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Functionality and specific membrane localization of transport GTPases carrying C-terminal membrane anchors of synaptobrevin-like proteins

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Ras-related guanine nucleotide-binding proteins of the Ypt/Rab family fulfill a pivotal role in vesicular protein transport both in yeast and in mammalian cells. Proper functioning of these proteins involves their cycling between a GTP- and a GDP-bound state as well as their reversible association with specific membranes. Here we show that the yeast Ypt1 and Sec4 proteins, essential components of the vesicular transport machinery, allow unimpaired vesicular transport when permanently fixed to membranes by membrane-spanning domains replacing their two C-terminal cysteine residues. Membrane detachment of the GTPases therefore is not obligatory for transport vesicle docking to or fusion with an acceptor membrane. It was also found that the membrane anchors derived from different synaptobrevin-related proteins have targeting information and direct the chimeric GTPases to different cellular compartments, presumably from the endoplasmic reticulum via the secretory pathway.

Keywords: GTPase/Rab proteins/synaptobrevin/tail-anchored proteins/vesicular protein transport/Ypt

Introduction

A large number of structurally related GTPases, termed Rab in mammals and Ypt in yeast, act as regulators of endo- and exocytic protein transport reactions (Novick and Brennwald, 1993; Strom and Gallwitz, 1993; Zerial and Stenmark, 1993). Proper functioning of these proteins involves their cycling between a GTP- and a GDP-bound state, as well as their reversible association with specific membranes. Geranylgeranylation of C-terminally located cysteines mediates membrane attachment of Ypt/Rab GTPases (Farnsworth *et al.*, 1991; Khosravi-Far *et al.*, 1991; Magee and Newman, 1992). Proteins, called GDI (for guanosine-diphosphate dissociation inhibitor), detach GDP-bound Ypt/Rab proteins from cellular membranes (Araki *et al.*, 1990; Garrett *et al.*, 1994) and target them to defined donor membranes for a new functional cycle (Soldati *et al.*, 1994; Ullrich *et al.*, 1994).

Of the several transport GTPases identified in the yeast *Saccharomyces cerevisiae* (Strom and Gallwitz, 1993), Ypt1p and Sec4p function in the secretory pathway. Ypt1p is a component of the endoplasmic reticulum-to-Golgi transport machinery (Schmitt *et al.*, 1988; Segev *et al.*, 1988; Rexach and Schekman, 1991) and Sec4p is required for targeting Golgi-derived transport vesicles to the plasma membrane (Walworth *et al.*, 1989). Ypt1p and Sec4p are essential for cell viability (Schmitt *et al.*, 1986; Salminen and Novick, 1987).

It was shown previously that the deletion of the two C-terminal cysteines or their substitution with serine rendered the Ypt1 and Sec4 proteins non-functional and unable to bind to membranes (Molenaar *et al.*, 1988; Walworth *et al.*, 1989). Both proteins are isoprenylated by the same geranylgeranyl transferase (Jiang *et al.*, 1993) and are found in a membrane-bound and a soluble form (Molenaar *et al.*, 1988; Jiang *et al.*, 1993). In GDI-deficient yeast cells, the pool of soluble Ypt proteins becomes depleted and, as a consequence, cells lose their viability (Garrett *et al.*, 1994). As, in addition, Ypt/Rab proteins have not been observed in docking complexes isolated from yeast (Søgaard *et al.*, 1994) or mammalian cells (Söllner *et al.*, 1993), the question arises whether the detachment of GTPases from the membrane is a requirement for vesicle docking or membrane fusion.

To address this question, we constructed mutant forms of Ypt1p and Sec4p by replacing their C-terminal cysteines with the hydrophobic tail of either Sly2p (Sec22p), Snc1p or Snc2p, synaptobrevin-like, integral membrane proteins involved in ER-to-Golgi and Golgi-to-plasma membrane transport, respectively (Dascher *et al.*, 1991; Ossig *et al.*, 1991; Newman *et al.*, 1992; Protopopov *et al.*, 1993). Sly2p (Sec22p), Snc1p and Snc2p, like synaptobrevins of synaptic vesicles (Trimble *et al.*, 1988; Südhof *et al.*, 1989), are characterized by a C-terminal hydrophobic domain which serves as a membrane anchor such that the N-terminal part of these proteins faces the cytoplasm. We found that the permanently membrane-fixed GTPases retained their capacity to function in vesicular protein transport, showing that membrane detachment *per se* is not a requirement for vesicle docking to and fusion with the respective target membranes.

Results

Tail-anchored Ypt1 proteins are functional

Initially, two chimeric mutant GTPases were created, Ypt1–TM2p with the C-terminal membrane anchor of Sly2p (amino acids 190–214) and Ypt1–TM3p carrying the Snc1 membrane anchor (amino acids 94–117) instead of the C-terminal cysteine residues of Ypt1p. Both hybrid proteins, when expressed from the centromere-containing vector pRS315 (Sikorski and Hieter, 1989), allowed yeast cells depleted of wild-type Ypt1p to grow almost as efficiently as wild-type cells (generation time of 2 versus

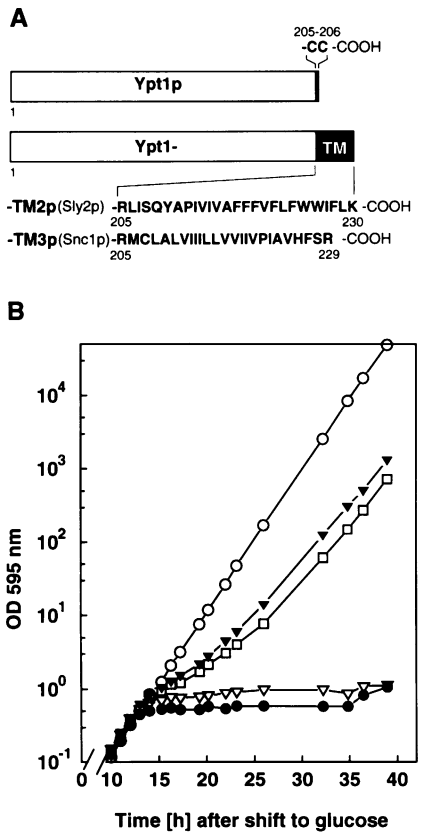

Fig. 1. Structure and functional analysis of membrane-anchored Ypt1 proteins. (**A**) Schematic representation of the structure of Ypt1p (upper part) and the chimeric Ypt1 transmembrane proteins (lower part). Membrane-spanning domains of Sly2p (Sec22p) or Snc1p are highlighted in black, C-terminal amino acid sequences of the proteins are shown in single-letter code. (**B**) Growth curves of yeast strains after switching off the *GAL10* promoter-controlled *YPT1* gene in glucose-containing medium. Individual strains carried different *YPT1* alleles, controlled by the *YPT1* promoter, on a centromeric plasmid: (○) wild-type *YPT1*; (▼) *YPT1–TM2*; (□) *YPT1–TM3*; (▽) *YPT1*ΔCC were transferred to YEP medium containing 8% glucose at 30°C. Cells of the same strain, transformed with the centromeric plasmid without insert (●), were used as a control.

