The EMBO Journal Vol. 19 No. 12 pp. 2834–2844, 2000

Increased protein kinase or decreased PP2A activity bypasses sphingoid base requirement in endocytosis

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Lipids have been implicated in signal transduction and in several stages of membrane trafficking, but these two functions have not been functionally linked. In yeast, sphingoid base synthesis is required for the internalization step of endocytosis and organization of the actin cytoskeleton. We show that inactivation of a protein phosphatase 2A (PP2A) or overexpression of one of two kinases, Yck2p or Pkc1p, can specifically suppress the sphingoid base synthesis requirement for endocytosis. The two kinases have an overlapping function because only a mutant with impaired function of both kinases is defective in endocytosis. An ultimate target of sphingoid base synthesis may be the actin cytoskeleton, because overexpression of the kinases and inactivation of PP2A substantially corrected the actin defect due to the absence of sphingoid base. These results suggest that sphingoid base controls protein phosphorylation, perhaps by activating a signal transduction pathway that is required for endocytosis and proper actin cytoskeleton organization in yeast.

Keywords: casein kinase I/endocytosis/protein kinase C/protein phosphatase 2A/sphingoid base

Introduction

Endocytosis is the process whereby eukaryotic cells internalize both plasma membrane and extracellular material. It is a commonly used pathway for recycling of components used in the secretory pathway, for the uptake of micronutrients and for the down-regulation of cell surface receptors. Certain viruses and microbes also exploit it to gain access to internal cellular compartments. Genetic studies of endocytosis using the yeast *Saccharomyces cerevisiae* have identified several components required for this pathway (Riezman *et al*., 1996; Geli and Riezman, 1998). Recently, not only proteins but also lipids, like sterols, were shown to be required for the internalization step of the endocytic pathway in yeast (Munn *et al*., 1999). Sterols also play a role in endocytosis in animal cells (Anderson, 1998; Kobayashi *et al*., 1998; Rodal *et al*., 1999; Subtil *et al*., 1999). Moreover, phosphatidic acid, diacylglycerol (DAG), ceramides and phosphoinositides have been implicated in several stages of membrane trafficking other than endocytosis in yeast (Bankaitis *et al*., 1990; Schu *et al*., 1993; Horvath *et al*., 1994; Kearns *et al*., 1997).

Sphingolipids and sphingoid bases and their phosphorylated derivatives, dihydrosphingosine-1-P (DHS-1P) and phytosphingosine-1-P (PHS-1P), are thought to be signaling molecules for regulating a variety of mammalian cellular processes including cell growth, stress, differentiation and apoptosis (Hannun, 1994, 1996; Cuvillier *et al*., 1996; Jayadev and Hannun, 1996; Kolesnick and Hannun, 1999). In mammalian cells, studies revealed that sphingosine induces *in vitro* phosphorylation of endogenous proteins through the activation of protein kinases (Pushkareva *et al*., 1992), and two unidentified sphingosine-activated protein kinases could be distinguished by their substrate specificity and their sphingosine requirement (Pushkareva *et al*., 1993). Sphingosine was also shown to have the ability to inhibit some kinases like protein kinase C (Hannun *et al*., 1986) and to activate some others, including casein kinase II (McDonald *et al*., 1991), the atypical protein kinase C isoform ζ (Muller *et al*., 1995), p21 activated kinase-1 (PAK1) (Bokoch *et al*., 1998) and 3-phosphoinositide-dependent kinase-1 (PDK1) (King *et al*., 2000). Thus, sphingoid bases appear to be bifunctional molecules with the ability to regulate protein phosphorylation.

In *S.cerevisiae*, sphingoid bases have been implicated in the heat stress response in yeast because they accumulate during heat stress (Jenkins *et al*., 1997; Skrzypek *et al*., 1999). Treatment of yeast cells with dihydrosphingosine (DHS), an intermediate in ceramide synthesis, leads to an accumulation of trehalose, a disaccharide that is required for full protection against heat stress. Moreover, DHS treatment induces transcription of a reporter gene containing the *TPS2* promoter or *STRE* (stress response element) sequences. The *TPS2* gene encodes a subunit of the trehalose synthase complex, suggesting that DHS acts as a signaling molecule to regulate trehalose accumulation during the heat stress response (Dickson *et al*., 1997). Recent reports show that heat shock induces an increase in the concentration of two phosphorylated sphingoid bases DHS-1P and PHS-1P (Skrzypek *et al*., 1999). In addition, several mutants accumulating these compounds have an increased survival at elevated temperature, suggesting that DHS-1P and PHS-1P may act as signals for resistance to heat stress (Mandala *et al*., 1998; Mao *et al*., 1999; Skrzypek *et al*., 1999).

Using the *S.cerevisiae lcb1-100* mutant, a requirement for sphingoid base synthesis for the internalization step of endocytosis and for proper actin cytoskeleton organization was revealed (Zanolari *et al*., 2000). The *LCB1* gene encodes a subunit of serine palmitoyltransferase that catalyzes the first step in sphingolipid synthesis: the condensation of serine and palmitoyl-CoA to yield the 3-ketosphinganine (Nagiec *et al*., 1994). Here we show that inactivation of a protein phosphatase, PP2A, or overexpression of one of two protein kinases, Yck2p or

Pkc1p, can abrogate this novel lipid requirement for endocytosis and restore a proper organization of the actin cytoskeleton. The two kinases have an overlapping function in endocytosis because only a mutant with impaired function of both kinases is defective in the internalization step of endocytosis. These results imply that the function of sphingoid base synthesis in endocytosis is to control protein phosphorylation. The ultimate target of the sphingoid base requirement may be the endocytic machinery and/or the actin cytoskeleton.

Results

Mutations in subunits of PP2A do not lead to a defect in endocytosis

Sphingolipids have been proposed to activate protein phosphatases (Dobrowsky *et al*., 1993; Wolff *et al*., 1994). The best candidate for a ceramide-activated protein phosphatase (CAPP) in yeast is PP2A (Fishbein *et al*., 1993; Nickels and Broach, 1996), which has two regulatory subunits, Cdc55p and Tpd3p (Healy *et al*., 1991; van Zyl *et al*., 1992), and a catalytic subunit. The identity of the catalytic subunit has been postulated to be Sit4p for the yeast CAPP (Nickels and Broach, 1996), but three other functionally overlapping genes, *PPH21*, *PPH22* and *PPH3*, encode the majority of the PP2A catalytic activity in yeast (Sneddon *et al*., 1990; Ronne *et al*., 1991). To determine whether PP2A plays a role in endocytosis, we constructed deletion strains for the non-essential regulatory subunit Cdc55p and temperature-sensitive strains for the essential catalytic subunits in our strain background. The strain *pph-ts* contains deletions of the catalytic subunits (*pph21Δ pph22Δ pph3Δ*) and is kept alive by a plasmid-borne temperature-sensitive *pph21-102* mutant allele. The strain *sit4-ts* contains a deletion of the catalytic subunit, *sit4Δ*, and harbors a temperature-sensitive *sit4-102* allele on a plasmid. In yeast, the internalization step of receptor-mediated endocytosis can be followed by using the α-factor pheromone that is bound and internalized by its G-protein coupled receptor, Ste2p (Riezman, 1998). α-factor uptake at 37°C was determined for *cdc55Δ*, *pph-ts* and *sit4-ts* strains and compared with wild-type cells (Figure 1A). All strains showed wild-type internalization kinetics, indicating that the internalization step of endocytosis is not affected by impaired function in a catalytic or a regulatory subunit of the PP2A.

