

Ric1p and Rgp1p form a complex that catalyses nucleotide exchange on Ypt6p

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Cells lacking the GTPase Ypt6p have defects in intracellular traffic and are temperature sensitive. Their growth is severely impaired by additional mutation of *IMH1*, which encodes a non-essential Golgi-associated coiled-coil protein. A screen for mutants that, like *ypt6*, specifically impair the growth of *imh1* cells led to the identification of *RIC1*. Ric1p forms a tight complex with a previously uncharacterized protein, Rgp1p. The Ric1p–Rgp1p complex binds Ypt6p in a nucleotide-dependent manner, and purified Ric1p–Rgp1p stimulates guanine nucleotide exchange on Ypt6p *in vitro*. Deletion of *RIC1* or *RGPI*, like that of *YPT6*, blocks the recycling of the exocytic SNARE Snc1p from early endosomes to the Golgi and causes temperature-sensitive growth, but this defect can be relieved by overexpression of *YPT6*. Ric1p largely colocalizes with the late Golgi marker Sec7p. Ypt6p shows a similar distribution, but this is altered when *RIC1* or *RGPI* is mutated. We infer that the Ric1p–Rgp1p complex serves to activate Ypt6p on Golgi membranes by nucleotide exchange, and that this is required for efficient fusion of endosome-derived vesicles with the Golgi.

Keywords: Golgi/Rab GTPase/*Saccharomyces cerevisiae*/vesicle fusion/Ypt6p

Introduction

A central feature of intracellular membrane traffic is the specific fusion of transport vesicles with their target organelles. Key components of this process are the SNAREs, membrane proteins found on both vesicles and target whose interactions are intimately involved in initiating bilayer fusion. Families of SNARE proteins exist, each with a characteristic distribution, and it is thought that the preferential formation of complexes comprising specific sets of SNAREs contributes to the specificity of membrane fusion (Rothman, 1994; Nichols and Pelham, 1998; Pelham, 1999). Further specificity is provided by additional proteins that aid the initial interaction of vesicles with their targets. These include both tethering factors with coiled-coil motifs and large protein complexes (Pfeffer, 1999; Waters and Pfeffer, 1999). Another set of proteins that may control the initial tethering or docking event are the small GTPases termed rabs (in animal cells) or Ypts (in yeast). Representatives of

this family are found at each step of transport (Lazar *et al.*, 1997; Chavrier and Goud, 1999; Gotte *et al.*, 2000). Examples in yeast are Sec4p, required for transport and fusion of exocytic vesicles with the plasma membrane, and Ypt1p, which plays a crucial role in the docking of endoplasmic reticulum (ER)-derived vesicles with early Golgi compartments containing the SNARE Sed5p.

Another GTPase associated with the Golgi is Ypt6p, the yeast homologue of mammalian rab6. Unlike Ypt1p, Ypt6p is not required (in most strains) for secretion or for viability, but null mutants have a number of phenotypes including temperature-sensitive growth, partial mis-sorting of the vacuolar protease carboxypeptidase Y and mis-localization of the late Golgi protein Kex2p to the vacuole (Li and Warner, 1996; Tsukada *et al.*, 1999). These phenotypes are quite similar to those of mutants lacking the SNARE Tlg1p, which has been shown to be involved in the recycling of proteins from early endosomes to the Golgi apparatus (Holthuis *et al.*, 1998a,b; Lewis *et al.*, 2000). Hence, a possible role for Ypt6p would be to assist the fusion of endosome-derived vesicles with the late Golgi. A role for Ypt6p in the Golgi is also consistent with some genetic evidence, which suggests a link between the functions of Ypt6p and Ypt1p (Li and Warner, 1998).

Despite these clues, rather little is known about the molecules with which Ypt6p might interact. Genetic studies have identified several genes that interact with *ypt6*, by suppressing its temperature-sensitive growth defect when they are overexpressed (Li and Warner, 1996; Tsukada and Gallwitz, 1996). One such gene was identified as a low-copy suppressor of a *ypt6* allele and named *IMH1* (Li and Warner, 1996). The same gene was isolated as a high-copy suppressor of a deletion allele of *ypt6*, and named *SYS3* (Tsukada and Gallwitz, 1996); for simplicity we use the name *IMH1* here. Since Imh1p has effects even in the absence of Ypt6p, it must be able to act independently of it. However, its function is clearly related, since loss of Imh1p, which normally has little effect on cells, severely reduces the growth of a *ypt6* mutant (Tsukada *et al.*, 1999).

In this paper we describe a search for proteins interacting with Ypt6p using two independent approaches. In the first, mutations were sought that, like *ypt6*, show synthetic interactions with *imh1*. In the second, proteins binding to Ypt6p were identified by protein purification and mass spectrometry. Both approaches led to the identification of a complex consisting of the products of the *RIC1* and *RGPI* genes. These are peripheral membrane proteins that colocalize with the Golgi marker Sec7p, and we show that they act *in vitro* as a nucleotide exchange factor for Ypt6p. Their activity does not seem to influence the ability of Ypt6p to bind to membranes, but in their absence the distribution of Ypt6p is distinct from that of the Golgi. We

suggest that Ypt6p is able to bind to endosome-derived vesicles, but becomes activated to the GTP-bound form by the Ric1p–Rgp1p complex only at Golgi membranes; Ypt6p–GTP then stimulates the fusion of endosome-derived vesicles with the Golgi. Proteins related to the C-terminal domain of Ric1p are found in a variety of species, including humans.

Results

Genetic screen for mutants showing synthetic lethality with *imh1*

Null mutants of *YPT6* are viable but temperature sensitive for growth. Though deletion of the *IMH1* gene alone has little or no effect on growth, *imh1Δ ypt6Δ* double mutants show a very severe growth defect (Tsukada *et al.*, 1999). We reasoned that mutations in genes with functions related to that of *ypt6* might also show synthetic lethality with *imh1*, and therefore undertook a screen for such mutants using a colony sectoring assay as described previously (Kranz and Holm, 1990).

An *imh1Δ* yeast strain that carried *IMH1* on a plasmid was mutagenized and some 13 000 colonies screened (see Materials and methods). Six mutants were identified that were unable to grow without the plasmid-borne *IMH1* gene. Of these, one could be complemented by *YPT6*, confirming the interaction between *imh1* and *ypt6*. From a yeast genomic library we identified a plasmid that was able to complement four of the remaining mutants; the fifth has proven, so far, refractory to cloning. We restricted the complementary activity of the recovered plasmid to a single intact open reading frame, which corresponded to the *RIC1* gene (YLR039c). Figure 1A shows typical data for one of the mutants, s1233. The mutant strain, which lacked the chromosomal copy of *IMH1* but carried a *URA3* plasmid with the *IMH1* gene, was transformed with *LEU2* plasmids carrying either *RIC1*, *RIC1–GFP*, *IMH1* or no inserted gene and the transformants plated on medium containing 5-fluoro-orotic acid (5-FOA) to select cells that had lost the *URA3–IMH1* plasmid. As shown in Figure 1, the strain was able to grow only if it contained a plasmid expressing either Imh1p or Ric1p, or the green fluorescent protein (GFP)-tagged version of Ric1p.

