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Swf1-dependent palmitoylation of the SNARE Tlg1 prevents its ubiquitination and degradation

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Protein palmitoylation is a post-translational modification that affects a great number of proteins. In most cases, the enzymes responsible for this modification have not been identified. Some proteins use palmitoylation to attach themselves to membranes; however, palmitoylation also occurs in transmembrane proteins, and the function of this palmitoylation is less clear. Here we identify Swf1, a member of the DHHC-CDR family of palmitoyltransferases, as the protein responsible for modifying the yeast SNAREs Snc1, Syn8 and Tlg1, at cysteine residues close to the cytoplasmic end of their single transmembrane domains (TMDs). In an *swf1*Δ mutant, Tlg1 is mis-sorted to the vacuole. This occurs because unpalmitoylated Tlg1 is recognised by the ubiquitin ligase Tul1, resulting in its targeting to the multivesicular body pathway. Our results suggest that one role of palmitoylation is to protect TMDs from the cellular quality control machinery, and that Swf1 may be the enzyme responsible for most, if not all, TMD-associated palmitoylation in yeast.

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Introduction

Many proteins are post-translationally modified by the addition of a palmitate molecule to a cysteine by thioesterification. This modification is better referred to as S-acylation because the cysteines can be modified by lipids of different chain lengths (Berthiaume and Resh, 1995; DeMar and Anderson, 1997). Several kinds of proteins have been found to be palmitoylated (Linder and Deschenes, 2004). Some hydrophilic proteins like Ras and G proteins make use of this modification to attach themselves to membranes, often in combination with prenyl groups. However, many transmembrane proteins are also modified by palmitate. Among these are the transferrin receptor (Omary and Trowbridge, 1981; Alvarez *et al*, 1990), the mannose 6-phosphate receptor (Schweizer *et al*, 1996), the human asialoglycoprotein receptor (Yik *et al*, 2002) and the calcium sensor synaptotagmin (Veit *et al*, 1996). The function of this kind of palmitoylation is less clear, but it has been shown to affect trafficking and function of several proteins (Bijlmakers and Marsh, 2003). Modification of transmembrane proteins occurs in the cytoplasmic domain, often near or within a transmembrane domain (TMD); there are no clear sequence requirements and neither the cytoplasmic nor the TMD sequences appear to have an influence (Yik and Weigel, 2002). However, adding a cysteine to a nonpalmitoylated protein does not necessarily result in palmitoylation (Ponimaskin and Schmidt, 1998).

Although palmitoylation of proteins has been known for many years, only a few palmitoyltransferases have been described and an enzyme responsible for modifying TMDs has not previously been identified. In the yeast *Saccharomyces cerevisiae*, Akr1 has been reported to modify Yck2 type I casein kinase, a hydrophilic protein (Roth *et al*, 2002), and Erf2, in combination with an additional subunit, Erf4, modifies Ras2 (Jung *et al*, 1995; Bartels *et al*, 1999; Lobo *et al*, 2002). In mammalian cells, the γ2 subunit of the $GABA_A$ receptor was found to be palmitoylated by GODZ (Keller *et al*, 2004). Very recently, Huntingtin interacting protein 14 (HIP14) was shown to have palmitoyltransferase activity and, upon overexpression, to transform cells to form aggressive tumours (Ducker *et al*, 2004). HIP14 and other related proteins modify the cytosolic neuronal protein PSD-95 (Fukata *et al*, 2004; Huang *et al*, 2004). All these proteins belong to a family of proteins that share a 50-residue-long DHCC cysteine-rich domain (CRD), which is a variant of the zinc-finger domain C2H2 (Putilina *et al*, 1999). Members of this family have been suggested to have a general role in palmitoylation (Roth *et al*, 2002). There are at least 23 distinct family members in the human genome and seven in yeast (Linder and Deschenes, 2004), which presumably differ in their substrate specificity, intracellular location or expression patterns. In addition, a protein lacking the DHCC-CRD, Ykt6, has recently been reported to possess palmitoyltransferase activity towards both Vac8 and itself (Dietrich *et al*, 2004; Fukasawa *et al*, 2004).

Members of the SNARE family of membrane fusion proteins (Sollner *et al*, 1993; Rothman, 1994) are known to be substrates for palmitoylation. Some of these lack a TMD and are anchored either by multiple palmitate residues, as in the case of SNAP-25 (Veit *et al*, 1996), or, for Ykt6, by a combination of prenylation and palmitoylation (Fukasawa *et al*, 2004). However, others contain TMDs with adjacent cysteine residues that could, in principle, be palmitoylated. In one case, that of the yeast Snc proteins, palmitoylation has been demonstrated. This modification occurs soon after synthesis, while the protein is still in the ER, and is of unknown function (Couve *et al*, 1995). Interestingly, viral glycoproteins that mediate membrane fusion are also often palmitoylated, and although this is not always necessary for function, in at least one case, that of Rous sarcoma virus, loss of palmitoylation causes the protein to be rapidly degraded in lysosomes (Ochsenbauer-Jambor *et al*, 2001).

Swf1 is a member of the *S. cerevisiae* DHCC-CRD family, and an *swf1* null mutant shows synthetic lethal interactions

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with many mutants that influence membrane recycling through endosomes and the Golgi apparatus, including Tlg1, Snc2, Ric1, Ypt6 and many others (Tong *et al*, 2004). Here we show that Swf1 is required for the modification not only of Snc1, but also of the endosomal SNAREs Tlg1 and Syn8. The most notable effects of Swf1 are observed for Tlg1, a SNARE involved in the recycling of proteins from endosomes to Golgi (Holthuis *et al*, 1998a, b; Lewis *et al*, 2000). Lack of Tlg1 palmitoylation results in its entry into multivesicular bodies (MVBs) and ultimately to its degradation in the vacuolar compartment. This occurs because nonacylated Tlg1 is recognised by the ubiquitin ligase Tul1 (Reggiori and Pelham, 2002) and becomes ubiquitinated, a signal for entry into MVBs (Katzmann *et al*, 2001; Reggiori and Pelham, 2001).

