The SNARE Ykt6 mediates protein palmitoylation during an early stage of homotypic vacuole fusion

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The NSF homolog Sec18 initiates fusion of yeast vacuoles by disassembling *cis*-SNARE complexes during priming. Sec18 is also required for palmitoylation of the fusion factor Vac8, although the acylation machinery has not been identified. Here we show that the SNARE Ykt6 mediates Vac8 palmitoylation and acts during a novel subreaction of vacuole fusion. This subreaction is controlled by a Sec17-independent function of Sec18. Our data indicate that Ykt6 presents Pal-CoA via its N-terminal *longin* domain to Vac8, while transfer to Vac8's SH4 domain occurs spontaneously and not enzymatically. The conservation of Ykt6 and its localization to several organelles suggest that its acyltransferase activity may also be required in other intracellular fusion events.

Introduction

The terminal step in membrane trafficking, fusion of lipid bilayers, is closely linked to the action of SNAREs (Rothman, 1994; Chen and Scheller, 2001; Rizo and Südhof, 2002; Jahn *et al*, 2003). These proteins have been implicated as either fusion catalysts or docking factors (Mayer, 1999). Little attention has been given to a possible role of SNAREs in the coordination of the fusion reaction.

Five SNAREs have been identified that are required for yeast homotypic vacuole fusion: the t- or Q-SNAREs Vam3, Vam7 and Vti1 and the v/R-SNAREs Nyv1 and Ykt6 (Nichols *et al*, 1997; Ungermann and Wickner, 1998; Ungermann *et al*, 1998a, b, 1999) There is, however, a discrepancy. The analysis of SNARE complexes revealed that four coiled-coil domains form the core of the SNARE complex (Katz *et al*, 1998; Poirier *et al*, 1998; Sutton *et al*, 1998; Ungermann *et al*, 1999; Antonin *et al*, 2002). Thus, vacuole fusion requires one SNARE too many. In addition, the fusion of liposomes is efficient with the vacuolar t-SNAREs on one membrane and

Nyv1 on the other membrane, but fails with Ykt6 as the sole v-SNARE (Fukuda *et al*, 2000). Ykt6 functioned as a v-SNARE in liposome fusion only after its farnesyl anchor had been replaced by a transmembrane domain (McNew *et al*, 2000). We decided to address this problem in detail.

Ykt6 is the most conserved SNARE in eucaryotes (Filippini *et al*, 2001; Tochio *et al*, 2001). It is required for trafficking to and within the Golgi (Lupashin *et al*, 1997; McNew *et al*, 1997; Tsui and Banfield, 2000; Zhang and Hong, 2002), for endocytic trafficking to the vacuole (Dilcher *et al*, 2001; Lewis and Pelham, 2002; Kweon *et al*, 2003) and for vacuole fusion (Ungermann *et al*, 1999). Ykt6 has a wide tissue distribution in rat and appears to have a specialized function in neuronal cells (Catchpoole and Hong, 1999; Hasegawa *et al*, 2003). It belongs to the *longin* class of v-SNAREs with a highly conserved N-terminal domain that suggests functionality (Filipini *et al*, 2001). Recently, the N-terminal domain of Ykt6 has been crystallized. It has a profilin-like fold very similar to the N-terminus of Sec22, a v/R-SNARE of the early secretory pathway (Gonzalez *et al*, 2001; Tochio *et al*, 2001). The interaction of the N-terminus with the SNARE domain suggests that Ykt6 is regulated in an autoinhibitory manner (Tochio *et al*, 2001).

The study of homotypic vacuole fusion allows the precise dissection of a fusion reaction. The reaction occurs in an ordered cascade of Sec18/17-dependent priming, a tethering reaction that requires the Rab GTPase Ypt7 and the HOPS/Class C Vps complex, SNARE-dependent docking and fusion. Several additional factors, like Ca^{2+}/calmodulin and V0-ATPase, have been implicated in the last fusion step (reviewed by Mayer, 1999; Wickner, 2002). Vacuole fusion also requires palmitoylation of the fusion factor Vac8 (Veit *et al*, 2001; Wang *et al*, 2001).

Protein palmitoylation is a post-translational modification that is required for membrane fusion reactions (Glick and Rothman, 1987; Pfanner *et al*, 1990; Sakai *et al*, 2002), although the machinery has remained elusive. Myristoylated Vac8 has to be palmitoylated at three N-terminal cysteines to support vacuole fusion *in vitro* and *in vivo* (Wang *et al*, 1998, 2001; Schneiter *et al*, 2000; Veit *et al*, 2001). Vac8 palmitoylation occurs early in the fusion reaction and is Sec18-dependent, presumably to release Vac8 from an initial association with the *cis*-SNARE complex (Veit *et al*, 2001). It has been shown that palmitoylated Vac8 is required for fusion at a stage following *trans*-SNARE pairing (Wang *et al*, 2001). Recently, we presented evidence that acylation of Vac8 on vacuole membranes is enzyme-mediated, although the identity of the protein remained unknown (Veit *et al*, 2003).

We now show that Ykt6 has the acyltransferase activity for Vac8. The reaction is mediated by the N-terminal domain of Ykt6 that presents Pal-CoA to Vac8 and thus promotes acylation. On the vacuole, the Ykt6 activity requires an early function of Sec18 that is independent of its cofactors Sec17. The conservation of Ykt6 and Sec18 and their localization to

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several compartments within eucaryotic cells suggest that the same mechanism is responsible for fusion-dependent palmitoylation in higher eucaryotes.