RIC1 is a non-essential gene originally isolated in a screen for mutants with altered regulation of ribosome synthesis (Mizuta *et al.*, 1997). This phenotype is common for secretory mutants (Mizuta and Warner, 1994), and indeed the same screen also yielded *ypt6* (Li and Warner, 1996). Recently, *RIC1* was also identified in a screen for mutants that are synthetically lethal with a temperature-sensitive clathrin heavy chain (Bensen *et al.*, 2000). A role for Ric1p in membrane traffic thus seemed likely, so we characterized it further. Deletion of the *RIC1* gene resulted in temperature-sensitive growth, which could be overcome by provision of *RIC1* on a plasmid (Figure 1B). We confirmed that a *ric1Δ imh1Δ* strain was unviable, but found that a *ric1Δ ypt6Δ* strain was no more defective in terms of growth than a *ypt6Δ* single mutant (data not shown). The fact that removal of Ric1p had no additional effect once Ypt6p was removed suggests that Ric1p performs a function that is not only related to that of Ypt6p, but is dependent on the presence of Ypt6p.

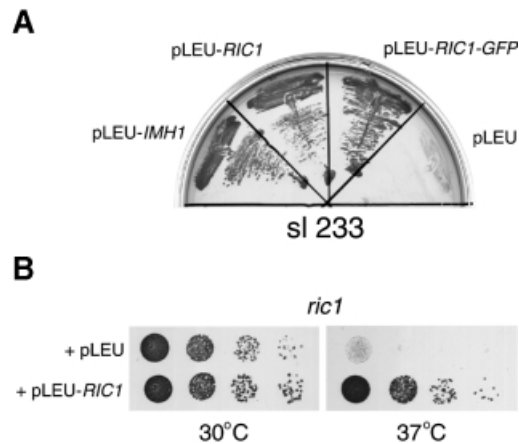


Fig. 1. Functional interaction between *IMH1* and *RIC1*. (A) Synthetic lethal interaction between *imh1Δ* and *ric1* mutants. The s1233 mutant was transformed with the indicated Yeplac111 plasmid (pLEU), either with no insert, or carrying *IMH1*, *RIC1* or a *RIC1–GFP* fusion. Transformants were grown on plates containing 5-FOA for 3 days at 30°C. (B) Growth properties of the *ric1Δ* deletion mutant (*ric1*). Cells from a *ric1Δ* strain transformed either with an empty *LEU2* plasmid or with the same plasmid carrying the wild-type *RIC1* gene were diluted in YEPD, spotted on to selective (–Leu) plates and grown at 30 and 37°C for 2 days.

Ric1p binds to Rgp1p

To identify proteins that might bind to Ric1p, we expressed a version of the protein tagged at its C-terminus with two IgG-binding domains of protein A (Ric1p–PtA). This was expressed from the *RIC1* promoter, on a centromere-containing vector, in *ric1Δ* cells; it fully complemented the temperature sensitivity of this strain (data not shown). Purification of the fusion protein on IgG–Sepharose revealed a second protein in roughly equimolar amounts (Figure 2, left panel, lane 2). Tryptic digestion of this protein and mass spectrometry of the products (see Materials and methods) showed that it corresponded to Rgp1p, the product of a largely uncharacterized gene reported to be required for efficient growth (Aguilera *et al.*, 1990). Protein A tagging and purification of Rgp1p, performed exactly as for Ric1p, resulted in the copurification of a single protein, which was identified by mass spectrometry as Ric1p (Figure 2, left panel, lane 1). Cleavage of either fusion protein with tobacco etch virus (TEV) protease at a site introduced between the proteins and the protein A tag yielded ~1:1 molar ratios of Ric1p to Rgp1p, regardless of which was originally tagged (Figure 2, right panel). This indicates that essentially all of the Ric1p and Rgp1p in the cell is in the form of a complex between the two proteins.

Deletion of Rgp1p in our standard wild-type strain resulted in a temperature-sensitive growth phenotype very similar to that of *ric1Δ* cells (data not shown, see also Figure 6). The similar properties of the deletion mutants makes it likely that both proteins are required for the activity of the Ric1p–Rgp1p complex.

Ric1p and Rgp1p bind to Ypt6p and act as a nucleotide exchange factor

In an independent series of experiments, we expressed a protein A–Ypt6p (PtA–Ypt6p) chimera in yeast cells, under the control of the *NOPI* promoter. This fusion protein was functional since it could complement the

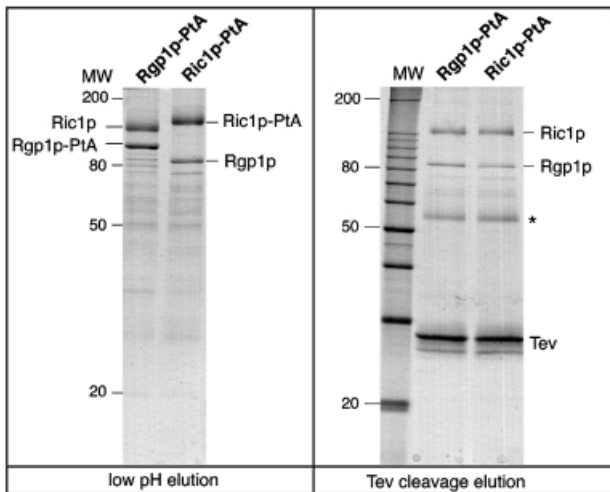


Fig. 2. Ric1p forms a complex with Rgp1p *in vivo*. (Left) Affinity purification of Rgp1p-PtA and Ric1p-PtA from *rgp1Δ* and *ric1Δ* strains, respectively. The purified proteins eluted from the IgG-Sepharose column with low pH were analysed by SDS-PAGE and Coomassie staining. The position of the protein A fusions and the copurifying proteins, identified as Ric1p and Rgp1p by mass spectrometry, are indicated. Molecular mass standards are indicated on the left side of the gel. (Right) The IgG-Sepharose beads carrying the Ric1p-PtA and Rgp1p-PtA fusions were digested by TEV protease and the released fractions containing the cleaved Ric1p and Rgp1p were analysed by SDS-PAGE and Coomassie staining. Molecular mass standards were resolved on the left side of the gel. The position of TEV protease is indicated; the asterisk indicates immunoglobulin heavy chains derived from the affinity column.

ypt6 deletion mutant (data not shown). We then isolated PtA-Ypt6p by affinity chromatography on IgG-Sepharose and looked for associated proteins. Since the interactions of the rab/Ypt GTPases are controlled by the state of the bound nucleotide, we incubated a portion of the cell extract with the non-hydrolysable GTP analogue, GTP- γ S, prior to purification and performed all subsequent steps in the presence of it. As shown in Figure 3A, two proteins that were present in very low amounts relative to Ypt6p were selectively lost when the extract was incubated with GTP- γ S. Mass spectrometry of tryptic digests of these proteins identified them as Ric1p and Rgp1p. To confirm this we co-expressed GFP-tagged Ric1p with PtA-Ypt6p, isolated the Ypt6p and detected Ric1p-GFP by immunoblotting. Figure 3B shows that Ric1p again copurified with Ypt6p, and that it could be released by addition of GTP- γ S in the lysis buffer. GDP also released Ric1p, but less efficiently. Thus, we conclude that the Ric1p-Rgp1p complex can bind to Ypt6p, and that it interacts preferentially with the nucleotide-free form and, to a lesser extent, with the GDP form, but not with the GTP form. These binding characteristics are typical for guanine nucleotide exchange factors (GEFs).