Results

SWF1 is required for the palmitoylation of Snc1

Snc1, the yeast homologue of synaptobrevin, is involved in vesicle docking and fusion with the plasma membrane. Intracellular traffic of this protein has been extensively studied and it is known to cycle between the plasma membrane, endosomes and the Golgi apparatus (Lewis *et al*, 2000). This protein and its very close homologue Snc2 are known to be post-translationally modified by the addition of a palmitate (Couve *et al*, 1995). In order to identify the enzyme(s) responsible, we examined the modification of Snc1 in wild-type (WT) cells and in mutants lacking each of the seven yeast members of the DHCC-CDR family of putative palmitoyltransferases. Under carefully controlled conditions (see Materials and methods section), palmitoylation of Snc1 can be detected by a reduction in electrophoretic mobility of the protein on polyacrylamide gels. Figure 1A shows that Myc-tagged Snc1 migrated slightly faster when expressed in the *swf1*Δ strain, compared to WT cells and all the other mutants. Identical results were obtained with myc-tagged Snc2 (data not shown).

To demonstrate that the shift was due to a change in the palmitoylation status of Snc1, we treated the samples with either 1 M hydroxylamine, pH 7.4, which selectively removes thioester-linked palmitates (Chamberlain and Burgoyne, 1998) or 1 M Tris–HCl, pH 7.4, as a control. After this treatment, the difference in mobility was abolished, and hydroxylamine-treated Snc1 expressed in a WT strain comigrated with Snc1 expressed in an *swf1*Δ mutant (Figure 1B).

Snc1 has only one cysteine in its sequence, in position 95, and therefore that is the only site susceptible to S-acylation. A mutant version of Snc1 in which cysteine 95 was mutated to alanine (Snc1ΔC) shows the same shift towards lower molecular weight that is observed for the nonmutated Snc1 expressed in an *swf1*Δ mutant, confirming that the shift reflects modification of the cysteine (Figure 1B).

It has been suggested that nonpalmitoylated Snc1 is less stable than the modified form (Couve *et al*, 1995). We did observe a slight reduction in the amount of Myc-tagged Snc1 in an *swf1*Δ mutant compared to WT cells. However, this does not seem to be due specifically to the lack of modification of Snc1, since we did not observe any reproducible reduction in the levels of Snc1ΔC in a WT strain. The effect may be an indirect one caused by the loss of Swf1-dependent modification of some other substrate(s). This is not inconsistent with the observations of Couve *et al*, since the unmodified Snc1 that they observed resulted from massive overexpression of the protein, which presumably saturates the transferase and would be expected to affect other substrates by competition.

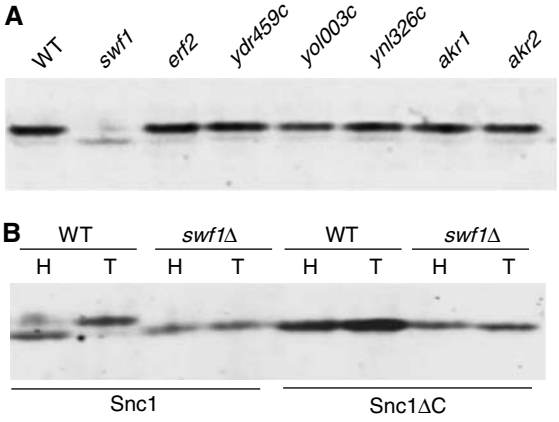

Figure 1 Palmitoylation of Snc1 requires Swf1. (**A**) Immunoblot of myc-tagged Snc1 expressed in the indicated strains. (**B**) Extracts of cells expressing myc-tagged Snc1 or the Cys–Ala mutant (Snc1ΔC) were treated with hydoxylamine (H) or, as a control, Tris (T) before gel electrophoresis and immunoblotting. Equal amounts of total protein were loaded in each lane in both (A) and (B), as confirmed by staining of the blot.

Yeast SNAREs		Location
Snc1	lkmkmclalviiillvviivpiavhfsr	PM/endo
Snc2	lkmrmclflvviillvviivpivvhfs	PM/endo
Sso1	nkirclwlivfaiivvvvvvvvvpavvktr	PM
Sso2	nkirclliicfiifaivvvvvvvpsvvetrk	PM
Tlg1	kyddcccglllivvlivllvlafia	TGN/endo
Syn8	dngncviilvlivvllllllvl	Endo
Tlg2	qkckvillltlcvialfffvmlkphgggsg..	TGN/endo
Vam3	kccgkvtliiiivvcmvvllavls	Vacuole
Human SNAREs		
VAMP1	kncckmmimlgaiccaiivvvivskyr	PM/endo
VAMP2	knlkmmiilgvicaiiliiiivyfss	PM/endo
VAMP3	kncckmwaigitvlvifiiiiivwvvss	Endo
VAMP4	ckikaimalvaaillliviilivmkyrt	TGN
VAMP5	iryricvglvvvgvlliilivllvvflpqs..	PM
VAMP8	knvkmivlicvivfiiilfivlfatgafs	Endo
syn1A	rrkkimiiiccvilgiviastvggifa	PM
syn4	rkkkvliaicvsitvvllaviigvtvvg	PM
syn6	drrqwccaiailfavllvvlilflvl	TGN
syn8	ksascgmimvillllvaivvvavwptn	Endosomes
syn10	drrqwccaiavlvgvlllvlillfsl	TGN

Figure 2 TMD sequences of yeast and human SNAREs. Cysteine residues are highlighted, and the hydrophobic TMD sequences underlined. The major locations of the proteins are indicated: PM, plasma membrane; endo, endosomes.

Swf1 modifies other SNARES

Having established an assay capable of detecting palmitoylation, we searched for additional substrates of Swf1. We focused on other SNAREs with an endosome/Golgi location, several of which have cysteines near or within their TMDs (Figure 2).