Results

A requirement for Ykt6 in palmitoylation of Vac8

Sec18 disassembles *cis*-SNARE complexes and triggers Sec17 release from the vacuole (Ungermann *et al*, 1998a; Mayer, 1999). It is also necessary for Vac8 acylation, possibly by releasing *cis*-SNARE-associated Vac8 (Veit *et al*, 2001). To gain further insight into the mechanism by which Sec18 regulates Vac8's palmitoylation, we tested antibodies directed against components of the *cis*-SNARE complex for their ability to interfere with the acylation reaction. In contrast to antibodies to Sec18, those against Sec17 did not prevent palmitoylation (Figure 1A). However, the same antibodies to Sec17 block *cis*-SNARE complex disassembly and thus fusion at an early stage (Mayer *et al*, 1996; Ungermann *et al*, 1998a), suggesting that Vac8 acylation can occur independently of this reaction. This observation also indicates a function of Sec18 independent from Sec17. Second, of all antibodies to vacuolar SNARE proteins, only those to Ykt6 inhibited palmitoylation. Recombinant Ykt6 protein that contains a mutation at a conserved arginine (R71G) also prevented palmitoylation (Figure 1A) and interfered with the fusion reaction (Figure 1B). In contrast, the recombinant wild-type Ykt6 protein did not inhibit the fusion reaction (Figure 1C), suggesting that Ykt6-R71G competes against endogenous Ykt6 on vacuoles, thereby preventing fusion and palmitoylation. In agreement with the observation that

palmitoylation is independent of SNARE complex disassembly, we found that Ykt6-R71G did not influence ATP-dependent Sec17 release from vacuoles (Figure 1D), an established assay to monitor priming (Mayer *et al*, 1996). Interestingly, all our antibodies to vacuolar SNAREs blocked Sec17 release, probably by masking the SNARE complex. Moreover, we found that excess recombinant Sec17 blocks Vac8 acylation, an effect that can be neutralized by adding exogenous Sec18 (Figure 1E). It was previously shown that excess Sec17 blocks vacuole fusion, but permits SNARE complex disassembly and release of endogenous Sec17 (Wang *et al*, 2000). Our data suggest that excess recombinant Sec17 competes for Sec18 that is required for palmitoylation, but not Sec18 attached to the SNARE complex. These results add to the picture that Sec18 together with Ykt6 performs a novel function in palmitoylation unrelated to Sec17-dependent disassembly of *cis*-SNARE complexes.

The longin domain of Ykt6 is involved in acylation and Pal-CoA binding

A role of Ykt6 in the acylation reaction was unexpected. Ykt6 is the most conserved SNARE, and bound to membranes via

Figure 1 Ykt6 is implicated in a Sec17-independent early reaction. (**A**) Palmitoylation of Vac8 is specifically inhibited by antibodies to Ykt6 and Sec18. Fusion reactions (300 µl) containing 60 µg of vacuoles from BJ3505 were incubated for 10 min at 26°C in the presence of ATP (0.5 mM), cytosol (0.5 µg/µl), coenzyme A (CoA, 10 µM), [³H]-palmitate (150 µCi), and either purified IgGs to the indicated proteins or recombinant Ykt6-R71G (15 µM) or an equal volume of PS buffer (20 mM PIPES/KOH, 200 mM sorbitol). Vacuoles were then isolated by centrifugation (10 min, 12 000 *g*, 4°C), washed with 500 µl of PSK buffer (20 mM PIPES/KOH, pH 6.8, 200 mM sorbitol, 150 mM KCl) and resuspended in SDS sample buffer without 2-mercaptoethanol. Palmitoylated Vac8 was identified by SDS–PAGE and fluorography. Bands were quantified by laser densitometry (IPLab GelH, Scientific Image Processing 1.5, Signal Analytics). The positive control was set to 100%. (**B**) Interference with Ykt6 blocks fusion. Standard fusion reactions were incubated for 60 min at 26°C in the presence of Ykt6-R71G (15 µM) or anti-Ykt6 antibodies. The inhibiting amounts of protein were determined by titration (see Figure 1C). Background activity was subtracted from all measurements. Fusion activity in the absence of inhibitor was set to 100%. (**C**) Inhibition of fusion by Ykt6-R71G, but not Ykt6-wt. Indicated amounts of purified Ykt6 proteins were added to standard fusion reactions. Alkaline phosphatase activity was determined after 90 min at 26°C. (**D**) Sec17 release is unaffected by Ykt6-R71G. BJ3505 vacuoles were incubated for 10 min at 26°C in a 150 µl reaction containing 200 ng/ml Sec18 in the presence or absence of Ykt6-R71G (15 µM), anti-Ykt6 (inactive), anti-Ykt6, anti-Vam3, anti-Nyv1, anti-Sec17 or anti-Sec18. Then vacuoles were placed on ice, centrifuged (5 min, 16 000 *g*, 4°C) and washed twice with 500 µl PSK buffer. Vacuoles were analyzed by immunoblotting with anti-Sec17 and anti-Vam3 antibodies. (**E**) Excess recombinant Sec17 blocks palmitoylation. Palmitoylation reactions were performed as in (A). Recombinant Sec17 (7 µmol) or Sec18 (12.5 µmol) was added where indicated. At the same concentration, Sec17 inhibited the fusion reaction completely (data not shown), in agreement with previous studies (Wang *et al*, 2000).
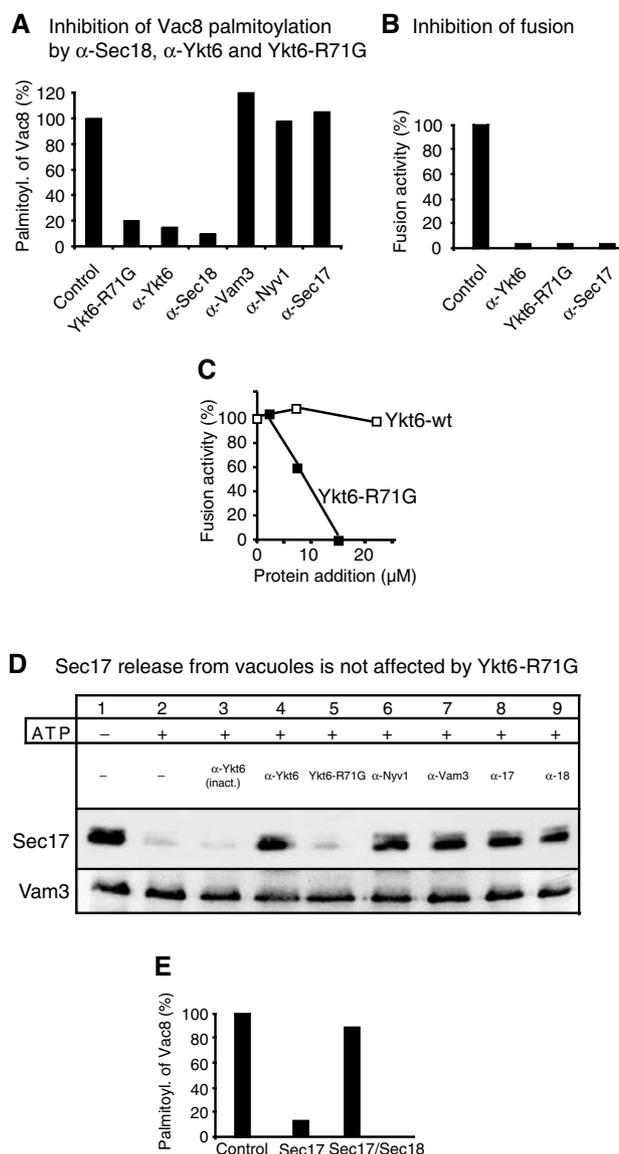
an isoprenoid anchor (Chen and Scheller, 2001; Tochio et al, 2001). It is required for multiple membrane fusion reactions along the secretory pathway (Lupashin et al, 1997; McNew et al, 1997; Dilcher et al, 2001) and to the yeast vacuole (Kweon et al, 2003). Because of its conserved extended N-terminal domain, Ykt6 has been classified as a member of the longin family (Filippini et al, 2001). This domain is characterized by a profilin-like fold that can interact with the C-terminal SNARE domain in an autoinhibitory manner (Tochio et al, 2001). To investigate whether this domain is involved in regulating palmitoylation of Vac8, we generated a recombinant Ykt6 N-terminal peptide (amino acids 1–140; Tochio et al, 2001) and antibodies to it. Strikingly, both were sufficient to inhibit vacuole fusion (Figure 2A) and Vac8 palmitoylation (Figure 2B), indicating a direct involvement of the N-terminal domain. We speculate that the Ykt6 N-terminal peptide inhibits vacuole fusion due to the lack of a C-terminal coiled-coil domain. Thereby, it might act as a competitor for components that are required for palmitoylation and fusion. Furthermore, a Psi-BLAST analysis revealed a short, but significant alignment of the N-terminal region of Ykt6 with the β-ketoacyl synthase subunit of fatty acid synthase (Figure 2C). This activity transfers C_2-fragments from malonyl-CoA to the growing acyl chain. The homology region shown does not include histidine 292 of the fatty acid synthase that has been implicated in catalysis (Witkowski

