

# The conserved Pkh–Ypk kinase cascade is required for endocytosis in yeast

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Internalization of activated signaling receptors by endocytosis is one way cells downregulate extracellular signals. Like many signaling receptors, the yeast  $\alpha$ -factor pheromone receptor is downregulated by hyperphosphorylation, ubiquitination, and subsequent internalization and degradation in the lysosome-like vacuole. In a screen to detect proteins involved in ubiquitin-dependent receptor internalization, we identified the sphingoid base-regulated serine–threonine kinase Ypk1. Ypk1 is a homologue of the mammalian serum- and glucocorticoid-induced kinase, SGK, which can substitute for Ypk1 function in yeast. The kinase activity of Ypk1 is required for receptor endocytosis

because mutations in two residues important for its catalytic activity cause a severe defect in  $\alpha$ -factor internalization. Ypk1 is required for both receptor-mediated and fluid-phase endocytosis, and is not necessary for receptor phosphorylation or ubiquitination. Ypk1 itself is phosphorylated by Pkh kinases, homologues of mammalian PDK1. The threonine in Ypk1 that is phosphorylated by Pkh1 is required for efficient endocytosis, and *pkh* mutant cells are defective in  $\alpha$ -factor internalization and fluid-phase endocytosis. These observations demonstrate that Ypk1 acts downstream of the Pkh kinases to control endocytosis by phosphorylating components of the endocytic machinery.

## Introduction

The internalization of plasma membrane proteins into the endocytic pathway is a key regulatory event in signal transduction, nutrient uptake, and ion homeostasis. The activity and interaction of the network of proteins and lipids that comprise the endocytic machinery is carefully controlled in response to intra- and extracellular signals. This control is exerted by many types of regulatory proteins, including protein and lipid kinases, phosphatases, GTPases, and proteins that regulate actin dynamics. Lipid messengers, such as phosphoinositides, sphingolipids, and sterols, also play key roles in endocytosis (for review see D'Hondt et al., 2000). Sphingolipids and their precursors, sphingoid bases and ceramides, have recently emerged as important but poorly understood activators of endocytosis. Exogenous addition of C<sub>6</sub> ceramides or liberation of ceramide from sphingomyelin by addition of sphingomyelinase promotes endocytic vesicle formation in mammalian cells (Zha et al., 1998; Li et al., 1999). In yeast, an *lcb1* (long chain base) mutant defective

in the first step of sphingolipid synthesis was isolated in a screen for endocytosis mutants, and the *lcb1* endocytic phenotype is rescued by exogenous addition of sphingoid bases (Munn and Riezman, 1994; Zanolari et al., 2000).

Ceramide and sphingoid bases may be crucial for endocytosis because they activate regulatory phosphorylation cascades. Inactivation of protein phosphatase 2A, or overexpression of either the Pkc1 or Yck2 kinase, suppresses the endocytosis defects of an *lcb1* mutant, suggesting that sphingoid base-stimulated kinase activity is important for receptor endocytosis (Friant et al., 2000). Endocytic proteins that are kinase substrates include clathrin (Wilde et al., 1999), amphiphysin (Bauerfeind et al., 1997), dynamin (Robinson et al., 1993), synaptojanin (McPherson et al., 1994), Eps15 (Fazioli et al., 1993), and epsin (Chen et al., 1999). The regulated phosphorylation of these proteins is likely to be critical for the assembly and disassembly of the network required for internalization (Slepnev et al., 1998).

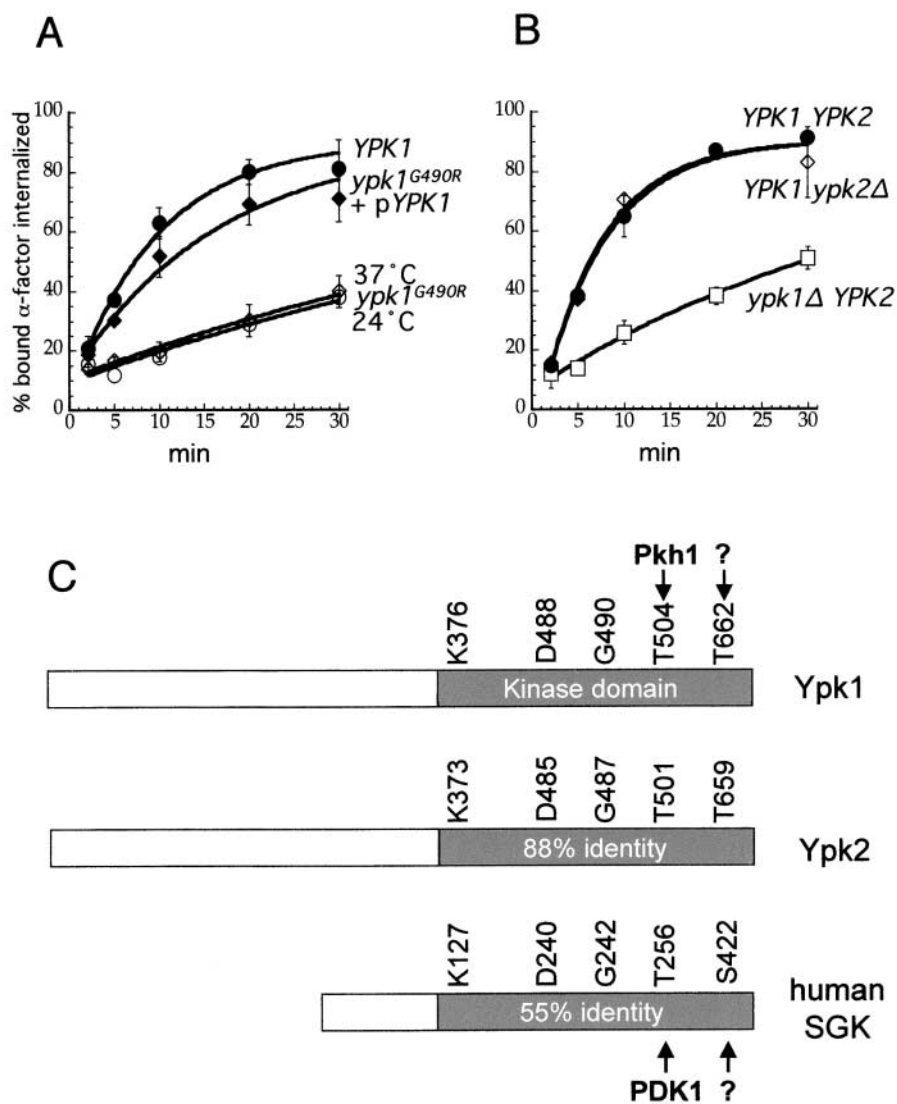
Many of the proteins comprising the internalization machinery are conserved from yeast to mammals, and yeast has been exploited to identify novel proteins that participate in receptor internalization (for review see D'Hondt et al., 2000). Receptor-mediated endocytosis has been studied in *Saccharomyces cerevisiae* using Ste2, a G protein-coupled signaling receptor that is rapidly internalized in response to binding its ligand,  $\alpha$ -factor (Jenness and Spatrick, 1986). The isolation of mutants defective in Ste2 internalization has

