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Transmembrane domain-dependent sorting of proteins to the ER and plasma membrane in yeast

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Sorting of membrane proteins between compartments of the secretory pathway is mediated in part by their transmembrane domains (TMDs). In animal cells, TMD length is a major factor in Golgi retention. In yeast, the role of TMD signals is less clear; it has been proposed that membrane proteins travel by default to the vacuole, and are prevented from doing so by cytoplasmic signals. We have investigated the targeting of the yeast endoplasmic reticulum (ER) t-SNARE Ufe1p. We show that the amino acid sequence of the Ufe1p TMD is important for both function and ER targeting, and that the requirements for each are distinct. Targeting is independent of Rer1p, the only candidate sorting receptor for TMD sequences currently known. Lengthening the Ufe1p TMD allows transport along the secretory pathway to the vacuole or plasma membrane. The choice between these destinations is determined by the length and composition of the TMD, but not by its precise sequence. A longer TMD is required to reach the plasma membrane in yeast than in animal cells, and shorter TMDs direct proteins to the vacuole. TMD-based sorting is therefore a general feature of the yeast secretory pathway, but occurs by different mechanisms at different points.

Keywords: endoplasmic reticulum/plasma membrane/protein sorting/SNAREs/transmembrane domains

Introduction

The eukaryotic secretory pathway consists of a number of discrete, membrane-bound organelles with distinct protein and lipid compositions; transport between these organelles occurs via transport vesicles which bud from one compartment and fuse specifically with the next. The location of membrane proteins within this system depends on their ability to be incorporated selectively into, or excluded from, the appropriate transport vesicles. This can be influenced not only by direct interactions between cytoplasmic sequences and vesicle coat proteins, but also by the nature of their membrane-spanning segments.

Key components of the transport system that themselves must be sorted are the SNARE proteins. These are membrane-bound receptors found on the target membranes (t-SNAREs) and on transport vesicles (v-SNAREs) that interact specifically during vesicle docking and promote vesicle fusion via the NSF and SNAP fusion machinery (Söllner *et al.*, 1993a,b). In the budding yeast *Saccharomyces cerevisiae*, t-SNAREs have been identified for four organelles: the plasma membrane (Sso1p and the closely related Sso2p), an endosomal/pre-vacuolar compartment (Pep12p), the *cis*-Golgi (Sed5p) and the endoplasmic reticulum (ER) (Ufe1p) (Hardwick and Pelham, 1992; Aalto *et al.*, 1993; Becherer *et al.*, 1996; Lewis and Pelham, 1996). t-SNAREs in different compartments are not highly conserved at the primary sequence level, although they share common secondary structural features: they are C-terminally anchored, with the bulk of the protein being in the cytosol, and they contain three stretches of heptad repeats which could form coiled-coil domains, the longest stretch being close to the transmembrane domain (TMD). This region has been shown to be crucial for the interaction of syntaxin with the incoming v-SNARE, synaptobrevin (Calakos *et al.*, 1994; Kee *et al.*, 1995).

The t-SNAREs, which 'define' a given compartment in that they determine which vesicles are able to fuse with it, must be localized correctly in order to prevent aberrant fusion of vesicles with the wrong compartment. They thus provide a model system for studying protein sorting, i.e. they are a family of similar proteins which are targeted to diverse locations. t-SNAREs lack well-characterized sorting signals, although we have shown previously that the localization of the Golgi t-SNARE, Sed5p, is influenced both by its TMD and by the cytoplasmic portion of the protein (Banfield *et al.*, 1994).

This study focuses on the ER t-SNARE, Ufe1p. In particular, we have investigated the importance of the TMD in the localization of this protein. Considerable evidence already supports a role for the TMD in the targeting of Golgi enzymes in mammalian cells (Munro, 1991; Nilsson *et al.*, 1991; Swift and Machamer, 1991). Plasma membrane proteins generally have longer TMDs than Golgi membrane proteins, and sorting has been postulated to be a result of the difference in thickness of the lipid bilayer between the Golgi apparatus and the plasma membrane (Bretscher and Munro, 1993). An alternative model has also been proposed in which the role of the TMD is to interact with other Golgi enzymes, to form large hetero-oligomers which are prevented by size from entering outgoing vesicles (Nilsson *et al.*, 1993).

Our results show that the Ufe1p TMD contains an ER targeting signal. Unlike those required for Golgi retention in animal cells, this signal is not simply determined by TMD length but is dependent on the amino acid sequence, and thus is likely to be recognized by a protein. Surprisingly, residues along one helical face of the TMD are also crucial for Ufe1p function, but the sequence requirements for function are distinct from those for ER retention. TMD sequences have also been implicated in the sorting of another yeast ER protein, Sec12p, and a putative sorting receptor, Rer1p, has been identified. However, targeting

by the Ufe1p TMD can occur in the complete absence of Rer1p, implying that it occurs by a novel mechanism.

Using Ufe1p as a reporter molecule, we have also investigated TMD-mediated sorting in the later parts of the yeast secretory pathway. Attempts to map the retention signals of yeast Golgi enzymes have focused on the distinction between a Golgi and a vacuolar location; unlike in animal cells, overexpressed or chimeric proteins do not seem to accumulate on the plasma membrane (Chapman and Munro, 1994; Lussier *et al.*, 1995). These findings, together with an apparent lack of localization signals in vacuolar membrane proteins, have suggested that the vacuole is the default destination for membrane proteins in yeast, and that some so far unspecified signal is required to reach the cell surface (Roberts *et al.*, 1992; Nothwehr *et al.*, 1993). We find that the choice between plasma membrane and vacuole is dictated by TMD length and (when length is limiting) by amino acid composition, but not by the precise sequence. This supports a lipid-based sorting mechanism as proposed for animal cells. However, yeast differs from animal cells in that a longer TMD is required to ensure transport to the surface, and that proteins with relatively short TMDs are not restricted to the Golgi apparatus but can also reach the endosome/vacuolar system.

Results

Ufe1p can be mistargeted by altering its TMD

Our strategy to identify the roles of TMDs in sorting was to prepare chimeras between the cytoplasmic and membrane domains of different SNAREs and examine their locations. All chimeras used were based on SNAREs tagged at their N-terminus either with a myc epitope (Munro and Pelham, 1987) or with green fluorescent protein (GFP) (Chalfie *et al.*, 1994). As outlined in Materials and methods, to facilitate the swapping of TMDs, either *Hin*dIII (encoding the amino acids KL) or *Kpn*I (encoding the amino acids RYQ) restriction sites were introduced near the TMDs of Ufe1p, Sft1p, Sso1p and Pep12p. Immunofluorescence microscopy confirmed that the distribution of these tagged molecules did not differ noticeably from the previously described locations of the wild-type SNAREs. To confirm that the proteins also retained function, we tested their ability to suppress appropriate temperature-sensitive mutations. In the case of Sso1p, we assayed its ability to suppress *sec1-1* (Aalto *et al.*, 1993). In no case did tagging or adding the *Hin*dIII or *Kpn*I sites interfere with the ability of Ufe1p, Sft1p and Sso1p to suppress the temperature sensitivity of *ufe1-1*, *sft1-1* and *sec1-1* mutants, respectively.