Syn8 is an endosomal SNARE, homologous to mammalian syntaxin 8 (Lewis and Pelham, 2002). Western blot analysis

of WT or *swf1*Δ cells probed with anti-Syn8 antibody showed increased mobility of the protein in the *swf1*Δ mutant. This difference was abolished by treatment of the protein extract from the WT strain with 1 M hydroxylamine (Figure 3A). Analysis of all seven members of the DHCC-CDR family showed that only Swf1 contributes to the modification of Syn8 (Figure 3B).

Next, we analysed Tlg1 (Holthuis *et al*, 1998a), a SNARE whose function overlaps that of Syn8. Again, there was a small but reproducible increase in the mobility of the protein from *swf1*Δ cells compared to WT cells, which was abolished by hydroxylamine treatment (Figure 3C).

As the mobility difference was small, we sought other ways to test the modification of Tlg1. Attempts to label the protein *in vivo* with [^{3}H]palmitate were unsuccessful, possibly because the palmitate does not turn over and the rate of synthesis of Tlg1 is relatively low. As an alternative, we used a maleimide derivative (Biotin-BMCC) that biotinylates free cysteine residues, allowing unpalmitoylated Tlg1 to be purified on streptavidin beads and detected by immunoblotting. This is possible because Tlg1 contains only two cysteines, which are the potential sites of palmitoylation. Figure 3D shows that, in *swf1* mutant cells, but not in WT cells, Tlg1 reacted well with this reagent, indicating free cysteines. These became inaccessible when the *swf1* cells were transformed with a plasmid expressing Swf1, but not with a version of Swf1 in which the cysteine residue of the DHHC sequence, known to be crucial for palmitoyltransferase activity (Lobo *et al*, 2002; Roth *et al*, 2002), was mutated to alanine. The faint bands detected at 38 and 45 kDa are discussed below.

In a complementary experiment, we treated cell extracts first with *N*-ethylmaleimide to block free cysteines, then used hydroxylamine treatment to remove palmitate, and finally the biotinylation reagent to detect the exposed cysteines. This is an established assay for palmitoylation (Drisdel and Green, 2004; Politis *et al*, 2005), and, as expected, gave a strong signal only when WT Swf1 was present (Figure 3D).

We conclude that both Syn8 and Tlg1 have the hallmarks of palmitoylation, and depend on Swf1 for their modification.

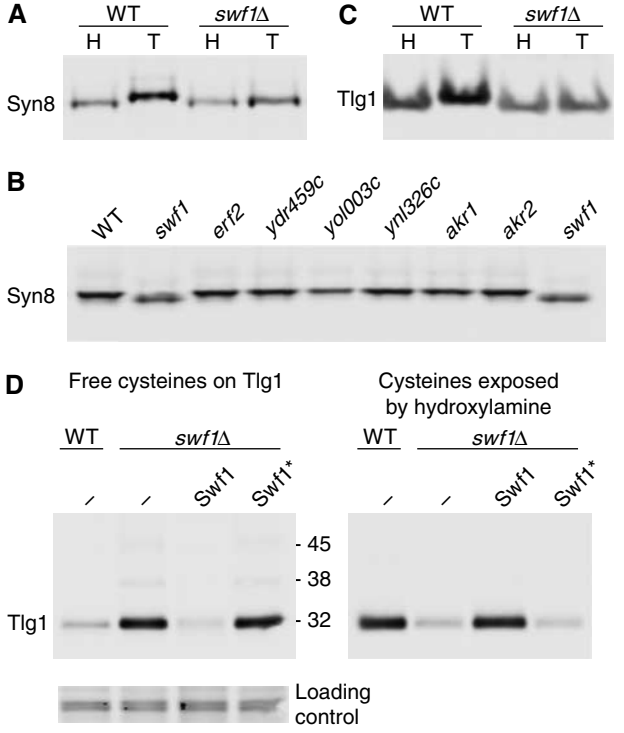

Figure 3 Swf1-dependent modification of Syn8 and Tlg1. (**A**) Extracts were treated with hydroxylamine (H) or Tris (T) as in Figure 1B, and immunoblotted using anti-Syn8 antiserum. (**B**) Syn8 mobility was compared in the indicated deletion mutants by immunoblotting. (**C**) Tlg1 was analysed as in panel A, using anti-Tlg1 antiserum. (**D**) The indicated strains were transformed with an empty vector (−), or a plasmid expressing either C-terminally GFP-tagged WT Swf1 or the equivalent DHHC mutant (Swf1*). In the left panel, extracts were treated with the cysteine-specific biotinylation reagents, and biotinylated proteins recovered with streptavidin beads before immunoblotting. In the right panel, samples were treated sequentially with *N*-ethylmaleimide to block free cysteines, then with hydroxylamine to remove palmitate, then with the biotinylation reagent. The loading control represents an example of a cysteine-containing protein detected by probing the same blot with fluorescent streptavidin, to demonstrate the reproducible recovery of biotinylated protein. Controls in which biotinylation was omitted resulted in no detectable Tlg1 being recovered from the streptavidin beads.

*GFP-Tlg1 is mislocalised and degraded in swf1*Δ *cells*

Despite their lack of palmitoylation, we found no evidence for substantial mis-sorting of either Snc1 or Syn8 in *swf1*Δ cells, as judged by examination of GFP-tagged proteins (not shown). However, the effects on Tlg1 were more dramatic. This SNARE is normally localised to the TGN/endosomal compartment, and, when expressed as a GFP-fusion protein, it showed the dotty distribution characteristic of this compartment in yeast. A fraction of the GFP-tagged protein reached the vacuole, where it could be seen on the outer vacuolar membrane (Figure 4A). In contrast, in *swf1*Δ cells, the fluorescence was mostly inside the vacuole (Figure 4A). Since Tlg1 was tagged with GFP at its amino-terminus, which is normally cytoplasmic, the appearance of GFP inside the vacuole can only occur if the protein enters the internal vesicles of MVBs, which are formed by invagination of the endosomal membrane. Following fusion of the MVB with the vacuole, these vesicles are delivered into the vacuole for degradation. Since GFP itself is resistant to proteolysis, whereas the attached Tlg1 protein is not, free GFP accumulates in the vacuole. In confirmation of this, Western blotting revealed an increase in the amount of free GFP in the *swf1*Δ background (Figure 4B). Strains lacking other members of the DHHC-CRD family showed much less vacuolar proteolysis of GFP-Tlg1 (Figure 4C).