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**Figure 1. Ypk1 is required for  $\alpha$ -factor internalization.** (A and B) Internalization of  $^{35}\text{S}$ - $\alpha$ -factor was measured by the continuous presence protocol at 37°C (except where noted) after growth in YPUAD. *ypk1<sup>G490R</sup>* cells are the same as *udi5-1* cells. (A) *YPK1* (LHY291, ●); *ypk1<sup>G490R</sup>* (LHY2543 ◇); *ypk1<sup>G490R</sup>* with pYPK1, a centromeric plasmid (LHY2712, ◆); *ypk1<sup>G490R</sup>* (LHY2543, ○, at 24°C). (B) *YPK1 YPK2* (LHY2632, ●); *ypk1 $\Delta$  YPK2* (LHY2536, □); *YPK1 ypk2 $\Delta$*  cells (LHY2633, ◇). (C) Schematic diagrams of Ypk1, Ypk2 (68% identical to Ypk1), and human SGK (50% identical to Ypk1). Residues mutated in this study and their counterparts in Ypk1 homologues are shown. The percent identity of the kinase domains is shown in the gray box. Phosphorylation sites are indicated with an arrow with known kinases noted.



revealed a novel role for the sphingoid base-regulated Pkh and Ypk kinases in the internalization step of endocytosis.