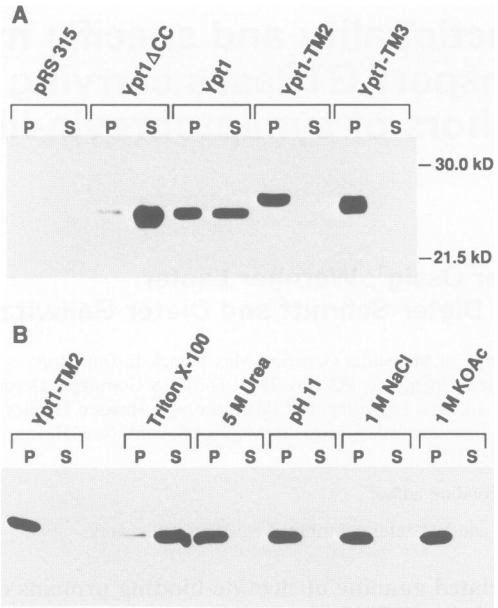

Fig. 2. Membrane localization of Ypt1–Sly2 and Ypt1–Snc1 hybrid proteins. (**A**) GFUI-6D cells carrying the centromeric plasmid pRS315 without insert or containing either the wild-type *YPT1* gene or mutant *YPT1* alleles as indicated were grown for 20 h in glucose medium to switch off chromosomal *YPT1* expression. Lysed cells were fractionated to obtain a crude membrane (P) and a soluble fraction (S), and Ypt1 proteins were detected by SDS–PAGE and immunoblotting as described in Materials and methods. Numbers to the right indicate positions of the molecular mass standards. (**B**) Cellular extracts of a *ypt1* deletion strain expressing Ypt1–TM2p from pRS315 were treated with the different reagents indicated prior to the separation of membrane and soluble fractions. Samples were processed as described above.

1.6 h). This was shown both in a yeast strain harboring the chromosomal *YPT1* gene under transcriptional control of the regulatable *GAL10* promoter (Figure 1B) and in a *ypt1* deletion strain. The vector without the insert or a recombinant plasmid expressing Ypt1p lacking the C-terminal cysteines did not sustain the growth of cells depleted of Ypt1p (Figure 1B).

To verify membrane binding of the chimeric proteins, soluble and membrane-bound proteins of yeast strains expressing either wild-type Ypt1p, Ypt1p lacking the C-terminal cysteines or Ypt1–Sly2 and Ypt1–Snc1 fusion proteins from centromere-containing plasmids were analyzed by Western blotting. Whereas the wild-type protein was distributed equally over the soluble and particulate fractions (i.e. 100 000 *g* supernatant and pellet), only traces of the non-functional Ypt1p lacking the C-terminal cysteines (Ypt1ΔCC) were pelletable. In contrast, Ypt1 proteins carrying the C-terminal membrane anchors were found exclusively in the pellet fraction (Figure 2A) and could be solubilized by treatment with detergent only (Figure 2B, shown for Ypt1–TM2).

Centromeric plasmids are limited to one to two copies per cell. We observed that, in comparison with the amount of Ypt1p in wild-type cells, *ypt1* deletion strains expressing the membrane-fixed Ypt1 proteins from a centromeric plasmid (Ypt1–TM2p or Ypt1–TM3p) exhibited moderately elevated levels (~2-fold) of these mutant GTPases. We also found that more than one copy of the gene encoding Ypt1–TM2p had to be integrated into the genome to obtain viable haploid strains (data not shown). This gene dosage effect could be explained either by a reduced stability or a restricted functionality of the mutant Ypt1 proteins due to their inability to cycle between a membrane-associated and a soluble form. To distinguish between these possibilities, the genes encoding the wild-type or the mutant Ypt1–TM2 protein were placed under transcriptional control of the *GAL10* promoter and the growth properties of yeast cells carrying the different genes on chromosome VI were followed after promoter switch-off in glucose-containing media. As already observed previously (Schmitt *et al.*, 1986), depletion of wild-type Ypt1p allowed unimpaired cell growth for up to 12 h. In contrast, depletion of Ypt1–TM2p led to an inhibition of cellular growth already after 3–4 h (Figure 3A). More importantly, the kinetics of the disappearance of wild-type Ypt1p and of the permanently membrane-anchored Ypt1–TM2 protein were comparable, and significant amounts of the mutant protein could still be detected several hours after the cessation of cellular proliferation (Figure 3B). This argues for an impaired functional efficiency rather than a reduced half-life of the mutant as compared with the wild-type Ypt1 protein.

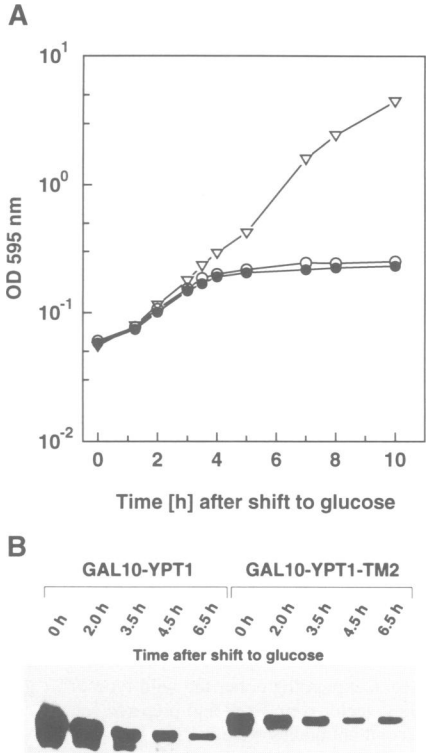

Fig. 3. (**A**) Growth curves of yeast strains, carrying the *GAL10* promoter-controlled *YPT1* wild-type (▽) or mutant *YPT1–TM2* genes (○, ●) at the *YPT1* locus of chromosome VI. Galactose-grown cells were transferred to medium containing glucose as carbon source at time zero. (**B**) Western blot analysis using polyclonal anti-Ypt1p antibodies as described in the legend to Figure 2 to verify the depletion of Ypt1p and Ypt1–TM2p after different periods of time (h) following the *GAL10* promoter switch-off in yeast strains shown in (A).

