Vps51p Links the VFT Complex to the SNARE Tlg1p*

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Intracellular membrane fusion requires the complex coordination of SNARE, rab/ypt, and rab effector function. In the yeast Saccharomyces cerevisiae, fusion of endosome-derived vesicles with the late Golgi depends on a cascade of protein-protein interactions that results in the recruitment to Golgi membranes of a conserved docking complex, VFT. This complex binds to Ypt6-GTP, which is necessary for its localization to the Golgi, and also to the SNARE Tlg1p. We show here that the VFT complex contains a fourth, previously uncharacterized, subunit, Vps51p (Ykr020w). Yeast cells lacking VPS51 have defects in vacuole morphology and recycling of the SNARE Sec1p to the plasma membrane, but still assemble a core VFT complex consisting of Vps52p, Vps53p, and Vps54p that localizes properly to the Golgi. Binding to Ypt6-GTP is a property of Vps52p. In contrast, binding to Tlg1p is mediated by a short sequence at the N terminus of Vps51p. Recent evidence suggests that components of a number of rab/ypt effector complexes share a common, distantly related helical coiled-coil motif. We show that each VFT subunit requires this coiled-coil motif for assembly into the complex.

Membrane traffic within eucaryotic cells requires an elaborate machinery to create transport carriers and to dock and fuse them to the correct membrane. Key components are the SNAREs, integral membrane proteins that, by the formation of helix bundles, draw membranes into close apposition (1–4). Earlier stages in the targeting process are regulated by peripheral membrane proteins, including the rab/ypt GTPases and their effectors, among which are a set of large protein complexes implicated in vesicle docking (5, 6).

Three such complexes, acting at different stages in the secretory pathway, have recently been shown to contain subunits with regions of weak but detectable similarity (for a review, see Ref. 6). These were first characterized in yeast, although each has an animal cell homologue. The exocyst, with eight subunits, is involved in the fusion of vesicles with other organelles, including the plasma membrane, and is required for the recycling of proteins from endosomes to the late Golgi (10, 11). It was originally described as a trimeric complex of Vps52p/Sac2p, Vps53p, and Vps54p/Luv1p. Like many subunits of the COG and exocyst complexes, each of these proteins contains a conserved short potential amphipathic helix. In addition, Vps52p shows homology to a domain of the exocyst component Sec3p (8).

The extent to which these complexes share structural and functional features remains to be determined. VFT is recruited to membranes by Ypt6p (11), and COG is an effector of Ypt1p (9). However, while some exocyst components are bound to vesicles via the GTPase Sec4p, others, notably Sec5p, are targeted to the plasma membrane by a separate mechanism that involves Rho1p and Cdc42p (7, 12, 13). Both VFT and COG complexes have been reported to bind SNAREs (9, 11), but this does not seem to be a feature of the exocyst. Despite some differences, the complexes may well share common features. It has been suggested that each may be based on a tetrameric organization, with the exocyst and COG complexes containing two tetramers (6).

We have previously shown that the VFT complex purified from yeast can bind independently to the GTP form of Ypt6p and to the N-terminal domain of the SNARE Tlg1p (11). These interactions suggest that VFT functions either to link endosome-derived vesicles containing Tlg1p with Golgi membranes marked by Ypt6p or to activate Tlg1p and associated SNAREs on the Golgi for subsequent complex formation and membrane fusion. To analyze the interactions in more detail, we attempted to map the binding site for Tlg1p and were surprised to find that none of the known VFT subunits, expressed alone, could bind this SNARE. Here we present further characterization of the VFT complex and show that although originally thought to be a trimer, it normally contains a fourth subunit, Vps51p. This subunit bears a short peptide sequence through which it interacts with Tlg1p. We also show that short amphipathic helices found in each of the four VFT components are necessary, although apparently not sufficient, for the assembly of the complex.

EXPERIMENTAL PROCEDURES

Yeast Strains, Microbiological Techniques, and Plasmids—The yeast strains used in this work are listed in Table I. Recombinant GST and His, fusions were expressed using pGEX6p2 (Amersham Biosciences) in MC1061 and pET30a (Novagen) in BL21DE3 cells, respectively. The following plasmids were used for yeast expression: YCplac111 and Ycplac33, ARS1/CEN4 vectors with the LEU2 and the URA3 marker respectively (14); YEpplac181, 2-μ vector with the LEU2 marker (14); YCplac111-VPS52-PtA, YCplac111-VPS54-PtA, YCplac33-VPS54-myc, YCplac111-VPS52-GFP, and YCplac111-VPS54-GFP (11).

