The $H_{abc}$ Domain of the SNARE Vam3 Interacts with the HOPS Tethering Complex to Facilitate Vacuole Fusion*

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Background: Tethering complexes such as HOPS bind SNAREs to coordinate fusion.

Results: HOPS binds the Vam3 $H_{abc}$ domain and the SNARE complex, which is required for membrane fusion.

Conclusion: Binding of the Vam3 $H_{abc}$ domain prepositions HOPS for optimal fusion support.

Significance: Our data reveal that HOPS coordinates SNARE assembly and fusion via two distinct SNARE binding sites.

Membrane fusion at vacuoles requires a consecutive action of the HOPS tethering complex, which is recruited by the Rab GTPase Ypt7, and vacuolar SNAREs to drive membrane fusion. It is assumed that the Sec1/Munc18-like Vps33 within the HOPS complex is largely responsible for SNARE chaperoning. Here, we present direct evidence for HOPS binding to SNAREs and the $H_{abc}$ domain of the Vam3 SNARE protein, which may explain its function during fusion. We show that HOPS interacts strongly with the Vam3 $H_{abc}$ domain, assembled Q-SNAREs, and the R-SNARE Ykt6, but not the Q-SNARE Vti1 or the Vam3 SNARE domain. Electron microscopy combined with Nanogold labeling reveals that the binding sites for vacuolar SNAREs and the $H_{abc}$ domain are located in the large head of the HOPS complex, where Vps16 and Vps33 have been identified before. Competition experiments suggest that HOPS bound to the $H_{abc}$ domain can still interact with assembled Q-SNAREs, whereas Q-SNARE binding prevents recognition of the $H_{abc}$ domain. In agreement, membranes carrying Vam3$\Delta H_{abc}$ fuse poorly unless an excess of HOPS is provided. These data suggest that the $H_{abc}$ domain of Vam3 facilitates the assembly of the HOPS/SNARE machinery at fusion sites and thus supports efficient membrane fusion.

Membrane fusion at endomembranes concludes the vesicular transport of proteins and lipids between membranes. This process depends on a conserved machinery, including a Rab GTPase, tethering factors, the Sec1/Munc18 (SM)2 family of proteins, and membrane-embedded SNAREs. The initiating tethering of membranes, which requires the interaction of Rabs and tethers, is followed by the assembly of membrane-embedded SNAREs on opposite membranes (1). SNAREs are distinguished by their conserved hydrophilic residue within the otherwise hydrophobic SNARE domain, have been termed accordingly $Q_{a-}$, $Q_{b-}$, $Q_{c-}$, and R-SNARE. Four SNAREs (three Q-SNAREs and one R-SNARE) are needed for successful fusion. These zipper via their membrane-proximal SNARE domains into a four-helix bundle, which pulls membranes close and thus promotes lipid-bilayer mixing (2–4). Furthermore, fusion critically depends on a Sec1/Munc18 protein, which likely chaperones SNARE assembly (3, 5). However, their precise role during SNARE-mediated fusion is still debated. After fusion, the assembled SNARE complex has to be disassembled by the co-factors $\alpha$-SNAP and N-ethylmaleimide-sensitive factor to recycle SNAREs for further rounds of fusion.

We are interested in membrane fusion at endosomes and the lysosome-like vacuole in yeast. At the yeast vacuole, the hexameric HOPS tethering complex combines Rab interaction and SNARE binding ability (6, 7). HOPS has two Rab-specific subunits, named Vps39 and Vps41, which directly interact with the Rab7-like protein Ypt7 (8–10). These are located at opposite ends of the seahorse-like hexamer. Regions of the tail are occupied by Vps11 and Vps18, whereas Vps16 and the SM-like Vps33 proteins are present in the larger head region (10). Recent crystallographic studies on human and fungal Vps33 demonstrated the strong similarity to other SM proteins and identified its interaction site to an $\alpha$-helix-like segment of Vps16 (11, 12). Consistent with this, Vps33 seems to bind preferentially to assembled SNAREs and the SNARE domains of Vam3, Nyv1, and Vam7 (13, 14). This suggests that Vps33 acts like any other SM protein in membrane fusion.