Tail-anchored Ypt1 proteins allow unimpaired invertase secretion

Invertase, induced in low glucose medium, is a commonly used marker protein in studies of the yeast secretory pathway. The secreted form of invertase is core-glycosylated in the ER, further glycosylated during its passage through the Golgi compartments and is then transported to the periplasmic space.

To assess whether the membrane-anchored Ypt1 proteins performed the proper function in ER-to-Golgi protein transport, glycosylation and secretion of invertase were followed in cells expressing either the wild-type or one of the two chimeric Ypt1 proteins only. As can be seen in Figure 4, permanent membrane anchoring of Ypt1p did not impair its function significantly, either in the early or in later steps of the secretory pathway: highly glycosylated invertase was secreted and there was no indication of intracellular accumulation of the core-glycosylated ER forms of the enzyme. In contrast, ER forms of invertase could be found to accumulate intracellularly in Ypt1p-depleted cells (Figure 4, lanes 1 and 2).

The membrane-spanning domains of synaptobrevin-like proteins have targeting information

Sly2p (Sec22p) has been localized to the ER (our unpublished observations) and to ER-derived transport vesicles

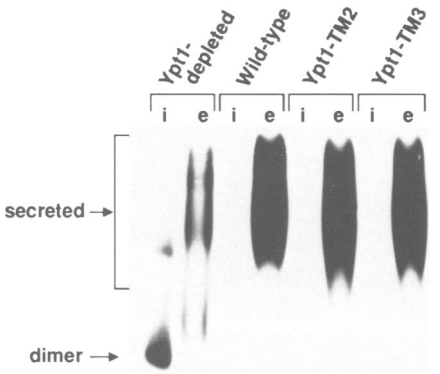

Fig. 4. Glycosylation and secretion of invertase in yeast cells with membrane-anchored Ypt1 proteins. Cells of a *ypt1* deletion strain carrying either *YPT1* (wild-type), *YPT1–TM2* or *YPT1–TM3* on a centromere-containing plasmid were derepressed for synthesis of secreted invertase. As a control, yeast cells expressing the chromosomal *YPT1* allele under transcriptional control of the *GAL10* promoter were depleted of Ypt1p by transfer to glucose-containing medium (25°C) 12 h before invertase derepression. Intracellular (i) and periplasmic (e) invertase were separated and extracts were treated as described in Materials and methods. Invertase activity was monitored after protein separation in a non-denaturing 6.5% polyacrylamide gel.

(Lian and Ferro-Novick, 1993; Rexach *et al.*, 1994), but Snc1p resides on Golgi-derived vesicles and on the plasma membrane (Protopopov *et al.*, 1993). From the proper functioning of the chimeric Ypt1–Sly2 and Ypt1–Snc1 proteins in ER-to-Golgi transport it might be concluded that the membrane anchors of Sly2p and Snc1p direct the chimeric proteins indiscriminately to any cellular membrane, including that of the ER and of ER-derived transport vesicles where Ypt1p is known to act. However, it might also be that proteins carrying the C-terminal membrane anchors of Sly2p, Snc1p and other related type II membrane proteins are first inserted into the ER, and from there reach their final destination (Jäntti *et al.*, 1994; Kutay *et al.*, 1995).

We therefore sought to determine the intracellular localization of the chimeric GTPases by indirect immunofluorescence using affinity-purified polyclonal anti-Ypt1p antibodies. We found that the localization of the two membrane-fixed proteins is not random. Both hybrid proteins, Ypt1–TM2p in particular, appeared to reside in the ER membrane, as evidenced by the staining of the nuclear envelope (Figure 5C and E). However, a significant fraction of the Ypt1–Snc1 protein, like Snc1p itself, is clearly attached to the plasma membrane but the Ypt1–Sly2 protein is not. Wild-type Ypt1p, as has been seen previously (Segev *et al.*, 1988), gave a more punctate staining pattern (Figure 5A) which appears to result from vesicular and Golgi localization.

Despite the limitations of this procedure, it seems likely from the results obtained that the membrane anchors of Sly2p and Snc1p by themselves contribute to specific membrane localization of the chimeric Ypt1 proteins.

Tail-anchored Sec4 protein is functionally active depending on the origin of the membrane-spanning domain

Do transport GTPases other than Ypt1p retain their regulatory function when permanently fixed to membranes?