To test whether the proteolysis of Tlg1 was caused by its lack of palmitoylation, or by some other effect of the *swf1*Δ mutation, we mutated the two adjacent cysteine residues located at the cytosolic end of the Tlg1 TMD. Mutation of both these residues to serine (construct Tlg1M2) resulted in delivery of Tlg1 to the vacuole interior in WT cells (Figure 4A and B). A single mutation of either cysteine was sufficient to trigger entry into the vacuole (Figure 4A, Tlg1M3 and Tlg1M4).

Palmitoylation of Tlg1 prevents recognition by Tul1

Degradation in the vacuole is normally associated with ubiquitination, which signals entry into MVBs. Indeed, when GFP-Tlg1 was concentrated by immunoprecipitation, we could detect faint bands in *swf1*Δ cells that were larger

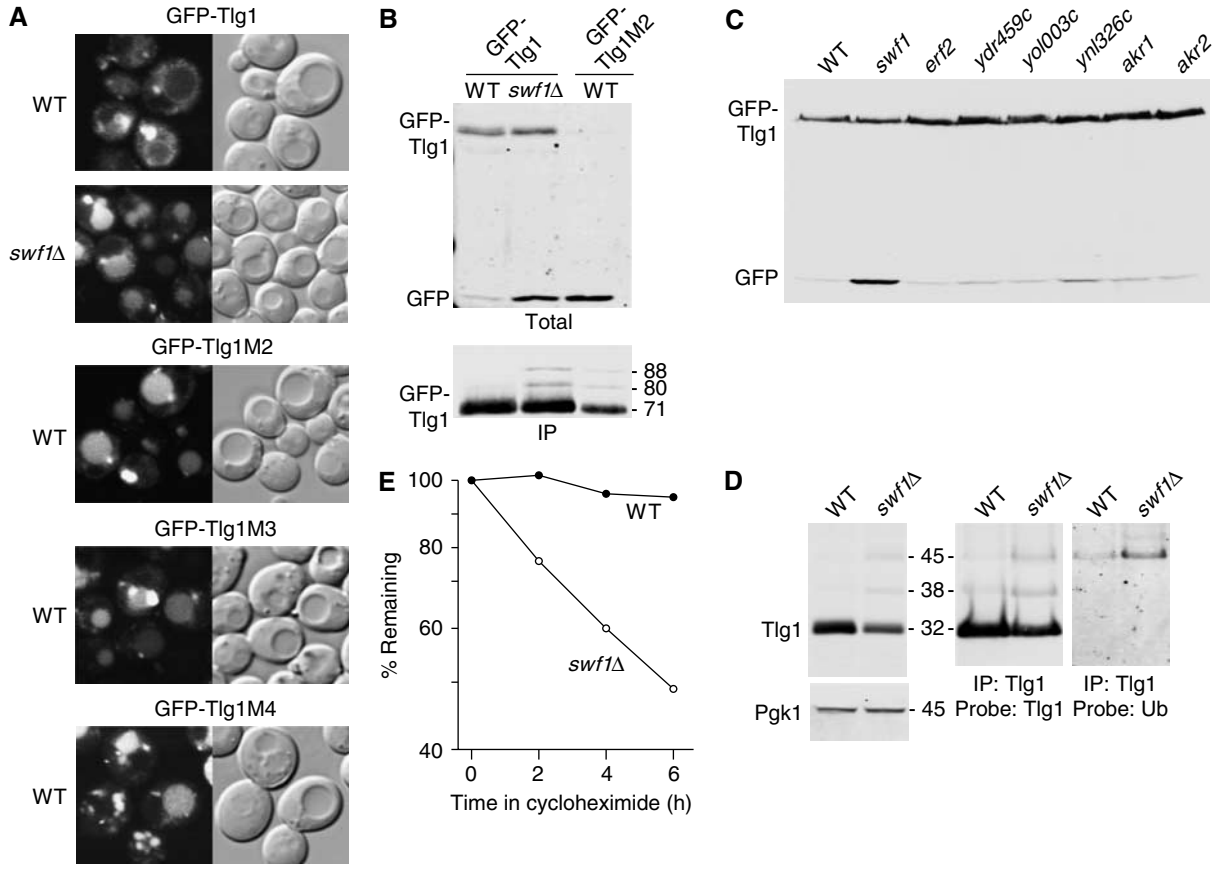

Figure 4 Vacuolar targeting of Tlg1 in *swf1*Δ cells. (**A**) GFP-Tlg1 visualised in WT and *swf1*Δ cells, and GFP-Tlg1M2, in which the two cysteine residues are replaced with serines, together with the single cysteine mutants M3 (CC-CS) and M4 (CC-SC), in WT cells. With the mutant proteins, or in *swf1* mutant cells, GFP is visible inside vacuoles. (**B**) Immunoblotting with anti-GFP shows the generation of free (vacuolar) GFP from GFP-Tlg1 in *swf1*Δ cells, and from GFP-Tlg1M2 in WT cells. The lower panel shows the same samples after immunoprecipitation with anti-Tlg1 antibodies. Note that full-length GFP-Tlg1M2 is not abundant enough to be clearly visible unless concentrated by immunoprecipitation. Numbers indicate apparent sizes in kilodaltons. (**C**) Immunoblotting shows that degradation of GFP-Tlg1 occurs preferentially in *swf1*Δ cells. (**D**) Immunoblotting with anti-Tlg1 shows reduced levels of endogenous protein in *swf1*Δ cells, and the appearance of bands with reduced mobility corresponding approximately in size to the addition of one and two ubiquitins. Equivalent loading was demonstrated by probing the same blot with antibodies to Pgk1. Tlg1 was also immunoprecipitated from the cells, and the precipitates probed with anti-Tlg1 (centre panel) and anti-ubiquitin (right panel). (**E**) WT and *swf1*Δ cells were treated with cycloheximide and samples taken at intervals for the assay of Tlg1 by immunoblotting. Note the logarithmic scale of the vertical axis.