et al, 2002). We therefore speculated that the N-terminal domain of Ykt6 could be involved in fatty acid or CoA binding. Since we found that Vac8 and Ykt6 are proximal to each other on vacuoles (Figure 2D), we asked whether Ykt6 or its N-terminal domain could bind to palmitoyl-CoA or CoA, thus serving as an acceptor of activated palmitate during the acylation reaction. Full-length Ykt6 and the N-terminal fragment bind [³H]-Pal-CoA efficiently when compared to a control fatty acid binding protein, bovine serum albumin (BSA; Figure 2E). Furthermore, when we used [³H]-acetyl-CoA as a substrate, BSA did not bind, while Ykt6-R71G or just the N-terminus still did (Figure 2F). Ykt6-wt behaved like Ykt6-R71G in the binding assays (not shown). A control protein, Vti1-GST, did not bind to Pal-CoA, and heat inactivation of Ykt6 abolished binding completely (not shown). Preliminary experiments show that Ykt6 is also able to recognize palmitate specifically (not shown). This suggests that Ykt6 binds both the fatty acid and the CoA moiety, and therefore provides specificity for the transfer. Binding of Ykt6 to Pal-CoA or CoA is, however, not strong under these conditions since we required an excess of Ykt6 proteins to see efficient binding. These data suggest that the N-terminus of Ykt6 might itself support the acylation of Vac8 by directly interacting with the activated fatty acid. The N-terminus is