Gene Deletion of the VPS51 Gene—The complete VPS51 open reading frame was deleted by generating two unique BamHI sites via PCR.

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The abbreviations used are: COG, conserved oligomeric; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; GST, glutathione S-transferase; TEV, tobacco etch virus; GFP, green fluorescent protein.

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48318
mediated mutagenesis, one just after the start codon and the other just before the stop codon, removing the DNA between start and stop codon and inserting a BamHI fragment containing the HIS3 gene. The vps51::HIS3 alleles, carrying 5' and 3'-flanking sequences, were excised and transformed into the SEY210 strain.

Construction of VPS51/52/53 Fusion Genes and Truncation Mutants—To construct a VPS22 truncation mutant lacking its N-terminal coiled-coil domain, a PCR fragment coding for its promoter and four N-terminal residues was ligated with a second PCR fragment coding for residues 123 to 641. Similarly, the VPS33 truncation mutant was constructed by ligation of a PCR fragment coding for its promoter and four N-terminal residues with a second fragment coding for residues 104–801. The VPS45 mutant was constructed by ligating a fragment coding the promoter and the 267 N-terminal residues with a second fragment coding for residues 312–889. In all three cases, the last C-terminal residue was followed by the tobacco etch virus (TEV) protease cleavage site and the protein A tag. To delete the C-terminal coiled-coil domain of VPS51, a PCR fragment the 120 N-terminal residues was ligated with a second fragment encoding the protein A tag. All constructs were cloned into the LEU2-based YCplac111 vector.

To construct VPS51-Pta or GFP C-terminal fusions, a BamHI site was introduced just before the VPS51 stop codon by PCR and a BamHI fragment coding for the TEV protease cleavage site followed by two IgG protein A fusions or A GFP was inserted. In all experiments, VPS51-Pta or GFP fusions were expressed from the VPS51 promoter on centromere vectors.

To express recombinant VPS51, VPS52, or VPS53, the full-length genes were cloned in-frame as PCR fragments into the pGEK6P2 vector. To construct the various VPS51 truncation mutants as GST fusions, PCR fragments encoding residues 1 to 47, 40, 34, 30, or 27 or 121 to 164, were cloned into pGEK6P2. To express the VPS52/53/54 coiled-coil domains as recombinant His6 fusions, the corresponding residues (1–139 for VPS52, 1–110 for VPS53, and 216–382 for VPS54) were cloned as PCR fragments into the pET11a vector.

Affinity Purification of PA Fusions and in Vitro Binding Experiments—Affinity purification of Vps52p-, Vps54p-, and Vps51p-Pta from yeast extracts was the same as described previously (11). Eight tryptic peptides were obtained from the Vps51p band in the Vps45-Pta pull-down (sequence coverage 50%, mean mass error 9.8 ppm). 26 peptides were obtained from the Vps53p band (sequence coverage 36%, mean error 37 ppm) and 15 peptides from Vps54p (sequence coverage 19%, mean error 12 ppm).

RESULTS

Identification and Characterization of a Fourth VFT Subunit—To search for novel VFT-interacting proteins we expressed either Vps52p or Vps54p tagged with protein A and purified the complex from yeast cells on IgG-Sepharose. Fig. 1 shows a Coomassie-stained gel of the eluted proteins. Analysis by mass spectrometry indicated that many of the minor bands were either irrelevant contaminants or breakdown products (data not shown), but one small protein (Ykr020w) was consistently present. As seen in Fig. 1, tagging of Ykr020w with protein A resulted in co-purification of the other VFT components, confirming the interaction (see also below).