As a direct test of GEF activity, we purified Ypt6p from a yeast strain lacking both Rgp1p and Ric1p (see Materials and methods), loaded it with [3 H]GDP, incubated it with excess unlabelled GDP and monitored the release of labelled GDP from the protein. As shown in Figure 4A, the spontaneous rate of release was low but addition of the Ric1p-Rgp1p complex, purified by protein A affinity chromatography and TEV protease cleavage as in Figure 2, accelerated release in a dose-dependent manner. Identical

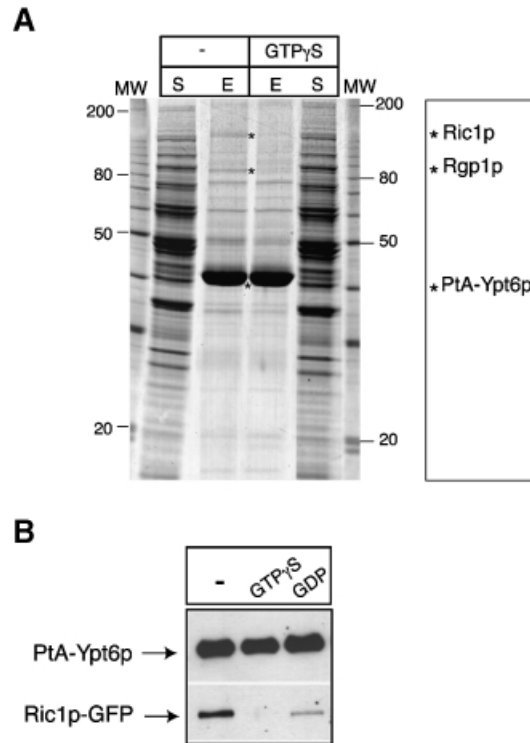


Fig. 3. The Ric1p-Rgp1p complex binds to Ypt6p *in vivo* in a nucleotide-dependent manner. (A) Affinity purification of a PtA-Ypt6p fusion and identification of proteins that bind to it. A detergent-solubilized supernatant from a *ypt6Δ* strain expressing a PtA-YPT6 fusion, in the absence (-) or presence of GTP- γ S, was loaded onto an IgG-Sepharose column and the purified fusion protein eluted at low pH. Samples from the supernatant loaded on to the column (S) and the eluted PtA-Ypt6p (E), (3700-fold concentrated as compared with the supernatant), were analysed by SDS-PAGE followed by Coomassie staining. The position of PtA-Ypt6p and the copurifying Ric1p and Rgp1p, identified by mass spectrometry, are indicated by asterisks. (B) PtA-Ypt6p was affinity purified from a *ypt6Δ ric1Δ* double deletion strain co-expressing a Ric1p-GFP fusion from a Ycplac33-URA3 plasmid, in the absence of nucleotide, or in the presence of GDP or GTP- γ S. The eluates from the IgG-Sepharose column were analysed by SDS-PAGE followed by western blotting using anti-protein A or anti-GFP antibodies.

results were obtained when unlabelled GTP was used as competitor (data not shown). This effect was clearly catalytic, since complete release of [3 H]GDP from 90 pmol Ypt6p could be induced by only 8 pmol of the complex; only 20% of the [3 H]GDP was released without the complex within the same time period.

The exchange activity was specific, since no effect of Ric1p-Rgp1p was seen when Ypt1p was used in place of Ypt6p (Figure 4B). We also tested Ric1p and Rgp1p separately, expressing each from a multicopy vector in the appropriate deletion strain. This was necessary because Rgp1p, in particular, appeared unstable in the absence of its partner. However, neither protein alone provided detectable exchange activity (Figure 4C).

Taken together these data show that the Ric1p-Rgp1p complex can bind to Ypt6p and function as an exchange factor. Like other exchange factors, it probably acts primarily by destabilizing the Ypt6p-GDP complex.

Role of Ric1p-Rgp1p in Ypt6p-mediated traffic

If Ric1p and Rgp1p are important for the formation of Ypt6p-GTP, the presumed active form of the protein, then

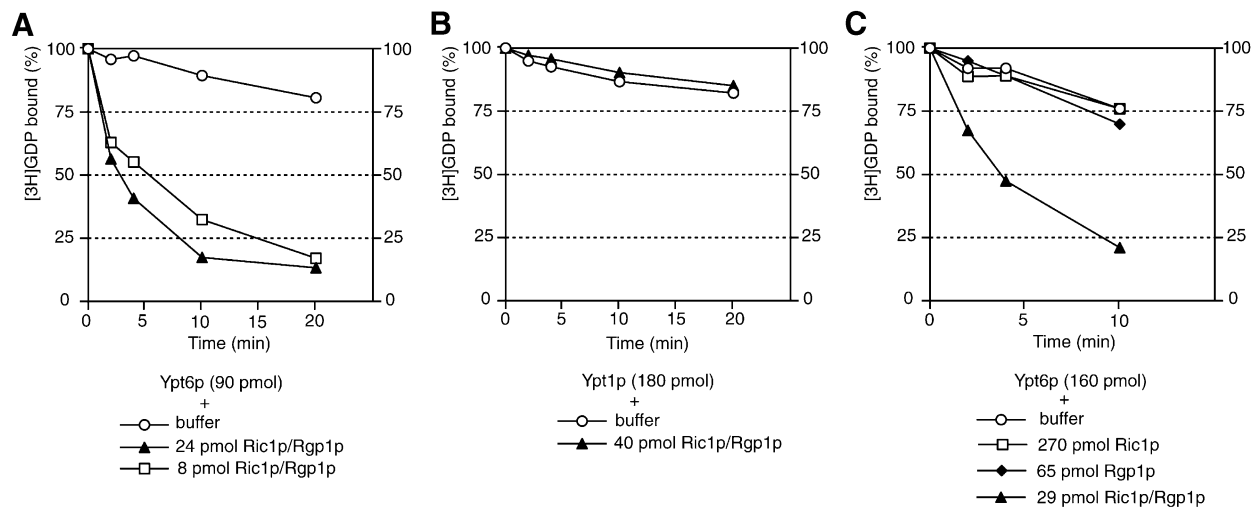


Fig. 4. The Ric1p–Rgp1p complex stimulates GDP exchange on Ypt6p. (A) Ypt6p and the Ric1p–Rgp1p complex were purified from yeast as described in Materials and methods. Ninety picomoles of Ypt6p pre-incubated with [³H]GDP were incubated with the indicated amounts of Ric1p–Rgp1p at 24°C, and protein-bound radioactivity was determined at intervals by filtration of aliquots through nitrocellulose. Background binding (with bovine serum albumin in place of Ypt6p) was equivalent to ~10% of the total and has not been subtracted. (B) Ypt1p preloaded with [³H]GDP was incubated with the Ric1p–Rgp1p complex at 24°C and nucleotide exchange was determined as in (A). (C) Ypt6p preloaded with [³H]GDP was incubated independently with either Ric1p or Rgp1p or the Ric1p–Rgp1p complex at 24°C. Nucleotide exchange was determined as in (A).

one would expect cells lacking one or both to have phenotypes similar to those of *ypt6* mutants (Li and Warner, 1996; Tsukada *et al.*, 1999). Indeed, like *ypt6* cells, a *ric1* mutant showed little defect in secretion of invertase or transport to the plasma membrane of an endocytosis-defective version of the exocytic SNARE Snc1p (data not shown), and only a mild mis-sorting of the vacuolar protease carboxypeptidase Y (Bensen *et al.*, 2000). *ypt6*, *ric1* and *rgp1* cells all showed a similar characteristic fragmentation of vacuoles, as visualized by uptake of the dye FM4-64 (Figure 5A). Since many of the phenotypes described for *ypt6* are similar to those of *tlg1*, a gene required for recycling of proteins such as Snc1p through early endosomes to the Golgi, we also examined the distribution of GFP–Snc1p in the various mutants. In wild-type cells this protein travels from Golgi to plasma membrane, is endocytosed and returns to the Golgi for reuse (Lewis *et al.*, 2000); in the steady state it is visible on the plasma membrane and in internal dots corresponding to endosomes and/or Golgi cisternae. In *ypt6*, *ric1* and *rgp1* cells it was entirely intracellular, in an indistinct hazy or dotted pattern that probably corresponds at least in part to transport vesicles that are unable to fuse with their target (Figure 5B). Hence, as previously proposed for Ypt6p (Tsukada *et al.*, 1999), all three proteins are likely to be involved in endosome–Golgi recycling.