Figure 2 Essential function of the N-terminal domain of Ykt6. (**A**) Inhibition of fusion by the N-terminal domain. Fusion reactions were performed as described in Materials and methods in the presence of antibodies to Ykt6N or recombinant Ykt6N (22 μM) according to the titration (see the inset). (**B**) Inhibition of palmitoylation by N-terminal antibodies or recombinant Ykt6N. Palmitoylation in the absence or presence of Ykt6N (22 μM) or anti-Ykt6N was performed as in Figure 1A. (**C**) Sequence alignment of Ykt6 (accession number P36015) and human fatty acid synthase (P49327) as revealed by Psi-BLAST. The alignment of homologous regions is shown. Identical residues are in black and conserved ones in gray (32% identical, 49% conserved). (**D**) Ykt6 is proximal to its substrate Vac8 on vacuoles. Isolated BJ3505 vacuoles (60 μg) were incubated with 200 μM of the cleavable crosslinker DSP for 30 min on ice. Vacuoles were then reisolated, lysed in 10 μl 10% SDS, boiled for 5 min at 95°C and then detergent solubilized in 1 ml IP buffer (1% Triton X-100, 300 mM NaCl, 10 mM Tris/HCl, pH 7.4). Immunoprecipitation and analysis was carried out with protein A-coupled antibodies to Vac8 as described (Veit et al, 2001). Immunoblots were decorated with antibodies to Vti1 and Ykt6. (**E**, **F**) The N-terminal domain of Ykt6 binds to CoA. [³H]-Pal-CoA (1.7 fmol (0.1 μCi); American Radiolabeled Chemicals, Inc. (E)) or [³H]-acetyl-CoA (same concentration (F)) was incubated alone or together with 20 μg of the indicated protein in 500 μl PS buffer at room temperature for 10 min. Then, a piece of nitrocellulose (1 cm²; Protran, Schleicher and Schuell) was added to the reaction. After 30 min, the nitrocellulose was washed three times for 10 min in PSK buffer. The amount of [³H]-Pal-CoA/[³H]-acetyl-CoA attached to the nitrocellulose was determined by scintillation counting. Unspecific binding of Pal-CoA/acetyl-CoA to nitrocellulose (no protein) was subtracted and set to 0% (CoA: 0% = 30 cpm, 100% = 170 cpm; Pal-CoA: 0% = 2800 cpm, 100% = 5000 cpm). The signal obtained for BSA, which binds at least six acyl chains per protein, was divided by six in (E) and set to 100%. BSA was not able to bind to acetyl-CoA (F). (**G**) Growth kinetics. BJ Gal-Ykt6 (full-length) strains containing chromosomally integrated pRS406 plasmids encoding full-length Ykt6, a deletion mutant lacking amino acids 60–90 (Ykt6Δ60–90) or one lacking the N-terminus (Ykt6ΔN) were grown overnight in galactose-containing YP medium, and then diluted to an OD_{600} of 0.002 in fresh YP medium containing glucose in order to switch off the expression of Gal-Ykt6. The cultures were incubated at 30°C and OD_{600} was determined at the indicated time points.
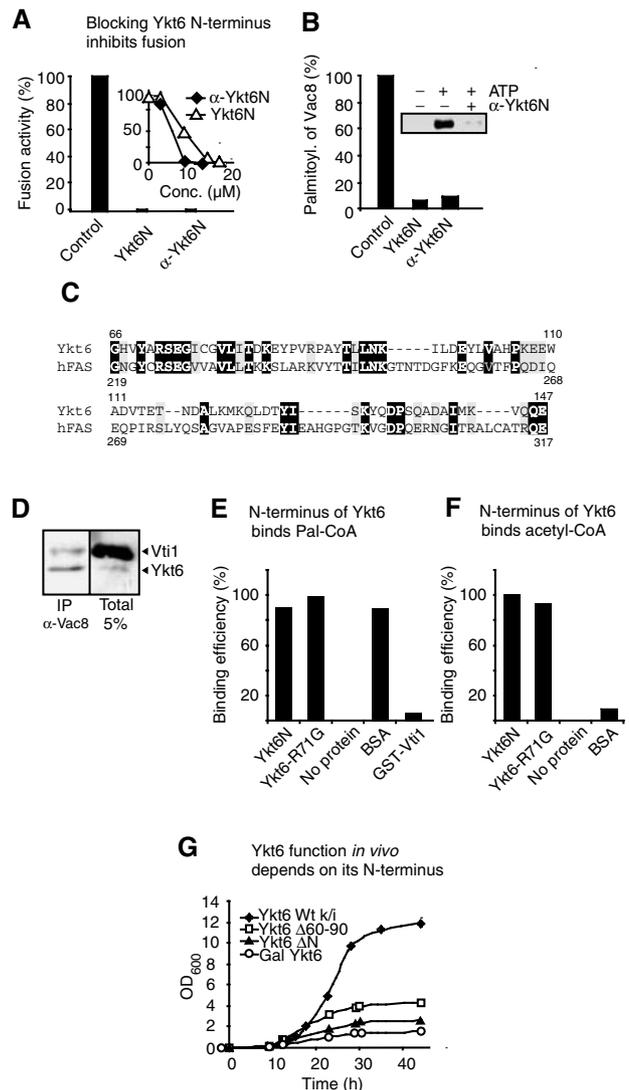
indeed important for Ykt6 function within yeast. Mutants lacking the N-terminal domain of Ykt6 or deleted in 30 amino acids of the conserved region (residues 60–90) are not viable (Figure 2G). Thus, our results point toward a new function of the N-terminal *longin* domain.

The N-terminus of Ykt6 is required early in fusion

Ykt6 is known to be involved in a docking-related stage of vacuole fusion (Ungermann *et al*, 1999). Our results indicate an unexpected early function of Ykt6. To address this seeming contradiction, we performed kinetic studies of the fusion reaction in order to determine the stage at which the N- and C-termini of Ykt6 function (Figure 3). The last step, at which an inhibitor acts, can be determined by adding the inhibitor at various time points to an ongoing fusion reaction and monitoring when resistance against the inhibitor is gained. The priming inhibitors anti-Sec17 and anti-Sec18 block fusion only within the first 10 min, whereas inhibitors of docking like anti-Vam3 do so until about 30 min (Mayer *et al*, 1996). The tethering inhibitor Gdi1, which extracts Ypt7, inhibits after priming, but before docking (not shown). An aliquot set on ice at each time point shows the progression of the fusion reaction until that time. This assay allows a distinction between different curves that represent stages of the fusion reaction. Due to variations in vacuole preparations, the kinetics of the reaction may change, but not the order of the curves. Antibodies to full-length Ykt6 block the reaction slightly before docking, as did recombinant Ykt6-R71G or just the C-terminal part (Figure 3A,B, data not shown), similar to the tethering inhibitor Gdi1 (not shown). In contrast, the N-terminal fragment of Ykt6 or antibodies to the Ykt6 N-terminus only inhibited when added during the first 15 min of the reaction, that is, during priming (Figure 3C, D).

This confirms an early function of the N-terminus and correlates with the kinetics of Vac8's palmitoylation (Veit *et al*, 2001). The C-terminal part of Ykt6 appears to function at tethering and not at docking (*trans*-SNARE pairing) like the other antibodies to vacuolar SNAREs (Ungermann *et al*, 1998a, 1999), a function that we are now exploring.