We recently learned that Ykr020w is identical to the product of ALG9, since at that time its identity with VPS51 was unknown (18). These genetic studies indicate that Vps51p, like the other components of the VFT, is a group 1 SNARE glycoprotein important for efficient recycling of the exocytic SNARE Snc1p from the plasma membrane back via the Golgi and for proper vacuolar-mediated transport of vacuolar enzymes back to the plasma membrane.
Vps51p (although no obvious homologues exist in the genome) has functions similar to those of the other VFT subunits. Fig. 2B shows that the mutant has fragmented morphology (10, 11, 15). We therefore tested these phenotypes for vps51. Fig. 2A shows that the mutant has fragmented vacuoles and accumulates GFP-Snc1p within cells. Thus, Vps51p has functions similar to those of the other VFT subunits.

We next examined the localization of a functional GFP-tagged version of Vps51p, expressed from its own promoter in the absence of endogenous Vps51p. Fig. 2B shows that it had a punctate distribution characteristic of Golgi compartments in yeast, consistent with its association with the VFT complex on Golgi membranes.

Formation of a VFT Complex without Vps51p—Despite its apparent role as an integral part of the VFT complex, loss of Vps51p had a milder effect on growth than did deletion of any of the other components. Fig. 2C shows that although deletions of vps51, vps52, and vps54 were all temperature-sensitive for growth at 37°C, at lower temperatures vps51 cells grew much more strongly than vps52 or vps54 cells. This suggests either that a form of the VFT complex lacking a fourth subunit retains some function or that some other protein can substitute for Vps51p (although no obvious homologues exist in the S. cerevisiae genome).

That some of the properties of the VFT complex do not depend on Vps51p was borne out by examination of the intracellular distribution of GFP-tagged proteins. We have previously shown that the complex has a punctate Golgi distribution and that this is dependent on Ypt6p, without which the complex is dispersed throughout the cell (11). Significantly, GFP-tagged Vps52p, Vps53p, and Vps54p each retain their punctate distribution in a vps51 null strain (Fig. 3A). In contrast, deletion of VPS52 resulted in complete dispersal of Vps54-GFP (Fig. 3A), and deletion of VPS54 had a similar effect on Vps53-GFP (data not shown).

To examine whether a VFT complex could form in the absence of Vps51p, we purified Vps52-protein A from a vps51 deletion strain. Fig. 3B (left lane) shows that Vps53p and Vps54p could still be found associated with Vps52p, but that the level of the complex was substantially reduced relative to cells that contain also Vps51p (right lane). We could detect no new protein associated with the VFT complex in the vps51 null strain; because of the low yield of the complex, it is difficult to exclude the possibility of a minor band. We conclude that a trimeric core VFT complex consisting of Vps52p, Vps53p, and Vps54p can form and that this is sufficient for correct targeting to the Golgi, but that for full stability and function of the complex the presence of Vps51p is essential.

Vps51p Mediates the Binding of VFT to the SNARE Tlg1p via a Short N-terminal Peptide—We have previously shown that the VFT complex, purified from yeast cells, can directly bind to the central portion of the proteins which is much more divergent. In addition, the last 40 residues have the potential to form an amphipathic coiled-coil (Fig. 5A). Like a number of other
SNAREs, the N-terminal domain of Tlg1p is predicted to form a three-helix bundle (19), and thus one possible mode of binding would be for a helix from Vps51p to pack against this bundle. To map the domain responsible for binding, we expressed various portions of Vps51p as GST fusions and checked binding as for the full-length protein. As shown in Fig. 4B, the Tlg1p binding site mapped not to the C-terminal coiled-coil, but to the N terminus of Vps51p. More detailed mapping showed that residues 1–30, which are highly conserved, bound to Tlg1p as efficiently as did the full-length protein. In contrast, residues 1–27 were not sufficient for binding. Residues 15–30 are predicted to be helical, and if helical the whole region would have an amphipathic character (Fig. 4B). Binding of such a structure to Tlg1p might be sufficient to alter the conformation, and hence activity, of this SNARE (see “Discussion”).