Despite these similarities, we had observed a more severe growth defect for *ypt6Δ* cells than for *ric1Δ*, *rgp1Δ* or a *ric1Δ rgp1Δ* double mutant, especially on selective medium at elevated temperatures. This implied that Ypt6p was capable of some function even without the GEF activity provided by Ric1p and Rgp1p. In confirmation of this, we found that even very mild overexpression of Ypt6p (from a low-copy plasmid and the *YPT6* promoter) was sufficient to improve growth of *ric1Δ*, *rgp1Δ* and the double mutant at 37°C, and when a stronger promoter was used to drive *YPT6* expression, growth was restored to wild-type levels (Figure 6). This could be accounted for by

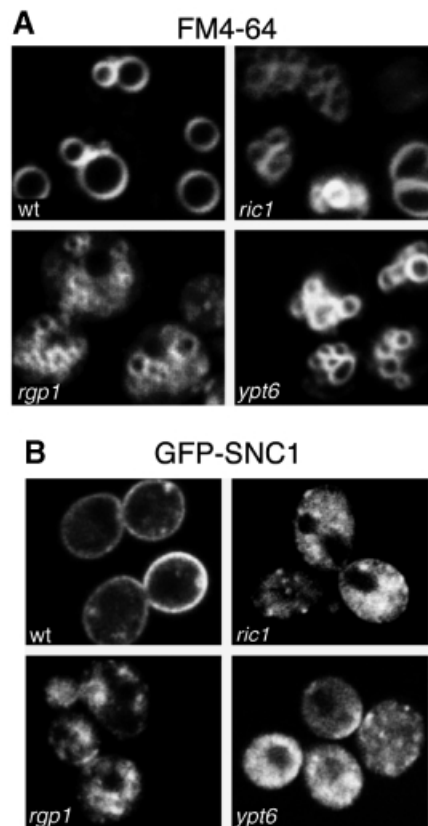


Fig. 5. Defects in vacuolar morphology and GFP–Snc1p recycling in *ric1Δ*, *rgp1Δ* and *ypt6Δ* mutants. (A) The *ric1Δ*, *rgp1Δ* and *ypt6Δ* deletion mutants and a wild-type strain were grown at 30°C to exponential phase, labelled with the endocytic tracer dye FM4-64 for 15 min and chased for 1 h. Note that only vacuoles are visible under these conditions. (B) The deletion mutants were transformed with a plasmid expressing GFP–Snc1p. Transformants were grown to exponential phase at 30°C and inspected by confocal microscopy.

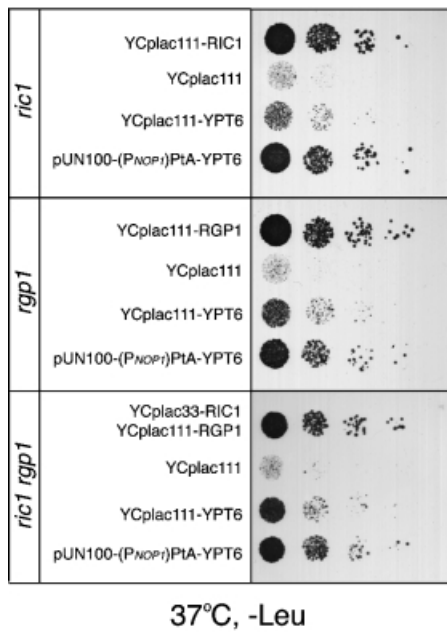


Fig. 6. *YPT6* suppresses the temperature-sensitive growth defect of the *ric1*, *rgp1* and *ric1 rgp1* deletion mutants. The *ric1*, *rgp1* and *ric1 rgp1* deletion mutants were transformed with an empty centromeric plasmid (YCplac111), YCp plasmid(s) carrying the complementing gene(s) as indicated, or with a centromeric plasmid over-expressing PtA-YPT6 [pUN100-(P_{NOP1})PtA-YPT6]. Transformants were spotted onto selective (-Leu) plates and incubated for 2 days at 37°C.

a low level of Ypt6p-GTP being generated spontaneously, or by the action of some other GEF. It strongly suggests that the major role of Ric1p and Rgp1p *in vivo* is to activate Ypt6p. Conversely, we can conclude that once activated by nucleotide exchange, Ypt6p does not require Ric1p or Rgp1p to stimulate membrane traffic.

Location of Ypt6p and Ric1p-Rgp1p in cells

We used functional GFP chimeras, expressed at endogenous levels in the absence of the corresponding untagged protein, to localize Ypt6p, Ric1p and Rgp1p. All of these fusion proteins retained their function (Figure 1A and data not shown). None are integral membrane proteins, but confocal microscopy showed that each was present in a dotted pattern typical of the yeast Golgi (Figure 7A). To identify the dots, we colocalized Ric1p-GFP with a YFP-tagged version of Sec7p (Figure 7; see Materials and methods for details). Analysis of fields of cells showed that 75–80% of the Ric1p dots also contained Sec7p. The dots containing the two proteins sometimes showed slight differences in shape or position, suggesting segregation of the proteins within a single structure. Since Sec7p is a late Golgi marker (Rossanese *et al.*, 1999; Lewis *et al.*, 2000), the colocalization confirms that the Ric1p-Rgp1p complex is present on Golgi membranes. A similar analysis showed that Ypt6p also overlapped with Sec7p (Figure 7), but interestingly, the degree of overlap appeared lower: only 50–60% of the Ypt6p dots contained Sec7p. Hence, Ypt6p may not be restricted to late Golgi membranes alone.

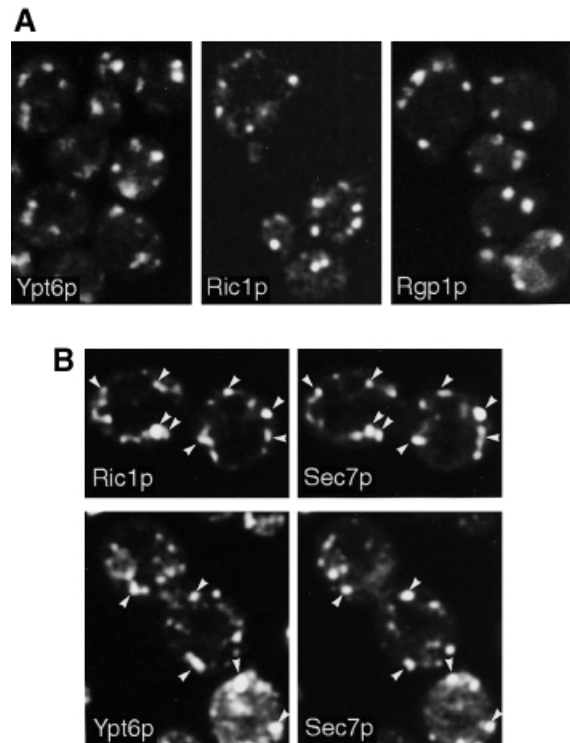


Fig. 7. Localization of Ric1p-GFP, Rgp1p-GFP and GFP-Ypt6p fusions. (A) *RIC1-GFP*, *RGPI-GFP* and *GFP-YPT6* were expressed from centromeric plasmids under the control of their own promoters, in the corresponding deletion mutants. (B) The Ric1p-GFP and GFP-Ypt6p strains were mated with a strain bearing a chromosomal copy of *YFP-SEC7*, under the control of the TPI1 promoter. YFP and GFP images of diploid cells were obtained as described in Materials and methods. Arrows indicate identical positions in the two images.