The vacuolar acyltransferase activity co-migrates with Ykt6

Our data are so far consistent with a role of both Ykt6 and Sec18 during palmitoylation. Moreover, the N-terminal domain of Ykt6 appears to have a direct influence on palmitoylation. To investigate how direct the involvement of both proteins is, we took advantage of our recent observations that palmitoylation activity can be recovered from a vacuolar detergent extract by using activated palmitate ($[^3H]$-Pal-CoA) as a substrate (Veit *et al*, 2003). Incubation of the detergent extract with recombinant Vac8, but not with a mutant lacking the N-terminal cysteines, results in efficient palmitoylation (Veit *et al*, 2003). To characterize the palmitoylation activity in more detail, we fractionated the extract in a glycerol gradient and analyzed the fractions for acylation activity (Figure 4). Palmitoylation efficiency of recombinant Vac8 protein peaked in fractions #2–3, at an estimated molecular weight of 15–40 kDa, and was suppressed by an antibody to Ykt6 (not shown). Ykt6 was recovered in the same fractions as the acylation activity, whereas Sec18 had its peak in fractions #4–6 at its molecular weight of 80 kDa. Thus, our data suggest that Sec18 is not required for palmitoylation. Ykt6 alone could mediate the reaction and not as part of a large protein complex.
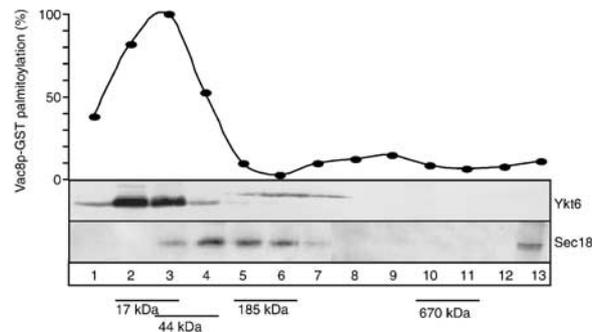
Figure 4 Acyltransferase activity *in vitro* corresponds to Ykt6. Sizing of the acyltransferase activity. Vacuoles (60 μg) were preincubated in PSK buffer for 10 min at 26°C without ATP, reisolated and solubilized in 300 μl 0.1% Triton X-100, 20 mM Tris/HCl (pH 7.4) and 150 mM NaCl for 10 min on ice. Unsolubilized material was removed by centrifugation (10 min, $14\,000 \times g$, 4°C). The cleared detergent extract was loaded on top of a 10.5 ml 10–34% continuous glycerol gradient in PSK buffer and centrifuged (SW41, 40 000 g, 4°C, 18 h). Equal fractions (750 μl) were collected from the top of the gradient. Aliquots of the fractionated extract (10 μl) were collected and analyzed. To determine PAT activity, 10 μl extract and recombinant myr-Vac8-GST were incubated for 30 min at 26°C in a 100 μl reaction in the presence of $[^3H]$-Pal-CoA (Pal-CoA) and then processed by SDS–PAGE of precipitated proteins. Fluorographs were quantified by laser densitometry. Aliquots of the same fraction were resolved on SDS–PAGE gels and blotted onto nitrocellulose to analyze the size of Sec18 and Ykt6 in the gradient. The distribution of Sec18 and Ykt6 is shown. For size determination, a control gradient with marker proteins was run in parallel. Sizes are indicated.

Figure 3 Ykt6 is involved in early and later steps of fusion. A 30 × scale fusion reaction was started in the presence of 0.5 mM ATP and 10 μM CoA at 26°C. Aliquots (30 μl) were removed at the indicated times, added to recombinant Ykt6-R71G, an N-terminal peptide of Ykt6 (Ykt6N) or antibodies to Ykt6, Ykt6N, Sec17, Sec18 or Vam3, and incubated at 26°C or placed on ice. Reactions were incubated for a total of 90 min before assaying for alkaline phosphatase activity. Inhibition by anti-Ykt6 (**A**), Ykt6-R71G (**B**), anti-Ykt6N (**C**) and Ykt6N (**D**).

Transfer of Pal-CoA to Vac8 in vitro depends on Ykt6

We directly tested this hypothesis by incubating recombinant Vac8-GST with purified Ykt6 proteins or Sec18 in the presence of [^{3}H]-Pal-CoA (Figure 5A). Indeed, addition of Ykt6-wt alone was sufficient for *in vitro* acylation of Vac8-GST (lane 2). In contrast, Sec18 had no acyltransferase activity (lane 3), and did not stimulate Ykt6-mediated acylation significantly (not shown). The N-terminal domain of Ykt6 alone, fused to GST, was sufficient for palmitoylation, while the N-terminus of Vti1 as a GST fusion protein was not. This implies that the acylation activity is confined to the N-terminus of Ykt6 as suggested by our deletion and antibody analysis (Figure

Figure 5 Ykt6 is sufficient for Vac8 palmitoylation. (**A**) *In vitro* reconstitution of the acyltransferase reaction depends on Ykt6. Indicated combinations of recombinant Vac8-GST (0.5 μM) and Ykt6-wt (2 μM) were incubated with 0.5 μM [^{3}H]-Pal-CoA for 60 min at 26°C in the buffer of the standard fusion reaction. For inactivation, Ykt6 was heated for 5 min at 95°C before being added to the assay. In lanes 5 and 6, Ykt6(1–120)-GST (Ykt6 N-term.) or GST-Vti1(1–110) (Vti1-N-term.) were used instead of Ykt6-wt. After the assay, proteins were precipitated by chloroform/methanol, dried, resuspended in SDS sample buffer, resolved on SDS–PAGE and analyzed by fluorography. (**B**) Kinetics of the *in vitro* acylation reaction. Reactions were performed as described in (A). Either purified Ykt6 or a vacuolar lysate (Figure 4A) was used as the enzyme source. Fluorograms were quantified by laser densitometry. Maximal palmitoylation observed in the reaction was set to 100%. (**C**) Determination of the stoichiometry of the reaction. Increasing amounts of Ykt6 were titrated against 1.5 μM Vac8. The reaction was performed and analyzed as described in (A). (**D**) Indicated amounts of Vac8 were titrated against 0.25 μM Ykt6. (**E**) Ykt6 stimulates acylation under reducing conditions. Acylation was performed as in (A). Where indicated, 1 mM DTT was added to the reaction. Ykt6-mediated palmitoylation in the absence of DTT was set as 100%.

