than in the *ypt1-1* strain, which shows an accumulation of transport intermediates and secretion of a small amount of Golgi-modified (p2) CPY even at the permissive temperature. Figure 4*b* shows the transport of invertase in the same three strains. Here, mistargeting of ER vesicles to the plasma membrane would result in secretion of the core-glycosylated form of invertase. The transport of invertase appears normal in both *YPT1-* and *Sec4-(L7, HV)*^{YP}-containing strains. Both show rapid secretion of fully glycosylated invertase, unlike *ypt1-1*, which shows transient accumulation of the ER form and secretion of an underglycosylated form of invertase[1]. Taken together, these data suggest that Sec4 and Ypt1 do not, by themselves, act as molecular 'tags' to specify the acceptor compartment for a given vesicle. Rather, we believe, their primary function is as a switch to regulate the interaction of components required for the docking and fusion of transport vesicles with the appropriate membrane. □

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Specificity domains distinguish the Ras-related GTPases Ypt1 and Sec4

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THE essential Ras-related GTPases[1,2] Ypt1 and Sec4 act at distinct stages of the secretion pathway in the yeast *Saccharomyces cerevisiae*: Ypt1 is required for vesicular transport from the endoplasmic reticulum to the Golgi apparatus, whereas Sec4 is required for fusion of secretory vesicles to the plasma membrane[3–6]. Here we use chimaeras of the two proteins to identify a 9-residue segment of Ypt1 that, when substituted for the analogous segment of Sec4, allows the chimaera to perform the minimal functions of both proteins *in vivo*. This segment corresponds to loop L7 of the p21^{ras} crystal structure[7]. Substitution of a 24-residue Ypt1 segment, including the residues just mentioned, together with 12 residues of Ypt1 corresponding to the 'effector region' of p21^{ras} (loop L2; refs 7, 8), transforms Sec4 into a fully functional Ypt1 protein without residual Sec4 function.

To determine which domain(s) within these two homologous proteins (which have ~50 per cent amino-acid identity) enables them to perform distinct cellular functions, we made precise reciprocal fusions between their coding sequences using a polymerase chain reaction (PCR) technique[9,10] (Fig. 1). Three conserved motifs, here termed GxSGVGK, DTAGQ and NKxD (also designated G1, G3, and G4; ref. 11), were used as fusion joints. The ability of fusion proteins on low-copy plasmids to function as Ypt1 and/or Sec4 *in vivo* was tested by their ability to complement the conditional-lethal *ypt1-1*, or *sec4-8*, mutations or a *ypt1*-deletion allele; results of similar experiments are presented in an accompanying paper[12].

Reciprocal exchange of 5′ or 3′ non-coding seqences had no effect on the *in vivo* function of either gene (SY1, SY5, YS1, YS5; see Fig. 2) and the full-length genes complement only their cognate mutations (Fig. 2). The functional specificity must thus reside in the protein sequences themselves rather than in their regulation.

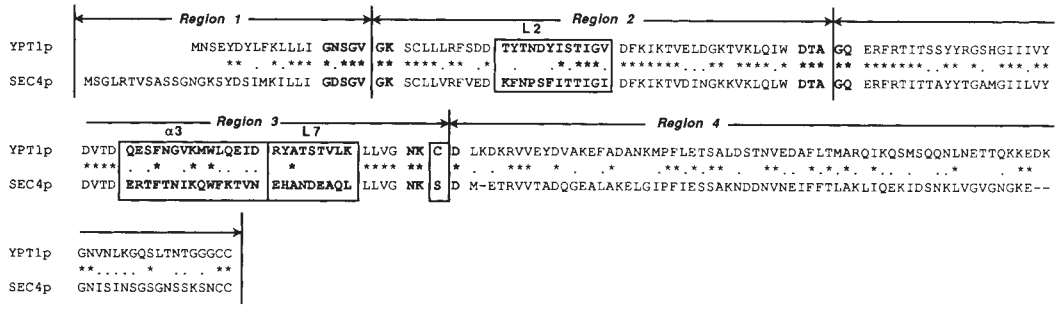

FIG. 1 The aligned amino-acid sequences of Ypt1 (ref. 22) and Sec4 (ref. 5) are shown. Asterisks indicate perfect amino-acid matches; dots indicate amino-acid similarities. The GxSGVGK, DTAGQ and NKxD motifs are shown in bold letters, and the fusion junctions within the motifs are shown as vertical lines. The amino-acid regions exchanged in chimaeric proteins Y3A, Y3B, Y23A and Y23B are shown in bold letters that are boxed and labelled; L2 refers to the 'effector region' (loop L2) region, α3 refers to the helix α3 region, and L7 to the loop L7 region, as described in the text. Note that the boxed residue within the NKxD motif is included in the L7 exchange region. Chimaeric protein Y3A consists of the entire Sec4 amino-acid sequence except for the Ypt1 residues within the α3 and L7 boxes; likewise, Y3B contains just the Ypt1 residues in box L7; Y23A contains just the Ypt1 residues shown in boxes L2, α3 and L7; and Y23B contains just the Ypt1 residues in boxes L2 and L7.