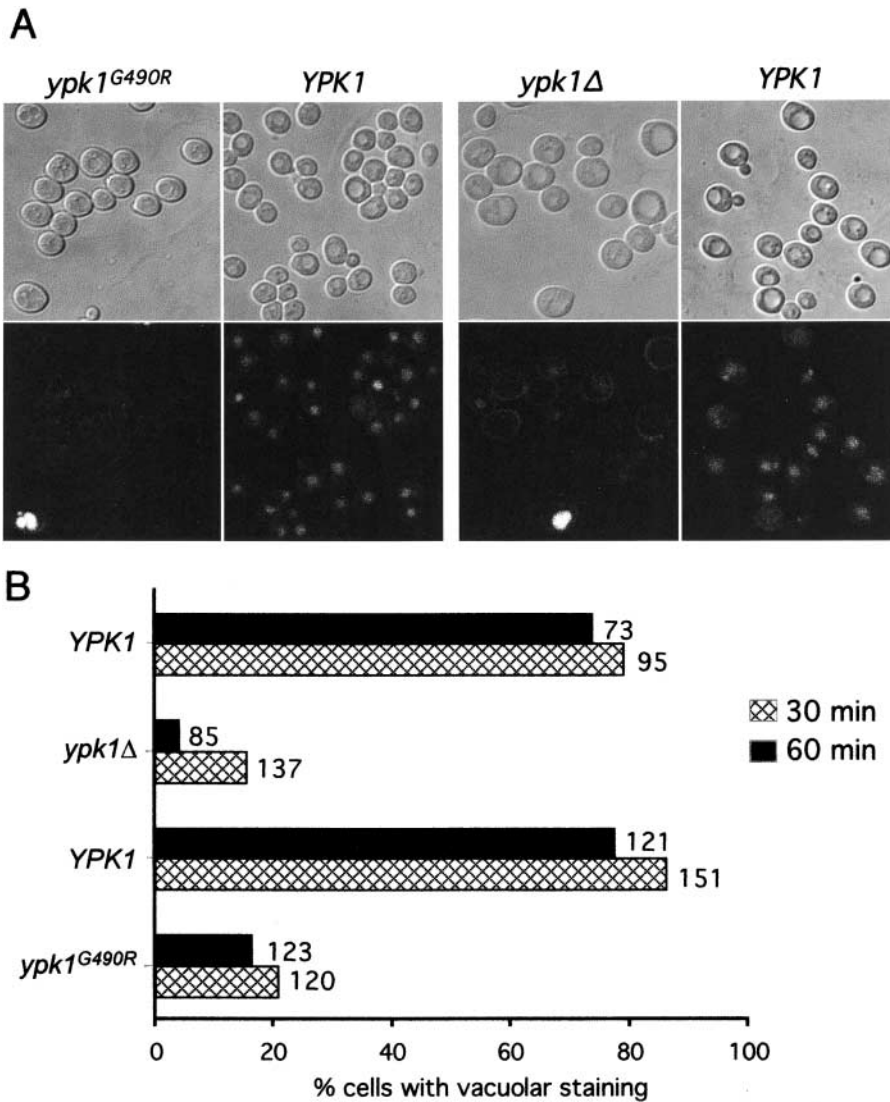
## Results and discussion

### Ypk1 is required for endocytosis

Ubiquitination of the Ste2 cytoplasmic tail is required before internalization (Hicke and Riezman, 1996). We performed a screen of ethyl methanesulfonate-mutagenized cells to identify novel proteins involved in ubiquitin-dependent receptor internalization. One mutant, *udi5-1* (ubiquitin-dependent internalization), that was defective in  $\alpha$ -factor internalization at both 24°C and 37°C (Fig. 1 A), showed reduced growth on YPUAD + 2 mM EGTA. We screened a genomic DNA library for plasmids that rescued this growth defect and identified a plasmid carrying the *YPK1* gene. A centromere-based plasmid carrying *YPK1* restored the ability of *udi5-1* both to grow on YPUAD + 2 mM EGTA (unpublished data) and to internalize  $\alpha$ -factor (Fig. 1 A). A *ypk1 $\Delta$*  strain had an internalization defect similar to the *udi5-1* strain (Fig. 1 B), suggesting that the mutation in the *udi5-1* strain was in

*YPK1*. We rescued the *ypk1* gene from *udi5-1* cells, and found that it had a single point mutation in the coding region for the Ypk1 catalytic domain that changed glycine 490 to arginine. Expression of Ypk1<sup>G490R</sup> in *ypk1 $\Delta$*  cells did not rescue internalization, whereas expression of Ypk1 did (unpublished data). These results demonstrate that *UDI5* is allelic to *YPK1*, and hereafter we refer to *udi5-1* as *ypk1<sup>G490R</sup>*.

Ypk1 is a serine-threonine kinase involved in sphingolipid signaling (Sun et al., 2000). Ypk1 has a *S. cerevisiae* homologue, Ypk2 (68% identical), and a mammalian homologue, SGK (50% identical) (Casamayor et al., 1999) (Fig. 1 C). The amino acid mutated in *ypk1<sup>G490R</sup>* cells, G490, is conserved in Ypk1 homologues. Unlike *ypk1 $\Delta$*  cells, *ypk2 $\Delta$*  cells exhibited no  $\alpha$ -factor internalization defect (Fig. 1 B). Ypk1 is important for endocytosis and cannot be replaced by Ypk2, despite their high degree of conservation, whereas Ypk2 plays either no role in endocytosis or a role that can be fully assumed by Ypk1. Ypk1 and Ypk2 perform at least one redundant, essential function because *ypk1 $\Delta$  ypk2 $\Delta$*  cells are dead, even though each single-null mutant is viable (Chen et al.,



**Figure 2. Ypk1 is required for fluid-phase endocytosis.** (A and B) Lucifer yellow (LY) localization was assayed in *ypk1<sup>G490R</sup>* (LHY2543), *ypk1Δ* (LHY2536), and wild-type cells from the same tetrad as each mutant (LHY2761 and LHY2537, respectively). Cells were grown to early logarithmic phase in rich medium at 24°C, shifted to 37°C for 15 min, and then allowed to internalize LY at 37°C for 30 or 60 min (A) Images were taken using DIC optics (top) and fluorescence optics (bottom). (B) The percent of cells that accumulated LY in their vacuoles was quantified for the 30- and 60-min time points by blind counting of each sample. The number of cells counted is indicated to the right of the corresponding bar.

1993; Casamayor et al., 1999). *ypk1<sup>G490R</sup> ypk2Δ* cells are also dead, suggesting that the Ypk1<sup>G490R</sup> protein is completely inactive.

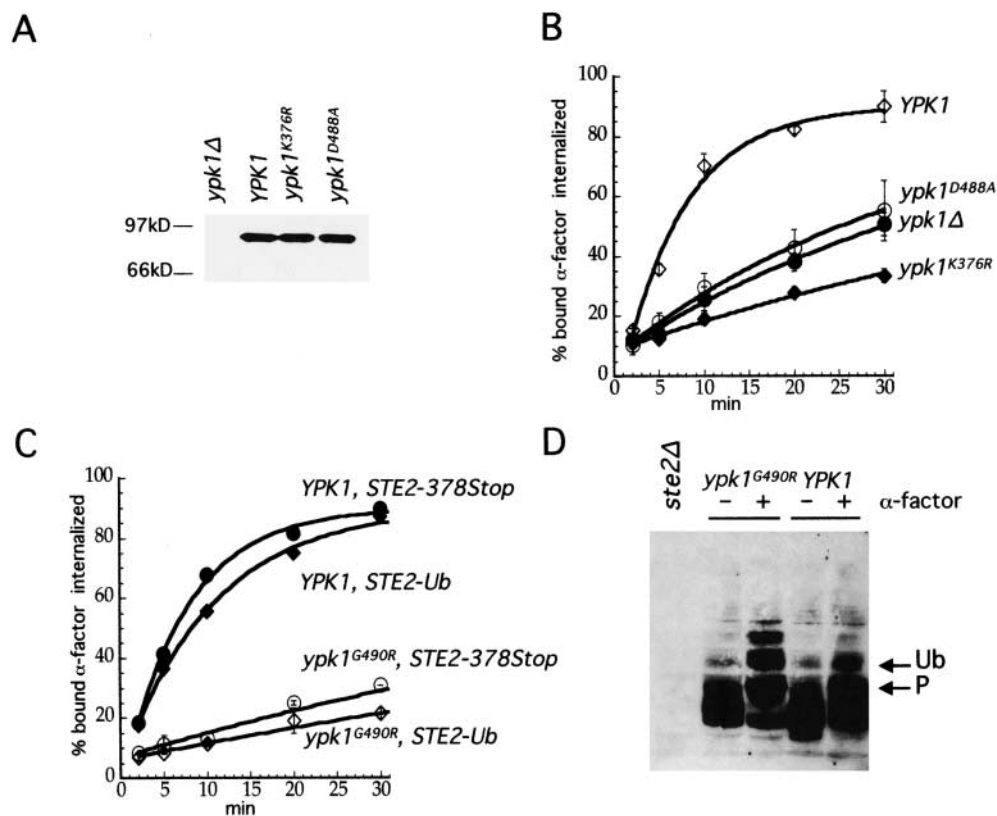
To investigate the role of Ypk1 in fluid-phase endocytosis, we assayed the ability of *ypk1* cells to deliver Lucifer yellow (LY)\* to the vacuole (Fig. 2, A and B). Both *ypk1<sup>G490R</sup>* and *ypk1Δ* cells were significantly impaired in LY transport compared with their congenic wild-type strains. Ypk1 was also required for internalization of receptors carrying the linear peptide internalization signal NPF<sub>XD</sub> (Tan et al., 1996), instead of a ubiquitin signal (unpublished data). Ypk1 is not generally required for membrane trafficking, because carboxypeptidase Y was transported through the biosynthetic pathway to the vacuole with normal kinetics in *ypk1<sup>G490R</sup>* cells incubated at the restrictive temperature (unpublished data). These observations indicate that Ypk1 is necessary for fluid-phase endocytosis and for the internalization of plasma membrane proteins carrying different types of internalization signals.