To test whether the TMD of Ufe1p is required for its localization in the ER, we replaced it with those of Sed5p, Pep12p and Sso1p, yeast t-SNAREs for the *cis*-Golgi, endosome and plasma membrane respectively (Figure 1A). Ufe1p normally displays a predominantly perinuclear distribution, coincident with the ER marker BiP (Lewis and Pelham, 1996; Figure 1B), and when its TMD was replaced with that of Sed5p, no change in its distribution was detectable (Figure 1B). The Ufe1p–Pep12p chimera was also mostly in the ER (Figure 1B). Thus, the TMD of Ufe1p is not strictly necessary for its correct localization. Interestingly, however, we found that these chimeras were

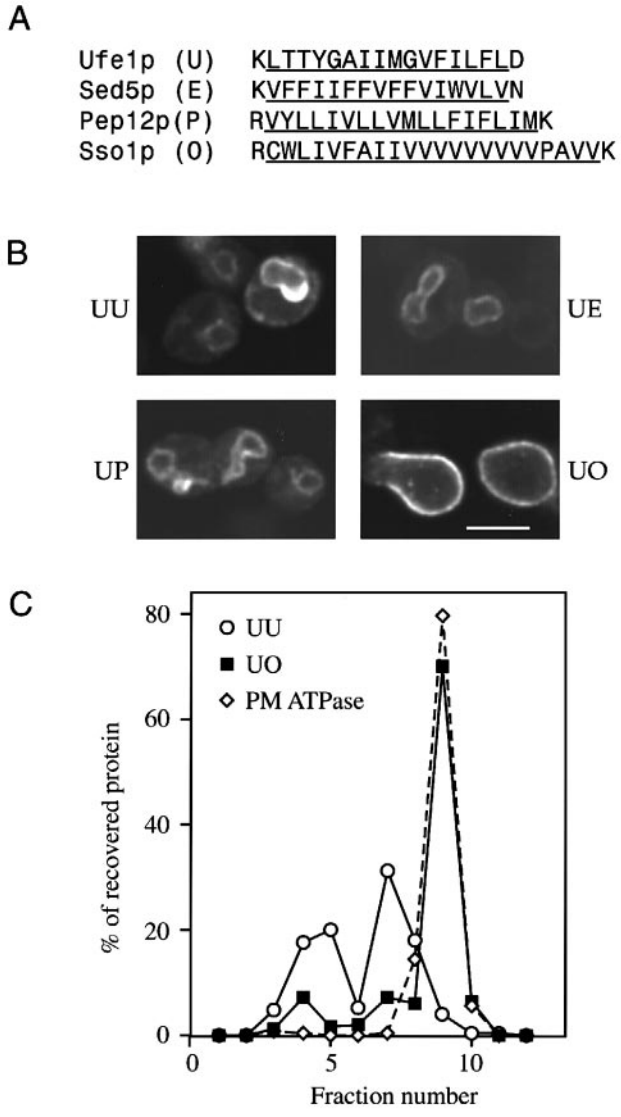


Fig. 1. The Sso1p TMD is sufficient to direct the ER t-SNARE, Ufe1p, to the plasma membrane. (**A**) TMD sequences. (**B**) Immunofluorescence of N-terminally myc-tagged Ufe1p with its own TMD (UU) or the TMD of Sed5p (UE), Pep12p (UP) or Sso1p (UO), recognized by the anti-myc monoclonal 9E10. Bar is 5 μm. (**C**) Sucrose gradient fractionation shows localization of the Ufe1p–Sso1p chimera to the plasma membrane. Cells expressing tagged Ufe1p (UU) or the chimera (UO) were fractionated as described in Materials and methods, and the proteins, together with the plasma membrane ATPase, quantitated by immunoblotting. The ER, indicated by the UU signal, forms a characteristic double peak on these gradients. Some of the UO construct is detectable in the ER fractions, but most is in a peak that co-fractionates with the plasma membrane marker.

incapable of suppressing the ts phenotype of *ufe1-1*, implying that the normal TMD is important for function.

In contrast to the Ufe1p–Sed5p and Ufe1p–Pep12p constructs, a chimera containing the Sso1p TMD was found solely in the periphery of the cell, in a distribution similar to that of Sso1p itself at the plasma membrane (Figure 1B; compare Figure 6A, construct OO). This striking result was confirmed by density gradient fractionation. Membranes from cells expressing the UU (Ufe1p) and UO (Ufe1p–Sso1p) constructs were pelleted at 10 000 *g*, resuspended and separated by flotation through a discontinuous sucrose gradient (Figure 1C). The UU

construct, acting as a marker for the ER, showed the double peak that is typical of ER proteins on these gradients (Bowser and Novick, 1991). In contrast, with the UO construct, >80% of the material that floated up into the gradient was found in a sharp peak coincident with the plasma membrane ATPase. The expression of the chimeric ER t-SNARE on the plasma membrane had no obvious effect on cell growth; like the previous constructs it appeared to be non-functional. We conclude that although the native Ufe1p TMD is not strictly necessary for correct targeting to the ER, changes to it can result in mislocalization.

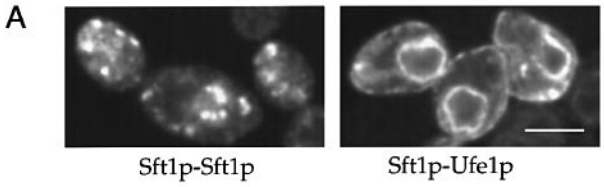
The Ufe1p TMD contains an ER targeting signal that is sequence dependent

The above results indicate that targeting of Ufe1p is in part dependent upon its TMD, but they do not address the question of whether the TMD can act as an ER sorting signal in its own right. To answer this question, we fused it to the cytoplasmic part of Sft1p, a v-SNARE normally found in a late Golgi compartment (Banfield *et al.*, 1995). This change was sufficient to redirect Sft1p from the Golgi (shown by the punctate staining in Figure 2A) to the ER (Sft1p–Ufe1p construct, Figure 2A).