Ric1p-Rgp1p affects the localization of Ypt6p

We next examined Ric1p-GFP and GFP-Ypt6p individually in a *ypt6Δ ric1Δ* strain; since the GFP-tagged proteins are functional, this allowed the effects of removal of one on the distribution of the other to be tested. As might be expected, since the Golgi complex remains functional, the dotted pattern of Ric1p-GFP was unchanged in *ypt6Δ* cells (Figure 8A). This shows that binding of the Ric1p-Rgp1p complex to membranes does not require Ypt6p. However, removal of Ric1p had a dramatic effect on the distribution of GFP-Ypt6p: the pattern changed from dots (when *RIC1* was provided on a plasmid) to an even haze (Figure 8B). Similar results were obtained with an *rgp1* deletion mutant (data not shown). Since removal of Ric1p is not likely to have any greater effect on Golgi structure than removal of Ypt6p, the absence of a dotted pattern for GFP-Ypt6p suggests that this protein is absent from Golgi membranes in a *ric1* mutant.

A possible explanation would be that only the GTP-bound form of Ypt6p associates with membranes. To test this, we fractionated the cells by lysis and centrifugation at 13 000 g and then 100 000 g and analysed the pellets and supernatant by immunoblotting (Figure 8C). As controls, we checked the soluble enzyme phosphoglycerokinase (P_{gk1p}), which was found primarily in the supernatant, and the late Golgi/early endosomal SNARE Tlg1p, which was found partly in the P13 fraction and partly in the P100 fraction. In wild-type cells, GFP-Ypt6p was distributed

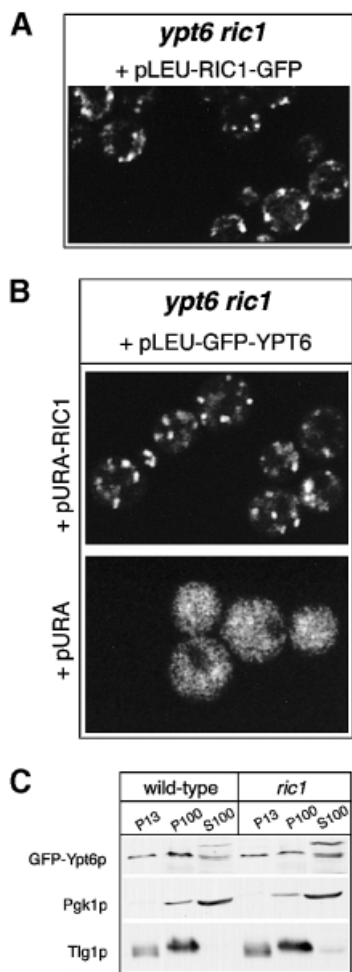


Fig. 8. Subcellular distribution of GFP-Ypt6p is drastically altered in *ric1* deletion mutants. (A) The *ypt6 ric1* double deletion mutant was transformed with a centromeric plasmid expressing Ric1p-GFP and examined under the confocal microscope. (B) A *ypt6 ric1* double deletion mutant transformed with the indicated plasmids was examined under the MRC-600 confocal microscope. (C) The GFP-YPT6 plasmid was transformed into *ypt6* cells, thus complementing them ('wild-type') or into the double *ypt6 ric1* deletion mutant ('*ric1*') and fractionated into 13 000 and 100 000 g pellets (P13 and P100, respectively) and 100 000 g supernatant (S100). Equivalent amounts of each fraction were analysed by SDS-PAGE followed by western blotting with anti-GFP, anti-Pgk1p and anti-Tlg1p antibodies. The upper band visible in the S100 fraction probed with anti-GFP is a cross-reacting endogenous protein.

between all three fractions, with ~80% being present in the combined membrane pellets and the remainder in the supernatant. Strikingly, however, this distribution was almost identical whether or not Ric1p was present. Thus, the change in distribution of GFP-Ypt6p is unlikely to be due to its release from membranes.

To summarize, in the absence of its exchange factor complex, Ypt6p changes its punctate Golgi localization to a haze, yet appears to be still membrane bound. It is possible that in a *ric1* mutant, Ypt6p is bound to small endosome-derived transport vesicles, which, because they are numerous and scattered, appear as a haze. This could occur if Ypt6p binds initially to endosomal membranes and is delivered to the Golgi on vesicles, or if it is permanently membrane associated and cycles through

early endosomes in a similar manner to resident late Golgi membrane proteins. By the same argument, the fact that the distribution of Ric1p is unaltered in *ypt6Δ* cells implies that it does not cycle through early endosomes.

Discussion

In this paper we have shown that Ric1p and Rgp1p form a complex that is peripherally associated with Golgi membranes and that has the ability to catalyse, *in vitro*, guanine nucleotide exchange on Ypt6p. This *in vitro* evidence is supported by the observations that small amounts of Ric1p and Rgp1p can be isolated from cells in a complex with Ypt6p, which dissociates upon addition of GTP. Furthermore, *ric1* and *rgp1* mutants have similar phenotypes to a *ypt6* mutant, and like *ypt6Δ*, a *ric1Δ* mutant shows synthetic lethality with an *imh1Δ* mutation.

A *ric1* mutant was originally isolated in a screen for genes affecting ribosome synthesis, and Ric1p was postulated to be a transcription factor (Mizuta *et al.*, 1997). However, the same screen yielded a *ypt6* mutant, and it has been established that a variety of secretory mutants affect ribosome synthesis (Mizuta and Warner, 1994; Li and Warner, 1996). *RIC1* also shows genetic interactions with clathrin mutants (Bensen *et al.*, 2000). Hence, the effects of Ric1p on ribosome synthesis are likely to be secondary to its effects on Ypt6p. Removal of Ric1p from a *ypt6Δ* strain does not result in any additional growth impairment, and the growth defects of *ric1* and *rgp1* mutants can be fully rescued by overexpression of *YPT6*. This argues that the major and perhaps only role of Ric1p and Rgp1p is in the activation of Ypt6p.

Why two proteins should be required for GEF function is unclear. However, the single proteins seem relatively unstable, and preliminary results indicate that neither Ric1p nor Rgp1p is able to localize to Golgi membranes efficiently in the absence of its partner. Thus, the complex forms a functional unit. Further analysis will be required to determine the precise residues responsible for the GEF activity.

Neither Ric1p nor Rgp1p shows any detectable homology to other known GEFs, including those active on rab-like GTPases such as Sec2p (a GEF for Sec4p), Vps9p (a GEF for Vps21p) or the rab5 GEF rabex 5. However, database searches show that Ric1p contains a C-terminal region that is clearly homologous to proteins in *Schizosaccharomyces pombe* and other species, including humans (Figure 9). The proteins from higher eukaryotes that contain this domain are obvious candidates for rab6 exchange factors.