than the fusion protein; similar bands were detectable with the Tlg1M2 mutant in WT cells, despite the low levels of intact protein in this case (Figure 4B, lower panel). Immunoblotting of endogenous Tlg1 also revealed not only a reduction in the amount of protein in *swf1*Δ cells but also proteins migrating at 38 and 45 kDa (Figure 4D), as previously detected in Figure 3D. Immunoprecipitation of Tlg1 followed by immunoblotting with anti-ubiquitin antibody showed that the 45 kDa band, at least, is a ubiquitinated form of Tlg1 (Figure 4D). The 38 kDa band did not react with our monoclonal anti-ubiquitin, though it is not clear whether this is due to a genuine lack of ubiquitin or due to a particular property of the antibody. Some ubiquitination of endogenous Tlg1 could also be detected in WT cells. This may represent molecules that fail to be modified with palmitate, because we were unable to detect ubiquitinated bands when palmitoylated Tlg1 was selectively purified using the hydroxylamine-dependent biotinylation procedure (right-hand panel of Figure 3D; data not shown).

Since ubiquitination results in degradation, the clear prediction is that Tlg1 should turn over more rapidly in *swf1*Δ cells than in WT cells. To test this, we added cycloheximide to block protein synthesis, and followed the fate of Tlg1 by immunoblotting. Figure 4E shows that, under these conditions, the SNARE was very stable in WT cells, but in the *swf1*Δ strain it was degraded with a half-life of about 6 h.

Our results indicate that Tlg1 is ubiquitinated in *swf1*Δ cells. We therefore sought a ubiquitin ligase capable of recognising unpalmitoylated Tlg1. Tul1 is a ring domain ubiquitin ligase that recognises polar TMDs and targets them for degradation (Reggiori and Pelham, 2002). Another protein, Bsd2, also recognises TMDs and mediates protein ubiquitination by recruiting the HECT domain ubiquitin ligase Rsp5 (Hettema *et al*, 2004). To see whether these proteins contribute to the degradation of unpalmitoylated Tlg1, the Tlg1 cysteine to serine mutant Tlg1M2 was transformed into *bsd2*Δ, *tul1*Δ and *bsd2*Δ *tul1*Δ strains and the transformants analysed by fluorescence microscopy and Western blotting. Figure 5A shows that Tlg1M2 still enters the vacuolar lumen in the absence of either Tul1 or Bsd2, but not in the double mutant. In agreement with this, Western

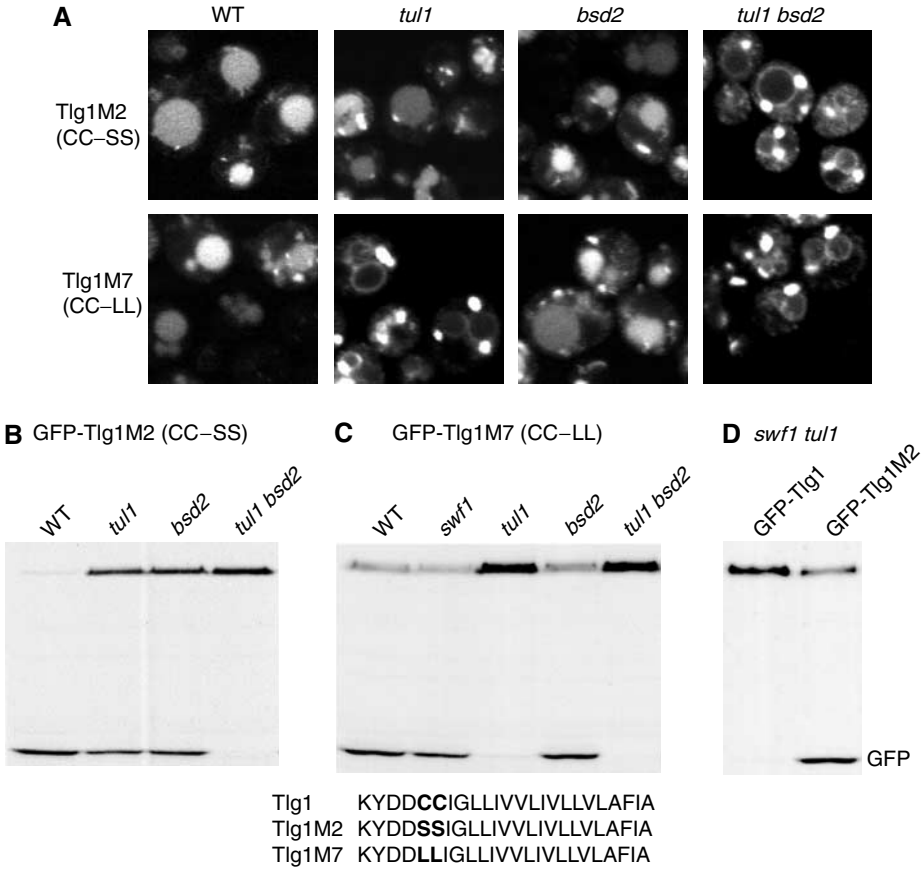

Figure 5 Unpalmitoylated Tlg1 is recognised by Tul1 and Bsd2. (**A**) GFP-tagged Tlg1 mutants with the cysteines replaced by serines (Tlg1M2) or leucines (Tlg1M7) were visualised in the indicated strains. (**B**) Immunoblotting with anti-GFP antibodies of the strains shown in (A) expressing GFP-Tlg1M2. (**C**) GFP-Tlg1M7 analysed as in (B) in the indicated strains. (**D**) GFP-Tlg1 and GFP-Tlg1M2 analysed in an *swf1 tul1* double mutant. Sequences of the C termini of the various forms of Tlg1 are shown below.

blot analysis showed that, although degradation of GFP-Tlg1 was reduced in the single mutants, only in the double mutant was it blocked completely (Figure 5B). Thus, Tlg1M2 is a substrate for both Tul1 and the Bsd2 system.