1A,2G). The Ykt6-mediated acylation was specific, since heat inactivation of the protein abolished labeling of Vac8-GST. Thus, the N-terminal domain of Ykt6 is sufficient for acylation of Vac8, providing an unexpected connection between a reversible protein lipidation and SNARE function. Sec18 is not necessary for palmitoylation, but may provide an important regulatory function on the vacuole.

To characterize the mechanism by which Ykt6 palmitoylates Vac8, we measured stoichiometry and kinetics of the *in vitro* palmitoylation reaction. Acylation of endogenous Vac8 on vacuoles occurs within a few minutes, indicating that Ykt6 is positioned such that it acylates Vac8 immediately (LEP Dietrich, M Cristodero and C Ungermann, unpublished; see Figure 2D). A vacuolar detergent extract used as an enzyme source palmitoylates recombinant Vac8-GST, although the reaction requires 20–30 min until completion (Veit *et al*, 2003; Figure 5B). Consistently, Ykt6-mediated *in vitro* acylation of Vac8-GST has comparable kinetics (Figure 5B). To determine stoichiometry, we titrated Ykt6 into a reaction with a defined concentration of Vac8-GST (1.5 μM; Figure 5C). (Micromolar amounts of Vac8-GST are required to detect a palmitoylation signal, representing a technical limitation of the assay (our unpublished observations).) Interestingly, we observed that the reaction saturated at approximately equimolar concentrations of Ykt6 and Vac8, while limiting Ykt6 amounts were insufficient for *in vitro* acylation (Figure 5C). To confirm this, we did the converse experiment, and titrated Vac8-GST into the acylation reaction while keeping Ykt6 constant (Figure 5D). Indeed, at approximately equimolar amounts of Vac8 and Ykt6 acylation was most efficient, while increasing the Vac8 concentration did not result in more acylation. Accordingly, with a five-fold higher Ykt6 amount, approximately five-fold more Vac8 could be modified (not shown). This confirms that similar micromolar amounts of Ykt6 and Vac8 are required for efficient *in vitro* acylation. The ratio of Vac8 to Ykt6 and the slow kinetics make it unlikely that Ykt6 is enzymatically involved in Vac8 palmitolyation. Based on this and Ykt6's ability to bind Pal-CoA, we propose that Ykt6 serves acylation by specifically presenting activated palmitate to Vac8. How is the acylation itself mediated? A feature of most palmitoylated proteins is that *in vitro* they become acylated at the authentic cysteines independently of any enzymatic protein source (Duncan and Gilman, 1996; Bano *et al*, 1998; Veit, 2000; Bizzozero *et al*, 2001). In Figure 5E we show that the same is true for Vac8 if the reducing environment of the cytosol is simulated by adding 1 mM of the reducing agent DTT (lane 2), and is stronger than Ykt6-mediated acylation in the absence of DTT (lane 1). This indicates that in the presence of abundant activated palmitate (Pal-CoA), palmitoylation can occur spontaneously in the reducing atmosphere of the cytosol. However, within the cell, the pool of free Pal-CoA is too small (in the nanomolar range) to allow the reaction to take place (Faergeman and Knudsen, 1997). A possible mechanism for protein palmitoylation is that protein-bound Pal-CoA is specifically targeted to its substrate, followed by spontaneous thioesterification. Indeed, addition of Ykt6 to the reaction in the presence of DTT increases Vac8 acylation synergistically (lane 3), implying that Ykt6 is able to stimulate the transfer also under reducing conditions. Our data thus indicate that Ykt6 is the protein presenting Pal-CoA to Vac8. This model is very attractive since it would bring together the conflicting results

on the mechanism of protein acylation that were published over the last 20 years. Our model agrees with the assumption that thioesterification between palmitate and protein occurs spontaneously (Bano *et al*, 1998; Bizzozero *et al*, 2001; Qanbar and Bouvier, 2003). But it also demonstrates the need for a specific protein that enables and controls this reaction *in vivo*. It should be noted that none of the recently published palmitoyl transferases were demonstrated to act enzymatically (Lobo *et al*, 2002; Roth *et al*, 2002), and that Ykt6-mediated palmitoylation of Vac8 occurs with similar efficiency when we used the buffer conditions described by Lobo *et al* (not shown). In agreement with our results, similar amounts of PAT and substrate are required in all cases.

Ykt6-mediated acylation of Vac8 occurs in a novel subreaction of vacuole fusion

Finally, we directly tested the idea that Ykt6 and Sec18 participate together in a mechanism that is independent of *cis*-SNARE disassembly. When fusion reactions were incubated in the presence of antibodies to Sec18 to inhibit priming, then reisolated and resuspended in the presence of purified Sec18 protein to reverse the inhibition, reactions were still sensitive to Ykt6-R71G and to antibodies to Sec17, Vam3 and Vam2, a subunit of the HOPS/Class C Vps tethering complex (Price *et al*, 2000; Seals *et al*, 2000; Wurmser *et al*, 2000). In contrast, if recombinant Ykt6-R71G was applied in a first incubation in the presence of ATP and removed later by re-isolating the vacuoles, reactions were still sensitive to antibodies to Sec18 (Figure 6B). While remaining sensitive

to anti-Sec18, though, the reaction gained resistance to anti-Sec17. Resistance was also gained to anti-Vam2 and anti-Vam3, suggesting that a block in Ykt6 function allows tethering and docking, even though it prevents fusion of yeast vacuoles. This nicely fits the observation that a block in palmitoylation of Vac8 allows *trans*-SNARE pairing, but prevents fusion (Wang *et al*, 2001). Thus, acylation mediated by Sec18 and Ykt6 is triggered independently of the established priming reaction and marks a novel pathway in vacuole fusion.

Discussion

Our data reveal several surprising new insights into fusion-dependent palmitoylation. First, we have shown that Sec18 is linked to Ykt6-mediated palmitoylation in a mechanism independent of Sec17. This novel subreaction of vacuole fusion is initiated in parallel to the established priming and docking reaction. Second, we have identified and characterized the SNARE Ykt6 as the factor responsible for palmitoylation of Vac8. Our data suggest that the N-terminal domain of Ykt6 binds activated palmitate and promotes its transfer to Vac8. This reaction appears to proceed by an unexpected chaperone-like mechanism.