The phenotypes of *ypt6*, *ric1* and *rgp1* mutants are fully consistent with a role for Ypt6p in transport from early endosomes to the Golgi complex. Indeed, the properties of cells lacking Ypt6p are strikingly similar to those of cells lacking the SNARE Tlg1p, which has an established role in this process (Holthuis *et al.*, 1998b). The most characteristic features are loss of late Golgi markers, such as Kex2p, to the vacuole and a failure to recycle Snc1p, but the mutants also share temperature-sensitive growth, partial mis-sorting of carboxypeptidase Y and similar ultrastructural abnormalities (Holthuis *et al.*, 1998a; Tsukada *et al.*, 1999; Lewis *et al.*, 2000). Interestingly, in one yeast strain (W303), both Tlg1p and

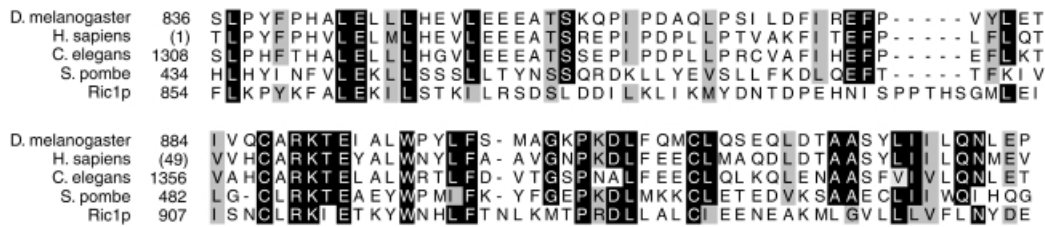


Fig. 9. Multiple sequence alignment of the C-terminal domain of Ric1p with related sequences found in proteins from other species: *Drosophila melanogaster* (DDBJ/EMBL/GenBank accession No. AF181626.1); *Homo sapiens* (AB037853.1; brackets in the residue number indicate that it is a partial sequence); *Caenorhabditis elegans* (Q09417); *S.pombe* (AL133361.1). Sequences were aligned with Clustal W1.8 (Thompson *et al.*, 1994) and displayed using the program Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

Ypt6p are also required for transport of secretory proteins through the Golgi (Li and Warner, 1996; Coe *et al.*, 1999). The reason for this strain-specific effect is unclear, but it may be that delivery of components from endosomes to the Golgi is important for maintenance of secretory function.

The localization of Ric1p–Rgp1p on Golgi membranes suggests that the GTP form of Ypt6p acts on the Golgi, to catalyse fusion of vesicles with it. This fits well with previous observations on Ypt1p, the GTPase that catalyses ER–Golgi transport. Though the GEF for Ypt1p has not been identified, its activity cofractionates with the Golgi (Jones *et al.*, 1998). Furthermore, although Ypt1p itself can be found on both Golgi and ER, and is likely to be present on vesicles that move between them, recent *in vitro* experiments show that its function requires it to be only on Golgi membranes, not on the transport vesicles themselves (Cao and Barlowe, 2000). Thus, both Ypt1p and Ypt6p may be activated to their GTP-bound state on Golgi membranes, and act there. Since Ric1p colocalizes quite well with Sec7p, which seems to be associated mostly with late Golgi cisternae (Rossanese *et al.*, 1999; Lewis *et al.*, 2000), Ypt6p may preferentially catalyse fusion with these later compartments.

Overexpression of Ypt6p allows it to function in the absence of its GEF, perhaps through spontaneous GTP binding or interaction with another GEF such as the unidentified one for Ypt1p. Under these circumstances, Ypt6p must perform its function through effectors that are distinct from Ric1p and Rgp1p. Ypt1p is thought to recruit the tethering factor Uso1p to membranes (Cao *et al.*, 1998), and it is tempting to speculate that Ypt6p performs a similar function for Imh1p, which shows some similarity with Uso1p. Indeed, Imh1p contains a GRIP domain, which has been shown to target proteins to the Golgi and has been reported to bind to rab6 on blots (Barr, 1999; Kjer-Nielsen *et al.*, 1999; Munro *et al.*, 1999). However, we have not been able to detect any interaction between Ypt6p and Imh1p by coprecipitation, and the fact that Imh1p can function (and indeed is essential for growth) in the absence of Ypt6p, and vice versa, argues that any such interaction is of minor functional importance. The genetic relationships are nevertheless striking, and suggest that Imh1p either contributes to the same fusion event as Ypt6p, albeit independently, or is involved in an alternative pathway from endosomes to Golgi. But the strong conclusion remains that Ypt6p must have an effector other than Imh1p, which remains to be identified.

Removal of its GEF did not prevent Ypt6p from binding to membranes, as judged by fractionation of the cells, suggesting that GDP–GTP exchange is not needed for membrane association of Ypt6p. This is in agreement with previous studies that detected transient membrane-bound rab–GDP intermediates, indicating that membrane binding precedes and is distinct from nucleotide exchange (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). Similarly, in the absence of its GEF, Sec2p, Sec4p still associates with late secretory vesicles (Walch-Solimena *et al.*, 1997). Surprisingly, however, Ypt6p has a dispersed, presumably vesicular distribution in *ric1Δ* cells, suggesting that it may be bound to small vesicles rather than to Golgi membranes. One possibility is that Ypt6p in its GDP-bound state binds initially to early endosomes or to vesicles that have budded from them (which is in agreement with our observation that, even in wild-type cells, a substantial proportion of the Ypt6p does not colocalize with Sec7p). Interaction of vesicle-bound Ypt6p with the Ric1p–Rgp1p complex could then occur as vesicles dock with the late Golgi, leading to the generation of Ypt6p–GTP and activation and/or recruitment of other components of the docking and fusion machinery. Of course, free Ypt6p may also be recruited directly to Golgi membranes by the Ric1p–Rgp1p complex, if this is present. Once fusion is complete, a Ypt6p GTPase-activating protein, Gyp6p (Strom *et al.*, 1993), presumably returns Ypt6p to the GDP state. It might then dissociate from the Golgi membrane and rebound to endosomes, or share the same transport pathway as other late Golgi membrane proteins that recycle through early endosomes.

Such a mechanism would ensure that active Ypt6p is generated where and when it is needed. However, it is not clear whether precise control of this is actually necessary for Ypt6p to function, and, since the site at which Ypt6p hydrolyses GTP is unknown, an additional function for Ypt6p in early endosomes cannot be ruled out. In practice, it has proven very difficult to distinguish early endosomal from late Golgi membranes in yeast (Lewis *et al.*, 2000).