Mutations in PP2A subunits restore endocytosis in the lcb1-100 mutant

The yeast *lcb1-100* mutant has a temperature-sensitive defect in the internalization step of endocytosis, witnessed by a defect in accumulation of the fluid-phase marker, lucifer yellow carbohydrazide (LY), in the vacuole (Figure 3) and a defect in internalization of the yeast α-factor pheromone (Figure 1B) (Munn and Riezman, 1994). To check whether inactivation of PP2A can affect the sphingoid base synthesis requirement for endocytosis, we created *lcb1 cdc55Δ*, *lcb1 pph-ts* and *lcb1 sit4-ts* mutant strains. We assayed these strains for internalization of α-factor at 37°C (Figure 1B). Interestingly, the *lcb1 cdc55Δ* and *lcb1 pph-ts* mutants internalized α-factor to a similar extent to the wild-type strain but the *lcb1 sit4-ts* mutant was still clearly defective in α-factor uptake. These

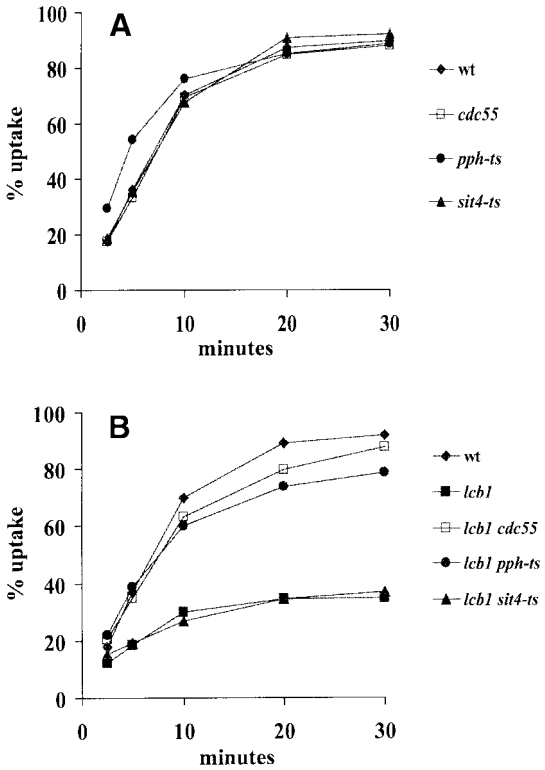

Fig. 1. Loss of PP2A function suppresses the *lcb1-100* mutation for endocytosis. (**A**) Wild-type RH1800 (wt) and single mutant strains RH3745 (*cdc55*), RH4137 (*pph-ts*) and RH4183 (*sit4-ts*) were assayed for α-factor internalization at 37°C. All mutants showed almost wild-type internalization kinetics. (**B**) The RH3809 strain (*lcb1-100*) and double mutant strains, RH3807 (*lcb1 cdc55*), RH4191 (*lcb1 pph-ts*) and RH4195 (*lcb1 sit4-ts*) were assayed for α-factor internalization at 37°C.

results indicate that inactivation of the *CDC55* or the *PPH* catalytic subunits of PP2A restores receptor-mediated endocytosis in the *lcb1-100* mutant.

These results were further supported by fluid-phase endocytosis assays. The *lcb1 cdc55Δ* and *lcb1 pph-ts* mutants accumulated LY in the vacuole to similar levels to wild-type cells (data not shown). These results show that loss of *CDC55* and *PPH21/22/3* function completely suppresses the *lcb1-100* mutation for endocytosis. Thus, the loss of PP2A activity abrogated the sphingoid base synthesis requirement for endocytosis, suggesting that the major role of sphingoid base is control of the protein phosphorylation status.

Overexpression of one of two kinases restores endocytosis in the lcb1-100 mutant

To identify a kinase that could control protein phosphorylation for endocytosis, we searched for a protein kinase whose overexpression would suppress the endocytic defect of the *lcb1-100* mutant. The *lcb1-100* strain was transformed with high copy (2μ) plasmids bearing genes encoding protein kinases (Table I). The yeast protein kinases can be subdivided into distinct families based on structural similarity in their catalytic domains (Hunter and Plowman, 1997). The kinases tested in this study belong to several families of protein kinases (Table I). None of the protein kinases tested was able to suppress the temperature-sensitive growth phenotype displayed by the *lcb1-100*

Table I. Plasmids used in this study

Plasmid	Yeast ori	Insert	Function	Group	Reference
pLJ721	2μ	*YCK1*	casein kinase I isoform	CK1	Robinson *et al.* (1993)
pL2.3	2μ	*YCK2*	casein kinase I isoform	CK1	Robinson *et al.* (1992)
YEp24-*YCK3*	2μ	*YCK3*	casein kinase I isoform	CK1	L.C.Robinson
pSH24	2μ	*PKC1*	protein kinase C	AGC	Helliwell *et al.* (1998b)
YCp-PKC1(R398P)	*CEN*	*PKC1*(R398P)	protein kinase C dominant activating mutant	AGC	Nonaka *et al.* (1995)
pSMA10	2μ	*BCK1*	member of the Pkc1p MAP kinase module	STE11/STE20	Irie *et al.* (1991)
YEp352-MKK1	2μ	*MKK1*	MEK, MAP kinase kinase	STE7/MEK	D.E.Levin
YEp352-MPK1	2μ	*MPK1*	MAP kinase	CMGC	Kamada *et al.* (1995)
YEp352-YPK1	2μ	*YPK1*	protein kinase with similarity to Pkc1p	AGC	Chen *et al.* (1993)
pSH22	2μ	*MSS4*	phosphatidylinositol-4-phosphate 5-kinase	PI-kinase	Helliwell *et al.* (1998a)
pSEY18-*CMK2*	2μ	*CMK2*	calcium–calmodulin-dependent kinase type II	CaMK	T.Beck
pJK20	2μ	*NPR1*	Ser/Thr kinase, nitrogen permease reactivator	NPR	Schmidt *et al.* (1998)
pSEY18-MCK1	2μ	*MCK1*	meiosis and centromere regulatory kinase	CMGC	T.Beck

The classification of protein kinases in different groups is based on Hunter and Plowman (1997).

mutant (data not shown). The strains overexpressing different protein kinases were tested for internalization of [^{35}S]α-factor at 37°C. As a positive control, we measured α-factor internalization by *lcb1-100* cells at 24°C, because this mutant shows only a partial defect in endocytosis at 24°C (Figure 2A). Most of the protein kinases tested did not suppress the *lcb1-100* defect in endocytosis. However, high copy vectors with two protein kinases from different families, Yck2p and Pkc1p, did. To characterize further the suppressor effects of these two kinases, more assays were performed.