To confirm this, we prepared subcellular fractions by differential centrifugation. Under the conditions used, essentially all of the ER (detected by immunoblotting Sec61p) is found in the 13 000 *g* pellet, whereas the Golgi membranes are split between the 13 000 *g* pellet and the 100 000 *g* pellet (p100; Lewis and Pelham, 1996). Analysis of the fractions (see Materials and methods) revealed that 50% of Sft1p was present in the p100 fraction when it carried its own TMD. This was reduced to 11% for the Sft1p–Ufe1p chimera, the bulk being present in the ER fraction. Thus, in this context, the Ufe1p TMD can indeed provide sorting information for the ER.

Having established that the Ufe1p TMD contains an ER targeting signal, we were interested in determining the nature of this signal. The best characterized TMD sorting signals are in Golgi enzymes, where length seems to be the primary determinant in sorting decisions between the Golgi and the plasma membrane (Munro, 1995). Such a model cannot be applied to this case, however, as the Sft1p and Ufe1p TMDs are the same length (see Figure 2B) yet target Sft1p to two different locations. One noticeable difference between the two TMDs is that Ufe1p has an acidic residue (Asp) at the C-terminal end, an unusual feature for a SNARE, whereas Sft1p has a lysine residue at this position. To test whether this residue was involved in ER targeting, we changed the aspartic acid to lysine by site-directed mutagenesis. This change had no effect on the ability of the Ufe1p TMD to target Sft1p to the ER (Figure 2B), as judged by both immunofluorescence and fractionation.

Since neither length nor flanking residues account for the ER targeting signal in the Ufe1p TMD, it must be provided by the specific sequence of the hydrophobic region or perhaps by some more general property conferred by its amino acid composition. Examination of a helix wheel representation of the sequence (Figure 2C) revealed a relatively non-hydrophobic face, characterized by the Thr, Ala and Gly residues at positions 4, 7 and 11. To test the significance of this feature for ER retention, we



B

TMD	Sequence (1 4 7 11 14 18)	Location (Sft1p)	Doubling time (min) (Ufe1p)
Sft1p	KLVGLALLIFFILYTLFK	Punc.	ND
Ufe1p	KLTTYGAIIMGVFILFLD	ER	160
A	----------K	ER	>1000
B	----------E	ER	160
C	--LL----------	ER	160
D	-----LL----------	ER	160
E	----------L-------	ER	280
F	--LL------L-------	ER	>1000
G	------LLL-L-------	ER	>1000
H	----------L--VVV--	ER	>1000
I	--LLLLL---L-------	Punc.	ND
J	--LFLIFVGMIIAGYTT-	Punc.	>1000
K	--LIFTGMFTIALVIGY-	Punc.	>1000

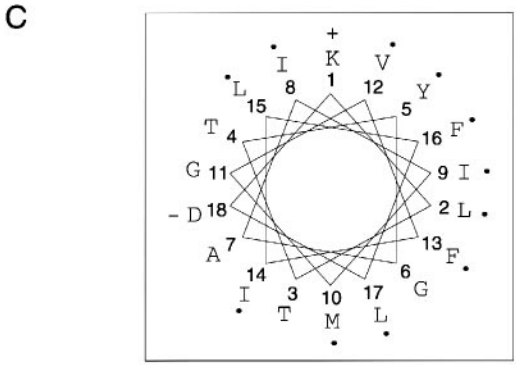


Fig. 2. An ER targeting signal in the Ufe1p TMD. The late Golgi v-SNARE, Sft1p, was fused to the Ufe1p TMD or mutated versions of it. (**A**) Myc-tagged Sft1p has a punctate distribution, previously shown to be coincident with the Golgi enzyme Mnt1p (Banfield *et al.*, 1995). When the Sft1p TMD is exchanged for that of Ufe1p (Sft1p–Ufe1p), its distribution is shifted to the ER. Bar is 5 μm. (**B**) Various TMD sequences were fused to Sft1p and the localization of the protein to the ER or to punctate Golgi structures determined by immunofluorescence as in (A). Dashes indicates residues identical to Ufe1p. The same sequences were attached to Ufe1p, and the constructs expressed in a *ufe1-1* mutant. The doubling times of the resultant strains at 37°C (a non-permissive temperature for *ufe1-1*) are given in minutes; ND indicates not done. Constructs A, G, H and J were also tested by a plasmid-shuffling procedure that removed the *ufe1-1* allele at 25°C; none supported growth at this temperature. (**C**) Helix wheel plot of the Ufe1p TMD. Note the relatively polar face centred on the Asp residue at position 18.

prepared a series of derivatives of the Sft1p–Ufe1p chimera in which Leu residues were introduced along this face of the helix. As shown in Figure 2B, many such changes were tolerated without loss of ER targeting, but changing the entire sequence TTYGA together with a second Gly residue to leucines (construct I) finally abolished ER retention.

The changes in construct I significantly increased the

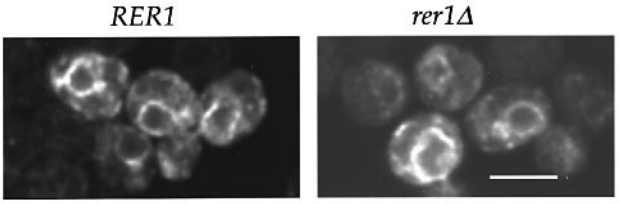


Fig. 3. *RER1* is not required for the Ufe1p TMD to act as an ER targeting signal. The Sft1p–Ufe1p chimera containing the Ufe1p TMD was localized in a *rer1*Δ strain and an isogenic *RER1* control. Both strains gave typical ER staining.

overall hydrophobicity of the TMD, and the possibility remained that it was this property, rather than a localized feature, that influenced sorting. However, two further constructs, in which the TMD sequence was reversed (construct J) or randomized (construct K), also failed to be retained in the ER but instead accumulated in Golgi-like structures (Figure 2B), a result that was confirmed by subcellular fractionation (not shown). Since the TMDs in these constructs have exactly the same amino acid composition and hence overall hydrophobicity as the Ufe1p TMD, we can conclude that this global property does not constitute the targeting signal. It seems, rather, that the TMD is recognized specifically. The relatively polar residues in the cytoplasmic half of the TMD are important for this, although the requirements for recognition are evidently not very stringent.

The TMD features required for Ufe1p function and ER sorting are distinct

Our observation that a Ufe1p variant carrying the Sed5p TMD was inactive, despite being correctly localized, suggested that the Ufe1p TMD participates in a specific functional interaction with some other protein. Conceivably, this same interaction could also account for the targeting properties of the TMD. To investigate this, we compared the functional activity (in the context of Ufe1p) and the targeting activity (assayed using Sft1p as a reporter) of the various mutant forms of the TMD. Figure 2B shows that mutations that affect the relatively polar face of the TMD helix, including the C-terminal Asp residue, could abolish Ufe1p function without affecting localization (constructs A, F, G and H). It appears, therefore, that localization and t-SNARE function depend on two different features of the Ufe1p TMD.