Our data suggest the following model (Figure 6C). At priming, Sec18 fulfills two functions: (a) together with Sec17 it causes *cis*-SNARE disassembly (Mayer *et al*, 1996; Ungermann *et al*, 1998a), and (b) together with Ykt6 it is required for palmitoylation of Vac8. Palmitoylated Vac8 then functions at a step following *trans*-SNARE complex formation (Wang *et al*, 2001). This observation extends previous pictures of the priming reaction (Wickner, 2002), now demonstrating that multiple, parallel reactions are initiated at this stage.

A function of Sec18 or NSF has previously been associated with its ability to bind to the SNARE complex (Wilson *et al*, 1992; Hanson *et al*, 1997; Hohl *et al*, 1998), and trigger its disassembly via ATP hydrolysis (Söllner *et al*, 1993). But there are data that suggest that Sec18/NSF has multiple roles: NSF, together with its cofactor α-SNAP, has a role distinct from ATP-dependent SNARE complex disassembly by catalyzing the association of GATE-16 with the v-SNARE GOS-28 in the re-formation of the postmitotic Golgi (Müller *et al*, 1999, 2002). NSF is also required with α-SNAP to activate the glutamate receptor at the postsynaptic membrane. The activation of the glutamate receptor is unrelated to the conventional role of NSF in fusion reactions (Nishimune *et al*, 1998; Osten *et al*, 1998; Song *et al*, 1998; Hanley *et al*, 2002). We do not yet know how Sec18p regulates palmitoylation, a focus that will be the subject of a separate manuscript (LEP Dietrich and C Ungermann, in preparation).

The identification of a novel function of Ykt6 in addition to its role as a SNARE is of major importance. It has been puzzling why vacuole fusion requires five SNAREs (Ungermann *et al*, 1999) even though the core complex contains only four helices (Katz *et al*, 1998; Poirier *et al*, 1998; Sutton *et al*, 1998; Ungermann *et al*, 1999; Antonin *et al*, 2002). Our data suggest that Ykt6 does not function as a classical SNARE in homotypic vacuole fusion. In fact, Ykt6 addition inhibits liposome–liposome fusion reconstituted with the vacuolar SNAREs Nyv1, Vam3, Vam7 and Vti1 (Fukuda *et al*, 2000). Moreover, Ykt6 does not function as
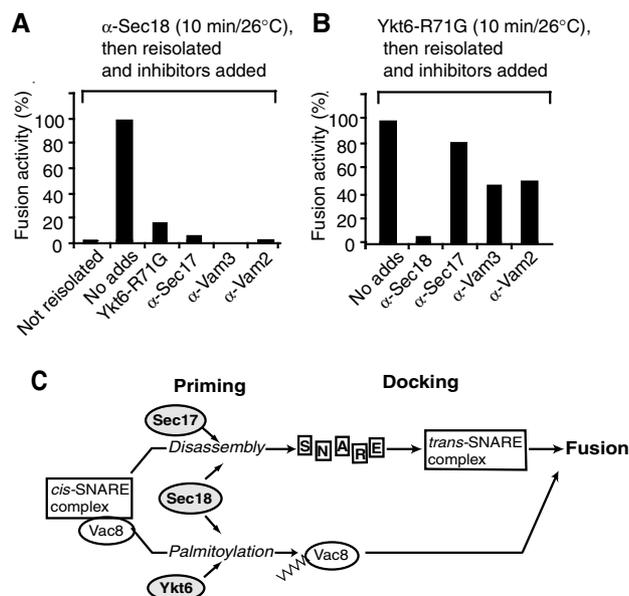
Figure 6 Role of Sec18 beyond Sec17-dependent priming. (**A**) Vacuoles were incubated with anti-Sec18 antibody in a standard fusion reaction with cytosol at 26°C. After 10 min, one sample (no reisolation) was set aside, and the others were diluted 10-fold in PSK buffer, centrifuged (5 min, 9000 *g*, 4°C) and resuspended in the original volume of reaction buffer with cytosol and 0.2 μg Sec18, containing the indicated inhibitors. After an additional 60 min at 26°C, alkaline phosphatase activity was determined. Background activity was subtracted. The activity of control samples (*no adds*) was set to 100%. (**B**) His-Ykt6 was added instead of anti-Sec18 to vacuoles, and incubations and reisolation were performed as before. (**C**) Working model of vacuole fusion. For details, see text.

v-SNARE in liposome-to-liposome fusion, unless its farnesyl anchor, a key feature of this protein, is replaced by a transmembrane domain (McNew *et al*, 2000). Indeed, we observed that Ykt6 is not found in the *trans*-SNARE complex (LaGrassa *et al*, in preparation). Hay and colleagues show that Ykt6 is more stable if purified from the cytosol than from membranes (Hasegawa *et al*, 2003), suggesting that Ykt6 adopts a closed conformation once released from vacuoles. This conformational switch might regulate Ykt6's acylation activity.

Ykt6 is required at the Golgi (Lupashin *et al*, 1997; McNew *et al*, 1997; Tsui and Banfield, 2000; Zhang and Hong, 2002), on late endosomes and on the vacuole (Ungermann *et al*, 1999; Lewis and Pelham, 2002; Hasegawa *et al*, 2003; Kweon *et al*, 2003). It is thus possible that the SNARE domain also targets Ykt6 to fusion complexes to fulfill its role as an acyltransferase. Recently, it was demonstrated that Ykt6 could replace Sec22 in ER to Golgi transport *in vivo* and *in vitro* (Liu and Barlowe, 2002). Sec22 and Ykt6 have similarly folded N-termini (Filippini *et al*, 2001; Gonzalez *et al*, 2001; Tochio *et al*, 2001), and thus have the ability to replace each other as SNAREs in the SNARE complex (Liu and Barlowe, 2002), or, potentially, as key players in palmitoylation. The conservation of Ykt6 and Sec18 across species makes it likely that palmitoylation of fusion factors is of general importance. Indeed, where analyzed, a role of Pal-CoA in fusion had been observed (Glick and Rothman, 1987; Pfanner *et al*, 1990; Haas and Wickner, 1996; Veit *et al*, 2001).