We and others previously analyzed the interaction of HOPS with SNAREs in the context of fusion, and we could correlate the fusion activity of HOPS with its interactions. Indeed, HOPS binds to assembled vacuolar Q-SNAREs, consisting of the SNARE domains of Vam3, Vam7, and Vti1, and to the Vam7 PX domain (15–20). It also binds via Vps33 (13) the entire vacuolar SNARE complex (18). Vps33/HOPS interaction with SNAREs protects the assembled SNARE complex from Sec17/Sec18 (20), although Sec17 also seems to positively promote the recruitment of Vps33 (21). We previously showed that the N-terminal domain of Vam3 is involved in efficient fusion and in HOPS recruitment (22), in agreement with a role of this...
domain in promoting efficient fusion (23). This nevertheless left the question open, how HOPS binding to the H_{abc} domain of Vam3 and the SNARE complex might be coordinated. Here, we present evidence that the fusion-active HOPS complex has two distinct but communicating binding sites: to the Vam3 H_{abc} domain and assembled SNAREs. We were able to localize these sites on the HOPS structure, show that assembled SNAREs are the strongly preferred binding partner, and provide further support that H_{abc} binding of HOPS facilitates efficient fusion. Our data thus nicely extend our knowledge on a tethering complex, which combines Rab binding and SNARE chaperoning.

**EXPERIMENTAL PROCEDURES**

**SNARE Expression Plasmids**—SNARE constructs with C-terminus fused GST tag for pull-down experiments were cloned into pETGEX vector using Ncol/Sacl digestion and cloned into bacterial expression vectors without their transmembrane domain. SNARE constructs for reconstituted proteoliposomes were published (32). The Vam3\Delta H_{abc} fragment also encoding the transmembrane domain was cloned with a coding region for an N-terminal 3C-protease cleavage site into pET32c(-Trx) using BglII/XhoI digestion. All expression plasmids used for this study are listed up in Table 1.

**SNARE Purification**—All proteins for pull-down experiments were expressed in *Escherichia coli* BL21 (DE3) Rosetta cells and induced at A_{600} 0.4 with 0.75 mM isopropyl-\beta-D-thiogalactopyranoside overnight at 16 °C. Harvested cells were lysed in buffer containing 150 mM NaCl, 50 mM HEPES, 1 mM PMSF and 0.5-fold protease inhibitor mixture, pH 7.5. The lysis buffer of Vam3-H_{abc}-GST contained in addition 0.15% Igepal to facilitate solubility of the protein, and His-Vam3\Delta N \Delta TMD was lysed in 400 mM KCl, 50 mM HEPES, 2 mM MgCl\textsubscript{2}, 1 mM PMSF, and 0.5-fold protease inhibitor mixture, pH 7.5. Lysates were centrifuged for 20 min at 25,000 × g. The supernatants were incubated either with glutathione-Sepharose fast flow beads (GE Healthcare) for 1 h at 4 °C for GST-tagged proteins, or Ni-NTA-agarose (Qiagen) for His-tagged proteins. Ni-NTA beads were washed with 25 ml of lysis buffer lacking PMSF and protease inhibitor mixture and containing 20 mM imidazole. For elution, the same buffer contained 0.3 M imidazole. Elution buffer of GST-tagged proteins contained 15 mM reduced glutathione. Proteins were stored at −80 °C after buffer exchange containing 10% glycerol via a PD10 column. The protein isolation for transmembrane containing SNARE proteins used for reconstitution assays was purified as described before (32).