Since Tul1 and Bsd2 recognise polar TMDs, it could be that their effect on Tlg1M2 is a consequence of the presence of the serines, rather than the absence of palmitoylation. Indeed, the M2 mutant, with serines, was less stable than WT Tlg1 in an *swf1* mutant, a situation in which the protein should have free cysteines. We therefore generated a new construct (Tlg1M7) in which the cysteines were replaced by hydrophobic leucines. This mutant also entered the vacuole in WT cells, though to a lesser extent than the serine-containing Tlg1M2. Interestingly, the leucine mutant remained a substrate for Tul1, but was completely unaffected by Bsd2 (Figure 5A and C). It was also unaffected by Swf1, confirming that Swf1 stabilises Tlg1 by acting directly on the cysteine residues, rather than indirectly through some other change to the protein-sorting machinery.

Finally, we constructed an *swf1*Δ *tul1*Δ double mutant to test whether unpalmitoylated WT protein, with free cysteines, is also recognised by Tul1. In this strain, GFP-Tlg1 was completely stable, as shown by the absence of free GFP. However, the serine-containing M2 mutant was still degraded, due to the presence of Bsd2 (Figure 5). We conclude that it is the absence of palmitoylation that renders Tlg1 sensitive to Tul1. In contrast, recognition by Bsd2 occurs only when the polarity of the TMD is increased by the introduction of serine residues.

Although removal of Tul1 prevented degradation of Tlg1 in *swf1*Δ cells, it did not suppress *swf1* phenotypes that we have observed, such as the inability of the cells to grow on lactate or the reduced level of Snc1 (data not shown). Thus, these other phenotypes must reflect additional functions of Swf1, or of unpalmitoylated SNAREs, and are not simply a consequence of low Tlg1 levels.

Swf1 is localised to the ER

Palmitoylation of Snc1 occurs rapidly after synthesis (Couve *et al*, 1995). To locate Swf1, we tagged it at the N and C termini with a triple HA epitope tag or GFP, and confirmed that the tagged proteins retained the ability to prevent degradation of GFP-Tlg1 (Figure 6A). Direct observation of both N- and C-terminal GFP tagged versions under the fluorescence microscope showed a weak signal, localised to the ER and most prominently the nuclear envelope (Figure 6B; data not shown). Immunofluorescence of HA-tagged Swf1 yielded similar results, although in this case some additional intracellular dots were observed (Figure 6B). Thus, Swf1 is in a position to modify newly synthesised SNAREs before they exit the ER. This is consistent with the observation that blocking ER exit with a temperature-sensitive *sec18* mutation does not prevent palmitoylation of Snc1 (Couve *et al*, 1995). It is likely that the modification persists for the lifetime of the

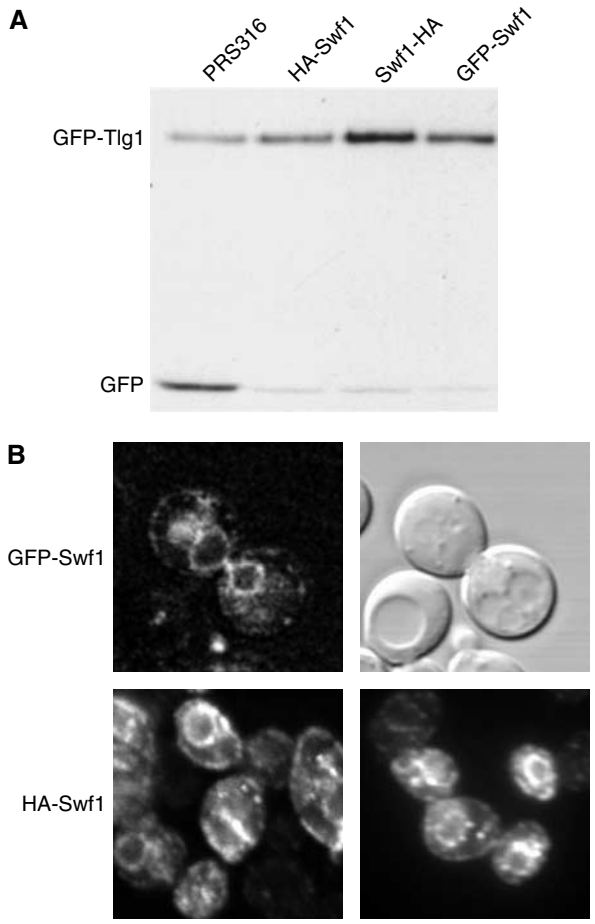

Figure 6 Localisation of Swf1. (**A**) Tagged Swf1 is functional. Immunoblotting of GFP-Tlg1 in *swf1*Δ cells transformed also with an empty plasmid (PRS316), or plasmids expressing Swf1 tagged with HA at the N-terminus (HA-Swf1) or C-terminus (Swf1-HA), or GFP at the N-terminus (GFP-Swf1). (**B**) Fluorescence and immunofluorescence of the N-terminally GFP-tagged Swf1 in *swf1*Δ cells, and HA-tagged Swf1 in WT cells. Two different fields are shown for Swf1-HA. Identical results were obtained with GFP and HA tags at either the N- or C-terminus, in either *swf1*Δ or WT cells.

protein, but we cannot rule out the possibility that some Swf1 is present in the Golgi or endosomes, and can act there to repalmitoylate proteins that lose their modification.

Discussion

In this paper, we have presented evidence that at least three different yeast SNAREs, Snc1, Tlg1 and Syn8, are palmitoylated on cysteine residues close to the cytoplasmic end of the TMD. Since several other SNAREs, both in yeast and in vertebrates, contain cysteine residues in a similar position (Figure 2), it is likely that they are all similarly modified. We have also shown that modification of all three of the SNAREs that we have studied requires the ER-localised DHHC-CRD protein Swf1. By analogy with other DHHC-CRD proteins, Swf1 is likely to be the palmitoyltransferase that acts directly on newly synthesised SNAREs, and presumably also on other proteins with appropriately positioned cysteine residues. In support of this, we have shown that the cysteine residue in the DHHC motif of Swf1 is essential for modification of Tlg1 *in vivo*. The equivalent residues in Erf2 and Akr1 have been shown to be required for palmitoyltransferase activity both *in vivo* and *in vitro* (Lobo *et al*, 2002; Roth *et al*, 2002).