To check whether suppression by Yck2p was specific, two other kinases, Yck1p and Yck3p, which also belong to the casein kinase I family (Robinson *et al.*, 1992; Wang *et al.*, 1996), were tested. Neither of these two kinases was able to restore α-factor internalization in the *lcb1-100* mutant (Figure 2A). The ability of *lcb1-100* cells overexpressing *YCK2* or *YCK1* to carry out fluid-phase endocytosis at 37°C was also tested and compared with wild-type and *lcb1-100* cells (Figure 3). The *lcb1-100* mutant showed a few small vacuoles when viewed by Nomarski optics and was defective for accumulation of LY in the vacuole. Consistent with the α-factor uptake results, overexpression of *YCK2* allowed the *lcb1-100* mutant to accumulate LY in the vacuole, while overexpression of *YCK1* did not (Figure 3). To determine whether other endocytosis mutants could also be suppressed by *YCK2* overexpression, the *end4-1* (*sla2-41*) and *end6-1* (*rvs161*) strains were transformed by the 2μ plasmid bearing the *YCK2* gene and assayed for α-factor uptake at 37°C (Figure 2B). These two mutants are defective for α-factor internalization at 37°C (Raths *et al.*, 1993; Munn *et al.*, 1995). The high copy expression of *YCK2* did not restore endocytosis in these strains (Figure 2B). We conclude that Yck2p overexpression specifically suppresses the *lcb1-100* endocytic defect.

The other protein kinase that restored endocytosis in the *lcb1-100* mutant was protein kinase C. Overexpression of *PKC1* permitted *lcb1-100* cells to internalize 50% of the α-factor by 30 min at 37°C. The *lcb1-100* mutant, with or without multicopy vectors bearing other kinases, was defective for α-factor uptake at 37°C (25–30% uptake after 30 min; Figure 2A). We also overproduced protein kinase C activity by transformation with a low copy number plasmid bearing a dominant, activated allele of

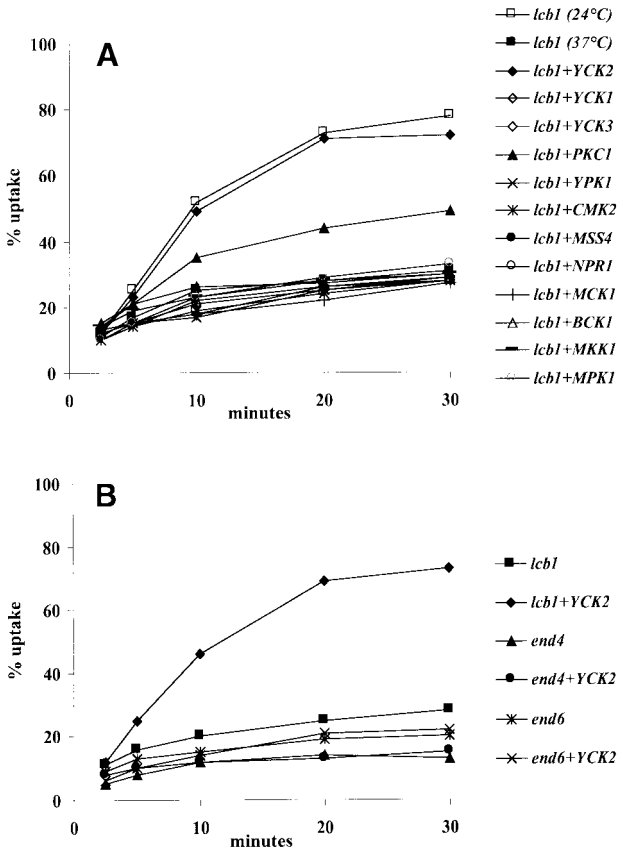

Fig. 2. Overexpression of *YCK2* and *PKC1* specifically suppresses the *lcb1-100* endocytic defect. (**A**) Strain RH3802 (*lcb1*) was transformed with high copy plasmids carrying the indicated genes (Table I) and the corresponding transformants were assayed for α-factor internalization at 37°C and compared with *lcb1-100* cells at 24 and 37°C. (**B**) The *lcb1-100*, *end4-1* (*sla2-41*) and *end6-1* (*rvs161*) temperature-sensitive mutants (RH3802, RH1597 and RH2082) were transformed by a high copy number plasmid bearing *YCK2* and assayed for α-factor uptake at 37°C.

PKC1 (*PKC1-R398P*) (Nonaka *et al.*, 1995) and measured α-factor uptake at 37°C. *PKC1-R398P* expression was able to restore α-factor uptake by *lcb1-100* cells to similar levels to overexpression of *PKC1* (data not shown). These results suggest that the *lcb1-100* mutant could be impaired

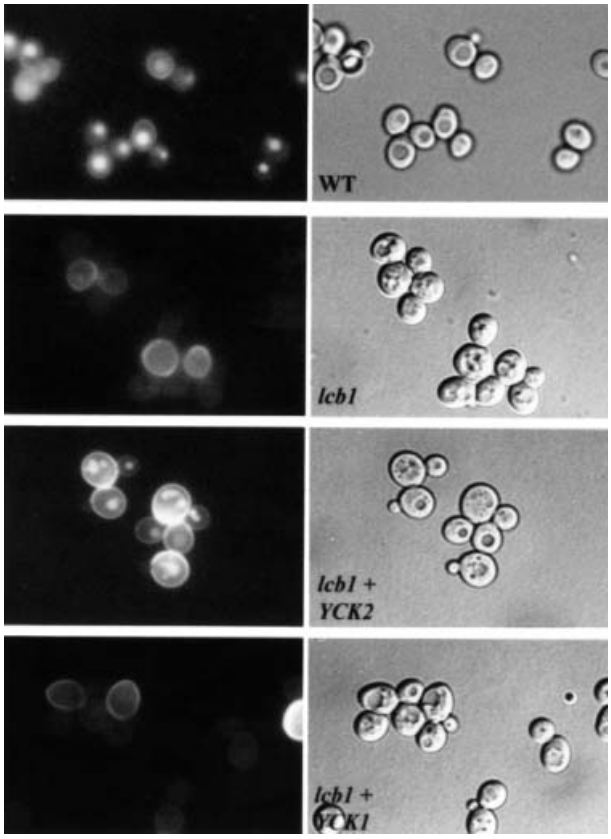

Fig. 3. *YCK2* overexpression suppresses the fluid-phase endocytic defect of the *lcb1-100* mutant. Wild-type cells (WT, RH448) and *lcb1-100* (*lcb1*, RH3802) cells carrying either a *YCK2* or a *YCK1* high copy number plasmid (*lcb1+YCK2* or *lcb1+YCK1*) were assayed for LY accumulation in the vacuole at 37°C. The same field of cells viewed by fluorescence (left panels) and by Nomarski optics (right panels) is shown. Note that *lcb1-100* cells have fragmented vacuoles when compared with wild-type cells (right panels).