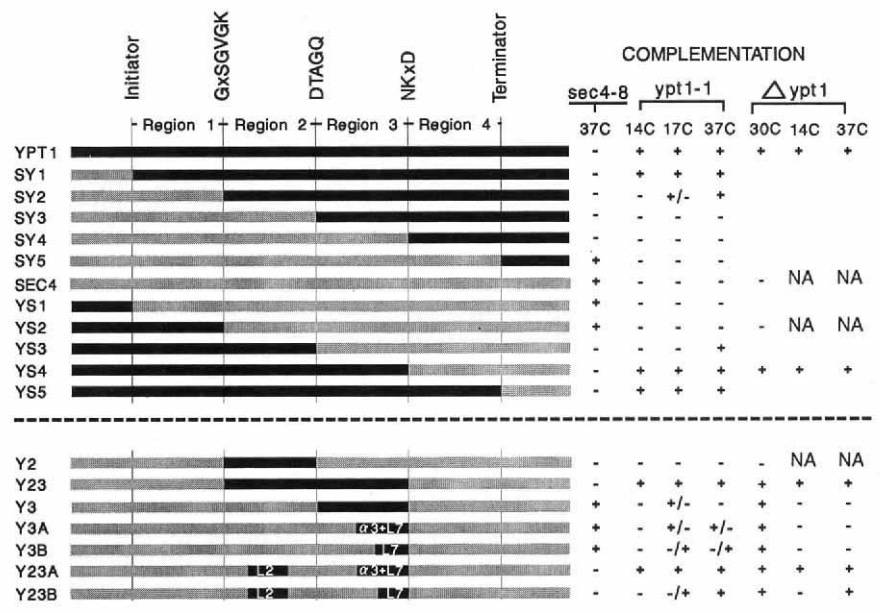

FIG. 2 Diagrams of chimaeric genes and results of complementation assays. Solid black regions represent *YPT1* sequences and grey stippled regions represent *SEC4* sequences. The amino-acid sequences of the three conserved GTP-binding/hydrolysis motifs used as fusion junctions are shown at the top. For chimaeric proteins with fusion regions smaller than those between the conserved motifs, the exchanged regions are labelled L2, α3 or L7, as described in Fig. 1. Relative growth rates are indicated as follows: a plus sign indicates robust growth on Ura drop-out plates[23] after 5–7 days at 14–17 °C or 2–3 days at 30–37 °C; +/− and −/+ represent increasingly less vigorous growth after these incubation times; and a minus sign indicates no growth after these incubation times. N/A, not applicable.

METHODS. We used a two-step PCR-based method[9] to create fusion genes using a protocol similar to that in ref. 10. The final fusion products were cloned into the polylinker of the *URA3*-marked CEN plasmid pRS316 (ref. 24). Each construction was sequenced across the PCR junction. Plasmids were transformed using lithium acetate[25] into two haploid strains with conditional lethal alleles: DBY1803 (**a** *ypt1-1 ura3-52 his4-539 lys2-801*), which carries the cold- and heat-sensitive *ypt1-1* allele[26], and NY405 (**a** *sec4-8 ura3-52*), which carries the heat-sensitive *sec4-8* allele[27]. The ability of each chimaeric protein to support growth at the relevant restrictive temperatures (14 °C, 17 °C and 37 °C for *ypt1-1* and 37 °C for *sec4-8*) was assayed. In addition, we assayed the ability of a subset of the fusion genes to complement the lethality of a deletion of the *YPT1* gene. This was done by transforming the plasmids into a diploid strain heterozygous for a *ypt1* deletion marked with *HIS3* (NY 921; ref. 12). Recovery of His^+ spore indicated that the chimaeric protein was able to rescue the spore; such spores were also tested for growth at 14 °C and 37 °C on YPD.

Reciprocal exchange of the small N-terminal region (to the GxSGVGK motif; Fig. 2, SY2 and YS2) had little or no effect. Moreover, almost the entire C-terminal half of Ypt1 (from NKxD to the C terminus; Fig. 2, YS4) can be swapped with that of Sec4 without loss of Ypt1 function *in vivo*. Any 'specificity domain' of Ypt1 must thus reside internally, between the GxSGVGK and the NKxD motifs. Although localization signals have been identified in the C-terminal regions of two mammalian members of the Ypt1/Sec4 family[13], this need not contradict our result, because we assayed function rather than localization. The reciprocal hybrid (SY4) failed to complement any of the *ypt1* or *sec4* mutations; the protein may, however, be trivially defective.