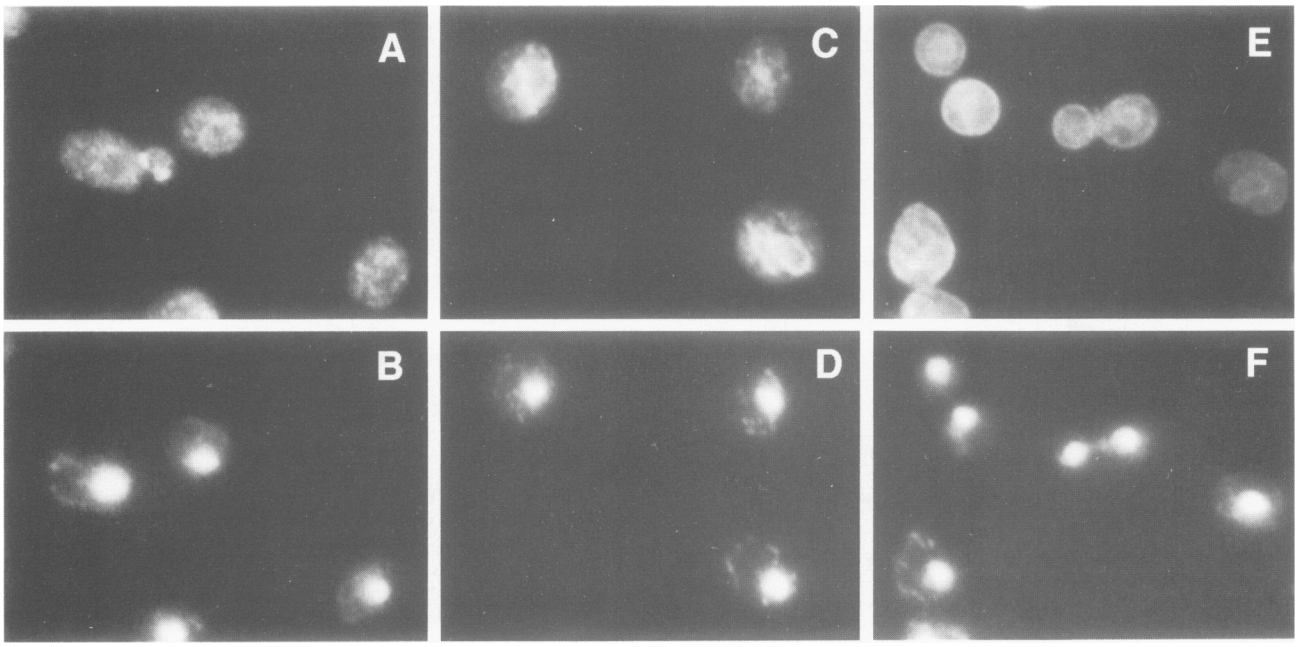

Fig. 5. Indirect immunofluorescence to localize wild-type and chimeric Ypt1 proteins. Yeast cells expressing either the wild-type *YPT1* gene (**A** and **B**) on a centromeric vector or *YPT1–TM2* (**C** and **D**) and *YPT1–TM3* (**E** and **F**) on a multicopy vector were fixed and prepared for immunofluorescence using an affinity-purified anti-Ypt1p antibody. Rhodamine-conjugated second antibodies were used to visualize the different Ypt1 proteins (A, C and E). DAPI staining of DNA was used to localize the nuclei (B, D and F).

To answer this question, Sec4p was chosen because it is essential for yeast cell viability and is involved in a late step of the secretory pathway (Walworth *et al.*, 1989). It was reasoned that a study of tail-anchored Sec4 GTPases could, in addition, be helpful to substantiate the assumption that the C-terminal domains of various synaptobrevin-like proteins direct the chimeric GTPases to the ER membrane from where they would reach their final destination.

As outlined in Figure 6, the C-terminal cysteines of Sec4p were deleted and replaced by either the membrane anchor of Sly2p/Sec22p (C-terminal 23 amino acids) or that of Snc2p (C-terminal 21 or 31 amino acids). Snc2p was chosen to see whether its membrane anchor, whose primary sequence is similar to that of Snc1p (compare Figures 1 and 6), would also direct fusion GTPases to the plasma membrane. A tag of 15 amino acid residues derived from the Sendai virus L-protein, flanked by short glycine- and proline-containing linkers, was placed between the Sec4p and the Sly2p- and Snc2p-derived sequences. This allowed the easy identification of the chimeric GTPases by Western blotting and indirect immunofluorescence, as a monoclonal antibody against this epitope is available (Einberger *et al.*, 1990).

Wild-type *SEC4* and the different mutant genes were integrated into centromere-containing and 2μ-based multicopy vectors. The empty or recombinant vectors were transformed into either a yeast strain with the chromosomal *SEC4* gene under transcriptional control of the *GAL10* promoter or into a strain with a temperature-sensitive *sec4* allele. The functionality of the membrane-fixed GTPases could thus be investigated in cells depleted of wild-type Sec4p or in cells with a defective Sec4 protein at the non-permissive temperature. Under both conditions, the Sec4–Snc2 proteins (Sec4–TM3p and Sec4–TM4p) but not the Sec4–Sly2 protein (Sec4–TM2p) proved to be functional

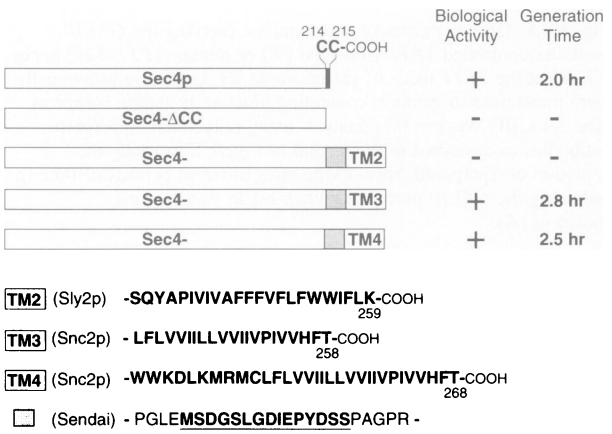

Fig. 6. Structure and functional analysis of membrane-anchored Sec4 proteins. Schematic representation of the structure of Sec4p, Sec4ΔCCp and the chimeric Sec4 transmembrane proteins. The Sendai virus protein tag placed between the Sec4p sequences and the membrane-spanning domains of Sly2p (Sec22p) or Snc2p are highlighted in black. The C-terminal amino acid sequences of the membrane-spanning domains are shown in single-letter code. The 15 amino acid residues of the Sendai virus protein tag are underlined and flanked by short linker sequences. The hybrid genes expressed from multicopy vectors allowing cellular growth are marked by (+). The generation times given are for strains depleted of wild-type Sec4p but producing the proteins shown to the left.

(Figure 7A and B). In contrast to the tail-anchored Ypt1 proteins, the membrane-fixed Sec4 proteins had to be expressed from a multicopy vector in order to rescue Sec4p-depleted cells and *sec4-2* mutant cells at the restrictive temperature. This was not due to the Sendai virus epitope as the chimeric Sec4 proteins lacking the viral epitope also required high expression from a multicopy plasmid to substitute for wild-type Sec4p function. Further-

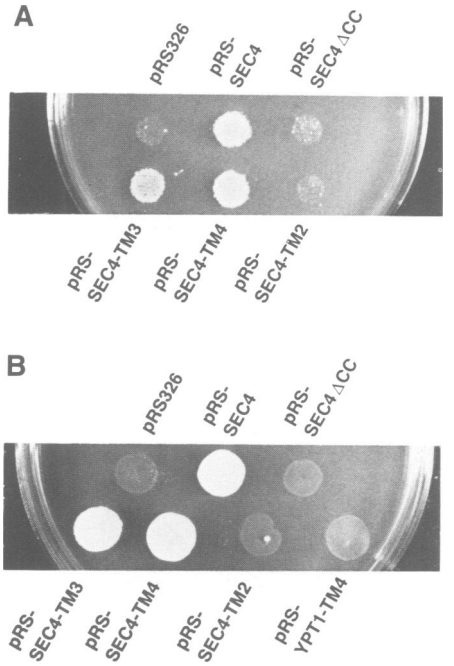

Fig. 7. Growth assay on YEPD plates. Yeast cells expressing either the chromosomal *SEC4* gene under the control of *GAL10* promoter (**A**) or *sec4-2* mutant cells (**B**) carried the vectors indicated. Cells were spotted onto agar plates with glucose as carbon source. The plates were either incubated at 30°C and replica-plated twice (A) or incubated for 2 days at the non-permissive temperature of 37°C (B).