The Ufe1p TMD ER targeting signal is not dependent on the Rer1p retrieval system

The best studied example of an ER targeting signal in a TMD is that of Sec12p (Sato *et al.*, 1996). A genetic screen for mutants that mislocalize Sec12p identified Rer1p, a predicted four TMD protein that is necessary for the Sec12p TMD-dependent localization of a heterologous protein to the ER (Sato *et al.*, 1996). We were interested in establishing whether correct targeting of the Ufe1p TMD was also Rer1p dependent. We found that deletion of the *rer1* gene had no obvious impact on the Ufe1p TMD-dependent targeting of Sft1p to the ER by immunofluorescence, with clear perinuclear staining visible both in *rer1*Δ cells and in an isogenic control strain (see Figure 3). This result was confirmed by fractionation, which showed that 90% of the protein was in the p13 fraction in each strain (data not shown). The distribution of Ufe1p itself was also unchanged in the *rer1*Δ strain, as judged by immunofluorescence and fractionation (data not shown). The Ufe1p TMD ER targeting signal appears, therefore, to operate by a previously uncharacterized mechanism.

Table I. Sequences of constructs used in Figures 4–6

Fig.	Chimera	C-terminal sequence	Location
4	UUK (Ufe1p)	raagrtakLttygaiimgvfilfldyvgKTR*	ER
	U18	raagrtakLttygaiimgvfilflVLdyvgKTR*	ER
	U20	raagrtakLttygaiimgvfilflVLVLdyvgKTR*	End/Vac
	U22	raagrtakLttygaiimgvfilflVLVLVLdyvgKTR*	End/Vac
	U24	raagrtakLttygaiimgvfilflVLVLVLVLdyvgKTR*	PM
	U26	raagrtakLttygaiimgvfilflVLVLVLVLVLdyvgKTR*	PM
5	UORev	raagrtakLVVAPVVVVVVVVVIIAFVILWKTR*	PM
	UUO1	raagrtakLttygaiiIVVVVVVVVVPAVVKTR*	PM
	UUO2	raagrtakLttygaiimgvfilfVVVPAVVKTR*	End/Vac
6a	OO (Sso1p)	karknkiKLwlivfaiivvvvvvvvvpavvktr*	PM
	OP	karknkiKLYLLIVLLVMLLFIFLIMKL*	PM/Punc
6b	PP (Pep12p)	amryqkrtsrwrvyllivllvmllfiflimkl*	End
	PO	amryqRKARKNKIKMWLIVFAIIVVVVVVVVVPAVVKTR*	End

Underlined regions indicate the predicted TMDs; lower case letters indicate the wild-type sequence (relative to the cytoplasmic domain of the chimera). The primary location of the constructs is also indicated (ER, endoplasmic reticulum; PM, plasma membrane; Punc, punctate; End, endosome; Vac, vacuole).

Lengthening the Ufe1p TMD disrupts the ER localization of Ufe1p

Having established that the ER targeting signal in the Ufe1p TMD is sequence dependent, we sought to determine whether the same was true for the plasma membrane targeting conferred by the Sso1p TMD (see UO, Figure 1B). Little is known about targeting to the yeast plasma membrane, and it has been hypothesized that a specific signal is needed for plasma membrane targeting whereas traffic to the vacuole is by default (Roberts *et al.*, 1992). At 22 amino acids, Sso1p has a noticeably longer TMD than those of Ufe1p or Sft1p, which are both 16 amino acids long (see Figure 1A). To establish whether it is simply the increase in length of the TMD that causes Ufe1p to move to the plasma membrane in the UO chimera, we gradually extended the Ufe1p TMD length by insertion of the amino acids VL at the C-terminal end of the TMD, resulting in a series of constructs from 16 amino acids (wild-type) to 26 amino acids (see Table I).

With TMDs of 16 or 18 amino acids (UUK and U18), Ufe1p gave perinuclear staining typical of the ER (see Figure 4), and the localization was confirmed by double staining with the ER marker BiP (data not shown). However, a functional assay of U18 (performed as in Figure 2B) showed that despite its correct localization it was incapable of supporting the growth of a *ufe1-1* mutant at 37°C. With a TMD of 20 or 22 amino acids (U20 and U22), Ufe1p staining was found predominantly in ringed structures distinct from BiP and either adjacent to or coincident with vacuolar ATPase staining (Figure 4). The exact location of these constructs was not determined,

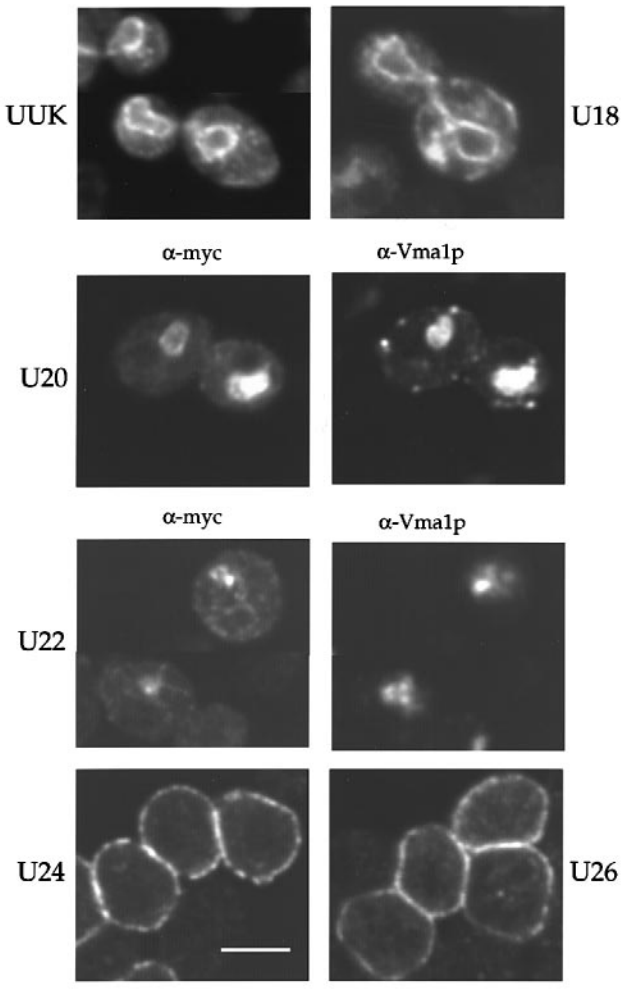


Fig. 4. Effects of lengthening the TMD of Ufe1p. Ufe1p with its normal 16 residue TMD (UUK) or an 18 residue TMD shows typical ER staining, while extension to 24 or 26 residues results in plasma membrane staining. The intermediate lengths (U20, U22) show staining of presumptive endosomes and/or vacuoles, the latter being revealed by double labelling with antibodies to the vacuolar ATPase subunit Vma1p. The TMD sequences are shown in Table I. Bar is 5 μm.

but they are likely to be in the pre-vacuolar/endosomal compartment where Pep12p is resident. Thus extension of the Ufe1p TMD to 20 or 22 residues causes the protein to leave the ER, but it is mislocalized to the vacuolar/endosomal branch of the late secretory pathway rather than to the plasma membrane.