To define the putative Ypt1 'specificity domain' more precisely, we substituted internal portions of Ypt1 into analogous regions of Sec4. A chimaeric protein containing a 104-residue internal region of Ypt1 and the N- and C-terminal regions of Sec4 (Fig. 2, Y23) functions as well as the wild-type Ypt1 at all restrictive temperatures, including rescue of *ypt1*-deletion spores. Y23 actually functions better as Ypt1 than SY2, which has more Ypt1 sequences (Fig. 2), suggesting that the N- and C-terminal regions of Sec4 may interact.

Next we split the internal Ypt1 region of Y23 into two parts, using DTAGQ as a junction, thus yielding chimaeric proteins Y2 and Y3 (Fig. 2). Y2 contains 46 residues of Ypt1 sequence, including the 'effector loop' in small GTPases[8,14,15], but fails to complement any of these mutations (Fig. 2), allowing no conclusion to be drawn. Y3 contains 58 residues of Ypt1 between the DTAGQ and NKxD motifs, of which only 27 amino acids are different in the two proteins (Fig. 1). Surprisingly, Y3 can rescue *ypt1*-deletion spores, showing that it carries out the minimal functions of Ypt1, but it can complement *ypt1-1* only at the semi-permissive temperature of 17 °C (Fig. 2). Likewise, the *ypt1*-deletion spores rescued by Y3 (which had been germinated at 30 °C) are heat- and cold-sensitive. Y3 thus has a minimal Ypt1 function. Strikingly, it can also complement the *sec4-8*

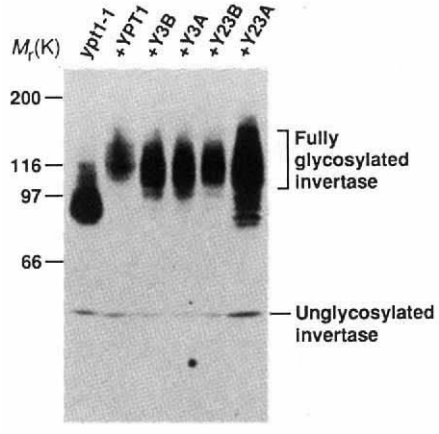

FIG. 3 Glycosylation of invertase. Strain DBY1803 (*ypt1-1*; ref. 26) with or without plasmids carrying an intact *YPT1* gene or chimaeras Y3B, Y3A, Y23B or Y23A (same plasmids as for Fig. 2) were grown in liquid Ura drop-out medium[23] to mid-log phase at 30 °C (Y23A was grown at 25 °C), then shifted to low (0.1%) glucose conditions for 1.5 h. Whole cell extracts were prepared, then assayed by immunoblotting as described[3], except that enhanced chemiluminescence (Amersham) was used for detection. Positions of molecular size ($M_r \times 10^{-3}$) markers (BioRad) are indicated on the left and of fully glycosylated and unglycosylated invertase on the right. The amount of Y23A extract loaded was inadvertently ~2–3 fold higher.

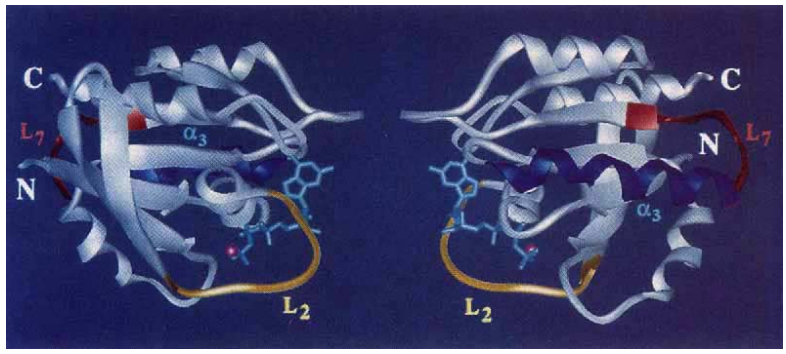

FIG. 4 Crystal structure of p21^{ras}. The molecule is shown in two views: the molecule on the right is rotated ~180° about the vertical axis relative to the molecule on the left. The regions of p21^{ras} corresponding to the loop L2, helix α3 and loop L7 exchange regions (Fig. 1) are highlighted: L2 in yellow, α3 in blue, and L7 in red (note that the residue corresponding to the Cys 123 of Ypt1 is not highlighted). The GTP is shown in cyan, and Mg^{2+} in magenta. The N and C termini are indicated. Figures were drawn with MidasPlus, developed by the Computer Graphics Laboratory at UCSF, using coordinates[7] provided by A. Wittinghofer.

mutation completely at 37 °C (Fig. 2). This bifunctionality means that the specificity-bearing domain(s) of Ypt1 and Sec4 are not entirely overlapping, otherwise a swap would necessarily cause loss of one function while gaining the other. This result also shows that Y3 is stable at 37 °C; therefore its failure to function as Ypt1 at this temperature must reflect a specific feature of Ypt1 function.