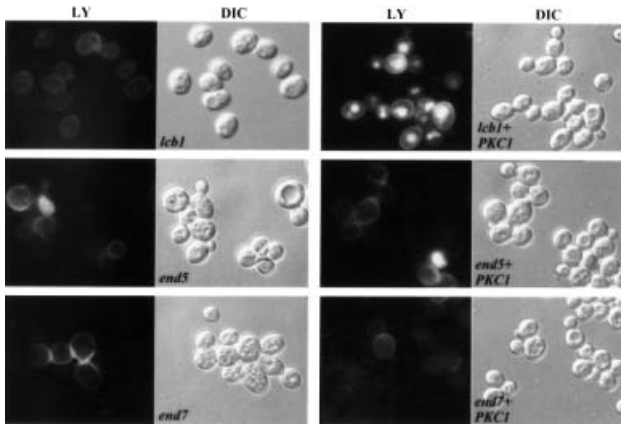

Fig. 4. Overexpression of *PKC1* specifically suppresses the LY accumulation defect of the *lcb1-100* mutant. The *lcb1-100*, *end5-1* (*vrp1*) and *end7-1* (*act1*) temperature-sensitive mutants (RH3802, RH2077 and RH2069), which are defective in fluid-phase endocytosis (left panels) (Raths *et al*., 1993; Munn *et al*., 1995), were transformed by a high copy number plasmid containing *PKC1* (right panels), assayed for LY accumulation at 37°C and observed by fluorescence (LY) and Nomarski (DIC) microscopy.

in activation of Pkc1p and furthermore in the characterized *PKC1*-mediated signaling pathway.

Genetic studies have shown that one of the downstream signaling pathways of Pkc1p is the mitogen-activated protein (MAP) kinase cascade (Herskowitz, 1995). This MAP kinase signaling pathway is composed of four downstream effectors, Bck1p, Mkk1p/Mkk2p and Mpk1p (Lee and Levin, 1992; Irie *et al*., 1993; Lee *et al*., 1993), which are homologs of MAP kinase kinase kinase, MAP kinase kinase and MAP kinase in mammalian cells, respectively. The *lcb1-100* mutant was transformed with high copy number plasmids bearing *BCK1*, *MKK1* or *MPK1* genes, and α-factor uptake was assayed at 37°C (Figure 2A). None of these three kinases was able to suppress the endocytic defect of the *lcb1-100* cells, indicating that the suppressor effect of the Pkc1p kinase is not mediated through the known MAP kinase pathway.

To assay fluid-phase endocytosis, *lcb1-100* cells bearing the *PKC1* 2μ plasmid were incubated with LY for 1 h at 37°C (Figure 4). Overexpression of *PKC1* restored LY accumulation by the *lcb1-100* mutant very efficiently. To test whether the suppression is specific for the sphingoid base synthesis requirement, LY accumulation was measured in other temperature-sensitive endocytosis deficient mutants, *end4-1* (*sla2-41*) (Raths *et al*., 1993), *end6-1* (*rvs161*), *end5-1* (*vrp1*) and *end7-1* (*act1*) (Munn *et al*., 1995) transformed with the *PKC1* plasmid (data not shown and Figure 4). The endocytic defect of these mutants was not suppressed by *PKC1*. Taken together, the above results show that overexpression of two kinases, Yck2p and Pkc1p, can suppress both receptor-mediated and fluid-phase endocytosis defects of the *lcb1-100* mutant. This suppressor effect is specific for the sphingoid base synthesis requirement, because other endocytic mutants were not suppressed by high copy expression of these kinases. Therefore, sphingoid base synthesis may activate a protein phosphorylation pathway that is required for the internalization step of endocytosis.

Genetic interaction between lcb1-100 and yck-ts

To investigate further the relationship between Yckp functions and sphingoid base synthesis, we tested for genetic interactions between the *lcb1* and *yck* mutations. *YCK1* and *YCK2* have an overlapping function for cell growth (Robinson *et al*., 1992). Preliminary experiments showed that they also have an overlapping function in endocytosis, because single *yck1Δ* or *yck2Δ* mutant strains showed wild-type internalization (data not shown). Therefore, we crossed a *yck-ts* mutant (*yck1Δ yck2-2-ts*) with the *lcb1-100* mutant to create a *yck-ts lcb1-100* mutant (*yck1Δ yck2-2-ts lcb1-100*) that is conditional for both casein kinase I activity and sphingoid base synthesis. Next, we measured α-factor internalization in these strains at 24 and 37°C (Figure 5A). At 24°C, *yck-ts* cells internalized α-factor rapidly and to a similar extent to *lcb1-100* cells. In contrast, *yck-ts lcb1-100* cells showed a strongly reduced rate of α-factor internalization at 24°C (Figure 5A). Consistent with previous reports (Hicke *et al*., 1998), the *yck-ts* mutant showed a defect in α-factor internalization at 37°C, because the Yck kinases are required for phosphorylation of the α-factor receptor (Hicke *et al*., 1998). Phosphorylation and ubiquitylation of the receptor are required for internalization. At 37°C the *yck-ts lcb1-100* cells were even more defective in α-factor uptake than either *yck-ts* or *lcb1-100* strains (Figure 5A).