\*Abbreviations used in this paper: DIC, differential interference contrast; HA, hemagglutinin; LY, Lucifer yellow.

### The kinase activity of Ypk1 is required for endocytosis downstream of receptor phosphorylation and ubiquitination

To test whether the kinase activity of Ypk1 is required for its function in internalization, we made two mutant forms of epitope-tagged Ypk1 that abolish its kinase activity, Ypk1<sup>K376R</sup> and Ypk1<sup>D488A</sup> (Casamayor et al., 1999; Sun et al., 2000). We assayed cells expressing similar levels of the different Ypk proteins (Fig. 3 A) for their ability to internalize  $\alpha$ -factor. Both *ypk1<sup>K376R</sup>* and *ypk1<sup>D488A</sup>* mutants showed internalization defects similar to that of *ypk1Δ* (Fig. 3 B), demonstrating that the kinase activity of Ypk1 is required for its role in endocytosis.

We used two approaches to test whether Ypk1 is involved in Ste2 phosphorylation, which is a prerequisite to receptor ubiquitination and internalization (Hicke et al., 1998). First, we assayed  $\alpha$ -factor internalization by a Ste2-Ub chimeric protein that does not require posttranslational ubiquitination for endocytosis (Terrell et al., 1998; Dunn and Hicke, 2001). In *ypk1<sup>G490R</sup>* cells, internalization of Ste2-Ub was as defective as the internalization of a similar receptor carrying posttranslational ubiquitination sites but no fused ubiquitin (Ste2-378Stop) (Fig. 3 C), consistent with the



**Figure 3. The kinase activity of Ypk1 is required for receptor internalization, but not for receptor modifications.** (A) Extracts of *ypk1Δ* cells expressing hemagglutinin (HA)-tagged wild-type Ypk1, Ypk1<sup>K376R</sup>, or Ypk1<sup>D488A</sup> were subjected to immunoblotting with  $\alpha$ -HA antibodies. (B and C) Cells were grown and assayed for  $\alpha$ -factor internalization as in Fig. 1. (B) The same cells used in A: *ypk1Δ* (LHY2536, ●); YPK1 (LHY2563, ◇); *ypk1<sup>K376R</sup>* (LHY2564, ◆); *ypk1<sup>D488A</sup>* (LHY2565, ○). (C) YPK1 cells expressing Ste2–378Stop (LHY825, ●) or Ste2-Ub (LHY558, ◆); *ypk1<sup>G490R</sup>* cells expressing Ste2–378Stop (LHY2684 ○) or Ste2-Ub (LHY2690 ◇). (D) *ste2Δ* (LHY10), *ypk1<sup>G490R</sup>* (LHY2543), or YPK1 (LHY1) cells were treated (+) or not (–) with 1  $\mu$ M  $\alpha$ -factor for 8 min at 37°C. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with  $\alpha$ -Ste2 antibodies. Phosphorylated (P) and monoubiquitinated (Ub) species are denoted with arrows.

idea that Ypk1 functions after receptor ubiquitination. Second, we demonstrated that Ste2 was phosphorylated and ubiquitinated normally in *ypk1<sup>G490R</sup>* and *ypk1Δ* cells (Fig. 3 D; unpublished data). The modified forms accumulated in *ypk1<sup>G490R</sup>* cells when compared with wild-type cells, as observed in other endocytic mutants (Hicke and Riezman, 1996). These results indicate that the kinase activity of Ypk1 is required for receptor internalization, and that Ypk1 is not involved in ligand-stimulated receptor modifications.

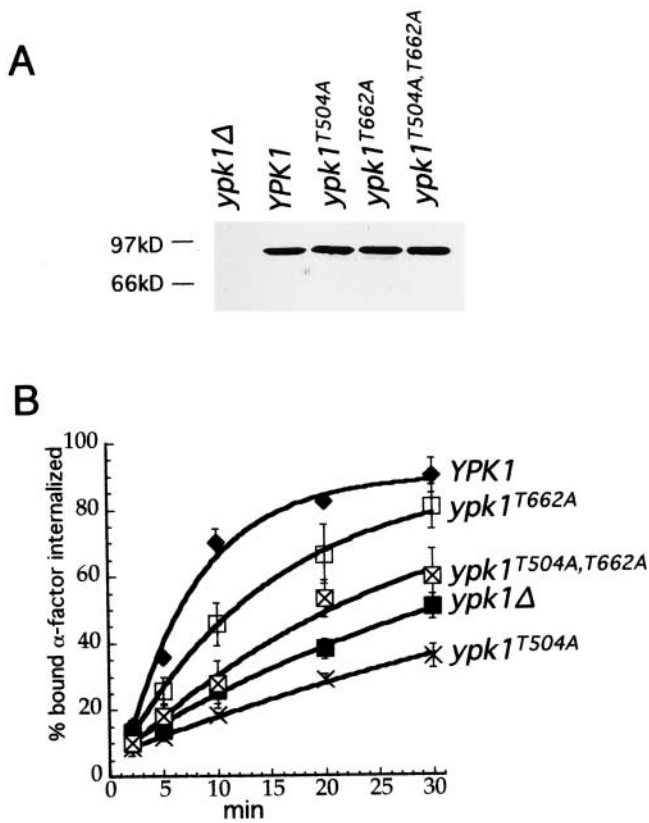
#### Pkh-dependent phosphorylation of Ypk1 is required for efficient internalization

