex functions in fusion of Golgi-derived vesicles at the ER4 membrane and consists of the three subunits Dsl1, Dsl3/Sec39, and Tip20. It forms a stable complex with the ER SNAREs Sec20, Ufe1, and Use1 (2, 3). In addition, Dsl1 and Tip20 are linked to the coatamer, which implies a role in the recognition and/or uncoating of the COPI vesicle (4–7). In agreement with this, Dsl1 depletion leads to a massive accumulation of COPI-coated vesicles (8).

The Dsl1 complex is closely linked to the SNAREs Ufe1, Use1/Slt1, Sec20, and Sec22, which are required for fusion at the ER membrane (9–12). The R-SNARE Sec22 is generally accepted as the v-SNARE on COPI vesicles. However, Sec22 has not been previously identified as part of the Dsl1 complex and is dispensable for yeast survival. In addition, it can be functionally replaced by the R-SNARE Ykt6 in anterograde (13) and potentially also in retrograde transport. Ykt6, which lacks a transmembrane domain and thus is unlikely to function as the sole v-SNARE, is found in multiple SNARE complexes at the Golgi, endosomes, and the vacuole (10, 14).

Here, we present additional insights into the interactions and functions of the Dsl1 complex. We show that the two R-SNAREs Sec22 and Ykt6 are associated with the Dsl1 complex, with Sec22 being the preferred subunit. Only Ykt6 is sensitive to Sec18/NSF, whereas the remaining interaction between SNAREs and the Dsl1 complex is unaffected. Reconstitution approaches reveal that the Dsl1 complex contains several interfaces for SNAREs, and in vivo studies suggest that subunits of the Dsl1 complex and the Q-SNAREs are ER-resident proteins. Our data support a model of tethering via coat recognition, followed by SNARE assembly and fusion.

**EXPERIMENTAL PROCEDURES**

Yeast Strains and Plasmid Construction—Yeast strains used in this study are listed in supplemental Table S1. These were either generated by homologous recombination of PCR-ampli-
Interactions between SNAREs and the Dsl1 Complex

fied fragments or by transformation with plasmids (see below). For yeast two-hybrid analysis, full-length or truncated ORFs were cloned into both pACT2 (Clontech) and pFBT9 (15) plasmids and co-transformed into yeast strain Pf69-4A (as shown in supplemental Table S2). Transformants were selected on synthetic dextrose complete-Leu-Trp plates, and four clones of each tested interaction were restreaked to quadruple drop-out plates (synthetic dextrose complete-Leu-Trp-His-Ade). Growth was assayed after 4–7 days. Plasmids for purification of recombinant proteins were cloned either into pGEX-2TK (GE Healthcare), pETHIS (14), or pET32c(-Trx) (modified from pET32c(+), Novagen, by removal of the thioredoxin tag) and are listed in supplemental Table S3. The SNAREs were cloned without their transmembrane domain. PCR amplification was performed using Pfu polymerase (Fermentas GmbH), and all Y2H plasmids were sequenced (GATC Biotech AG). Restriction digest and cloning were performed according to the manufacturer’s instructions (Fermentas GmbH).

Microscopy—Yeast cells expressing either Tip20-monomeric GFP, Dsl1-GFP, or Dsl3-monomeric GFP were grown to early log-phase, harvested, washed once with PBS, and mounted on object slides. Visualization was performed on a fluorescence microscope (Leica DM5500 B; Leica Microsystems GmbH) equipped with a GFP filter (excitation, D480/30; emission, D535/40, beamsplitter 505dclp; Chroma Technology), captured with a digital camera (Spot Pursuit-4MP; Diagnostic Instruments, Inc.) and processed using Metamorph (Molecular Devices) and Autoquant X (Media Cybernetics). Strains bearing a temperature-sensitive allele were analyzed both after growth at 23 °C and after a temperature shift to 37 °C for 30 min. For Dsl1 and Ypt1 depletion experiments, strains were grown in YPG, washed once in PBS, and then grown in YPD for 9 h.

Tandem Affinity Purification—Tandem affinity purification (TAP) tag protein tandem affinity purification was performed as described in Refs. 16, 17 using the following buffer: 50 mM HEPES/KOH, pH 7.4, 150 mM NaCl, 0.15% Nonidet P-40 (Igepal CA-630; Sigma-Aldrich), and 1.5 mM MgCl2. The buffer was supplemented with 1 mM PMSE, 1 mM DTT, and 1× protease inhibitor mix FY (Serva). For washing of IgG-Sepharose (GE Healthcare), the buffer was supplemented with 0.5 mM DTT. Additional methods are provided in the supplemental data.