It is striking that the distribution of Ric1p in a *ypt6* mutant remains punctate rather than dispersed. This argues that Ric1p–Rgp1p itself does not cycle through endosomes, as one would expect it to be trapped in vesicles under these conditions if it did. The Ric1p–Rgp1p complex may therefore be one component that functionally differentiates Golgi membranes from endosomes, perhaps recognizing directly some characteristic feature of the membrane, such as its lipid composition. Indeed,

Table I. Yeast strains used in this study

Strain	Genotype	Reference/source
SEY6210	<i>MATα ura3 his3 leu2 trp1 suc2 lys2</i>	S.Emr
IAY25	<i>MATα ura3 his3 leu2 trp1 can1 ade2 ade3</i>	J.Kilmartin and I.Adams
MHY002	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 ypt6::HIS3</i>	Pelham laboratory
BNY01	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 imh1::HIS5 (Sp)</i>	Pelham laboratory
SSY01	<i>MATα ura3 his3 leu2 trp1 ade2 ade3 imh1::HIS5(Sp) [pRS316-URA3-ADE3-IMH1]</i>	this study
SSY02	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 ric1::TRP1</i>	this study
SSY03	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 rgp1::HIS3</i>	this study
SSY04	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 ypt6::HIS5(Sp) ric1::TRP1</i>	this study
SSY05	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 ypt6::HIS5(Sp) rgp1::TRP1</i>	this study
SSY06	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 ypt6::HIS5(Sp) [YCplac111-LEU2-GFP-YPT6]</i>	this study
SSY07	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 ric1::TRP1 [YCplac111-LEU2-RIC1-GFP]</i>	this study
SSY08	<i>MATα ura3 his3 leu2 trp1 suc2 lys2 ric1::TRP1 rgp1::HIS3</i>	this study
SSY09	<i>MATα ura3 leu2 trp1 ade2 sec7::(P_{TRP1})YFP-SEC7</i>	this study
SSY10	cross between SSY09 and SSY06	this study
SSY11	cross between SSY09 and SSY07	this study

attempts to fractionate membranes containing the complex showed that it was readily released *in vitro*, implying that it is not firmly anchored to some integral protein (our unpublished observations). Interestingly, the rab GEFs identified so far are all peripheral membrane proteins. These could, in principle, identify even continuously maturing compartments, binding and leaving as their membrane composition changes.

One remaining question concerns the relationship between the function of Ypt6p in yeast cells and that of rab6 in animal cells. Rab6 is found on the Golgi as well as on mobile structures that move around the cytoplasm (White *et al.*, 1999). It has been argued that it is required for retrograde transport of toxin molecules from the cell surface to the ER, and that it may mediate a novel pathway direct from the *trans*-Golgi network to the ER (Girod *et al.*, 1999). However, our results suggest that at least some of the rab6-positive structures may in fact be *en route* from early endosomes to the Golgi. The situation is complicated by the fact that there are at least three different isoforms of rab6 in humans; it is possible that these have distinct functions, some of which are unique to animal cells. Further insight may be provided by examining the location and function of the mammalian protein related to Ric1p.

Materials and methods

Yeast strains and plasmids

The yeast strains used in this work are listed in Table I. The following plasmids were used: YCplac111 and Ycplac33, *ARS1/CEN4* vectors with the *LEU2* and the *URA3* marker, respectively (Gietz and Sugino, 1988); YEplac181, 2 μ vector with the *LEU2* marker (Gietz and Sugino, 1988); pRS316-*ADE3-IMH1*, a pRS316 *ARS4/CEN6* plasmid (Sikorski and Hieter, 1989) with the *ADE3* gene (inserted at the *SmaI-SpeI* sites) and the *IMH1* gene (inserted at the *SacI-XbaI* sites); pUN100-(P_{NOP1})PtA, an *ARS1/CEN4* plasmid expressing the protein A tag (Senger *et al.*, 1998). The integrative (P_{TRP1})YFP-*SEC7* construct was kindly provided by Drs S.Reichelt and T.Levine. The yeast genomic library was from ATCC (ATCC no. 77164).

Synthetic lethal screen with an *imh1* deletion mutant

To perform a synthetic lethal screen with the *imh1 Δ* deletion mutant, the red/white colony sectoring assay (Kranz and Holm, 1990) was used. The screening strain was constructed by mating the *imh1 Δ* strain with the IAY25 strain, selecting after tetrad dissection the *ade2 ade3 imh1 Δ* haploid progeny and transforming it with a pRS316-*ADE3-IMH1*

plasmid. This sectoring strain was grown in selective medium (-Ura) and UV-mutagenized (with a killing rate ranging between 97 and 99%). Thirteen-thousand-and-one-hundred colonies were analysed for a red, non-sectoring phenotype at 30°C, on YEPD plates containing 4% glucose. After three successive rounds of selection, six mutagenized colonies that were synthetically lethal with *imh1 Δ* , but not *IMH1*, were isolated (sl6, 9, 61, 68, 78 and 233). Sl233 was transformed with a *TRP1/CEN*-based yeast genomic library and seven transformants that exhibited red/white sectoring and could grow on 5-FOA plates were isolated. Restriction analysis of the recovered plasmids demonstrated that five of them contained overlapping genomic inserts that did not correspond to the *IMH1* gene. Sequencing of the inserts, followed by subcloning of the ORFs present, demonstrated that the *RIC1* gene complemented the sl233 mutant. The synthetic lethality between *imh1 Δ* and the *ric1 Δ* deletion mutants was verified by deleting the *RIC1* gene in the starting *IMH1* sectoring strain. Sl6, 9 and 68, but not sl78, regained both the sectoring phenotype and growth on 5-FOA plates upon transformation with *RIC1*. Transformation of sl78 with the genomic library, followed by similar analysis as described above, demonstrated that three sectoring transformants were complemented by the same overlapping genomic fragments and that the complementing activity resided in the *YPT6* gene.

Deletion of the *RIC1*, *RGPI*, *YPT6* and *IMH1* genes

For the deletion of the *RIC1* gene, an internal *NheI-XhoI* fragment (Ala100 to Leu1052) was removed and replaced by a filled-in fragment coding for the *TRP1* gene. The *ric1::TRP1* allele, with 5' and 3' flanking sequences, was excised as an *EcoRI* fragment and transformed into either the SEY6210 wild-type strain or the *IMH1* sectoring strain used for the synthetic lethality screen. The complete *RGPI* ORF was deleted by generating two unique *BamHI* sites via PCR-mediated mutagenesis, one just after the start codon and the other just before the stop codon, removal of the DNA between start and stop codon and insertion of a *BamHI* fragment containing either the *HIS3* or the *TRP1* gene. The *rgp1::HIS3*, or *rgp1::TRP1* alleles, carrying 5' and 3' flanking sequences, were excised as *SacI-SphI* fragments and transformed into the SEY6210 strain. The *ypt6::HIS5* and *imh1::HIS5* strains were kindly provided by M.van Horssen and B.Nichols. Deletion of *YPT6* and *IMH1* in these strains was achieved by replacing the entire coding regions of these genes by the *HIS5* gene of *S.pombe*.

Construction of *RIC1*, *RGPI*, *YPT6* and *YPT1* fusion genes

In order to tag *RIC1* with protein A, a naturally occurring *XhoI* site present three codons before the stop codon of the *RIC1* ORF was used to insert an *XhoI* fragment coding for the three last residues of Ric1p, followed by the TEV protease cleavage site and two IgG-binding domains from *Staphylococcus aureus* protein A. The same C-terminal *XhoI* site was used to insert in-frame the S65T/V163A variant of GFP and construct the *RIC1-GFP* fusion. To tag *RGPI* with protein A or GFP, a *BamHI* site was introduced just before the stop codon by PCR and a *BamHI* fragment coding for TEV-PtA, or GFP, respectively, was inserted. The *GFP-YPT6* fusion protein was constructed by introducing a *BamHI* site after the start codon of *YPT6* by PCR and in-frame insertion of a *BamHI* fragment

coding for GFP. Most of the *RIC1* and *RGPI* fusion proteins and the *GFP-YPT6* fusion were expressed under the control of their respective authentic promoters from centromeric plasmids with the *LEU2* (YCplac111) or *URA3* (YCplac33) markers and could complement the phenotypes of the *ric1*, *rgp1* or *ypt6* deletion mutants, respectively. In order to obtain sufficient amounts of Ric1p and Rgp1p from the *ric1 rgp1* mutant, the *RIC1-PtA* and *RGPI-PtA* fusions were also subcloned into high-copy vectors (YEplac181). To construct the *PtA-YPT6* and *PtA-YPT1* fusions, the *YPT6* or *YPT1* open reading frames were cloned into the *NdeI* and *BamHI* sites of the pUN100-(P_{NOPI})PtA plasmid. The *PtA-YPT6* and *PtA-YPT1* fusions were expressed under the control of the *NOPI* promoter from a centromeric plasmid with the *LEU2* marker (pUN100).