To define the Ypt1 specificity domain more exactly, we created the chimaeric protein Y3A (Fig. 2), which contains the C-terminal 24 of the 58 Ypt1 residues in Y3. These 24 residues (21 of which differ in Ytp1 and Sec4; see Fig. 1) correspond to helix α3 and loop L7 in p21^{ras} (Fig. 4; ref.7). We also created Y3B (Fig. 2), which contains only nine Ypt1 residues preceding the NKxD motif. These nine residues (8 of which differ in the two proteins; see Fig. 1) correspond to loop L7 in p21^{ras}. Both Y3A and Y3B also contain the Ypt1 Cys 123 within the NKxD motif, rather than the serine of Sec4. Both proteins are able to complement *ypt1* mutations (including the *ypt1* deletion) as well as the parent Y3, although Y3 and Y3A complement *ypt1-1* at 17 °C better than Y3B. Thus 9 Ypt1 residues (number 108, 109, 111–116, and 123) at most are required to confer minimal Ypt1 function on a related, but functionally distinct, GTPase. Both Y3A and Y3B still fully complement *sec4-8* (Fig. 2), indicating that they, like Y3, are bifunctional.

The results with Y23 indicated that we could confer full Ypt1 function upon Y3A and Y3B by restoring 12 residues of Ypt1 corresponding to the 'effector region' (loop L2) of p21^{ras} (eight residues of which differ in Ypt1 and Sec4), to create chimaeras Y23A and Y23B (Figs 1 and 2). Like Y23, Y23A complements the *ypt1-1* and *ypt1*-deletion mutants at all restrictive temperatures, but has lost the ability to complement *sec4-8*. Sec4 can thus be converted to a fully functional 'Ypt1' protein through the transfer of two short Ypt1 segments containing 36 residues (of which 29 differ in Ypt1 and Sec4). In contrast, Y23B (21 total residues with 17 differences), when compared to its parent Y3B, has gained Ypt1 function only at 37 °C, not at 14–17 °C. The loop L2 'effector region' thus seems to modulate the specificity of Ypt1 and Sec4 function at 37 °C, whereas the Ypt1 helix α3 region influences Ypt1 function at 14–17 °C.

To assess how far our chimaeric proteins restored function, we examined glycosylation in *ypt1-1* strains with and without plasmids bearing Ypt1 or several of the chimaeras. All the functional chimaeras that we tested completely suppress the mis-glycosylation of invertase characteristic of *ypt1-1* (ref. 3; Fig. 3), showing that they correct at least one secretion defect in the *ypt1-1* mutant. As *ypt1-1* mis-glycosylates invertase even at a permissive temperature[3], this criterion is quite rigorous.

Although at least four unlinked genes (*SLY* genes) have been identified that appear to bypass Ypt1 function[16–19], three do so only when highly expressed. Our complementing chimaeras differ in that they are highly homologous to Ypt1, almost certainly encode functional GTPases, and complement when expressed from a normal promoter at low copy number. (The *SEC4* gene, in contrast, cannot complement *ypt1* mutations even at high copy number[5].) Most tellingly, the *SLY* genes are unable to suppress fully the misglycosylation of invertase[16,17]. The chimaeras are therefore unlikely to bypass Ypt1 or Sec4 functions the way the *SLY* genes do.

The structures of Ypt1 and Sec4 are probably very similar to that of p21^{ras} (Fig. 4) as their sequences are homologous[20]. Twenty-one of the 29 amino acids that distinguish Ypt1 functions from those of Sec4 lie in the region corresponding to loop L7 and helix α3 in p21^{ras} (shown in Fig. 4 in red and blue, respectively). The putative 9-residue Ypt1 'specificity domain' (loop L7) is surface-accessible, and lies on the opposite side of the molecule from the remaining 8 of the 29 amino acids, which fall near the region corresponding to the GTP-binding pocket. (Fig. 4; loop L2, in yellow). It has been proposed[21], on the basis of analogous studies with α-subunits of signalling GTPases, that the face corresponding to that surrounding loop L7 in p21^{ras} interacts with membranes or membrane-localized proteins. Results in the accompanying letter[12] make it unlikely that cellular localization alone is responsible for the different functions of the two proteins; for Ypt1, such specificity may well be achieved by interactions between these two regions and particular downstream ligands. □

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