RESULTS

Isolation of Dsl1 Complex Identifies R-SNARE Ykt6—The SNARE Ykt6 is distributed between cytosol and membranes. To identify a potential receptor of Ykt6, we tagged Ykt6 with GFP in a loop contained within its N-terminal longin domain and isolated the protein using antibodies against GFP coupled to protein A-Sepharose. Proteins that co-eluted with Ykt6-GFP were identified by mass spectrometry (Fig. 1A). Besides known interaction partners (Sed5, Sec17, and Syl1), we were able to identify Dsl1, Dsl3, and Tip20 in the eluate. All three proteins belong to the Dsl1 complex at the ER, localize similarly to punctate structures at the cortical and perinuclear ER (Fig. 1B), and are equally abundant (Fig. 1C), as reported (2).

To confirm the interaction between the Dsl1 complex and Ykt6, we tagged Dsl1 and Dsl3 with a C-terminal TAP tag and performed a tandem affinity purification via IgG Sepharose and CaM beads (Fig. 1D and supplemental Fig. S1A). Using mass spectrometry, we identified not only the three Dsl1 complex subunits, but also all of the components of the ER SNARE complex Ufe1, Use1, and Sec20. This stable complex of tethers and SNAREs was reported previously but lacked the appropriate R-SNARE (2). When we subjected the eluate of the IgG beads to gel filtration, we recovered the Dsl1 complex together with the previously identified Q-SNAREs and Ykt6 in a high molecular mass complex of ~700 kDa in fractions 11, 12 (Fig. 1D and supplemental Fig. S1A), and a subcomplex of Dsl1 and Dsl3 in fractions 13 and 14 (Fig. 1D), in agreement with recent structural work (3).

Sec22 and Ykt6 Interaction with Dsl1 Complex Differs—Sec22 was previously described to be the R-/v-SNARE in trans-
port between the Golgi and ER (11, 18, 19), though we found Ykt6 (Fig. 1). However, we detected both Sec22 and Ykt6 in association with Dsl3-TAP and the other subunits of the Dsl1 complex on Western blots (supplemental Fig. S1B), whereas control beads did not precipitate any SNARE (Fig. 2E). We therefore wondered whether we could detect differences in the composition of the Dsl1 tether complex with SNAREs and thus determine the precise interactions. We tested different temperature-sensitive mutants of the Dsl proteins, Dsl1 and Tip20, the SNARE Sec20 and Sec18, the ATPase required for SNARE complex disassembly. The composition of the Dsl1 complex and its interaction with SNAREs were unchanged in the sec18-1 mutant, which is blocked in SNARE disassembly at the restrictive temperature (Fig. 2A, lane 14, supplemental Fig. S1B). In contrast, mutants in dsl1 (lanes 2–6), or tip20 (lanes 9–12) strongly affected the stability of the Dsl1 complex (Fig. 2A). This indicates that the interaction of the Dsl1 complex with all five SNAREs is affected by Dsl1 complex mutations, but not by alterations in the SNARE chaperone Sec18.

To unravel whether the two R-SNAREs compete for the same binding site on the complex, we overexpressed either Sec22 or Ykt6 and purified the Dsl1 complex via Dsl3. Overexpression of Ykt6 did not have any significant effect on Sec22 binding to the Dsl1 complex, whereas Sec22 overexpression completely abolishes binding of Ykt6 (Fig. 2B, lane 3 versus 4). We then asked whether the competition of Ykt6 and Sec22 may be enhanced if the turnover of the SNARE complex was impaired. Indeed, overexpression of Ykt6 in the sec18-1 strain was sufficient to displace Sec22 from the Dsl1 complex (Fig. 2C), indicating that Ykt6 can compete with Sec22 under these conditions. Moreover, these cells grew more slowly at higher temperatures than cells with the sec18-1 allele alone (Fig. 2D). It
was demonstrated previously that Ykt6 functionally replaces Sec22 in transport between the Golgi and the ER (13). We therefore reasoned that Ykt6 might be enriched in the Dsl1 complex if Sec22 is lacking. Contrary to our assumption, the pulldown of Dsl3-TAP in a sec22/H9004 strain did not show a significant accumulation of Ykt6 in the Dsl1 complex (Fig. 2B, lane 2). We conclude that association of Ykt6 with the Dsl1 complex occurs upstream of Sec22 binding and the latter prevents Ykt6 from re-entering the complex.