**Tandem Affinity Purification**—Cells expressing HOPS, Vps33 (from *vps16Δ*), or the Vps33–16 subcomplex were grown to an A_{600} of 12, pelleted, and then resuspended in TAP buffer (300 mM NaCl, 50 mM HEPES/NaOH, pH 7.5, 1.5 mM MgCl\textsubscript{2}, 1 mM DTT, 1 mM PMSF, 1× FY protease inhibitor mix (Sera), 10% glycerol). Lysis was performed in a Disrupter Genie three times for 10 min in the presence of glass beads. Beads were removed by centrifugation at 3,000 × g for 20 min at 4 °C. The supernatant was centrifuged for 1 h at 100,000 × g. Afterward the clear lysate was incubated with 300 μl of prewashed IgG beads (GE Healthcare) for 2 h at 4 °C. Beads were washed with 15 ml of TAP buffer without PMSF and FY and only 0.5 mM DTT. Proteins were eluted from the IgG beads by TEV protease treatment for 1 h at 16 °C. All yeast strains used in this study are listed in Table 2.

**Pull-down Experiments**—100 μg of GST-tagged proteins or GST alone were incubated with purified HOPS or HOPS subcomplexes (25–30 μg) for 2 h. Afterward, proteins were coupled to GSH beads for 1 h. Beads were washed four times (300 mM NaCl, 50 mM HEPES/KOH, pH 7.4, 1 mM DTT, 0.15% Igepal), before proteins were eluted by boiling. For analysis, 20% of eluate was used for Coomassie staining and 80% for Western blot against the calmodulin-binding peptide (CbP) tag. To test whether HOPS binds to assembled SNAREs, 30 μg of GST-Vam7SD was assembled with 80 μg of His-Vti1 and 80 μg of His-Vam3\Delta NTD for 1 h before they were incubated with the HOPS. For Vps16 interaction with SNAREs, vps33Δ cells expressing Vps16-TAP were frozen in liquid nitrogen and lysed in a Retsch MM301 mill for 30 s with a frequency of 29.9/s. Purification via IgG was done as described. 200 μl of Vps16-loaded IgG beads were used for pull-down assays and incubated with 300 μg of purified GST or Vam3 for 2 h at 4 °C. Afterward the beads were washed extensively with TAP buffer. Elution was performed by TEV protease cleavage for 1 h at 16 °C. For analysis the eluate was TCA-precipitated, and 80% was used for Western blot against the GST or the CbP tag.

**On-grid Nanogold Labeling and EM**—The purified HOPS complex was incubated with His-tagged Q5, Ykt6, Vam3-H_{abc}, Vti1, or Vam7 and cross-linked on a glycerol gradient. 4 μl of the respective samples were applied to a glow-discharged carbon-coated copper grid and incubated for 1 min. After washing with 20 μl of water, 4 μl of 5 mM Ni-NTA-Nanogold® (Nanoprobe) were applied to the grid and incubated for 30 s. Subsequently the grid was incubated twice for 1 min on 20 μl of 0.1 M imidazole, washed with 20 μl of deionized water, and stained with two 10-μl drops of freshly prepared 0.035 mg/ml uranyl formate solution. Images were collected using a Jeol JEM-1400 equipped with a LaB6 cathode operated at 120 kV. The micrographs were taken on a CMOS camera (TVIPS TemCam F-416, 4000 × 4000).

**V Hague Fusion Assay**—Vacuoles were purified from tester strains BJ3505 (pep^4A) and DKY6281 (pho8Δ3) and harvested from a Ficoll gradient as described (33). Isolated vacuoles were