The specificity of Swf1 function is striking. Six other DHHC-CRD palmitoyltransferases are present in yeast. Of these, Erf2 is localised to the ER (Bartels *et al*, 1999), and we have found that Yol003c is also in the ER, yet neither can substitute for Swf1 even when overexpressed (our unpublished observations). Since the actual transfer of palmitate from palmitoyl-CoA to cysteine residues can occur spontaneously, it has been suggested that the role of the transferases is simply to present a palmitoyl-thioester to the substrates, either as palmitoyl-CoA or as an enzyme-linked intermediate (Roth *et al*, 2002; Dietrich and Ungermann, 2004), in much the same way that E3 ubiquitin ligases present ubiquitin thioesters to their substrates. The conserved DHHC-CRD is likely to be involved in the palmitoylation reaction itself (Roth *et al*, 2002), while other features of the individual transferases recognise specific substrates. For example, prenylated Ras proteins may be recruited by Erf4, a cytoplasmic protein that associates tightly and specifically with Erf2, while the soluble casein kinase Yck2 may be recruited via the ankyrin repeats that are a feature of its specific transferase, Akr1. In the case of Swf1, an unusual feature is the presence of five predicted TMDs, the first close to the amino-terminus; most DHHC-CRD proteins have four, or in some cases two, three or six. It may be that the 5-TMD structure includes a pocket for interaction with the single TMDs of SNARE proteins, or potentially with any free TMD. This feature may be conserved: humans and mice have only one DHHC protein (DHHC-4) that fits the Swf1 topology, and it is more closely related to Swf1 than to any other yeast protein. However, not all species have an Swf1-like protein with five TMDs. Thus, there may be some structural diversity in the palmitoyltransferases that recognise TMDs.

Once palmitoylated, the SNARES leave the ER and thus are largely separated from the transferase. The modification is therefore likely to be permanent, rather than a transient change associated with each membrane fusion event. Its contribution, if any, to the membrane fusion activity of the SNAREs is evidently subtle, since unpalmitoylated mutants of both Snc1 and Tlg1 can support growth under conditions in which the SNAREs themselves are essential (our unpublished observations; Lewis *et al*, 2000). Furthermore, even the multiple palmitoylation defects of *swf1*Δ cells cause only a mild defect in the sorting of vacuolar proteases, a sensitive assay for membrane traffic between Golgi and endosomes (Bonangelino *et al*, 2002). Nevertheless, the synthetic lethality of an *swf1* mutation with others that affect such traffic does indicate an important role for palmitoylation of one or more substrates.

Since TMDs are already firmly embedded in the membrane, it seems unlikely that palmitoylation is involved in anchoring them. However, it might help to increase the solubility of the TMD in a sterol-rich bilayer. TMD-associated cysteines are present almost exclusively in SNAREs associated with the plasma membrane, endosomes and TGN, membranes rich in sterols (Figure 2). Increasing bilayer solubility may help SNAREs to partition into, and be sorted with, sterol-rich regions of membrane. It may also help the TMDs to dissociate from each other when the SNARE complex is disrupted following a fusion event. Indeed, we have

observed that, upon lysis of *swf1*Δ mutant cells, Tlg1 readily forms disulphide linkages with other SNAREs to which it is bound, implying that the cysteines, and hence normally the palmitoyl groups, are located close to the interfaces between TMDs in the SNARE complex.

In the case of Tlg1, one clear effect of palmitoylation is to prevent interaction with the ubiquitin ligase Tul1, which results in its ubiquitination and targeting to the vacuole. Tul1 normally seeks out aberrant proteins whose TMDs expose polar residues to the lipid bilayer—that is, TMDs that do not interact well with lipid. However, we found that it can act on a Tlg1 mutant in which the cysteine residues have been replaced by leucine, creating an almost entirely hydrophobic TMD sequence. Tlg1 is unusual in having a pair of acidic residues immediately preceding the TMD, rather than the more typical basic residues which can interact with phospholipid headgroups. Palmitoylation in this case may fix the position of the transmembrane helix relative to the bilayer, preventing the acidic residues from slipping into the membrane. Acidic residues exposed to lipid are known to be recognised by Tul1 (Reggiori and Pelham, 2002). Another curiosity is that replacement of even one of the cysteines with serine rather than leucine was sufficient to make Tlg1 a substrate for Bsd2-dependent ubiquitination. This shows that Tul1 and Bsd2 have subtly different specificities, and also highlights the danger in assuming that the apparently minor change of cysteine to serine in a palmitoylated protein will have no effect other than blocking palmitoylation.

Though animal cells lack Tul1, they do contain Bsd2 homologues. Moreover, the A1 adenosine receptor, the CCR5 chemokine receptor and Rous sarcoma virus glycoprotein have all been shown to be palmitoylated close to a TMD, and to be rapidly degraded when palmitoylation is blocked by mutation (Gao *et al*, 1999; Ochsenbauer-Jambor *et al*, 2001; Percherancier *et al*, 2001). By analogy with Tlg1, we suggest that such alterations either cause a subtle conformational change in the protein, or create a more polar feature, resulting ultimately in less favourable interaction of the protein with lipids and recognition by the cellular quality control machinery. It will be interesting to see whether the Bsd2 homologues N4WBP5 and N4WBP5A are involved in the degradation of these proteins.

Materials and methods

Plasmids and strains

The strains used in this study were derivatives of BY4742 from the EUROSCARF consortium, containing complete deletions of *BSD2*, *TUL1*, *SWF1*, *ERF2*, *YDR459C*, *YOL003C*, *YNL326C*, *AKR1* and *AKR2*. The *bsd2*Δ *tul1*Δ strain has been described (Hettema *et al*, 2004). The *swf1*Δ *tul1*Δ strain was obtained by deleting the *TUL1* ORF in the EUROSCARF *swf1*Δ strain, using the *Schizosaccharomyces pombe HIS5* gene flanked by *TUL1* sequences introduced by PCR. Expression of tagged versions of Tlg1 was driven by the *TPI1* promoter from a *URA3 CEN* plasmid PRS416, which also contains the *PEP12* terminator. Mutations in Tlg1 and Swf1 were generated using Quickchange Mutagenesis (Stratagene) and checked by sequencing. The Snc1- and Syn8-expressing plasmids have been described (Lewis *et al*, 2000; Lewis and Pelham, 2002). The *SWF1* ORF was amplified from the BY4742 strain and cloned in a Ycplac33 vector containing the *TPI1* promoter, either GFP or triple HA tags, and a *PGK1* terminator.