The Coiled-coil Domains of the VFT Subunits Are Required for Complex Assembly—One intriguing recent finding is that a number of tethering complexes, including VFT, may be structurally related to each other (8). All VFT subunits contain short regions predicted to form amphipathic helices or coiled coils (see Fig. 5, A and B). The Vps53p and Vps54p N-terminal helices exhibit distant homology with similar domains found in components of the Sec34/35 complex and the exocyst. Furthermore, the Vps52p coiled-coil is related to that of the exocyst component Sec3p. The function of these short helices is not understood. Since coiled-coils tend to assemble with each other, and each VFT subunit contains such a domain, one possibility is that they form a four-helix bundle that is required for complex assembly. Alternatively, these coiled-coil domains might interact with other proteins involved in membrane docking and fusion, such as Rabs/Ypts or SNAREs. To address these questions we performed a systematic deletion of the coiled-coil domains found in the four VFT subunits (Fig. 5, A and C). We did not include in this analysis the second predicted coiled-coil found in the middle of Vps53p, because the amphipathic nature of this region is not conserved in related yeast species and also because, unlike the other helical domains, we were unable to express it in bacteria.

The short coiled-coil domains within the different VFT subunits are evidently important for function because their removal abolished the ability of Vps51p, Vps52p, Vps53p, and Vps54p to complement the growth of the respective null mutants at 37 °C (data not shown). The truncated proteins did, however, accumulate to the same levels in cells as the full-length proteins and thus were unlikely to be grossly misfolded, since this usually leads to rapid degradation (Fig. 5C). We next tested the ability of the protein A-tagged full-length and truncated Vps51p, Vps52p, and Vps53p to co-precipitate Myc-tagged Vps54p or of protein A-tagged full-length and truncated Vps54p to co-precipitate Myc-tagged Vps53p. As shown in Fig. 5C, in each case removal of the coiled-coil domain from a

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The coiled-coil domains within the VFT subunits are necessary but not sufficient for complex assembly. All yeast strains used in this experiment were co-expressing two proteins: Vps51p-Myc (lanes 1–6) or Vps53p-Myc (lanes 7 and 8) together with full-length (FL) protein A fusions of Vps51p (lane 1), Vps52p (lane 3), Vps53p (lane 5), and Vps54p (lane 7) or the respective truncation mutants lacking the predicted coiled-coil domains Vps51ΔCC (lacking residues 121–164, lane 2), Vps52ΔCC (lacking residues 5–122, lane 4), Vps53ΔCC (lacking residues 5–103, lane 6), and Vps54ΔCC (lacking residues 268–311, lane 8). Protein A fusions were purified and eluted with low pH (lanes 1 and 2) or TEV-protease digestion (lanes 3–8) as described previously (15). Eluates were analyzed by Western blot. Stars denote the position of the protein A fusions in the solubilized extracts (upper panel). The position of Vps51-Myc and Vps53-Myc in the extracts or eluates (lower panel) is indicated. Note that in lanes 5, 7, and 8 the Pia and Myc fusions co-migrate. All fusion genes were expressed under the control of their own promoters from centromeric plasmids. D, the coiled-coil domains of the VFT complex do not assemble in a four-helix bundle. Solubilized supernatants from bacteria expressing the predicted coiled-coil domains of Vps52p (residues 1–139; His652CC), Vps53p (residues 1–110; His653CC), and Vps54p (residues 216–382; His654CC) as His6 fusions were mixed with a bacterial supernatant containing either the Vps51p-predicted coiled-coil domain (residues 121–164; GST-51CC) as a GST fusion or GST alone. GST proteins were purified as described under “Experimental Procedures.” Samples of the supernatants of the His6 fusions (left panel) and the bound fractions (middle panel) were analyzed by Western blot using an anti-His6 antibody. Right panel, samples of the bound fractions stained with Coomassie Blue.

indicating that at least under these conditions spontaneous assembly of the four helices did not occur (Fig. 5D).

Overall, these data show that the coiled-coil domains mediate interactions that are necessary for the assembly and function of the VFT complex in vivo. However, it seems that interactions outside the helical regions are also required for the formation of a tetrameric structure.

Vps52p Binds to Ypt6-GTP—An important interaction of the VFT complex is with Ypt6p in its GTP-bound form on Golgi membranes (11). Since Golgi targeting of the complex occurs in the absence of Vps51p, the Ypt6p interaction must involve one of the other subunits. To identify which one, we expressed protein A-tagged versions of Vps52p in a vps53 mutant and Vps53p and Vps54p in a vps52 mutant. The absence of an essential VFT subunit in each case ensured that assembly of the complex could not occur, thus allowing the binding proper-
ties of the single proteins to be assessed. Cell extracts were then incubated with beads containing GST-Ypt6p. As shown in Fig. 6, Vps52p had the ability to bind to Ypt6p and retained a preference for the GTP form, whereas the other subunits showed only background binding. This confirms our previous conclusion that binding to Tlg1p and Ypt6p are independent properties of the complex, now revealed to be mediated by different subunits, Vps51p and Vps52p.