We have shown that Ykt6 is sufficient to promote *in vitro* palmitoylation of Vac8 via its N-terminus. This reaction requires roughly an equimolar ratio of Ykt6 and its substrate Vac8 to be efficient. Taking into account the reaction kinetics (Figure 5C,D), we suggest that Ykt6 presents Pal-CoA to Vac8 while transfer under the chosen conditions occurs spontaneously. These conditions favor the target cysteine sulfhydryl group to be reduced and be present as a deprotonated thiolate, which are the requirements for an efficient transfer (Bizzozero *et al*, 2000). This is in agreement with our analysis using the vacuolar detergent extract as an enzyme source. Modification occurs with similar kinetics, and acylation efficiency depends on the ratio of the added extract to the Vac8-GST substrate (Veit *et al*, 2003). An alternative explanation would be that Ykt6 alters the conformation of Vac8 to facilitate spontaneous acylation. Such a model does not take into account that Ykt6 binds Pal-CoA and has homology to the β-ketoacyl synthase. Moreover, we did not find any evidence for a complex between Ykt6 and Vac8 *in vitro* (not shown). We therefore consider this unlikely. Our proposal of a mechanism of directed palmitate transfer would be consistent with all previous reports on putative acyltransferases that transfer palmitate to cysteine residues. Among these are DHHC-CRD proteins that have been introduced as acyltransferases for C-terminal CCaax and CC sequences (Lobo *et al*, 2002; Roth *et al*, 2002). Both Erf2/Erf4 and Akr1 have been reconstituted *in vitro* with their respective substrate. However, in these studies, neither stoichiometry nor kinetics of the transferase reaction were assayed. Interestingly, as in our *in vitro* assay, similar protein concentrations were used to measure acyltransferases activity *in vitro* (Lobo *et al*, 2002; Roth *et al*, 2002). Importantly, the kinetics of the acylation reaction reported in other studies agree with our observations (Veit *et al*, 1998; Dunphy *et al*, 2000). Taking

these criteria into account, Ykt6 could also be named an acyltransferase.

During vacuole fusion, Ykt6 has two separable functions. The complete Ykt6 protein is required until tethering, possibly as a chaperone for other SNAREs, a function that we are exploring further. The N-terminal *longin* domain of Ykt6 acts as the acyltransferase for the fusion factor Vac8 at an early stage of the fusion reaction. This novel step requires Sec18 but not other components necessary for SNARE disassembly, tethering or *trans*-SNARE pairing. In summary, our data extend the function of SNAREs beyond that of fusion catalysts by demonstrating a role for a SNARE prior to the actual fusion in an essential modification reaction. A single ATPase, NSF/Sec18, controls both pathways.

Materials and methods

Yeast strains and recombinant proteins
The Gal-inducible BJ3505 and DKY6281 strains were generated by chromosomal integration of the Gal promotor in front of the YKT6 open reading frame via PCR (Longtine *et al*, 1998). Other yeast strains were as described (Haas and Wickner, 1996).

Polyclonal antibodies to Sec17, Sec18, Vam3, Vam2 and Ykt6 were generated as described (Haas and Wickner, 1996; Nichols *et al*, 1997; Ungermann *et al*, 1999; Price *et al*, 2000), and antibodies to the N-terminal 120 amino acids of Ykt6 were generated to a GST fusion protein. Ykt6-wt, Ykt6-R71G, Ykt6N, GST-Vti1, GST-Vti1(1– 110), His-Sec17 and His-Sec18 were purified as described (Ungermann *et al*, 1999; Tochio *et al*, 2001). Sequencing of His-Ykt6 uncovered a mutation (R71G) within the N-terminus (termed Ykt6-R71G). For the *in vitro* assay, a wild-type version of His-Ykt6 (Ykt6-wt) was generated. C-terminally tagged Vac8-GST or Ykt6 (1–120)-GST were overproduced from a pETGEX vector in *E. coli* cells, which, in the case of Vac8-GST, carried a plasmid encoding the human *N*-myristoyl transferase (Veit *et al*, 2003).

Vacuole purification and fusion assay
Vacuoles were purified as described (Mayer *et al*, 1996; Ungermann *et al*, 1998a). Standard fusion reactions (30 μl) containing 3 μg of each vacuole type were incubated at 26°C in reaction buffer (125 mM KCl, 5 mM MgCl$_2$, 20 mM PIPES/KOH, pH 6.8, 200 mM sorbitol), a protease inhibitor cocktail (Xu and Wickner, 1996), 10 μM CoA, an ATP-regenerating system and cytosol (Haas and Wickner, 1996). One unit of fusion activity is measured as 1 μmol *p*-nitrophenol phosphate hydrolyzed per min per μg BJ3505 at 30°C.

Palmitoylation assay
[^{3}H]-palmitate labeling of Vac8 on isolated vacuoles was performed as described (Veit *et al*, 2001). For *in vitro* acylation, Vac8-GST (2 μM) was incubated in a 100 μl reaction containing reaction buffer (20 mM PIPES/KOH, pH 6.8, 120 mM KCl, 0.015% Triton X-100), His-Ykt6 (2 μM) and [^{3}H]-Pal-CoA (4 μCi; 0.5 μM) for 60 min at 26°C. Proteins were precipitated by the addition of 300 μl of MeOH, 100 μl of chloroform and 400 μl of H$_2$O, vortexed and centrifuged for 5 min at room temperature. The aqueous phase was removed carefully and discarded. Then, 300 μl of MeOH was added, the sample was vortexed and centrifuged again. The protein pellet was dried briefly, resuspended in sample buffer without 2-mercaptoethanol, and analyzed by SDS–PAGE and fluorography.

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