Protein electrophoresis and Western blots

Protein samples were prepared according to Volland *et al* (1994). In order to detect palmitoylation, it was necessary to prepare and analyse proteins without exposure to sulphydryl agents. For these experiments, protein samples were prepared as follows: 10–20 OD of logarithmically growing cells were collected and resuspended in 300–500 μl of lysis buffer (PBS, protease inhibitor tablets and 5 mM EDTA). Then, 100 μl of glass beads was added and cells were broken using a 30-s pulse in a bead beater. SDS was added to a final concentration of 1% and the tubes were rotated for 15 min at 4°C or 5 min at room temperature. Protein concentration was measured and the samples were heated for 10 min at 65°C with sample buffer without β-mercaptoethanol or dithiothreitol.

For the hydroxylamine treatment, 100 μg of protein extract, usually in no more than 50 μl, was transferred to a separate tube containing 600 μl of either 1 M hydroxylamine, pH 7.4, or 1 M Tris, pH 7.4, as a control, and incubated at room temperature for 1 h. A mixture of chloroform–methanol (600–150 μl, respectively) was added, samples were vortexed and then centrifuged for 2 min at maximum speed in a tabletop centrifuge. The upper phase was discarded, without disturbing the interphase where proteins are. Then, 450 μl of methanol was added, samples vortexed and centrifuged again, the supernatant discarded and the pellet air-dried and resuspended in 100 μl of sample buffer without β-mercaptoethanol or dithiothreitol. Typically, 15–20 μl was loaded on the gel.

To detect the molecular weight shifts, we used either 10% Bis–Tris acrylamide gels or 4–20% Tris-glycine acrylamide gradient gels (Novex) for Snc1 and Syn8. Tlg1 palmitoylation was better observed using 4–12% Bis–Tris gradient gels (Novex). Proteins were transferred to nitrocellulose membranes and detected using 9E10 anti-Myc monoclonal antibody, anti-GFP antibodies (Roche), rabbit anti-Tlg1 (Holthuis *et al*, 1998a) or rabbit anti-Syn8 (kindly provided by David Banfield).

The blots were probed using secondary antibodies coupled to either Alexafluor 680 (Molecular Probes) or IRdye800 (Rockland Immunochemicals, Inc.) and then scanned using an Odyssey Infrared imager (LICOR Bioscience, UK). Some blots were probed with secondary antibodies coupled to HRP and developed by ECL (Amersham). For the ubiquitination of Tlg1, the blot was probed first with anti-ubiquitin monoclonal antibody (Santa Cruz Biotech), scanned, and then re-probed with rabbit anti-Tlg1 to avoid any possible bleed-through between the channels. Loading controls were similarly visualised by re-probing the same blot after initial scanning.

Biotinylation assays

To identify Tlg1 with free cysteines, 20 OD units of cells were lysed with glass beads in 300 μl of lysis buffer: PBS containing 2 mM EDTA and protease inhibitor tablets (Roche). Lysis buffer (700 μl) was added, the samples centrifuged for 4 min at 300 *g*, supernatant removed and the pellet re-extracted with a further 300 μl buffer. Pooled supernatants were centrifuged for 15 min at 13 000 *g* and the membrane pellet resuspended in 550 μl lysis buffer. Octylglucoside (1%) and the biotinylating reagent Biotin-BMCC (Pierce) (300 μM) were added. The samples were incubated for 2 h at 4°C and then proteins precipitated with methanol/chloroform. The pellet was air-dried and then resuspended by sonication in 70 μl of resuspension buffer (2% SDS, 8 M urea, 100 mM NaCl, 50 mM Tris, pH 7.4). Samples were diluted to 2 ml with lysis buffer containing 0.1% Triton X-100, and 100 μl of streptavidin-agarose beads (Sigma) were added. Samples were rotated for 1 h at room temperature and then washed three times with PBS containing 0.5 M NaCl and 0.1% Triton X-100, and once with PBS. Proteins were eluted by heating for 5 min at 95°C with 20 μl resuspension buffer plus 40 μl of 4 × SDS sample buffer. A volume of 20 μl of this was analysed by immunoblotting. Loading was controlled by probing the membrane with streptavidin linked to Alexafluor 680.

To detect only palmitoylated Tlg1, *N*-ethylmaleimide was added to the lysis buffer at 25 mM. Cell lysates were obtained as above, but the membrane fraction resuspended in lysis buffer containing 25 mM *N*-ethylmaleimide and 1% octylglucoside, incubated for 10 min at 4°C and immediately precipitated with methanol/chlorophorm. The pellet was taken up in 50 μl dissociation buffer with sonication, diluted with 600 μl of 1 M hydroxylamine, 300 μM biotin-BMCC and rotated for 2 h at 4°C. Proteins were again precipitated using methanol/chloroform, then resuspended and purified on streptavidin beads as described above.

Tlg1 immunoprecipitation

Total lysates prepared as above were solubilised with 1% Triton X-100. For the detection of ubiquitinated bands, 10 mM NEM was added to the lysis buffer. Anti-Tlg1 serum was crosslinked to protein A-Sepharose beads using dimethylpimilimidate as described (Harlow and Lane, 1998). In all, 50 µl of these beads was incubated for 2 h at 4°C, washed three times with lysis buffer, once with PBS, and proteins eluted from the beads by heating for 10 min at 65°C with sample buffer containing no reducing agent.

Microscopy

Cells were grown to early log phase and imaged in water with a BioRad Radiance confocal microscope, using a single slow scan.

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