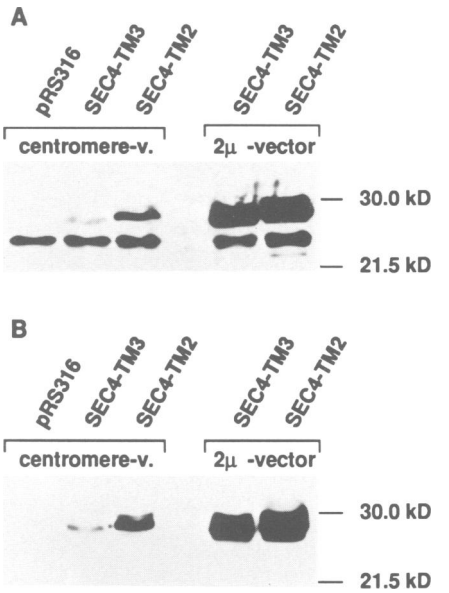

Fig. 8. Immunoblot analysis of the chimeric Sec4 proteins. *SEC4–TM3* and *SEC4–TM2* were expressed from the centromeric vector pRS316 (lanes 2 and 3) or from the multicopy vector pRS326 (lanes 4 and 5) in a wild-type strain (GFUI-5B). After cell lysis, crude protein extracts were separated by SDS–PAGE and subjected to Western blot analysis to show the relative amounts of wild-type Sec4p (faster moving bands in A) and the tail-anchored Sec4 proteins. Sec4 proteins were identified using (**A**) anti-Sec4p antibodies or (**B**) monoclonal anti-Sendai tag antibodies. As a control, lanes 1 of (A) and (B) display extracts of cells transformed with the pRS316 without insert. The position and molecular mass (kDa) of reference proteins is indicated to the right.

more, Ypt1–Sly2 and Ypt1–Snc2 fusion proteins containing the virus epitope sequence proximal to the membrane-spanning domain could be expressed from a single copy vector to allow yeast cells depleted of Ypt1p to grow (data not shown). As judged from the generation time of the different yeast strains, the Sec4–TM4p with the longer Snc2p C-terminus seemed to be somewhat more potent as the substitute for wild-type Sec4p than was Sec4–TM3p (Figure 6). As expected, Sec4p without its C-terminal cysteine residues (Sec4ΔCC) or a Ypt1–Snc2 fusion protein (Ypt1–TM4p) did not permit *sec4* mutant cells to grow (Figure 7A and B).

To assess to what extent the tail-anchored Sec4 GTPases were synthesized in wild-type cells transformed with the recombinant single- and multicopy vectors, a Western blot analysis was performed with total cellular proteins and anti-Sec4p (Figure 8A) or anti-Sendai epitope (Figure 8B) antibodies. From the centromere-containing vector, Sec4-TM3p, the functional membrane-fixed GTPase appeared to be present in smaller amounts than Sec4 wild-type protein expressed from the chromosomal gene. Most importantly, the non-functional Sec4–Sly2 fusion protein (Sec4–TM2p) apparently reached even higher levels than the functional Sec4–TM3p, showing that it is the membrane-spanning domain and the cellular location which decide on the functionality of the chimeric GTPases.

Differential localization of Sec4–Snc2 and Sec4–Sly2 fusion proteins

Having shown that the Sec4–Snc2 protein can substitute for wild-type Sec4p function but that Sec4–Sly2 protein cannot, it might be concluded that Sec4–Sly2p does not reach the scene of action of Sec4p, the post-Golgi transport vesicles and the plasma membrane.

As in the case of the tail-anchored Ypt1 proteins, indirect immunofluorescence was performed to localize the Sec4 fusion proteins. The Sec4–Snc2p, like the Ypt1–Snc1p (Figure 5E) and the Ypt1–Snc2p (data not shown), was clearly seen on the plasma membrane, but the Sec4–Sly2p, like the Ypt1–Sly2p (Figure 5C), was not (Figure 9C). Instead the latter was localized primarily on intracellular structures often surrounding the nucleus (Figure 9C). This shows again that the C-terminal membrane anchors determine the intracellular localization of the fusion GTPases.

Discussion

The precise mode of action of guanine nucleotide binding proteins involved in vesicular protein traffic is presently unknown. It has been argued that small GTPases might ensure unidirectionality of transport vesicle movement through successive membrane-enclosed compartments either by directly participating in vesicle docking to specific target membranes (Bourne, 1988; Walworth *et al.*, 1989) or by catalyzing the assembly of docking/fusion complexes (Rothman, 1994; Søgaard *et al.*, 1994). Whatever the exact role of Ypt/Rab GTPases might be, their shuttling between a membrane-bound and a soluble state is regarded to be a requirement for proper function (Garrett *et al.*, 1994; Soldati *et al.*, 1994; Ullrich *et al.*, 1994).

The main conclusion from the results we have presented here is that such a cycling of GTPases between a mem-

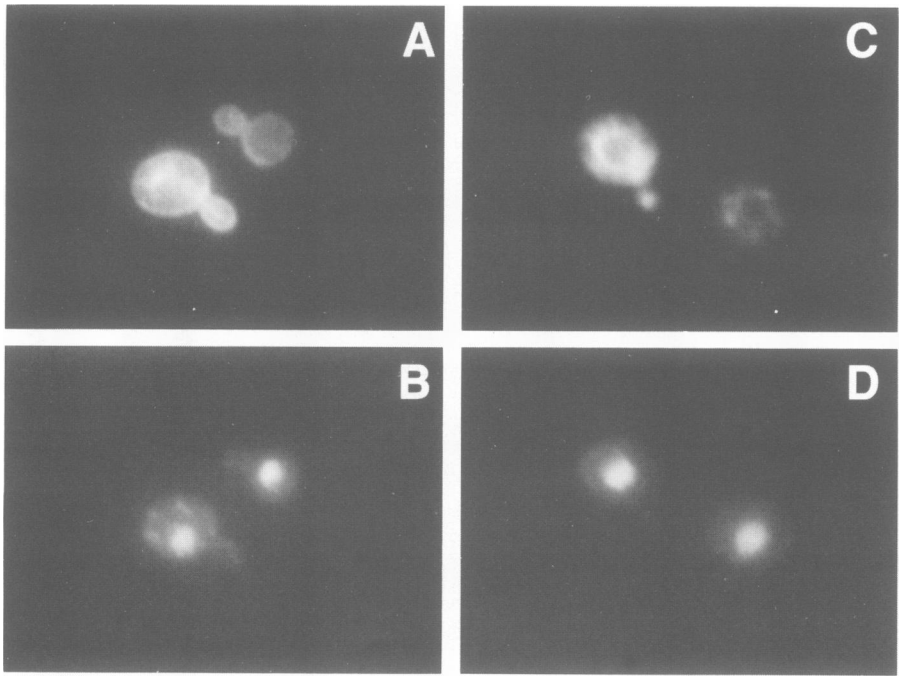

Fig. 9. Indirect immunofluorescence, tracing the cellular localization of the chimeric Sec4 proteins. Wild-type yeast cells expressing either *SEC4–TM3* (**A** and **B**) or *SEC4–TM2* (**C** and **D**) on a multicopy vector were fixed and prepared for immunofluorescence (see Materials and methods). The hybrid Sec4 proteins were labeled with monoclonal anti-Sendai tag antibodies and indocarbocyanin-conjugated secondary antibodies (A and C) while staining of DNA with DAPI was used to image the localization of the nuclei (B and D).