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**TABLE 1**

| Plasmids used in this study | Name | Protein | Source |
|----------------------------|------|--------|--------|
| pET28a-Vam3               | His-Vam3 (1–316) | Langosch lab (TU Munich) |
| pETGEX Vam3aa127-end      | Vam3 (127–264)-GST | This study |
| pETGEX Vam3aa27-end       | Vam3 (27–264)-GST | This study |
| pETGEX Vam3-N-pep         | Vam3-N-peptide-GST | This study |
| pETGEX Vam3-NTD           | Vam3 (1–126)-GST | This study |
| pETGEX Vam3               | Vam3 (1–264)-GST | This study |
| pETGEX Vt6                | Vt6 (1–189)-GST | This study |
| pETGEX Ykt6               | Ykt6 (1–201)-GST | This study |
| pETGEX Vam3-H_{abc}       | Vam3 (27–126)-GST | This study |
| pET28a Ykt6               | His-Ykt6 (1–200) | Ref. 18 |
| pET28a Vt6                | His-Vt6 (1–189) | Ref. 18 |
| pET28a Vam3               | His-Vam3 (1–264) | Ref. 18 |
| pET28a-VAM3\DeltaNATMD    | His-Vam3 (145–264) | Ref. 18 |
| pGEX-2k-VAM7SD            | GST-Vam7 (190–316) | Ref. 18 |
| pGEX-KT-VAM3ATMD          | GST-Vam3 (1–260) | Ref. 18 |
| pET32c(+)/Vam3H_{abc}     | His-Vam3 (27–126) | This study |
| pET28a Vam7SD             | His-Vam7 (191–316) | Ref. 18 |
| pET32c(-Trx)His–3C-Vam3\DeltaNTD | This study |

**Plasmids used in this study**

| Name | Protein | Source |
|------|--------|--------|
| pET28a-Vam7               | His-Vam7 (1–316) | Langosch lab (TU Munich) |
| pETGEX Vam3aa127-end      | Vam3 (127–264)-GST | This study |
| pETGEX Vam3aa27-end       | Vam3 (27–264)-GST | This study |
| pETGEX Vam3-N-pep         | Vam3-N-peptide-GST | This study |
| pETGEX Vam3-NTD           | Vam3 (1–126)-GST | This study |
| pETGEX Vam3               | Vam3 (1–264)-GST | This study |
| pETGEX Vt6                | Vt6 (1–189)-GST | This study |
| pETGEX Ykt6               | Ykt6 (1–201)-GST | This study |
| pETGEX Vam3-H_{abc}       | Vam3 (27–126)-GST | This study |
| pET28a Ykt6               | His-Ykt6 (1–200) | Ref. 18 |
| pET28a Vt6                | His-Vt6 (1–189) | Ref. 18 |
| pET28a Vam3               | His-Vam3 (1–264) | Ref. 18 |
| pET28a-VAM3\DeltaNATMD    | His-Vam3 (145–264) | Ref. 18 |
| pGEX-2k-VAM7SD            | GST-Vam7 (190–316) | Ref. 18 |
| pGEX-KT-VAM3ATMD          | GST-Vam3 (1–260) | Ref. 18 |
| pET32c(+)/Vam3H_{abc}     | His-Vam3 (27–126) | This study |
| pET28a Vam7SD             | His-Vam7 (191–316) | Ref. 18 |
| pET32c(-Trx)His–3C-Vam3\DeltaNTD | This study |
diluted to 0.3 mg/ml in 0% Ficoll (10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol, 0.1 mM protease inhibitor mixture). Standard fusion reaction contained 3 μg of B) and DKY vacuoles incubated for 90 min at 26 °C in fusion reaction buffer (125 mM KCl, 5 mM MgCl₂, 20 mM sorbitol, 1 mM PIPES/KOH, pH 6.8) with 10 μM CoA, 0.01 μg of His-Sec18. Dependent on the different reactions, fusion reactions contained an ATP-regenerating system, 2 μM His-Vam7, or 150 mM HOPS. The colorimetric substrate p-nitrophenolphosphate of the phosphatase was added, and the activity was determined by absorbance measurement of the generated nitrophenol at 400 nm.

Reconstitution of Proteoliposomes—Reconstitution of SNARE proteins to liposomes was done as described before (16, 32, 34) with variations. Phosphoinositides were from Echelon, non-fluorescent lipids were from Avanti Polar lipids, and ergosterol with variations. Phosphoinositides were from Echelon, non-