Ypk1 contains two conserved phosphorylation sites, T504 and T662 (Fig. 1 C). T504 is phosphorylated by Pkh1, a yeast homologue of the phosphoinositide-dependent kinase, PDK1 (Casamayor et al., 1999; Inagaki et al., 1999). To determine if either T504 or T662 is involved in the endocytic function of Ypk1, we mutated these residues to alanine alone or in combination. We integrated the constructs into *ypk1Δ* cells, identified transformants with equivalent expression levels of epitope-tagged Ypk1 variants (Fig. 4 A), and performed  $\alpha$ -factor internalization assays on these cells. Cells expressing Ypk1<sup>T662A</sup> showed a slight defect in internalization, whereas the cells expressing Ypk1<sup>T504A</sup> or

Ypk1<sup>T504A,T662A</sup> were more strongly impaired (Fig. 4 B). These results indicate that the conserved phosphorylation sites of Ypk1 are required for efficient internalization. T504 appears to play a critical role in internalization, suggesting that phosphorylation by Pkh1 is important for endocytosis. The modest but significant internalization defect observed with cells expressing Ypk1<sup>T662A</sup> suggests Ypk1 endocytic activity may also be regulated by phosphorylation at T662.

Due to the importance of T504, we investigated the role of Pkh1 in endocytosis. Pkh1 shares significant homology with two other yeast kinases, Pkh2 and Pkh3. Functions of these kinases are at least partially redundant because null mutations in individual *PKH* genes are not lethal, whereas a *pkh1Δ pkh2Δ* mutant is dead or slow growing (Casamayor et al., 1999; Inagaki et al., 1999; see below). We tested the ability of *pkh1Δ*, *pkh2Δ*, and *pkh3Δ* cells to internalize  $\alpha$ -factor and deliver LY to the vacuole. None of the single *pkh* mutants showed a defect in either assay as compared with isogenic wild-type cells (unpublished data). We then created double mutants to examine if Pkh kinases function redundantly in internalization. It has been reported that cells carrying a deletion of both *PKH1* and *PKH2* are inviable (Casamayor et al., 1999; Inagaki et al., 1999). In our genetic background, *pkh1Δ pkh2Δ* cells were viable, but grew slowly at 24°C (Fig. 5 A). We found that the





**Figure 4. The conserved phosphorylation sites of Ypk1 are required for  $\alpha$ -factor internalization.** (A) Extracts of *ypk1Δ* cells expressing HA-tagged wild-type Ypk1, Ypk1<sup>T504A</sup>, Ypk1<sup>T662A</sup>, or Ypk1<sup>T504A,T662A</sup>. (B) The same cells used in A were grown and assayed as in Fig. 1: *ypk1Δ* (LHY2536, ■); YPK1 (LHY2563, ◆); *ypk1<sup>T504A</sup>* (LHY2568 X); *ypk1<sup>T662A</sup>* (LHY2567, □); *ypk1<sup>T504A,T662A</sup>* (LHY2569, ☒).

slow growth phenotype of *pkh1Δ pkh2Δ* cells could be suppressed substantially by 1.2 M sorbitol, even at 30°C (Inagaki et al., 1999; Fig. 5 A). *pkh1Δ pkh2Δ* cells grown in sorbitol displayed a strong defect in  $\alpha$ -factor internalization and in accumulation of LY in the vacuole (Fig. 5, B and C). By contrast, *pkh1Δ pkh2Δ* cells grown in sorbitol were not generally defective in protein transport processes because they efficiently transported carboxypeptidase Y to the vacuole (unpublished data) and they appeared morphologically normal (Fig. 5 C). To support these findings, we obtained a mutant strain that is temperature sensitive for Pkh function (*pkh1<sup>D398G</sup> pkh2Δ*) (Inagaki et al., 1999), but which grows normally at 24°C (Fig. 5 A). The *pkh1<sup>ts</sup>* strain was severely defective for  $\alpha$ -factor internalization at the nonpermissive growth temperature of 30°C (Fig. 5 D). Like *ypk1* mutants, *pkh* mutants showed no defect in Ste2 phosphorylation or ubiquitination (unpublished data). These results indicate that the Pkh family of kinases is required for endocytosis, and suggest that at least one of their roles is to activate Ypk1 by phosphorylating T504.

### A conserved, sphingoid base-regulated kinase cascade in endocytosis

Sphingoid bases are required for the internalization step of endocytosis in yeast. A *lcb1–100* strain defective in sphin-

golipid biosynthesis is defective in receptor and fluid-phase endocytosis, and the addition of sphingoid bases suppresses this defect (Munn and Riezman, 1994; Zanolari et al., 2000). In addition, our screen identified a *lcb2* mutant strain with an  $\alpha$ -factor internalization defect similar to that of *lcb1–100* (unpublished data). Pkh and Ypk kinases are two members of a kinase cascade that function downstream of sphingoid bases. Ypk1 was isolated as a suppressor of growth inhibition by ISP-1, a toxic compound that acts by depleting intracellular sphingoid base levels. Furthermore, phosphorylation of Ypk1, which is required for its kinase activity, is dependent on the level of sphingoid bases in cells (Sun et al., 2000). *ypk1* mutants are synthetically lethal with both *lcb1* and *lcb2* mutants (unpublished data), an observation that supports a connection between sphingoid base synthesis and Ypk activity. Unlike the Yck and Pkc kinases, overexpression of Ypk1 does not suppress the internalization defect of *lcb1* or *lcb2* (Friant et al., 2000; unpublished data). This may be because overexpression does not increase the level of active, phosphorylated Ypk1, or because sphingoid bases regulate other endocytic proteins whose activity is not increased in a Ypk1-overexpressing cell.