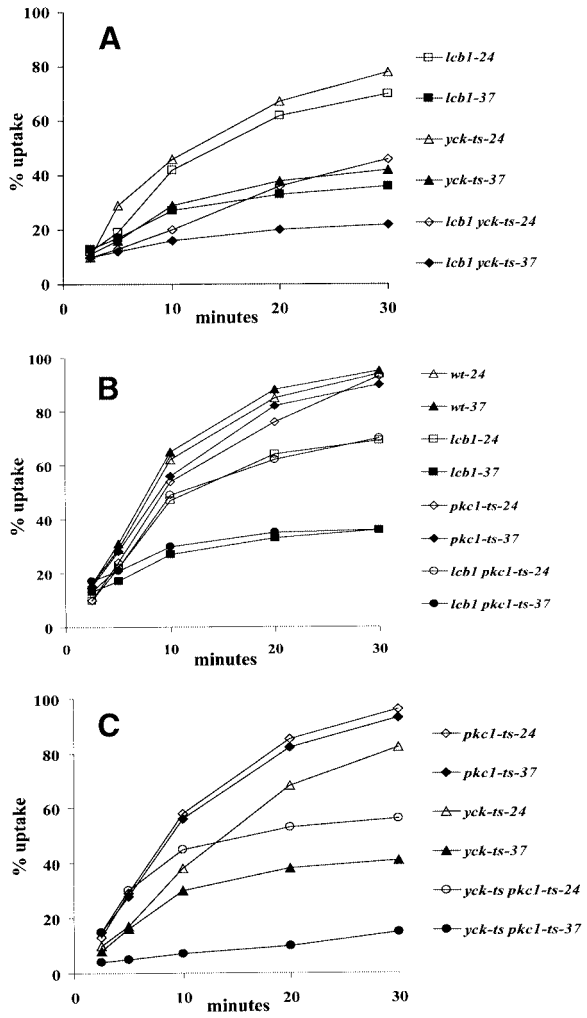

Fig. 5. (**A**) *lcb1-100* and *yck-ts* mutations have a synthetic effect on endocytosis. The single mutant *lcb1-100* (RH3802) and *yck-ts* (RH4336) cells were assayed for α-factor uptake at both 24°C (open symbols) and 37°C (closed symbols) and compared with the double mutant *lcb1 yck-ts* (RH4337) cells. (**B**) Radiolabeled α-factor uptake assays were performed at 24°C (open symbols) or 37°C (closed symbols) on wild-type (RH448), *pkc1-ts* (RH4325), *lcb1-100* (RH3802) and *lcb1 pkc1-ts* (RH4329) strains. (**C**) Internalization assays were performed at 24°C (open symbols) and 37°C (closed symbols) on *pkc1-ts* (RH4325), *yck-ts* (RH4742) and *yck-ts pkc1-ts* (RH4598) mutants.

These results reveal an enhancement of the endocytic defect at both permissive and restrictive temperature when *lcb1-100* and *yck-ts* mutations are combined, suggesting that the two functions may be interconnected for the internalization step of endocytosis.

Loss of Pkc1p activity does not affect endocytosis

As shown above, overexpression of *PKC1* restores the *lcb1-100* defect in endocytosis. To determine whether Pkc1p kinase activity is required for the internalization step of endocytosis and whether the two genes *LCB1* and *PKC1* showed a genetic interaction, endocytosis of *pkc1-2-ts* and *lcb1-100 pkc1-2-ts* strains was assayed (Figure 5B). The *pkc1-2-ts* strain harbors a chromosomal deletion of the *PKC1* gene (*pkc1::LEU2*) and is kept alive by a plasmid-borne temperature-sensitive *pkc1-2-ts* mutant allele. The single and double mutant strains were assayed for α-factor uptake at 24 and 37°C and compared with wild-type cells (Figure 5B). The *pkc1-2-ts* mutant cells internalized α-factor with the same rate as wild-type cells at 24 and 37°C, and the *lcb1-100 pkc1-2-ts* strain showed the same levels of internalization as *lcb1-100* cells at both 24 and 37°C (Figure 5B). Therefore, we conclude that the loss of Pkc1p kinase activity does not affect the internalization step of endocytosis and does not increase the *lcb1-100* defect in endocytosis at permissive or restrictive temperature.

Yck2p and Pkc1p have overlapping function in endocytosis

To determine whether Pkc1p and Yck2p have an overlapping role in endocytosis, we assayed α-factor uptake in single (*pkc1-2-ts* and *yck-ts*) and double (*pkc1-ts yck-ts*) mutant strains at 24 and 37°C. At 24°C, the *pkc1-ts yck-ts* strain showed a more severe α-factor internalization defect than either single mutant strain (Figure 5C). At 37°C, the double mutant strain was unable to internalize α-factor. The latter defect was again more severe than for either single mutant at this temperature.

As the *yck-ts* mutant is defective for α-factor receptor phosphorylation it was important to examine whether the double mutant is also more severely defective for another endocytic marker. Therefore, we tested for a synthetic defect using the LY accumulation assay. LY accumulation in the single and double mutants was assayed at 37°C (Figure 6). Either single mutant accumulated LY in the vacuole to a similar extent to wild-type cells. This confirms the lack of an endocytic phenotype for the *pkc1-2* mutant and suggests that the α-factor internalization defect in the *yck-ts* strain may be solely due to a lack of receptor modification, because there is no observable defect in LY accumulation. In contrast, the double mutant (*pkc1-ts yck-ts*) cells were completely defective in LY accumulation at 37°C (Figure 6). These results show that Pkc1p and Yck2p have an overlapping function in endocytosis, because only the cells with impaired activity for both kinases were defective for the internalization step of endocytosis.

Overexpression of Yck2p or Pkc1p or inactivation of PP2A corrects the actin defect of the lcb1-100 mutant

The *lcb1-100* mutant is defective in the organization of the actin cytoskeleton at 37°C, but this defect and the endocytic defect can be corrected by addition of PHS or DHS to the *lcb1-100* cells (Zanolari *et al*., 2000). Therefore, it is conceivable that overexpression of the kinases and inactivation of PP2A, which restored endocytosis by the *lcb1-100* mutant, may also correct the actin defect of the *lcb1-100* mutant. To test this, we examined whether the loss of *CDC55* and *PPH21/22/3* function or Yck2p and Pkc1p overexpression in the *lcb1-100* mutant could restore the polarized distribution of actin at 37°C. Wild-type, *lcb1-100*, *lcb1-100 cdc55Δ*, *lcb1-100 pph-ts*, *lcb1-100*+YEp-*YCK2* and *lcb1-100*+YEp-*PKC1* strains were grown at 24°C, shifted to 37°C for 2 h and the cells were fixed and stained with TRITC–phalloidin to visualize F-actin (Figure 7). A shift from 24 to 37°C causes a heat-induced reorganization of the actin