### TABLE 2

**Yeast strains used in this study**

| Strains               | Genotype                              | Reference   |
|-----------------------|---------------------------------------|-------------|
| CUY 6682              | MATa pep4Δ::HIS3 prb1Δ1Δ6.6 lys2::208 trp1Δ101 ura3::52 gal2 VAM3::NIP1pr-VAM3 | This study  |
| CUY 6863              | MATa leu2-3::LEU2 met15Δ0 trp1Δ63 ara3Δ0 VPS33::TRP1 GAL1 prVPS41::TAP URA3 VPS39::KanMX6-GAL1 pr | This study  |
| CUY 291               | BY4372 X Y VPS41::TAP1-GAL1 pr VPS41::TAP1 URA3 VPS39::KanMX6-GAL1 pr | Ref. 8      |
| CUY 5920              | MATa leu2-3::LEU2 met15Δ0 trp1Δ63 ara3Δ0 VPS33::TRP1 GAL1 pr VPS16::KanMX6-GAL1 pr | This study  |
| CUY 1275              | BY4732 X Y VPS41::TAP1-GAL1 pr VPS41::TAP1 URA3 VPS39::KanMX6-GAL1 pr | This study  |
| CUY 1275              | BY4732 X Y VPS41::TAP1-GAL1 pr VPS41::TAP1 URA3 VPS39::KanMX6-GAL1 pr | This study  |
| CUY 8912              | BY4732 X Y VPS41::TAP1-GAL1 pr VPS41::TAP1 URA3 VPS39::KanMX6-GAL1 pr | This study  |
| CUY 8913              | BY4732 X Y VPS41::TAP1-GAL1 pr VPS41::TAP1 URA3 VPS39::KanMX6-GAL1 pr | This study  |

is in agreement with the ability of Q-SNAREs to block HOPS for membrane fusion (18, 24). We could also observe binding to Vam7 or just its N-terminal PX domain, although we found no interaction to the Vam3. This was surprising because Vam3 seemed to bind purified Vps33 (13), and HOPS can be isolated with Vam3 from cells (13, 22).

To map the putative binding site of Vam3 with HOPS, we generated constructs of Vam3 fragments fused to GST, including the putative N-peptide, the Habc, and the SNARE domain (Fig. 1A). We reasoned that previous interaction studies of HOPS with Vam3 failed because of the use of N-terminal GST fusions (18) and therefore tagged all constructs C-terminally with GST. We then used these fusion proteins as a bait in pull-down analyses with the HOPS complex, which was purified via the C-terminally tandem affinity purification (TAP)-tagged Vps41. Consistent with our previous work, HOPS did not bind to GST-Vam3 at all (Fig. 1B), as shown both by Coomassie staining and by blotting against the TAP-tagged Vps41 in the bottom panel. However, HOPS bound strongly to C-terminally tagged Vam3. Further mapping revealed that the Habc domain alone interacted with HOPS as efficient as the entire cytosolic segment of Vam3 (Fig. 1B, lane 2 versus lane 5). In contrast to isolated Vps33 (13), we did not detect any binding to the SNARE domain of Vam3. Also the putative N-peptide was not recognized by HOPS (Fig. 1A, lane 7), even if we fused it to the SNARE domain (not shown), in agreement with the lack of an N-peptide binding site in Vps33 (11, 12). We thus conclude that purified HOPS binds strongly to the Habc domain of Vam3.

We next asked which subunit within HOPS would be required for the binding and used a HOPS subcomplex consisting of Vps33-Vps16, which contains the SM protein Vps33 and can be purified from yeast (10). When incubated with the same Vam3 fragments, we observed again selective binding to the Habc domain as detected by antibody staining against the tag on Vps16 (Fig. 1C). However, isolated yeast Vps33, which nicely interacted with a Q-SNARE complex consisting of Vam3, Vam7, and Vt11 (18), did not bind to any single SNARE or the Habc domain under our experimental conditions (Fig. 1D).

Because the full-length version of Vam3 could interact with immobilized purified Vps16 (Fig. 1E), our data suggest that Vps16 binds directly to the Habc domain.