Pkh kinases are likely to be the link between sphingoid bases and Ypk1 activation. Pkh-dependent phosphorylation is required for Ypk activity and the overexpression of Pkh1, like Ypk1, suppresses growth inhibition by ISP-1 (Sun et al., 2000). PDK1, the functional mammalian homologue of Pkh1 (Casamayor et al., 1999), is activated by sphingoid bases (King et al., 2000). Like Pkh1, Ypk1 has a mammalian homologue and the Pkh–Ypk kinase cascade is conserved in mammalian cells. PDK1 phosphorylates SGK at T256, a position equivalent to T504 in Ypk1 (Fig. 1 A) (Park et al., 1999). Although a role for PDK or SGK in endocytosis has not been reported, another Ypk homologue, PKB/Akt, is required to activate a small GTPase, Rab5, that is involved in endocytic trafficking (Barbieri et al., 1998).

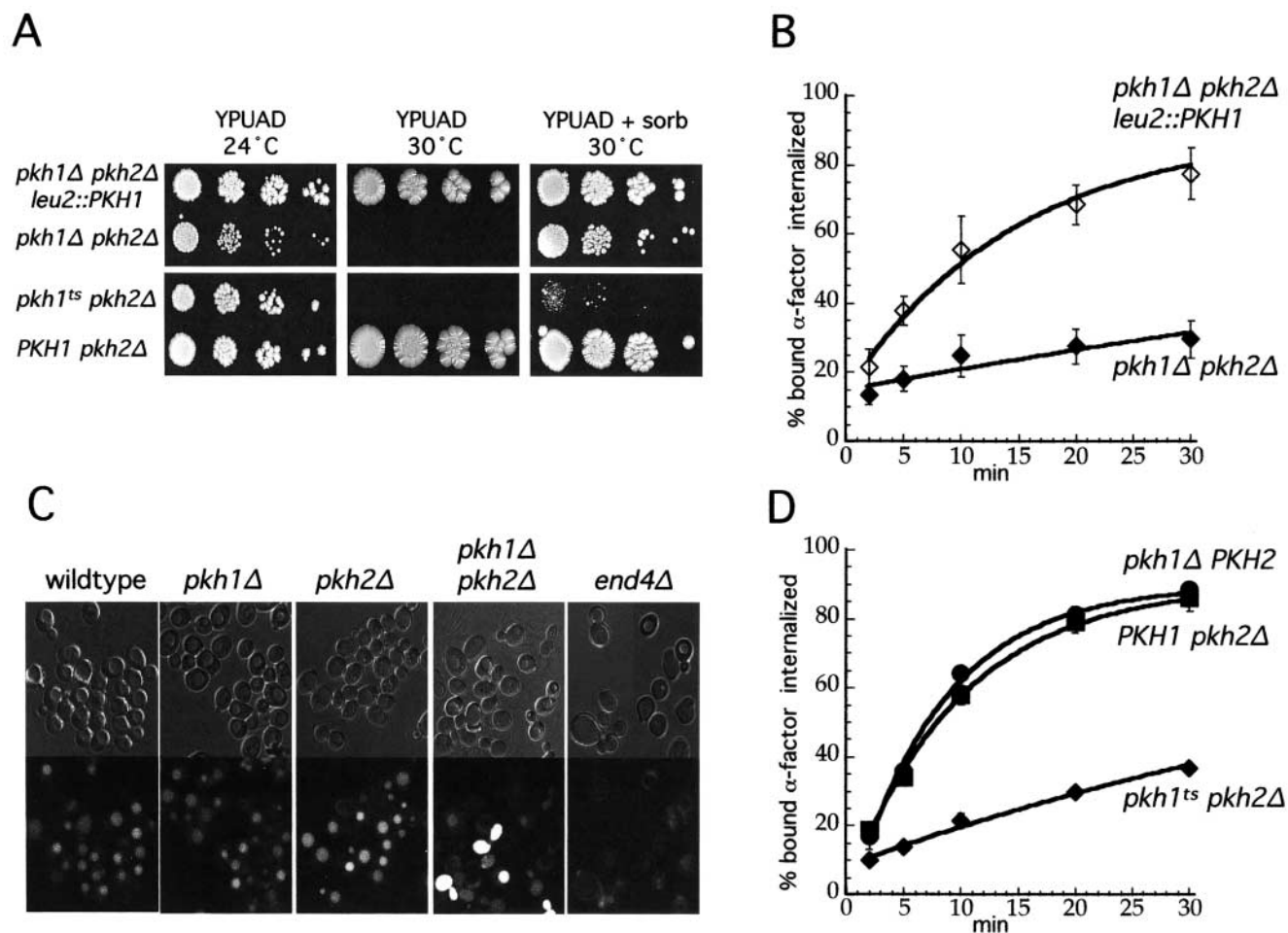
Sphingoid base-dependent phosphorylation may regulate Ypk1 by controlling its localization. Treatment of yeast cells with sphingoid bases appears to cause increased association of overexpressed GFP–Ypk1 with the plasma membrane (Sun et al., 2000); however, we did not see this effect using subcellular fractionation of Ypk1 expressed at normal levels. Cellular substrates of the Ypk kinases have not been reported. Friant et al. (2000) suggested that a target of sphingoid base regulation may be an actin-binding protein because the overexpression of sphingoid bases suppresses both the endocytic and the actin cytoskeleton defects in a *lcb1* mutant. One of the many proteins that link actin to endocytosis in yeast may be regulated by Ypk1-dependent phosphorylation.