We then asked whether the interaction of Sec22 and Ykt6 with the Dsl1 complex is altered by the addition of purified Sec18, which can disassemble SNARE complexes (20). When ER-enriched membranes were incubated with Sec18 and ATP, only Ykt6 was selectively displaced from Sec20 or Dsl3, whereas the remaining complex stayed intact (Fig. 2E and supplemental Fig. S1C, lane 4). Our data suggest that Ykt6 occupies a Sec18-sensitive binding site on the assembled SNARE-Dsl1 complex.

**Dsl1 Complex Binds Q-SNAREs Sec20 and Use1 via Their N-terminal Domains**—To map the interactions between components of the Dsl1 complex and SNAREs, we employed yeast two-hybrid analysis. Interestingly, several subunits like Use1 and Dsl1 showed multiple interactions, suggesting that they occur in the context of the partially assembled Q-SNARE-Dsl1 complex (supplemental Fig. S1D). We therefore focused on the N-terminal and the SNARE domains of each Q-SNARE (Fig. 3A). Our data indicate that the Dsl1 complex recognizes the N-terminal domains of the SNARE Use1 (residues 1–141) and Sec20 (residues 1–196) via its subunits Dsl3 and Tip20, in agreement with Hughson and colleagues (Fig. 3, A and B) (21).
Interactions between SNAREs and the Dsl1 Complex

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G

Diagram showing vesicle, Golgi, and ER.
Interactions between SNAREs and the Dsl1 Complex

For Tip20, we mapped the binding site for Sec20 to residues 377–611 (Fig. 3, C and D). C-terminal of the proposed binding site by Ren et al. (21). Whereas Tripathi et al. (3) mapped the interaction site of Tip20 to Dsl1 to the first 41 residues, an internal deletion of residues 80–110 also abolished the binding to Dsl1, which may be due to changes in secondary structure. Alternatively, the binding site between Tip20 and Dsl1 includes additional Tip20 segments.

To test the interactions directly, we purified the components of the Dsl1 complex and reconstituted the complex assembly. We detected direct binding of Dsl1 to Dsl3 and Tip20 but not for Tip20 to Dsl3 (Fig. 3E). This is in agreement with previously published structural findings (3, 21). By gel filtration, we showed that monomeric Tip20 (Fig. 3F) assembled into the large Dsl1 complex (fractions 45–50), if incubated with Dsl1 and Dsl3 (Fig. 3G) and some large complex in the void volume. When the concentrated Dsl1 complex was incubated with an excess of either Sec20 or Sec22, only Sec20 bound to the Dsl1 complex, presumably via Tip20 (Fig. 3H). The reconstitution of the Dsl1 complex with all SNAREs, including the R-SNARE, was very inefficient (data not shown), and the interaction may occur only transiently in vivo. Our data reveal that the Dsl1 complex has binding sites for the N-terminal domains of Sec20 and Use1 and plays a role in the assembly of the SNARE complex, consistent with our initial observation (Fig. 1).

Evidence for ER-resident Dsl1 Complex—If Sec22 were the retrograde v-SNARE, it appears unlikely that it is replaced by acylated Ykt6, which lacks the transmembrane domain that is required to drive fusion of the lipid bilayers. Moreover, the interaction of Dsl1 and COPII suggested that Dsl1 could be recruited to retrograde vesicles. We therefore took advantage of a COPII budding assay (22) to monitor the incorporation of selected subunits of the Dsl1 complex and SNAREs (Fig. 4A). Vesicles generated in the presence of COPII components contained Sec22 and the cargo receptor Erv26, but lacked Dsl1, Tip20, and Ufe1, consistent with the predominant ER-localization of the Dsl1 complex in vivo (Fig. 1B). As we lacked an antibody to Sec20, we turned to an in vivo assay, using functional GFP-tagged Sec20 (Fig. 4B). Sec20 has been previously reported to contain a luminal HDEL motif, which might be responsible of its ER localization (23). However, neither sec22 deletion, Ykt6 overexpression, Ypt1 deletion, mutations in the δ-COP (ret2-1), nor alterations in Dsl3 functionality affected the steady-state localization of Sec20 to the ER (Fig. 4, C and D). In the sec18-1 mutant, we observed accumulations of GFP-Sec20, which increased in size at the restrictive temperature (Fig. 4D). These dots colocalized with the ER marker Sec63 (supplemental Fig. S2D). They might be the result of additional Ykt6 associated with the (Dsl1-)SNARE complex, which may impair transport between ER and Golgi, and subsequently growth (Fig. 2D). Our data therefore suggest that it is unlikely that Sec20 functions as a v-SNARE on retrograde vesicles.