### Direct Localization of the SNARE Binding Site on the Isolated HOPS Complex—We then asked whether the same approach of C-terminal GST tagging would also reveal additional SNAREs as interaction partners of HOPS beyond the previously identified Vam7 and Vam7 PX domain (18, 25). By GST pulldown, we
observed strong interaction with Ykt6 but not Vti1 with purified HOPS (Fig. 2A), in agreement with observations on purified Vps33 (13). To observe the interaction site directly, we incubated purified HOPS with the Q-SNARE complex, the single SNAREs Ykt6, Vti1, Vam7, or the Habc domain of Vam3. The Q-SNARE complex consisted of the SNARE domains of Vam3 and Vam7, and the cytosolic domain of Vti1 and thus lacked the Habc domain of Vam3 (18). Ykt6, Vti1, Vam7, and Vam3 carried His6 tags, which allowed us to detect the SNAREs on the HOPS structure by electron microscopy after incubation.
with Ni-NTA-Nanogold. As shown in Fig. 2B, gold particles, visible as black dots, were observed in regions of the large head with assembled Q-SNAREs, Ykt6, or the Habc domain. In our assays, we did not observe a strong difference between the binding capacity of Q-SNARE complex and the QR complex (not shown), and both behaved similarly in interaction assays (18). This interaction with HOPS was observed best with the Q-SNARE complex, followed by the Habc domain and Ykt6 (Fig. 2C), which might suggest a preferred binding of the assembled SNARE complex over individual SNAREs. However, binding to Vti1 or Vam7 was not seen in this assay. These data nicely agree with our previous structural characterization because this is exactly the position within HOPS, where we previously localized the Vps33–Vps16 subcomplex (10). We should add that because of the variable appearance of the HOPS complex on the grid and the limited resolution, our data do not yet allow us to identify an exact region within the head region. Furthermore, HOPS partially disassembled upon SNARE binding, so only a

**FIGURE 2. Identification of SNARE-binding sites on isolated HOPS by electron microscopy.** A, HOPS interaction with Ykt6. GST-tagged SNAREs were incubated with HOPS and eluted by boiling. To the right, Vti1 with HOPS are shown in a separate experiment with Ykt6-GST as a control. Blots were decorated against the Cbp tag of Vps41. B, HOPS-SNARE-His complexes specifically labeled with Ni-NTA-Nanogold. Representative negatively stained single particles are shown for HOPS and HOPS incubated with His-tagged QS, Ykt6, or Vam3-Habc, respectively. For details see “Experimental Procedures.” C, for quantification the percentage of intact, labeled HOPS-SNARE particles was determined from 10 independent images. Significant differences of gold labeling between HOPS and the HOPS-SNARE complexes were estimated using t test. **p < 0.001; *p < 0.05. D, model of Vam3 Habc and Q-SNARE binding site on HOPS. HOPS structure is taken from Ref. 10. MW, molecular mass; QS, Q-SNARE.
Certain amount of HOPS bound to SNAREs as a stable complex that we could detect by Ni-NTA-Nanogold, suggesting that HOPS undergoes a conformational change when interacting with SNAREs. Importantly, binding of Vti1 was not observed under these conditions (Fig. 2C), in agreement with our pull-down analysis (Fig. 2A). The puzzling observation that Vam7 was not recovered on HOPS (Fig. 2C) will be discussed below.

A Role of the Habc Domain in HOPS Function during Fusion—Because HOPS binds both the Habc domain of Vam3 and the entire SNARE complex, we wondered whether binding of either partner would interfere with the binding of the other. We therefore preincubated HOPS with either the Habc domain or the assembled Q-SNAREs and then subjected it to a pull-down with the other binding partner (Fig. 3A). Surprisingly, the specific binding of HOPS to the Habc domain (lane 3 versus lane 5) was strongly reduced if the complex was preincubated with the Q-SNARE complex (lane 3 versus lane 4). However, when HOPS had been preincubated with the Vam3 Habc domain, interaction with the Q-SNARE complex was still possible (lane 6 versus lane 7). These data (Fig. 3, A and B) indicate that HOPS preferentially binds the Q-SNAREs, which prevents access of HOPS to interact with the Habc domain.