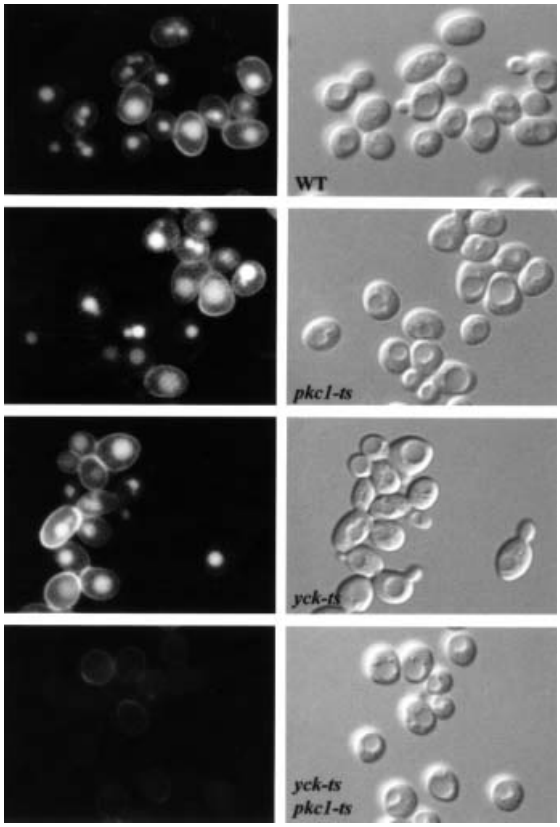

Fig. 6. Yck and Pkc1p kinases have redundant activity in fluid-phase endocytosis. Wild-type (WT, RH448), *pkc1-ts* (RH4325), *yck-ts* (RH4742) and *yck-ts pkc1-ts* (RH4598) cells were incubated with LY at 37°C. To visualize LY uptake, cells were viewed by FITC-fluorescence optics (left panels). The same fields of cells were viewed by Nomarski optics (right panels) to visualize the vacuoles.

cytoskeleton in wild-type yeast cells to a non-polarized distribution. Normal polarized actin localization is restored after 1.5–2 h at 37°C in wild-type cells (Figure 7). However, whereas the heat-induced reorganization of the actin cytoskeleton was transient in wild-type cells, this perturbation was irreversible in the *lcb1-100* mutant cells, as seen by the accumulation of actin patches in the mother cell of the budded cells (Figure 7) (Zanolari *et al*., 2000). In contrast, *lcb1-100* mutant cells that were suppressed for endocytosis either by PP2A mutations or by kinase overexpression, displayed a polarized distribution of actin that was similar to wild-type cells because cortical actin patches concentrated in the bud (Figure 7). Furthermore, *PKC1* overexpression in *lcb1-100* cells also restored the actin cables (Figure 7).

PKC1 has been shown to mediate signaling to the actin cytoskeleton in yeast (Helliwell *et al*., 1998b). To determine whether the suppression by *PKC1* was specific for the sphingoid base synthesis requirement of *lcb1-100* cells, another endocytic mutant that displays actin cytoskeleton defects, *end7-1* (*act1*) (Munn *et al*., 1995), was transformed by the YEp-*PKC1* plasmid and assayed for actin localization (Figure 7). Proper actin localization was not restored by *PKC1* overexpression in this mutant, showing that suppression is specific for sphingoid base synthesis. The above results suggest that one target of the sphingoid base synthesis requirement is likely to be the actin cytoskeleton, because overexpression of the kinases and mutation of the PP2A subunits specifically corrected the actin defect in the *lcb1-100* mutant.

Discussion

The major finding of this study is that overexpression of specific kinases or loss of PP2A activity suppresses the sphingoid base synthesis requirement for both endocytosis and actin cytoskeleton organization in yeast. Interestingly, neither the protein kinase overexpression nor the PP2A inactivation were able to suppress the temperature-sensitive growth phenotype displayed by the *lcb1-100* mutant (data not shown). Moreover, the loss of PP2A activity did not restore the ceramide synthesis defect observed in the *lcb1-100* mutant (Zanolari *et al*., 2000), since the *lcb1-100* strain and the double *lcb1-100 cdc55Δ* mutant showed the same reduction in sphingolipid synthesis at 37°C when compared with the wild-type strain or with the *cdc55Δ* mutant (C.Sütterlin and H.Riezman, unpublished data). Therefore, the suppressor effect was specific for the *lcb1-100* endocytic and actin defects. These results suggest that sphingoid bases could act by activating protein kinases that are required for the internalization step of endocytosis. Two likely candidates for this activation are Yck2p and Pkc1p, which have an overlapping function in endocytosis. The ultimate target of sphingoid base is likely to be the endocytic machinery and/or the actin cytoskeleton.

We demonstrated that loss of *CDC55* or *PPH21/22/3* function completely suppressed the endocytic defect present in the *lcb1-100* mutant strain. On the other hand, loss of *SIT4* activity had no effect on endocytosis in the *lcb1-100* strain. Previous studies have suggested that a yeast PP2A could be activated by C2-ceramide that induces a G_1 arrest of cells (Fishbein *et al*., 1993; Nickels and Broach, 1996). Yeast CAPP was proposed to be composed of two regulatory subunits, Cdc55p and Tpd3p, and a major catalytic subunit, Sit4p (Nickels and Broach, 1996). Our results suggest a common function for Cdc55p and Pph21p/Pph22p/Pph3p, but not Sit4p, as a possible antagonist of endocytosis. Other studies have shown a shared function between Cdc55p and Pph21p/Pph22p/Pph3p (Sneddon *et al*., 1990; Ronne *et al*., 1991). Interestingly, there is some evidence for partial redundant function between *SIT4* and *PPH22*. Elevated gene dosage of *PPH22* partially suppressed the *sit4-102* mutation (Sutton *et al*., 1991). Biochemical data suggest that Tpd3p and Cdc55p interact with Pph21p/Pph22p in cells. However, it is possible that these two regulatory subunits associate with a variety of different catalytic subunits, including Pph21p/Pph22p/Pph3p or Sit4p, for different functions, some activated by lipids and others not. Our study suggests that PP2A activity acts in opposition to the sphingoid base synthesis requirement in endocytosis. However, our study provides no information on the potential lipid activation of PP2A.