Furthermore, depletion of Ypt1 or Dsl1, the latter one causes a massive accumulation of COPI vesicles (8), did not affect Dsl3 localization to the ER (Fig. 4E). Upon depletion of Dsl1, Sec20 and Dsl3 showed colocalization with the ER marker Sec63 but not with the Golgi marker Mnn9 (supplemental Fig. S2, A and B). Also Dsl1 itself, which does not bind directly to a SNARE or any other ER transmembrane protein, seem to be stably localized at the ER. In ret2-1 cells or a temperature-sensitive mutant of the ER to Golgi v-SNARE bos1 (bos1-I), Dsl1 is found at the ER (Fig. 4E), similar to our observations upon Ypt1 deletion (data not shown). Our data are therefore consistent with a stable ER-resident Dsl1 complex, which is kept in place by binding ER-resident Q-SNAREs.

DISCUSSION

Fusion of Golgi-derived vesicles with the ER requires a close cooperation of the Dsl1 complex (2) with the SNAREs Ufe1, Use1, Sec20, and Sec22 (12, 19). Previous purification of the Dsl1 complex did not yield any R-SNARE but only the Q-SNAREs Use1, Ufe1, and Sec20 (2). However, Use1 interacts with Sec20, Ufe1, and the R-SNAREs Sec22 and Ykt6 (19). We now demonstrate that Sec22 and Ykt6 are found in substoichiometric amounts in association with the isolated Dsl1 complex, suggesting that Ykt6 may regulate vesicle fusion by binding not only the Q-SNAREs, but also the SNARE-Dsl1 complex. We could not identify a binding site between any Dsl1 complex subunit and Ufe1 or Sec22, indicating that both bind to the Dsl1 complex via the SNAREs Sec20 and Use1 (11), which bind Tip20 and Dsl3 via their N-terminal domains (Fig. 3, A and B) (21). Our data suggest that Sec22 is indeed the missing R-SNARE, as expected from previous studies (12, 19). Overexpression of Sec22 completely removes Ykt6 from its association with the Dsl1 complex, whereas Ykt6 could not do so in reverse. A similar association of Ykt6 with the Dsl1–SNARE complex was recently observed by Spang and colleagues (37). In addition, Sec18 addition displaced Ykt6, but not Sec22 or the Q-SNAREs, from the Dsl1 complex. It is therefore possible that Ykt6 acts as an acceptor for the preferred SNARE Sec22 at the ER. Our data are consistent with the view that Sec22 is the v-SNARE in vivo, whereas the Q-SNAREs seem to be resident proteins of the ER.

While our manuscript was in preparation, two studies provided insight into the structure of the Dsl1 complex (3, 21). Dsl1 and Tip20 resemble known structures of the exocyst complex.
In agreement with our results, Hughson and co-workers (21) demonstrate that Use1 and Sec20 bind via their N termini to Dsl3 and Tip20, respectively. Our data indicate that Ufe1 requires Sec20 for binding, whereas Sec22 only binds if all other SNAREs are present. This observation is consistent with our initial isolation of Sec22 and Ykt6 with the Dsl1 complex from yeast (Figs. 1 and 2). The purified Dsl1 complex is, however, very inefficient in promoting SNARE assembly (3), and it has not been tested whether the slight increase in complex formation correlates with increased fusion.

The Dsl1 complex is a critical factor involved in COPI vesicle recognition at the ER (8). Its subunit Dsl1 binds directly to the interaction of the HOPS complex with the AP-3 coat. Here, this would position the complex such that it directly couples the tethering of COPI vesicles and SNARE-mediated fusion, by bringing together the SNARE on the vesicle with the assembled t-SNARE complex on the ER membrane (Fig. 4G). Interestingly, such a scenario is reminiscent of the proposed interaction of the HOPS complex with the AP-3 coat. Here, the AP-3 subunit Ap15 binds the HOPS subunit Vps41 (31–33), which is regulated by phosphorylation (34). HOPS might get stabilized similarly by SNAREs on the vacuole (35) and could then tether AP-3 vesicles by binding the coat. We therefore postulate that the tether–coat interaction might be of general importance to drive fusion reactions.

Acknowledgments—We thank Fred Hughson for the Dsl3 expression plasmid and all members of the Ungermann laboratory for fruitful discussions.

REFERENCES


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