On the other hand, a small amount of the U20 protein must remain in the ER, because this mutant was able to sustain slow growth of the *ufe1-1* strain at 37°C, the doubling time being 340 min. The greater activity of U20 compared with U18 may reflect the fact that the critical C-terminal Asp residue has its normal helical orientation in U20. In any case, the different behaviour of these mutants re-emphasizes the distinction between the ability of the Ufe1p TMD to stimulate t-SNARE activity and to localize the protein to the ER: neither property is sufficient for the other.

In contrast to U20 and U22, lengthening the TMD even further to 24 or 26 amino acids (U24 and U26) resulted in plasma membrane staining (Figure 4). Thus, lengthening

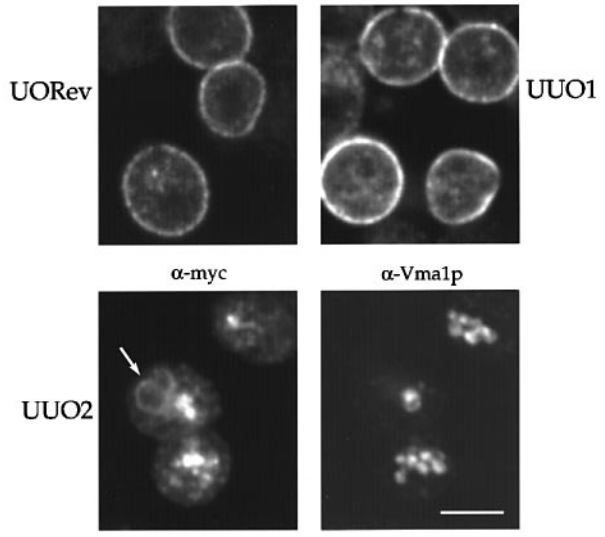


Fig. 5. Requirements for plasma membrane targeting. Ufe1p derivatives with a sequence-reversed version of the Sso1p TMD (UORev) or hybrid Ufe1p–Sso1p TMDs (UUO1, UUO2), each 22 residues long, were localized by immunofluorescence. UORev and UUO1 show plasma membrane staining, while UUO2 shows a typical endosomal/vacuolar pattern, as shown by double labelling with anti-Vma1p. In addition, faint ER staining was sometimes seen (arrow). Sequences of the TMDs are given in Table I. Bar is 5 μm.

its TMD moved Ufe1p through the secretory pathway in two steps, first from the ER to the vacuolar/endosomal system and then to the plasma membrane. It seems that a TMD of 24 or more residues is sufficient to target proteins to the plasma membrane. However, a striking feature of these results is that U22 was found in the endosome, in contrast to the Ufe1p–Sso1p chimera (UO) which is found at the plasma membrane. This suggests that the outcome does not depend solely on the length of the TMD, since this is the same in the U22 and UO constructs.

Plasma membrane targeting depends on both the length and composition of the TMD, but not on the precise sequence

To investigate the nature of the signal in the Sso1p TMD that transports Ufe1p to the plasma membrane, since it is clearly not only length, we reversed the sequence of the Sso1p TMD (UORev, see Table I). This TMD was still able to localize Ufe1p to the plasma membrane, as shown in Figure 5: UORev gave peripheral staining indistinguishable from that of the wild-type Sso1p TMD (UO, see Figure 1B). Similar staining patterns were observed when the normal and reversed Sso1p TMDs were attached to the Sft1p cytoplasmic domain (data not shown). Thus, unlike the Ufe1p TMD, the plasma membrane targeting signal in the Sso1p TMD is not dependent on the order of the residues within the membrane. Making chimeras between the Ufe1p TMD and the Sso1p TMD gave further information. Two TMDs both 22 residues long, one containing 14 residues from Sso1p and the other only seven (UUO1 and UUO2 respectively, see Table I), conferred very different localizations on Ufe1p (Figure 5). UUO1 accumulated on the plasma membrane, whereas UUO2 was found in the endosomal pathway (with occasional faint ER staining, as indicated by the arrow in Figure 5). This difference may reflect the

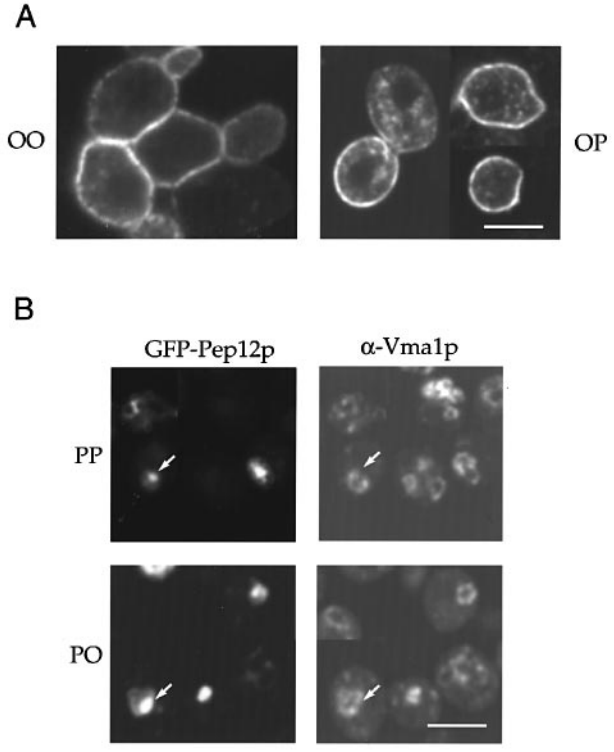


Fig. 6. Sso1p and Pep12p contain cytoplasmic sorting signals. (**A**) Immunofluorescence is shown of myc-tagged Sso1p with its own TMD (OO) or that of the endosomal t-SNARE, Pep12p (OP). Both constructs show plasma membrane staining, although OP also shows some internal punctate staining. (**B**) GFP-tagged Pep12p with its own TMD (PP), or the TMD of Sso1p (PO) co-stained with a mouse monoclonal antibody recognizing the 69 kDa vacuolar ATPase subunit, Vma1p. Both constructs stain a discrete structure often adjacent to, but distinct from, the vacuole (arrows emphasize the comparison of Pep12p staining and Vma1p staining in individual cells; two areas of the same slide are shown in each panel). In many cases, some faint Pep12p staining can be seen around the rim of the vacuole, although the majority of Pep12p does not co-stain with Vma1p. Identical staining was seen with N-terminally myc-tagged Pep12p constructs. Bar is 5 μm.

presence of two Phe residues in UUO2, since these have been shown to disfavour plasma membrane targeting in animal cells (Munro, 1995). Taken with the results from the gradual extension of the Ufe1p TMD, our data suggest that distinction between the plasma membrane and the vacuolar/endosomal system depends on both the length and the amino acid composition of the TMD.