Regulation of the internalization step of endocytosis requires multiple kinases that receive input from different sources for efficient assembly of endocytic vesicles. We have shown that the Pkh–Ypk1 kinase cascade is an important regulatory component of the endocytic machinery.

## Materials and methods

### Reagents

All strains were grown in minimal (SD) or rich (YPUAD) medium (Sherman, 1991) as indicated in the figure legends. Table 1 lists the strains used



**Figure 5. Pkh kinases are required for receptor-mediated and fluid-phase endocytosis.** (A) Growth of *pkh1Δ pkh2Δ* cells and *pkh1Δ pkh2Δ leu2::PKH1* cells from the same tetrad (LHY2716 and LHY2714, respectively) or *pkh<sup>ts</sup> (pkh1<sup>D398G</sup> pkh2Δ)* cells (LHY3030) and an isogenic *pkh2Δ* strain (LHY3032) on YPUAD at 24°C or 30°C or on YPUAD + 1.2 M sorbitol at 30°C after 4 d. The differences in strain background between the two *pkh* mutants are likely to account for the difference in suppression of the growth defect on sorbitol medium. (B) The same *pkh1Δ pkh2Δ* (◆) and *pkh1Δ pkh2Δ leu2::PKH1* (◇) strains as in (A) were grown overnight in YPUAD + 1.2 M sorbitol. Internalization of <sup>35</sup>S- $\alpha$ -factor was measured by the continuous presence protocol at 30°C in YPUAD + 1.2 M sorbitol. (C) LY localization was assayed for wild-type cells (LHY2762), *pkh1Δ* cells (LHY2759), *pkh2Δ* cells (LHY2760), *pkh1Δ pkh2Δ* cells (LHY2716), and *end4Δ* cells (LHY37). Cells were grown to early logarithmic phase in YPUAD + 1.2 M sorbitol at 24°C, shifted to 30°C for 15 min, and then incubated with LY at 30°C for 60 min. Images were taken using DIC optics (top) and fluorescence optics (bottom). The *pkh1Δ pkh2Δ* cells that are brightly stained throughout the whole cell are probably lysed cells. (D) *pkh1Δ* cells (LHY3031, ●), *pkh2Δ* cells (LHY3032, ■), and *pkh<sup>ts</sup>* cells (LHY3030, ◆) were grown overnight in YPUAD. Internalization of <sup>35</sup>S- $\alpha$ -factor was measured by the continuous presence protocol at 30°C in YPUAD.

in this study. BY4741 strains containing deletions in *YPK1*, *YPK2*, *PKH1*, *PKH2*, and *PKH3* were obtained from EUROSCARF. *pkh1<sup>ts</sup> pkh2Δ* and related strains were a gift from Kunihiro Matsumoto (Nagoya University, Nagoya, Japan). *bar1* derivatives were generated by single-step gene transplacement or by crossing to a *bar1* strain. Ste2 antiserum and <sup>35</sup>S- $\alpha$ -factor were purified as previously described (Dulic et al., 1991; Hicke and Riezman, 1996). HA monoclonal antibody 12CA5 was a gift of Robert Lamb (Northwestern University, Evanston, IL).

*STE2* plasmids used in this study have been described (Terrell et al., 1998; Dunn and Hicke, 2001). A triple HA-epitope was inserted in-frame at the NH<sub>2</sub> terminus of Ypk1 in pRS303 (LHP1113). The HA-*YPK1* plasmid rescued the growth and internalization defects of *ypk1<sup>G490R</sup>* cells. Mutations in LHP1113 were generated using site-directed mutagenesis (QuickChange™; Stratagene).

#### Screen for *udi* mutants

LHY1451, which expressed a variant  $\alpha$ -factor receptor with only ubiquitin-dependent internalization signals (Ste2-378Stop), was treated with 3% ethyl methanesulfonate for 90 min. Mutagenized cells were tested for their ability to internalize  $\alpha$ -factor using an assay modified from the method of Dulic et al. (1991). Cells were grown in YPUAD to mid logarithmic phase,

then incubated at 37°C for 15 min before the addition of <sup>35</sup>S- $\alpha$ -factor. 20 min after  $\alpha$ -factor addition, aliquots of cells were placed in either pH1 or pH6 buffer and cell associated radioactivity in each sample was determined as described (Dulic et al., 1991). Under these conditions, LHY1451 cells internalized 68 ± 8% of the surface-bound  $\alpha$ -factor. Mutagenized cells that consistently internalized <40% of bound  $\alpha$ -factor at 20 min were crossed to wild-type cells at least three times.

#### Cloning of *YPK1*

*YPK1* was cloned from a centromeric genomic library made in the YCpKan101 vector, provided by Jon Binkley and David Botstein (Stanford University, Stanford, CA) by complementation of the growth defect of *ypk1<sup>G490R</sup>* cells on YPUAD + 2 mM EGTA at 37°C. The *YPK1* gene was subcloned from this plasmid by ligating the 2.8-kb *BstBI* fragment into the *Clal* site of YCp50, resulting in LHP1086.

The mutation in *ypk1<sup>G490R</sup>* was determined by gapped plasmid repair (Rothstein, 1991). The gapped *YPK1* plasmid was purified and transformed into *ypk1<sup>G490R</sup>* and *YPK1* cells. Repaired plasmids were purified from yeast cells and transformed into *E. coli*. Bacterial plasmid DNA was purified and sequenced. Two plasmids recovered from *ypk1<sup>G490R</sup>* cells were each found to have a single point mutation in codon 490.