DISCUSSION

We have shown that the VFT complex is normally not trimERIC but tetrameric. The fourth subunit previously escaped attention, probably because of its small size and the fact that it lacks internal methionine or cysteine residues and thus cannot be labeled with 35S. This subunit, Vps51p, is unusual in that it is not absolutely required for assembly, localization, or even partial function of the VFT complex. Also, unlike the other subunits it is poorly conserved. Homologues can be found in eucaryotes.

Like the other subunits, Vps51p contains a coiled-coil motif. We have shown that each subunit requires its coiled-coil domain for assembly into the VFT complex, but that a Vps52p/Vps54p complex can form, albeit inefficiently, in the absence of Vps51p. Although indirect effects on the structures of the proteins cannot be ruled out, the simplest explanation is that the complex is stabilized by the formation of a four-helix bundle or in the absence of Vps51p a three-helix bundle. Since similar coiled-coil motifs are a feature of the COG and exocyst components, this may be a general feature of these complexes.

The coiled-coil motifs invite comparison with the SNAREs, which also form four-helix bundles. However, the isolated helical domains of the VFT components did not form bundles when tested under conditions in which SNAREs readily do so (21). There are also other significant differences. Whereas the coiled-coil "SNARE motif" is the most strongly conserved part of these molecules and easily recognizable, reflecting formation of stable bundles recognized by a universal dissociation machinery, the coiled-coils in the VFT complex are no more conserved than the rest of the proteins and seem to vary in length and exact position in different species. Furthermore, although some weak similarity between the subunits can be seen (Fig. 5B), its significance is unclear, and alignment of these yeast proteins with the mammalian COG and exocyst coiled-coil consensus is difficult. Thus, there may be additional interactions that drive assembly of these complexes, the coils adding a relatively nonspecific stabilizing influence. Indeed, we noticed that while complete removal of one subunit resulted in reduced yield of the others (10), removal of the coiled-coil from any one protein did not significantly destabilize either it or other components of the complex (Fig. 5C), perhaps because weak interactions between the proteins are still possible.

SNAREs undergo reversible assembly, and there is also evidence that the exocyst is assembled during the process of vesicle docking (7). Similarly, one could imagine that Vps51p binds to Tlg1p on vesicles, while the remaining VFT subunits are recruited to the Golgi membrane via the Ypt6p binding site of Vps52p and that docking is mediated by VFT assembly. We have not, however, been able to find circumstances under which the complex dissociates in vivo or under physiological conditions in vitro. Furthermore, the fact that vps51 mutants have a less severe growth phenotype than other VFT mutants implies that the complex has at least some activity that does not involve Vps51p.

Perhaps the most striking feature of Vps51p is its ability to bind, via a short N-terminal peptide, to the N-terminal domain of Tlg1p. Loss of this interaction may account for the observation that vps51 cells have severe trafficking defects even though they can still assemble and localize a core VFT complex. The N-terminal domain of Tlg1p is predicted to have a three-helix structure like that of syntaxin and thus might, as with the neuronal syntaxin 1a and yeast Syn1p, bind to and sequester the C-terminal SNARE motif (22, 23). In such a scenario the N terminus of Vps51p, which also has the potential to form an amphipathic helix, could compete for the same binding site as the SNARE motif and hence release this motif for interaction with other SNAREs. It has also been shown that interaction of a preformed complex of Tlg1p, Tlg2p, and Vt1p with Snc1p is markedly stimulated by peptides corresponding to the C-terminal portion of Snc1p (24), an effect that has been ascribed to subtle conformational changes in the SNARE helices (25). Further studies may reveal whether the N-terminal peptide of Vps51p has any effect on the assembly of Tlg1p-containing SNARE complexes and thus whether the VFT complex can directly mediate SNARE activity.

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Assembly of the VFT Complex

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