We next wondered whether we would find further support for this model by employing various fusion assays. Initially, we generated vacuole fusion strains, in which the endogenous copy of Vam3 was replaced by a Vam3/H9004 NTD variant, which lacks the entire Habc domain (see Fig. 1A), and observed reduced fusion rates (22). As before, we used vacuoles carrying a Vam3/H9004 truncation in our tester strains and measured fusion under different conditions. Vacuole fusion employs vacuoles from two different tester strains: one carries immature alkaline phosphatase proPho8 because of the deletion of the protease Pep4, and the other has Pep4 but lacks the phosphatase Pho8. Fusion is then detected by monitoring Pep4-driven Pho8 maturation and activity after luminal mixing. As before, we observed that vacuoles with Vam3ΔNTD had reduced fusion in the presence of...
ATP (Fig. 4A) (22, 23). This fusion deficiency could be rescued by titrating HOPS into the fusion reaction (Fig. 4B), suggesting that the Vam3 H$_{abc}$ domain is required to facilitate HOPS-assisted fusion.

Because this effect was rather small, we asked whether fusion conducted in the absence of ATP would reveal a stronger difference and clarify the role of the H$_{abc}$ domain. We thus monitored fusion in the absence of ATP by the addition of the Vam7 protein alone, which is a mobile SNARE (26, 27). In our initial analysis, we already observed a much stronger difference between wild-type fusion and fusion of vacuoles carrying Vam3NTD (Fig. 4, A and B). However, the addition of HOPS, which stimulated also wild-type fusion, strongly rescued the fusion defect of the Vam3NTD mutant vacuoles under these conditions (Fig. 4, A and B). We finally employed a proteoliposome fusion assay to compare the relative fusion efficiency. As

**FIGURE 4.** The Vam3 H$_{abc}$ domain is required for efficient fusion. A, fusion defect of vacuoles carrying Vam3 without the Vam3 H$_{abc}$ domain. Standard vacuole fusion reactions were incubated for 90 min at 26 °C either with ATP, 2 μM Vam7, or 2 μM Vam7 and 100 nM HOPS. Fusion reactions were developed as described under “Experimental Procedures.” Standard deviations correspond to three independent experiments. B, titration of HOPS into fusion reactions without ATP. Fusion reactions contained 2 μM Vam7 and the indicated amounts of HOPS or the corresponding buffer. Black and dark gray bars indicate fusion of WT vacuoles, and light gray and white bars indicate vacuoles with Vam3NTD. C, liposome fusion. Proteoliposomes carrying Vam3 or Vam3NTD, Vam7, Vti1, and Nyv1 were incubated in the presence or absence of HOPS for 30 min at 26 °C. Dequenching of the NBD-Rhodamin fluorophores was monitored as described (32). D, model of the HOPS function during SNARE-mediated fusion. The initial interaction of HOPS with Ypt7 is accompanied by Vam3 binding via its H$_{abc}$ domain. Additional binding sites in HOPS could collect Vam7 and Ykt6 or Nyv1. The assembling SNARE complex then interacts with Vps33 to promote bilayer mixing. preinc., preincubation; QS, Q-SNARE.
Interaction of HOPS with Vam3 Coordinates Fusion

in the vacuole fusion system, liposomes with Vam3ΔNTD were also less fusion competent (Fig. 4C). Because proteoliposome fusion depends on HOPS, this assay is only partially able to resolve the HOPS-dependent stimulation of fusion. In combination, our data provide further support that the interaction of HOPS with the Vam3 Habc domain promotes SNARE assembly and fusion, which is eventually dependent on Vps33 (Fig. 4D).

DISCUSSION

Our data provide evidence that the fusion-active HOPS complex has several SNARE binding sites, which are sequentially required to promote efficient membrane fusion (Fig. 4D). HOPS binds the Habc domain of Vam3, the R-SNARE Ykt6, and assembled SNAREs presumably via their SNARE domains. Consistent with this, we directly localized SNAREs to the large head of the HOPS complex by electron microscopy. Our data indicate that binding to the assembled SNAREs is stronger and preferred over binding to the Vam3 Habc domain. Even more surprisingly, once assembled SNAREs were incubated with HOPS, interaction with the Vam3 Habc domain was strongly reduced. This effect may reflect a conformational change within HOPS. It is also possible that the assembled SNAREs just block access to the Vam3 Habc binding site within HOPS, which otherwise would be available. Our data suggest a hand-over mechanism during fusion, in which HOPS is initially recruited to membranes via Ypt7-GTP, collects Vam3 and Vam7 via their N-terminal domains outside of their SNARE domain, and eventually promotes SNARE assembly via its bound Vps33 subunit.