The fact that the loss of PP2A activity completely abrogated the sphingoid base synthesis requirement for endocytosis suggests that the major role of sphingoid base synthesis in endocytosis is to stimulate protein phosphorylation. A similar suppression by overexpression of *YCK2* or *PKC1* supports this hypothesis. The suppression

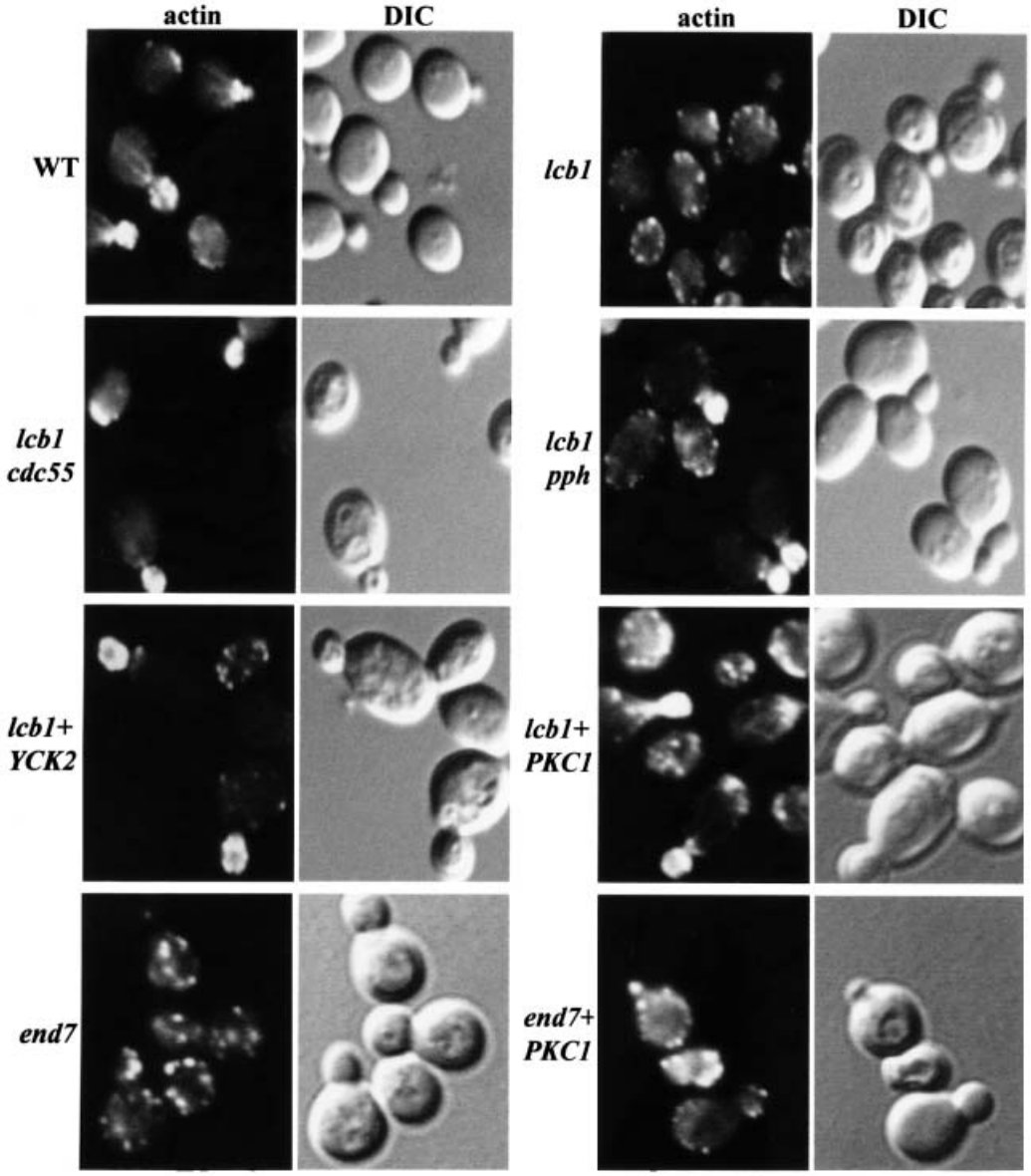

Fig. 7. Loss of function of P2A or kinase overexpression suppresses the *lcb1-100* actin organization defect. Logarithmic cultures of wild-type (WT, RH448), *lcb1* (RH3802), *lcb1 cdc55* (RH3807), *lcb1 pph-ts* (RH4191) and *lcb1-100* (RH3802) cells with high copy number plasmid containing either *YCK2* or *PKC1* genes (*lcb1+YCK2* or *lcb1+PKC1*) and *end7-1* (*act1*, RH2069) cells with or without the *PKC1* plasmid (*end7+PKC1*) were grown at 24°C, shifted to 37°C for 2 h, fixed, stained with TRITC–phalloidin and observed by fluorescence (actin) and Nomarski (DIC) microscopy.

is specific for these two kinase genes because overexpression of other kinase genes, some even belonging to the same kinase families, did not suppress the *lcb1-100* endocytic defect. Overexpression of *CMK2*, encoding a kinase that is activated by calmodulin, had no effect on the *lcb1-100* endocytic defect, even though the internalization step of endocytosis in yeast is dependent on calmodulin (Kübler *et al*., 1994; Geli *et al*., 1998). Yck1p, a kinase belonging to the same family of casein kinase I isoforms and 66% identical in amino acid sequence to Yck2p (Robinson *et al*., 1992), did not suppress *lcb1-100*, suggesting that some specificity, in either expression or substrate selection, is required to suppress *lcb1-100* for endocytosis. Yck1p and Yck2p have previously been implicated in endocytosis, due to their requirement for internalization of both **a**- and α-factor receptors (Panek *et al*., 1997; Hicke *et al*., 1998) and for down-regulation of the uracil permease, Fur4p (Marchal *et al*., 1998). It has been demonstrated that casein kinase I activity is required for ligand-induced phosphorylation and subsequent ubiquitylation of the α-factor receptor leading to its internalization (Hicke *et al*., 1998). The mode of action of Yck2p in suppression of *lcb1-100* is different because α-factor receptor phosphorylation is not defective in the *lcb1-100* mutant (Zanolari *et al*., 2000). On the other hand, Yck2p function is likely to be associated with the sphingoid base synthesis requirement for endocytosis, because the *lcb1-100* and *yck-ts* mutants had an additive effect on endocytosis at both permissive and restrictive temperature.

An increased gene dosage of *PKC1* or expression of a dominant, activated allele of *PKC1* suppressed the

endocytic defect of the *lcb1-100* mutant, suggesting that Pkc1p is required in its active form for this suppression. The Ypk1p kinase that has 44–46% identity to protein kinase C isozymes in the catalytic domain did not suppress the *lcb1-100* endocytic defect, suggesting that specificity in the substrate selection is required for this suppression. It has been shown that the kinase activity of an atypical mammalian PKC isozyme, PKC ζ, is regulated by sphingosine and by ceramide (Muller *et al*., 1995). This PKC ζ is not activated by Ca^{2+} or DAG like the classical PKCs (Ono *et al*., 1989). Interestingly, yeast Pkc1p has a similar pattern of activation, meaning that Pkc1p does not respond to Ca^{2+} or to DAG (Antonsson *et al*., 1994). It has been proposed that ceramide activates an atypical protein kinase C, Raf-1 and KSR kinases through binding to their cysteine-rich domains (CRDs) (van Blitterswijk, 1998). Sequence comparison of the CRDs revealed that all these ceramide-activated proteins share conserved Cys/His residues in their CRDs, but lack several residues known to be important for phorbol ester/DAG binding (van Blitterswijk, 1998). Interestingly, the sequence comparison of the C1-domain of the yeast Pkc1p with the CRDs of these proteins showed that yeast Pkc1p also lacks several of the residues essential for phorbol ester/DAG binding (data not shown).