Table I. Strains used in this study

Strain	Genotype <sup>a</sup>
LHY1	<i>his4 leu2 lys2 ura3 bar1-1</i>
LHY10	<i>ste2Δ::LEU2 his3 leu2 trp1 ura3 bar1-1</i>
LHY37	<i>end4::LEU2 his4 leu2 lys2 ura3 bar1-1</i>
LHY291	<i>his3 leu2 lys2 trp1 ura3 bar1-1</i>
LHY558	<i>ste2Δ::LEU2 his3 leu2 trp1 ura3::STE2<sup>4XR</sup>-Ub<sup>K48R</sup>::URA3 bar1-1</i>
LHY825	<i>ste2Δ::LEU2 his3 leu2 trp1 ura3::STE2<sup>378Stop</sup>::URA3 bar1-1</i>
LHY1451	<i>ste2Δ::TRP1 GAL2 ade2 his3 leu2 lys2 trp1 ura3 bar1-1 pGalSTE2-378Stop[URA3]</i>
LHY2536	<i>ypk1Δ::KAN his3 leu2Δ met15Δ ura3 bar1-1</i>
LHY2537	<i>his3 leu2 trp1 ura3</i>
LHY2543	<i>ypk1<sup>G490R</sup> GAL2 his3 leu2 lys2 trp1 ura3 bar1-1</i>
LHY2563	<i>ypk1Δ::KAN his3::YPK1::HIS3 leu2Δ met15Δ ura3 bar1-1</i>
LHY2564	<i>ypk1Δ::KAN his3::YPK1<sup>K376R</sup>::HIS3 leu2Δ met15Δ ura3 bar1-1</i>
LHY2565	<i>ypk1Δ::KAN his3::YPK1<sup>D488A</sup>::HIS3 leu2Δ met15Δ ura3 bar1-1</i>
LHY2567	<i>ypk1Δ::KAN his3::YPK1<sup>T662A</sup>::HIS3 leu2Δ met15Δ ura3 bar1-1</i>
LHY2568	<i>ypk1Δ::KAN his3::YPK1<sup>T504A</sup>::HIS3 leu2Δ met15Δ ura3 bar1-1</i>
LHY2569	<i>ypk1Δ::KAN his3::YPK1<sup>T504A, T662A</sup>::HIS3 leu2Δ met15Δ ura3 bar1-1</i>
LHY2632	<i>his3Δ leu2Δ met15Δ ura3Δ bar1Δ::URA3</i>
LHY2633	<i>ypk2Δ::KAN his3Δ leu2Δ met15Δ ura3Δ bar1Δ::URA3</i>
LHY2684	<i>ypk1<sup>G490R</sup> ste2Δ::LEU2 GAL2 his3 leu2 lys2 trp1 ura3::STE2<sup>378Stop</sup>::URA3 bar1-1</i>
LHY2690	<i>ypk1<sup>G490R</sup> ste2Δ::LEU2 GAL2 his3 leu2 lys2 trp1 ura3::STE2<sup>4XR</sup>-Ub<sup>K48R</sup>::URA3 bar1-1</i>
LHY2712	<i>ypk1<sup>G490R</sup> GAL2 his3 leu2 lys2 trp1 ura3 bar1 pYPK1[URA3]</i>
LHY2714	<i>pkh1Δ::KAN pkh2Δ::KAN his3Δ leu2::PKH1::LEU2 met15Δ ura3Δ bar1-1</i>
LHY2716	<i>pkh1Δ::KAN pkh2Δ::KAN his3Δ leu2 met15Δ ura3Δ bar1-1</i>
LHY2759	<i>pkh1Δ::KAN his3 leu2 trp1 ura3 bar1-1</i>
LHY2760	<i>pkh2Δ::KAN his3 leu2 trp1 ura3 bar1-1</i>
LHY2761	<i>MATα GAL2 his3 leu2 lys2 trp1 ura3 bar1-1</i>
LHY2762	<i>MATα leu2 his3 met15 ura3</i>
LHY3030	<i>pkh1<sup>D398G</sup> pkh2Δ::LEU2 ade1 his2 leu2 trp1 ura3 bar1Δ::URA3</i>
LHY3031	<i>pkh1Δ::LEU2 ade1 his2 leu2 trp1 ura3 bar1Δ::URA3</i>
LHY3032	<i>pkh2Δ::LEU2 ade1 his2 leu2 trp1 ura3 bar1Δ::URA3</i>

<sup>a</sup>All strains are MATα unless otherwise noted.

### Internalization assays

All α-factor internalization assays were performed essentially as described (Dulic et al., 1991) using the continuous presence protocol. Conditions for growth are indicated in the figure legends. Cells were harvested in early logarithmic phase and shifted to the assay temperature for 15 min before the addition of <sup>35</sup>S-α-factor. Each data point represents the average of at least three experiments. The error bars represent the standard deviation. LY localization assays were performed as described (Dulic et al., 1991). Cells were grown in YPUAD at 24°C to early logarithmic phase and 2 × 10<sup>7</sup> cells were harvested by centrifugation. After 30 min or 1 h incubation with LY (Sigma-Aldrich) at the indicated temperature, cells were washed and resuspended in phosphate buffer and mounted on a slide with an equal volume of 1.6% low-melt agarose. Cells were viewed using an LSM410 confocal microscope (Zeiss) with FITC or with differential interference contrast (DIC) optics. All FITC images within a figure were taken using identical parameters.

### Cell lysates and immunoblots

Lysates for HA-Ypk1 immunoblots were prepared after growth in YPUAD at 24°C (Horvath and Riezman, 1994). Lysates were resolved by SDS-PAGE on 10% gels and transferred to nitrocellulose. Membranes were first probed with α-HA antibodies, then with HRP-conjugated goat α-mouse antibodies (Sigma-Aldrich). The blots were developed using SuperSignal ECL reagents (Pierce Chemical Co.). Lysates for Ste2 immunoblots were prepared as described by Hicke and Riezman (1996), except cycloheximide treatment was not performed.

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