brane-bound and a cytoplasmic form in fact is not obligatory, although an elaborate and essential machinery for membrane association (geranylgeranyl transferase) and dissociation (GDI) of GTPases exists. We have shown this to be true for two essential GTPases, Ypt1p and Sec4p, which were permanently membrane-fixed with different C-terminally located hydrophobic membrane anchors. These were derived from synaptobrevin-related type II membrane proteins, Sly2p (Sec22p), Snc1p and Snc2p, which in part reside on ER-to-Golgi and post-Golgi transport vesicles, respectively (Lian and Ferro-Novick, 1993; Protopopov *et al.*, 1993; Rexach *et al.*, 1994). Synaptobrevin and its yeast counterparts are likely to perform a receptor-like function, as so-called v-SNAREs (Söllner *et al.*, 1993), in pairing with specific type II membrane proteins of the target membranes, designated t-SNAREs. The C-terminal membrane anchor of synaptobrevin has previously been shown to span the synaptic vesicle membrane in its entirety (Südhof *et al.*, 1989), and it is reasonable to assume that other tail-anchored proteins are permanent membrane residents as well. In keeping with this assumption, the tail-anchored GTPases studied here could be solubilized from cellular membranes only by treatment with detergents, and evidence for the existence of a cytoplasmic pool of GTPases fused to the C-terminal hydrophobic domains was not obtained. As the permanently membrane-anchored Ypt1 and Sec4 proteins allowed cellular growth and protein secretion without significant impairment, we assume that these structurally altered regulators retain their ability to interact with other components of the transport machinery, including nucleotide exchange factors and GTPase activators, and act as a 'molecular switch'. In particular, permanent membrane fixation of the GTPases does not appear to impede transport vesicle docking to or fusion with the correct target membrane, as shown by the largely unaffected glycosylation and secretion of invertase in cells relying solely on a transmembrane version of the essential Ypt1 GTPase.

What then is the function of GDI which removes the GDP-bound form of small GTPases from membranes (Araki *et al.*, 1990; Ullrich *et al.*, 1993) and which has been shown to be an essential protein for yeast cell viability (Garrett *et al.*, 1994)? It seems most likely that the reversibility of membrane association of transport GTPases is a device to enhance the efficiency of these regulator proteins by allowing their recruitment for several functional cycles (Figure 10). The ability of GDI to detach GDP-bound Ypt/Rab proteins from target membranes rather unspecifically (Ullrich *et al.*, 1993; Garrett *et al.*, 1994) and to assist their reassociation with (donor) membranes concomitantly with the required GDP→GTP exchange (Soldati *et al.*, 1994; Ullrich *et al.*, 1994) would, in addition, allow a vectorial use of the activated GTPases. The latter argument, in principle, might also apply to the functioning of a permanently membrane-bound GTPase provided the tail anchor fused to it would allow its cycling between a donor and an acceptor compartment (ER-derived vesicle→Golgi→Golgi-derived vesicle→ER, for instance). The results we obtained with a Ypt1 protein carrying the membrane-spanning domain of Sly2/Sec22p suggest that the membrane-fixed GTPase is functionally less efficient than the wild-type Ypt1 protein. This argument is based on the observation that the intracellular level of the Ypt1–Sly2 chimeric protein had to be higher than that of wild-type Ypt1p to allow efficient cellular growth, although Sly2p might potentially cycle between the ER and the Golgi and a significant amount of the

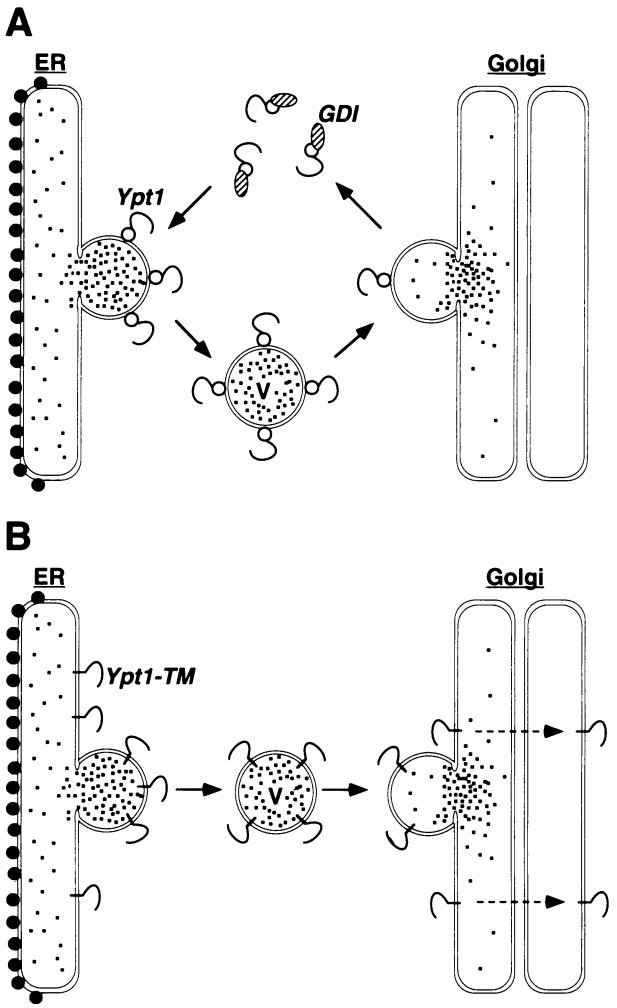

Fig. 10. Schematic representation of membrane association of Ypt1p during vesicular transport between the ER and the Golgi. (**A**) Wild-type Ypt1p in its GTP-bound form (Ypt1) is thought to associate specifically with ER-derived vesicles (V) during or after vesicle budding. This membrane association of Ypt1p is mediated by its isoprenylated C-terminal cysteine residues (white circle). After vesicle docking to or its fusion with the *cis*-Golgi membrane, the GDP-bound form of the protein is dissociated from the membrane through its interaction with GDI protein (striped ovals), ready for a new targeting cycle. (**B**) It is assumed that Ypt1–Sly2 as well as Ypt1–Snc1 hybrid proteins (Ypt1–TM), by means of their C-terminal membrane-spanning domains, are inserted into the ER membrane. As components of the transport vesicle membrane, they are functional in proper vesicle targeting to the *cis*-Golgi. Depending on the origin of the transmembrane domain, the chimeric Ypt1 protein either proceeds through the following compartments of the secretory pathway (indicated by an arrow) or it might recycle back to the ER. The same holds for the Sec4 transmembrane proteins, explaining why certain chimeric GTPases are functionally active and others are not.

chimeric GTPase, like Sly2p itself (our unpublished observation), appeared to reside on ER membranes.