Neither Sso1p nor Pep12p requires its own TMDs for correct targeting

It has been proposed that the vacuole represents the default destination for membrane proteins in yeast, while specific signals are required for targeting to the plasma membrane. However, our investigations of Ufe1p targeting have clearly established that the nature of the TMD can determine whether a membrane protein is localized to the vacuole/endosome or plasma membrane. Is this the way in which the t-SNAREs at these locations are sorted? To test this, we exchanged TMDs between Sso1p and Pep12p. Wild-type Sso1p was distributed to the plasma membrane, as expected (Figure 6A, construct OO). Changing its TMD to that of Pep12p (construct OP) resulted in a somewhat variable distribution: there was still clear cell surface staining, but in some cells the pattern was more punctate. The stained organelles were difficult to identify, but double labelling showed that they were distinct from the pre-vacuolar compartment defined by Pep12p itself (see Figure 6B, construct PP); they may correspond to a late Golgi compartment. The significant observation is that at least some of the Sso1p reached the cell surface even with a heterologous TMD, a conclusion reinforced by the finding that the OP construct retained the ability to suppress a *sec1* ts mutant (data not shown).

To address whether the same was true for Pep12p, we first examined the distribution of this t-SNARE. When overexpressed, both N-terminally myc-tagged (data not shown) and N-terminally GFP-tagged Pep12p showed a unique steady-state pattern, often spatially adjacent to but not coincident with the vacuole, and most often consisting of one to three discrete structures within the cell (see Figure 6B, comparison of vacuolar and Pep12p staining highlighted by arrows). This pattern is typical of exaggerated endosomal structures (Raymond *et al.*, 1992). When the TMD of Pep12p was replaced with that of Sso1p, joined at a naturally occurring *Kpn*I site nine residues N-terminal to the TMD, no change in the distribution of Pep12p was observed, the chimera remaining in the endosome (construct PO; Figure 6B). We conclude that targeting of these t-SNAREs to the plasma membrane and vacuolar branches of the secretory pathway is not wholly dependent on their TMDs; signals in their cytosolic portions also have a strong influence.

Discussion

Our studies of the localization of Ufe1p have revealed that TMDs can provide two different kinds of sorting information in yeast. First, the Ufe1p TMD is important for keeping the protein in the ER. Although some targeting information is also contributed by the cytoplasmic domain, transfer of the TMD to a Golgi protein, Sft1p, showed that it alone is capable of directing a protein to the ER. Second, we have shown that when Ufe1p is mislocalized to the late part of the secretory pathway, the choice between the plasma membrane and the vacuole/endosome system is strongly influenced by the length and composition of the TMD.

Perhaps surprisingly, Ufe1p and Sft1p proved to be convenient reporter molecules for these experiments. In particular, there was no evidence for the formation of complexes between the chimeric constructs and the endogenous wild-type SNAREs, which have to be present to maintain viability of the yeast strains. The distribution of the Ufe1p chimeras was similar whether they were expressed at low levels or at levels sufficiently higher than normal (up to 14-fold) to preclude the formation of 1:1 complexes. Furthermore, some forms of Ufe1p were mislocalized dramatically without toxic effect, which suggests that they neither carried with them the endogenous protein from the ER nor provided fusion activity at an inappropriate location. This may be because such constructs were intrinsically inactive, or simply because Ufe1p-mediated fusion depends on another membrane protein, Sec20p, which is restricted to the ER (Lewis and Pelham, 1996).

By retaining the overall structure of the SNAREs we

also avoided problems of misfolding and aggregation. In contrast, attempts to use alternative cytoplasmic domains, composed only of GFP or multimeric myc epitope tags, resulted in proteins that either failed to be incorporated stably into membranes or were found in multiple locations.

TMD-dependent localization of proteins to the ER

A significant conclusion from the present study is that a signal within the Ufe1p TMD aids its retention in the ER. Previous work has clearly established that Golgi proteins in animal cells can be prevented from moving to the plasma membrane by their TMDs, and that Golgi retention is more dependent on the physical properties of the TMD, notably its length, than on any particular sequence. To account for this, it has been proposed that sorting is achieved by the partitioning of proteins between domains of the lipid bilayer that differ in their composition and hence thickness, the transition from a phospholipid-rich thin membrane to a sterol- and sphingolipid-rich thick membrane occurring somewhere in the Golgi complex (Bretscher and Munro, 1993; Munro, 1995). In contrast, it is generally assumed that the lipid composition of the earliest Golgi compartment is similar to that of the ER, and there is no consistent difference in TMD length between ER and Golgi proteins. This argues that sorting of proteins between the ER and the Golgi apparatus is unlikely to be determined entirely by TMD length, and indeed we find that reversal or randomization of the sequence of the Ufe1p TMD, without changing its length or composition, is sufficient to disrupt ER targeting.

This issue is complicated by the observation that lengthening the Ufe1p TMD does result in release from the ER, and a similar phenomenon has been reported for cytochrome b_5 in animal cells (Pedrazzini *et al.*, 1996). It seems likely, however, that such length changes affect the sequence-specific signal indirectly, for example a long TMD would allow movement of individual residues relative to the boundaries of the lipid bilayer and may also allow the protein to move to a compartment which lacks the machinery necessary for retrieval to the ER. A particular length may thus be required for the retention signal to function, rather than itself being the signal.

Why should the Ufe1p TMD be used as a sorting signal? It may be significant that the TMD is also important for t-SNARE function, quite apart from its targeting role. One helical face of this domain is relatively polar, and residues along this face are crucial for activity. This suggests that Ufe1p interacts with another membrane protein via its TMD, and recently we have obtained good evidence that this protein is Sec20p (Lewis *et al*., 1997). It is unlikely that Sec20p is responsible for sorting Ufe1p, however. Only a small proportion of Ufe1p is associated with Sec20p at any one time, and although the abundance of Sec20p is quite low, ER retention of Ufe1p is not easily saturated. More importantly, several TMD mutations that we tested abolished Ufe1p activity completely without disrupting the ER localization signal, and one Ufe1p mutant (U20) was almost completely mislocalized despite retaining some function. It seems that functional interaction with Sec20p is neither necessary nor sufficient for ER localization. Nevertheless, it could be that the physical properties of the Ufe1p TMD that promote its interaction with Sec20p—effectively a poor solubility in the lipid phase—also facilitate its recognition, when in a free state, by a sorting receptor that spans the membrane. This fits with observations in animal cells, where unassembled subunits of multimeric cell surface proteins have in several cases been found to be retained in the ER by a TMD-dependent mechanism (e.g. Bonifacino *et al.*, 1991; Reth *et al.*, 1991; Hennecke and Cosson, 1993).