Our analyses on the Vam3 interaction of HOPS differ in some small but critical points from previous work on Vps33 (13, 14). Although we observe similar interactions of HOPS with Ykt6 and assembled SNAREs (Ref. 18 and this work), we find that HOPS binds exclusively the Habc domain but not the SNARE motif of Vam3. We thus consider it unlikely that the latter is critical for the initial HOPS interaction with Vam3, although it is possible that the weak binding to the Vam3 SNARE domain (13) supports handover from Vam3 Habc binding to SNARE complex interactions of HOPS. Curiously, Munc18 and other SM proteins also preferentially bind the N-peptide or the closed conformation of syntaxin proteins (28–31), which would be analogous to our findings on HOPS. It is also possible that the initial access of the SNARE domains to Vps33 is hampered by the large size of the complex or neighboring subunits. Because we do not yet have insights into the precise localization of the SNARE binding sites within the HOPS complex, let alone the position of the SNAREs relative to HOPS during fusion, this issue will only be clarified by future studies. Nevertheless, we were able to identify binding sites for the Habc domain, the Q-SNARE complex, and Ykt6 in the large head, which agrees with the localization of Vps33 and Vps16 (10).

We do not yet know where exactly the Habc domain binds within HOPS. Isolated Vps33, either recombinant or from yeast, does not bind it (13) (Fig. 1D), although we observed a clear interaction with the purified Vps33-Vps16 complex (Fig. 1C) and localize the binding site to the same site on HOPS, where this subcomplex resides (Fig. 2B). However, HOPS subunits from mutant cells lacking vps16 were still bound to the Habc domain (13). These data remain circumstantial because HOPS partially disassembles in class C mutants (8), which might reveal hydrophobic segments of HOPS subunits that interact nonspecifically. Because Vam3 could directly interact with purified Vps16 (Fig. 1F), we believe that this subunit recognizes the Habc domain. Future experiments need to identify the precise binding sites to understand the molecular interplay of this domain with the HOPS complex.

Our data show that the interaction with the Habc domain facilitates HOPS function. When we omitted ATP from the fusion reaction and just provided Vam7, vacuoles carrying Vam3 without the Habc domain were fusion-inactive. Importantly, the Vam7-triggered fusion still requires HOPS and Ypt7 (27). Because HOPS binds to the Vam3 Habc domain in solution and on isolated vacuoles (13, 22), our data indicate that this binding to the region outside of the SNARE domain may position HOPS on the vacuole proximal to at least one of the central SNAREs (Fig. 4D). The defect in the transition from hemifusion to fusion as observed before (23) might be due to the poor positioning of Vps33 relative to the SNARE domains under these conditions.

As shown in Fig. 2C, we did not detect binding to Vam7, which we and others reported before (13, 18, 25). It is possible that our EM analysis was too restrictive to detect this interaction, although we cannot exclude a nonspecific interaction of the PX domain with HOPS. If correct, the binding of the PX domain of Vam7 to HOPS may have a similar function (18, 25). Indeed, if both the PX domain of Vam7 and the Habc domain were deleted, fusion was strongly diminished (18).

In summary, our data provide an extended view how HOPS via its binding sites promotes SNARE assembly. Because Vps33 within HOPS does have a strong preference for the assembled SNARE complex (Refs. 13 and 21 and this work) but does not recognize the Habc domain or the N-peptide, other subunits within HOPS facilitate the initial binding of HOPS to SNAREs. It is possible that HOPS may rebind the Habc domain after successful fusion and thus is prepared for another round of fusion. Future studies will need to clarify the details of this process.

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