Antibodies

The antibody used to detect protein A fusions was from DAKO (catalogue no. Z0113). Anti-GFP polyclonal serum was a gift from R. Arkowitz (MRC LMB), the anti-Tlg1p polyclonal antibody as previously described (Holthuis *et al.*, 1998a) and the anti-Pgk1p monoclonal from Molecular Probes (catalogue no. A-6457).

Affinity purification of RIC1-PtA, RGP1-PtA and PtA-YPT6

For affinity purification of Ric1p-PtA, Rgp1p-PtA and PtA-Ypt6p, yeast strains expressing the corresponding protein A fusion proteins (Table I) were grown in selective medium (-Leu) (OD₆₀₀ 2.0–2.5). Cells were spheroplasted and kept frozen at -20°C. One gram of frozen spheroplast pellet was lysed in 16 ml ice-cold lysis buffer (150 mM KCl, 20 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 1% Triton X-100) supplemented with a cocktail of protease inhibitors (complete EDTA-free; Boehringer Mannheim). The extract was centrifuged for 15 min at 15 000 r.p.m. (SS-34 rotor, Sorvall) and the supernatant was first precleared by incubation with Sepharose Fast-flow beads (Pharmacia) and then loaded on to a column packed with 200 µl IgG-Sepharose beads (Pharmacia). Beads were washed with 20 ml of lysis buffer. For low pH elution of protein A fusions, beads were washed with 1 ml 5 mM ammonium acetate and bound proteins were eluted with 1 ml 0.5 M acetic acid/ammonium acetate pH 3.4. For TEV protease-dependent elution, beads were washed with 10 ml of TEV buffer [150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.8 mM dithiothreitol (DTT), 0.01% Triton X-100], then incubated with 20–40 U of TEV protease (Life Technologies) at room temperature for 90 min. Beads were then spun down and the soluble fraction containing the released proteins was collected. In all cases, both low pH and TEV digestion elution released most of the fusion proteins (>80% as judged by Coomassie staining), except for the PtA-Ypt6p TEV elution, where the efficiency was ~50%. Where indicated, lysis of the *PtA-YPT6* spheroplasts was carried out in the presence of 1 mM GDP or GTP-γS. Extracts were incubated for 15 min on ice and then processed, in the presence of GDP or GTP-γS, as described above.

Mass spectrometric analysis

Protein identification by mass spectrometry of tryptic fragments was performed as previously described (Jensen *et al.*, 1997). Samples were analysed with a Voyager-DE-STR MALDI-TOF mass spectrometer (PerSeptive Biosystems). Database searches using peptide masses were performed with the MS-Fit program (<http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm>). Fifteen tryptic peptides were obtained from Rgp1p (sequence coverage 31%) derived from the Ric1p-PtA purification. Thirteen tryptic peptides were obtained from Ric1p (sequence coverage 14%) derived from the Rgp1p-PtA purification. In the case of the PtA-Ypt6p purification, 26 tryptic peptides matched the protein identified as Rgp1p (sequence coverage 49%) and 12 peptides matched Ric1p (sequence coverage 14%). In all cases, the minimum required mass accuracy was 50 p.p.m.

Cell fractionation

Cells expressing the GFP-Ypt6p fusion protein were grown in selective medium, harvested at mid-logarithmic phase and spheroplasted as described above. Then, 0.5 g of spheroplasts were lysed in 1 ml of hypo-osmotic buffer (50 mM Tris-HCl pH 7.4, 200 mM sorbitol, 1 mM EDTA) at 4°C, in the presence of a cocktail of protease inhibitors. The cell lysate was centrifuged sequentially at 500 g (5 min), 13 000 g (10 min) and 100 000 g (60 min). Samples of each fraction were collected and analysed by SDS-PAGE followed by western blotting using anti-GFP, anti-Tlg1p and anti-Pgk1p antibodies.

Confocal microscopy

To view live cells expressing the various GFP fusions, cells were grown in the appropriate selective medium at 30°C to early log phase, and examined with an MRC-600 confocal microscope. For double-label GFP/YFP analysis, diploid cells expressing the Ric1p-GFP/YFP-Sec7p and GFP-Ypt6p/YFP-Sec7p pairs were grown to early log phase, fixed with 4% formaldehyde for 5 min, washed once with 0.1% Triton X-100 and twice with medium and then mounted on slides coated with concanavalin A. A Bio-Rad Radiance confocal microscope was used for imaging. YFP images were first collected using the 514 nm argon laser line for excitation and a 590/70 nm emission filter, and the cells then bleached by repeated slow scans with the same laser line until the YFP signal was no longer detectable. Finally, GFP images were collected using the 488 nm laser line and a 515/30 nm emission filter. This procedure was necessary because 488 nm light excites both YFP and GFP. Controls consisting of cells expressing the same YFP and GFP constructs singly confirmed that GFP was neither imaged nor bleached by the 514 nm laser line under these conditions, and that negligible YFP fluorescence could be detected using the 488 nm line following bleaching with 514 nm light. These controls were important for optimization of the scanning conditions.

Nucleotide exchange assay

The Rgp1p-PtA and the PtA-Ypt6p fusions were used to affinity purify the Ric1p-Rgp1p complex and Ypt6p from yeast cells, respectively, as described above. To ensure that no Ric1p-Rgp1p would be present in the Ypt6p preparation prior to the exchange assay, PtA-Ypt6p was isolated from a *ric1 rgp1* deletion strain. Proteins were released from the IgG-Sepharose beads by TEV protease digestion. For GDP exchange assay, 450 µl of Ypt6p (270 pmol) in 110 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.8 mM DTT, 0.005% Triton X-100 was incubated with 1.5 µM [³H]GDP (10.1 Ci/mmol, Amersham) for 60 min at 30°C. One-hundred-and-fifty microlitres of [³H]GDP-Ypt6p was mixed with an equal volume of 110 mM NaCl, 50 mM Tris-HCl pH 8.0, 12 mM MgCl₂, 0.8 mM DTT, 2 mM GDP, in the presence or absence of Ric1p-Rgp1p complex and incubated at 24°C. At the times indicated, aliquots were removed, diluted into ice-cold stop buffer (110 mM NaCl, 50 mM Tris-HCl pH 8.0, 6 mM MgCl₂) and filtered through nitrocellulose membranes (0.45 µm, Millipore). Membrane-bound radioactivity was measured by a liquid scintillation counter. Ypt1p purification and GDP exchange in the presence of the Ric1p-Rgp1p complex was carried out in the same way. To test the activities of Ric1p or Rgp1p separately, Ric1p-PtA or Rgp1p-PtA was expressed from high-copy vectors and purified independently from the *ric1 rgp1* double mutant as described above. Ypt6p preloaded with [³H]GDP was incubated with Ric1p, Rgp1p or the Ric1p-Rgp1p complex and nucleotide exchange was assayed as above. In all cases protein concentrations were determined both by the Bio-Rad assay and by comparison of the Coomassie staining of purified proteins with a BSA standard.

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