In yeast, the only other well-characterized TMD-dependent ER retention signal is found in Sec12p and, while this paper was undergoing revision, a detailed study of the Sec12p system was published (Sato *et al.*, 1996; see also Boehm *et al*., 1994). The Sec12p TMD does not have a polar helical face, but it does contain polar residues that are important for ER retention. As with Ufe1p, the requirements for retention are not very stringent, a variety of amino acid substitutions being tolerated (Sato *et al.*, 1996). However, the precise mechanism of Sec12p retention appears different from that of Ufe1p, because it is dependent on the *RER1* gene, whereas deletion of this did not alter the retention activity of the Ufe1p TMD. Yeast cells may, therefore, contain multiple receptors that can recognize particular features of TMDs.

Whether retention of Ufe1p, like that of Sec12p, involves retrieval from the Golgi complex is unclear. Versions of the protein extended at the C-terminus with glycosylatable sequences showed little sign of Golgi modification, but we could not rule out the possibility of low levels of modification, or of efficient retrieval prior to modification (unpublished observations). Insights into the mechanisms involved may follow from a genetic analysis of the requirements for Ufe1p targeting.

TMD-dependent sorting between the plasma membrane and vacuolar pathways

Our work also shows that forms of Ufe1p that leave the ER are sorted between the endosome/vacuolar pathway and the plasma membrane by a mechanism that is again dependent on the TMD. Length clearly plays a major role in this sorting step, as TMDs of 24 or 26 residues target Ufe1p effectively to the plasma membrane. However, for shorter TMDs (22 residues), some less easily definable physical property becomes important, such as hydrophobicity and the nature of the amino acid side chains. The clearest demonstration of this is the construction of two Ufe1p-based chimeras (UUO2 and UUO1) whose TMDs are identical in length and differ at only six positions within the hydrophobic portion: one chimera is targeted to the vacuole and the other to the plasma membrane. The presence of two bulky phenylalanines in the centre of the TMD may explain the fate of the vacuole-targeted construct; such residues are under-represented in mammalian plasma membrane TMDs and they have been shown to reduce transport to the cell surface (Munro, 1995). In contrast to the ER sorting signal, plasma membrane localization does not seem to require a specific amino acid sequence or an asymmetrical distribution of residues across the membrane, since reversal of the Sso1p TMD had no effect on its targeting properties.

These results are entirely consistent with the lipid-based sorting model proposed for animal cells, but there are some significant differences. First, the TMD length needed to ensure transport to the cell surface (24 residues) is longer than that required in animal cells (21 residues),

and indeed the known yeast plasma membrane proteins have TMDs of at least 25 residues (S.Munro, personal communication). This probably reflects the lipid composition and hence thickness of yeast plasma membranes, and it may have important implications for experiments in which expression of mammalian cell surface proteins is attempted in yeast.

Second, proteins with shorter TMDs do not appear to be retained in the Golgi apparatus, but move to the endosome and/or vacuole. This may reflect a difference in the lipid composition of yeast vacuolar membranes as compared with mammalian endosomes and lysosomes. The latter are thought to have a sterol-rich composition similar to that of the plasma membrane, but vacuolar membranes are phospholipid-rich and have relatively low amounts of sterols (Zinser *et al.*, 1991, 1993). Thus, shorter TMDs may be tolerated in vacuoles but excluded from lysosomes.

Our results suggest that some previous studies of membrane protein sorting in yeast should be re-interpreted. It was proposed that in yeast cells membrane proteins lacking a positive sorting signal travel by default to the vacuole rather than to the plasma membrane. This suggestion was based on the analysis of chimeras between a Golgi and a vacuolar protein (dipeptidyl aminopeptidases A and B respectively); removal of a cytoplasmic signal from dipeptidyl aminopeptidase A caused it to move to the vacuole (Roberts *et al.*, 1992). In a subsequent study, Nothwehr *et al.* (1993) tested the role of the dipeptidyl aminopeptidase A TMD in this relocalization by replacing it with the synthetic sequence $L(LALV)_5$, and found that the construct still reached the vacuole. They concluded that no particular TMD sequence is required for transport to the vacuole, and that some special signal is required to reach the cell surface. Our interpretation is that the TMDs of the vacuolar and Golgi proteins tested, and the 21 residue synthetic sequence, lack the length or other features needed for transport to the plasma membrane.

It seems that in yeast the major role of the TMD is not to determine whether a protein is targeted to the later regions of the Golgi apparatus or to the vacuole, but to distinguish both of these locations from the plasma membrane. Retention in the Golgi apparatus or the endosome would then depend largely on other signals. It has been reported that the TMDs of the Golgi enzymes Mnt1p and Mnn1p influence their retention in the Golgi apparatus (Chapman and Munro, 1994; Graham and Krasnov, 1995), although others have found that retention of Mnt1p is more strongly dependent upon cytoplasmic sequences (Lussier *et al.*, 1995). Our results show that Pep12p contains a strong endosome targeting signal that is independent of its TMD, and cytoplasmic signals are well documented in the sorting of TGN proteins such as Kex2p and dipeptidyl aminopeptidase A (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993).

In conclusion, our studies indicate that t-SNAREs (and presumably other membrane proteins) can be sorted by their TMDs at at least two distinct steps in the yeast secretory pathway. Sorting to the plasma membrane is determined largely by TMD length, as in animal cells, and the data are consistent with a lipid-based mechanism. ER targeting is sequence dependent and probably involves a novel protein receptor. TMDs are not the only determinants of location; as has been observed with other proteins (e.g. Ponnambalam *et al.*, 1994; Sato *et al.*, 1996), Ufe1p and the other t-SNAREs use a combination of TMD-dependent and cytosolic signals to find their places in the cell.

Materials and methods

Yeast strains

All immunofluorescence was carried out in a SEY6210/6211 diploid strain (MAT**a**/MATα *ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 +/ade2-101 lys2-801/+*). Membrane fractionation was carried out in SEY6210 (MATα *ura3-52 his3-Δ200 leu2-3,112 trp1-Δ901 lys2-801 suc2-Δ9*). The *ufe1-1* strain used to test the function of Ufe1p chimeras was MLY101 [MATα *ura3-52 trp1-1 Δufe1::TRP1 leu2* containing pUT1 (*CEN6, LEU2, ufe1-1*)], kindly donated by Mike Lewis. The effect of the *rer1* mutation was tested in strain SKY7 (MATα *rer1::LEU2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 lys2 ade2*), the control being provided by the congenic strain SNY9 (MATα *mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 lys2 ade2*). Both strains were kindly provided by Akihiko Nakano.