In studying the functionality of the membrane-fixed Ypt1 and Sec4 proteins, we made the observation that the final intracellular localization of the different chimeric GTPases is not random and depends on the tail anchor they carry. More importantly, the membrane-spanning domains of the synaptobrevin-related proteins directed the hybrid GTPases primarily to those membrane compartments where the parent type II membrane proteins reside. That transmembrane domains of tail-anchored yeast proteins belonging to the syntaxin family have targeting information when studied in mammalian cells has been shown recently (Banfield *et al.*, 1994; Jäntti *et al.*, 1994). In addition to presenting evidence for targeting information of the C-terminal transmembrane domains of yeast proteins that are members of the synaptobrevin family, we would conclude from our studies that the tail-anchored GTPases are inserted into the ER and travel along the secretory pathway to their final destination. This is a realistic assumption as the Ypt1–Snc proteins (Ypt1–Snc1p and Ypt1–Snc2p), which reside predominantly in the plasma membrane, are functionally active in ER-to-Golgi transport, whereas a Sec4–Sly2 hybrid protein cannot substitute for Sec4p function because it does not appear to reach the plasma membrane. This would also be compatible with the recent studies of Kutay *et al.* (1995) showing that, in mammalian cells, synaptobrevin is post-translationally inserted into the ER by an SRP-independent mechanism and is then transported through the compartments of the secretory pathway to become a resident of synaptic vesicles.

Although a significant part of the chimeric GTPases appears to be localized in a compartment-specific manner (suggested by indirect immunofluorescence), it is quite possible that membrane association of a fraction of these GTPases occurs at random. This then might be another reason for the need for their accelerated production to allow efficient cellular growth.

As Ypt/Rab proteins have not been found in isolated multimeric docking complexes, perhaps because of their transient association with one or several components of such complexes, the membrane-fixed GTPases might prove helpful in identifying interacting partners.

Materials and methods

Yeast genetic techniques

Yeast strains used in this study were: GFUI-6D: *MAT***a** *GAL10-YPT1::HIS3 ura3 leu2 his3 trp1*, GFUI-5B: *MAT*α *ura3 leu2 his3 trp1*, GFSI-5B: *MAT*α *GAL10-SEC4::LEU2 ura3 leu2 his3 trp1* and RH220-7D: *MAT*α *sec4-2 ura3 leu2 his4 lys2* (H.Riezman, Basel).

Yeast media were prepared essentially as described by Sherman *et al.* (1986). For induction or repression of the *GAL10* promoter either 2% galactose (YEPGal) or 8% glucose (YEPD) was added to YEP media (2% Bacto-peptone, 1% Bacto-yeast extract). Transformants were selected on SD agar plates containing 0.67% Difco yeast nitrogen base, supplemented either with peptone 140 (GIBCO-BRL) for selecting Ura^+ transformants or with a mixture of appropriate amino acids + uracil to select Leu^+ transformants. 2% glucose (SD-medium) or 2% galactose (SD-Gal medium) were added as carbon sources.

Yeast transformation and genetic manipulations were performed following established protocols (Ito *et al.*, 1983; Sherman *et al.*, 1986).

Nucleic acids techniques

DNA manipulations were performed according to Sambrook *et al.* (1989). To create the hybrid gene *YPT1–TM2*, plasmid pUC81-YPT1 carrying the *S.cerevisiae YPT1* gene (Gallwitz *et al.*, 1983) as a 1.1 kb *Bam*HI fragment was cut with *Age*I and *Bss*HII to remove a 25 bp fragment (ranging from 17 bp 5′ to 5 bp 3′ of the stop codon). A chemically synthesized 96 bp fragment was then inserted, thus fusing codon 204 of *YPT1* with codons 190–214 of *SLY2*. The two parts of the chimeric gene were joined by an additional arginine codon. To create *YPT1–TM3*, a *Sal*I–*Bss*HII DNA fragment extending from codon 205 to 4 bp downstream of the stop codon of the *YPT1–TM2* sequences was replaced by a 83 bp long synthetic oligonucleotide encoding the C-terminal 24 residues of Snc1p. To create the *YPT1–TM4* hybrid gene, a 90 bp DNA fragment was removed from *YPT1–TM2* by cleaving with *Sal*I and *Bss*HII. This short *SLY2*-derived segment was then replaced by

a *Sma*I–*Bss*HII fragment consisting of *YPT1* (up to codon 204), the Sendai virus tag and the last 31 codons of the *SNC2* gene (see below).

The genes encoding wild-type Ypt1p, the Ypt1–TM proteins and Ypt1p lacking the C-terminal cysteines (Ypt1ΔCC) (Molenaar *et al.*, 1988) were then subcloned into the *Bam*HI site of the centromeric vector pRS315 (CEN6-ARSH6, *LEU2*; Sikorski and Hieter, 1989) or the multicopy vector YEp511 (2μ, *LEU2*; Dascher *et al.*, 1991), respectively.

A strain expressing *YPT1–TM2* under control of the *GAL10* promoter was obtained by replacing the chromosomal *GAL10*-controlled *YPT1::HIS3* sequence in GFUI-6D by a *YPT1–TM2::LEU2* fragment. *Ypt1* deletion strains were obtained as described (Schmitt *et al.*, 1988).

Using standard PCR techniques, the *SEC4* gene was isolated as a 1.4 kb *Kpn*I–*Eco*RI fragment and cloned into the centromeric vector pRS316 (CEN6-ARSH6, *URA3*; Sikorski and Hieter, 1989) or the multicopy vector pRS326 (CEN6-ARSH6, *URA3*, 2μ; Strom *et al.*, 1993). To produce Sec4p lacking the C-terminal cysteine residues, codon 214 of *SEC4* (TGC) was replaced by an amber codon. Site-directed mutagenesis (Kunkel *et al.*, 1987) was used to introduce this mutation in pRS-SEC4 thus yielding pRS-SEC4ΔCC.

For the convenient insertion of sequences encoding membrane-spanning domains, a novel *Sma*I restriction site was created 3′ of codon 213 of the *SEC4* gene (pRS-SEC4ΔCC-Sma). To create the hybrid genes *SEC4–TM3* and *SEC4–TM4*, a 420 bp *Sma*I–*Sac*I fragment of pRS-SEC4ΔCC-Sma was replaced by *Sma*I–*Sac*I fragments containing 3′ parts of the *SNC2* gene. These fragments had been amplified by PCR, thereby creating additional *Sma*I and *Sac*II sites in front of the amplified *SNC2* region and a *Sac*I site in the 3′ non-coding region (120 bp downstream of the stop codon). The two inserts contained either the last 21 (pRS-SEC4-TM3) or 31 (pRS-SEC4-TM4) codons of the *SNC2* gene. For easier detection of the hybrid proteins, a chemically synthesized 66 bp *Sma*I–*Sac*II fragment coding for a tag of 15 amino acids of the Sendai virus L-protein (Einberger *et al.*, 1990), flanked by short glycine- and proline-containing linkers, was inserted between the *SEC4* and *SNC2* parts of the fusion genes (Figure 6). To construct the *SEC4–TM2* hybrid gene, an 81 bp *Eco*RV–*Bss*HII fragment obtained from *YPT1–TM2* was inserted into the *Sac*II site of the *SEC4–TM3* construct, which resulted in the replacement of the Snc2p membrane anchor by the Sly2p membrane-spanning domain (amino acid residues 192–214) of the fusion gene-encoded protein.

pRS-GAL10-SEC4 was obtained as follows. New restriction sites (*Sma*I, *Sal*I) were created close to the start codon of *SEC4* (pRS-SEC4). A 2.7 kb *Nde*I–*Sal*I fragment derived from YEp51 (2μ, *LEU2*; Broach *et al.*, 1983), containing the *LEU2* gene and the *GAL10* promoter, was inserted into these sites. A linear *Apa*I–*Eco*RI fragment was used to replace the *SEC4* gene of strain GFUI-5B by the *GAL10–SEC4::LEU2* fusion to generate strain GFSI-5B. The correct integration of these sequences was confirmed by Southern blot analysis.

Membrane localization of Ypt1–Sly2 and Ypt1–Snc1 hybrid proteins

Cells of strain GFUI-6D (*GAL10–YPT1*) carrying, in addition, the wild-type *YPT1* or one of the mutant alleles on a centromeric plasmid (pRS315), or the vector without insert, were grown to mid-logarithmic phase in selective galactose-containing medium. Twenty hours after shift to YEP medium containing 8% glucose, cells were broken with glass beads and a 500 g supernatant was centrifuged again at 100 000 *g* for 60 min to obtain a crude membrane and a soluble fraction (Ossig *et al.*, 1991). Ypt1 wild-type and mutant proteins were identified by Western blotting using an affinity-purified antibody raised in rabbits against bacterially produced Ypt1p. For detection of immobilized antigens, the ECL system (Amersham) was used.

To confirm tight membrane association of hybrid proteins, the cellular extracts of a *ypt1* deletion strain expressing Ypt1–TM2p from the centromeric plasmid pRS315 were treated for 30 min either with 1% Triton X-100, 5 M urea, 0.1 M sodium carbonate (pH 11), 1 M sodium chloride or 1 M potassium acetate, centrifuged to obtain the 100 000 *g* pellet and supernatant and subjected to immunoblot analysis (Ossig *et al.*, 1991).

Western blot analysis of the wild-type and chimeric Sec4 proteins

Wild-type cells (GFUI-5B) transformed with vector pRS316 or with pRS316 containing different *SEC4* hybrid alleles were grown to mid-logarithmic phase in selective medium. Extracts were prepared as described by Boehm *et al.* (1994). Solubilized proteins were separated by SDS–PAGE and transferred to nitrocellulose filters. Protein detection was performed using monoclonal antibodies raised against Sec4p or the Sendai virus protein tag and peroxidase-labeled secondary anti-mouse IgG following enhanced chemiluminescence (ECL system, Amersham).

Glycosylation and secretion of invertase

Cells of a *ypt1* deletion strain carrying either *YPT1* (wild-type), *YPT1–TM2* or *YPT1–TM3* on a centromere-containing plasmid were grown to mid-logarithmic phase. Synthesis of the secreted form of invertase was induced in YEP medium containing 0.1% glucose for 1.5 h at 30°C. In addition, GFUI-6D cells were shifted from galactose-containing medium to YEPD medium (25°C) 12 h before invertase derepression. Spheroplasts were prepared with lyticase (Sigma) and pelleted by centrifugation to separate intracellular and periplasmic invertase. After lysis of spheroplasts (Ossig *et al.*, 1991), samples of each fraction were heated at 50°C for 5 min to dissociate invertase oligomers (Baker and Schekman, 1989) and subjected to electrophoresis in a non-denaturing 6.5% polyacrylamide gel. Invertase was identified by activity staining (Baker and Schekman, 1989).

Growth assays

Growth of the different yeast transformants was followed at 30°C by measuring the optical density at 595 nm. Cells growing in selective galactose medium to the mid-logarithmic phase were harvested, washed in YEP medium and transferred to YEP medium containing 8% glucose to start the growth curves. After reaching a cell density equivalent to 1–3 OD_{595} units, cells were diluted into fresh YEPD.

For growth assays on plates, the galactose-grown transformants were washed three times with 1 M sorbitol, and 10 μl cells per strain equivalent to ~50 000 cells were spotted onto YEPD plates (2% glucose) and incubated for 3 days. During this time, plates were replica-plated twice to YEPD.

RH220-7D (*sec4-2*) transformants were incubated in selective medium at 25°C to mid-log phase. Cells were spotted onto YEPD plates as described above and incubated at a non-permissive temperature of 37°C for 2 days.

Indirect immunofluorescence

Yeast cells were fixed with 3.7% formaldehyde and prepared for immunofluorescence essentially as described by Kilmartin and Adams (1984). Fixed spheroplasts on slides were labeled either with affinity-purified anti-Ypt1p antibodies and rhodamine-conjugated anti-rabbit IgG (Sigma), or with anti-Sendai virus tag antibodies and Cy3 (Indocarbocyanin)-labeled secondary antibody (Dianova). For nuclear staining, the mounting medium contained 0.3 μg/ml 4′, 6′-diamidino-2-phenylindole (DAPI).

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