Plasmids

Plasmids expressing t-SNARE chimeric constructs were all based on JS209 (a 2 μ vector carrying the *URA3* gene and the TPI promoter; Semenza *et al.*, 1990), but including the 10 amino acid c-myc epitope MEQKLISEEDLNS cloned between *Hin*dIII and *Eco*RI sites and upstream of a *Bam*HI site, and with a further upstream *Eco*RI site removed; henceforth referred to as JS209R (a generous gift from D.Banfield). GFP-tagged constructs were made by replacing the c-myc epitope with a modified form of GFP kindly provided by K.Siemering and J.Haseloff. SNARE coding regions were obtained from previous studies for *SED5* (Hardwick and Pelham, 1992) and *UFE1* (Lewis and Pelham, 1996). The *PEP12* coding region was a kind gift from Tom Stevens, and the *SSO1* coding region was cloned by PCR amplification from yeast genomic DNA. SNAREs were cloned into JS209R as an *Eco*RI–*Bam*HI fragment to N-terminally tag all constructs with either GFP or the c-myc epitope. Sites were added by PCR mutagenesis and all constructs checked by DNA sequencing. All Sft1p constructs were expressed from pRS416 (*CEN6, URA3*) under the control of the TPI promoter. The *SFT1* coding region was obtained from earlier studies (Banfield *et al.*, 1995) and cloned in the same manner to the t-SNAREs described above. Sft1p constructs were N-terminally tagged with three consecutive copies of the c-myc epitope.

To alter TMDs, *Hin*dIII or *Kpn*I sites were introduced adjacent to the TMD by site-directed mutagenesis and checked by DNA sequencing, facilitating simple TMD swaps. The *Hin*dIII site introduces the amino acids KL, and the *Kpn*I site introduces RYQ. For the constructs extending the wild-type Ufe1 TMD, an *Mlu*I site, encoding the residues KTR, was introduced downstream of the TMD, as shown in Table I. Some TMD regions with appropriate sites were obtained by PCR mutagenesis. Annealing of complementary oligonucleotides was used to create the reversed Sso1p TMD, both reversed and randomized Ufe1p TMDs and the chimeric Ufe1p–Sso1p TMDs. Site-directed mutagenesis was used to create the majority of modified Ufe1p TMDs. In all cases, changes were confirmed by DNA sequencing.

Immunofluorescence

Cells were fixed and mounted on slides as described by Hardwick and Pelham (1992). Antibody incubations were carried out in phosphate-buffered saline (PBS) + 2% dried milk, primary antibodies were incubated overnight at 4°C and secondary antibodies for 2 h at room temperature. A tissue culture supernatant of the mouse monoclonal anti-myc, 9E10 (Evan *et al.* 1985), rabbit anti-c-myc (Santa Cruz), rabbit anti-BiP antiserum (a kind gift from M.Rose), rabbit anti-plasma membrane ATPase (a kind gift from R.Serano) and mouse anti-vacuolar ATPase 69 kDa subunit (Molecular Probes Inc.) were all used as primary antibodies. Fluorescein- and Cy3-conjugated secondary antibodies (Amersham) were used for visualization and images obtained using an MRC-600 confocal laser scanning microscope (Bio-Rad). In all cases, dual images were taken using separate excitation at the appropriate wavelengths to avoid bleed through.

Membrane fractionation

To separate the plasma membrane from the ER, fractionation was carried out essentially as in Bowser and Novick (1991). Briefly, SEY6210 expressing the relevant plasmid was grown to an OD_{600} of 1.0 at 30°C. Cells were pelleted, washed once in 10 mM NaN_3, resuspended in spheroplast medium (50 mM Tris–HCl pH 7.5, 10 mM NaN_3, 1.4 M sorbitol, 40 mM β-mercaptoethanol, 0.125 mg/ml Zymolyase-100T) and incubated for 45 min at 30°C. Spheroplasts were pelleted, cooled on ice, resuspended in lysis buffer [0.8 M sorbitol, 20 mM triethanolamine pH 7.2, 1 mM EDTA containing a protease inhibitor cocktail of 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 μg/ml leupeptin, 2 mM benzamidine, 3 μg/ml pepstatin] and pelleted at 450 *g*. The resuspension and spin were repeated, and the two supernatants pooled, made 50 mM MES pH 6.5 and spun at 10 000 *g* for 10 min. This 10 000 *g* pellet was resuspended in 2.5 ml of 55% sucrose in 10 mM MES pH 6.5, placed at the bottom of a 14×95 mm centrifuge tube (Beckman Instruments, Inc.) and overlaid with the following sucrose solutions: 1 ml 50%, 1 ml 47.5%, 1.5 ml 45%, 1.5 ml 40%, 1 ml 37.5%, 1 ml 35%, 1.5 ml 30%, all containing 10 mM MES pH 6.5. The gradients were spun at 170 000 *g* for 16 h in a SW40Ti rotor (Beckman Instruments, Inc.). Twelve 1 ml fractions were collected from the top. One hundred μl of each sample was spun at 100 000 *g* for 30 min in a TLA100.2 rotor (Beckman Instruments, Inc.) to collect membranes for immunoblotting. ER–Golgi separation was carried out by differential centrifugation of log-phase yeast lysates, at 13 000 *g* and then at 100 000 *g*, as described by Lewis and Pelham (1996).

Immunoblotting

Membrane pellets isolated from both fractionation procedures were resuspended in SDS sample buffer (80 mM Tris–HCl pH 6.8, 8% glycerol, 2% SDS, 5% β-mercaptoethanol, bromophenol blue, protease inhibitors as above) and run on a 12.5 or 15% polyacrylamide gel. Proteins were transferred to nitrocellulose (0.45 μm, Schleicher and Schuell) using a TE70 semi-dry blotting unit (Hoefer) according to the manufacturer's instructions. Blocking and antibody incubations were carried out in PBS + 2% dried skimmed milk, with primary antibodies as described. Immunoreactive bands were identified using peroxidase-conjugated anti-rabbit and anti-mouse (Amersham) and detection was carried out using chemiluminescence (ECL kit, Amersham). Bands were quantified using a Molecular Dynamics densitometer.

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We have recently shown that changing residue Y5 to L is not sufficient to abolish the ER targeting signal in the Ufe1p TMD. This confirms that no single residue in the cytoplasmic half of the TMD is crucial for sorting. Furthermore, changing the residues TTYG to AAAA (rather than LLLL) did not prevent ER retention, suggesting that the signal depends more on relative hydrophilicity than on a specific sequence.