In yeast, Pkc1p activates a MAP kinase cascade that is composed of several kinases, Bck1p, Mkk1p/Mkk2p and Mpk1p, acting sequentially in order to regulate the dynamics of the cell wall and actin cytoskeleton organization (Lee and Levin, 1992; Irie *et al*., 1993; Lee *et al*., 1993; Nonaka *et al*., 1995; Helliwell *et al*., 1998). None of the kinases that belong to this cascade was able to restore the endocytic defect of the *lcb1-100* mutant. This result suggests that the suppressor effect of Pkc1p overexpression is not mediated through the known Pkc1p MAP kinase signaling pathway. It has been shown previously that deletion of kinases of this cascade results in phenotypes that are less severe than that of *PKC1* deleted strains, also suggesting the existence of other pathways downstream of Pkc1p (Irie *et al*., 1993; Mazzoni *et al*., 1993). Moreover, a recent study showed that the transient depolarization of the actin cytoskeleton in response to environmental stress was controlled by a signaling pathway consisting of *PKC1* and an as yet unidentified *PKC1* effector branch (Delley and Hall, 1999).

It is interesting to note that *YCK2* overexpression suppressed the α-factor internalization defect of *lcb1-100* better than *PKC1* overexpression, whereas the opposite was true for the LY accumulation defect. This could be due to the nature of the assays. First, it should be noted that while the α-factor internalization assay apparently measures a single trafficking event, the internalization step of endocytosis, there are prerequisites for this step, including receptor phosphorylation and ubiquitylation. One effect that could contribute to better suppression by *YCK2* in this assay is its possible function to stimulate receptor phosphorylation. Secondly, the LY accumulation assay measures a series of events leading to and from the endocytic pathway and the vacuole. A possible explanation for an apparently better suppression by *PKC1* rather than *YCK2* overexpression would be if *PKC1* overexpression somehow inhibited another step of the pathway, such as LY recycling.

The *lcb1-100* mutant is defective in the uptake step of endocytosis and in the organization of the actin cytoskeleton at 37°C, and these defects can be suppressed by addition of PHS or DHS to the cells (Zanolari *et al*., 2000). As shown previously, the actin cytoskeleton plays an essential role in the internalization step of endocytosis in yeast, because yeast mutants in actin and actin-binding proteins are defective in endocytosis (Kübler and Riezman, 1993; Munn *et al*., 1995). Here we showed that overexpression of Yck2p or Pkc1p or mutations in PP2A restored the actin cytoskeleton organization defect of the *lcb1-100* mutant. It is interesting to note that neither *YCK2* nor *PKC1* overexpression restored the endocytic defect of other mutants that are defective in the internalization step of endocytosis and in actin cytoskeleton organization, indicating that this suppressor effect was specific for the *lcb1-100* mutation. Moreover, overexpression of the lipid kinase Mss4p, a phosphatidylinositol-4-phosphate 5-kinase that controls actin cytoskeleton organization in yeast (Desrivières *et al*., 1998), was not able to restore the endocytic (Figure 2A) or the actin (data not shown) defects of the *lcb1-100* cells.

Even though a *yck-ts* strain is defective for both **a**- and α-factor receptor internalization, we have shown here that the strain does not have a general endocytic defect, because fluid-phase endocytosis accumulation of LY in its vacuole is normal at non-permissive temperature. This suggests that the defect in receptor-mediated endocytosis is most likely due to inefficient receptor phosphorylation and ubiquitylation. The *pkc1-ts* mutant was not defective for fluid-phase endocytosis either. Only cells with impaired activity of both Yck2p and Pkc1p kinases were defective in both receptor-mediated and fluid-phase endocytosis, suggesting a functional redundancy. What could be this overlapping function? Recent work has shown that Yck2p kinase displays plasma membrane localization that is dynamic during the cell cycle and coincides with redistribution of actin structures (Robinson *et al*., 1999). Pkc1p is known to function in actin cytoskeleton organization. It is possible that a function, shared between the two kinases in actin dynamics, is required for endocytic internalization. This cannot simply be polarized actin distribution, because several mutants, including *pph-ts* (Lin and Arndt, 1995), show delocalized actin without affecting endocytosis (Figure 1).

It is interesting that the requirement for sphingoid base can be suppressed either by overexpression of *YCK2* or loss of *CDC55* activity. In other cases, mutations in these two activities lead to very similar phenotypes (Healy *et al*., 1991; Robinson *et al*., 1993), and the combination between *cdc55Δ* and *yck-ts* is lethal, suggesting a functional relationship between Yck2p activity and PP2A activity (Robinson *et al*., 1993). For endocytosis, their activities may act antagonistically on the same substrate, but other explanations are possible. It has been reported recently that some protein kinases and protein phosphatases form a protein complex, in which the two activities may regulate each other (Camps *et al*., 1998; Westphal *et al*., 1998). It could be that loss of Cdc55p would cause an increase in Yck2p (or Pkc1p) activity. If these two proteins do form such a complex an interesting possibility arises. Small

Table II. Yeast strains used in this study

Strain	Genotype	Source
RH448	*leu2 ura3 his4 lys2*	laboratory strain
RH1597	*leu2 ura3 his4 end4-1*	Raths *et al*. (1993)
RH1800	*leu2 ura3 his4*	laboratory strain
RH2069	*leu2 ura3 his4 end7-1*	Munn *et al*. (1995)
RH2077	*leu2 ura3 his4 end5-1*	Munn *et al*. (1995)
RH2082	*leu2 ura3 his4 end6-1*	Munn *et al*. (1995)
RH3745	*leu2 ura3 ade2 cdc55::LEU2*	this study
RH3802	*leu2 ura3 his4 his3 ade2 lys2 lcb1-100*	this study
RH3807	*leu2 ura3 his4 ade2 lys2 lcb1-100 cdc55::LEU2*	this study
RH3809	*leu2 ura3 his4 lcb1-100*	this study
RH4137	*leu2 ura3 his3 lys2 trp1 pph21::HIS3 pph22::LEU2 pph3::URA3* (CB3138[*pph21–102*])	this study
RH4183	*leu2 ura3 his3 lys2 trp1 sit4::HIS3* (CB213[*sit4-102*])	this study
RH4191	*leu2 ura3 his3 lys2 trp1 lcb1-100 pph21::HIS3 pph22::LEU2 pph3:: URA3* (CB3138[*pph21–102*])	this study
RH4195	*leu2 ura3 his3 his4 ade2 trp1 lcb1-100 sit4::HIS3* (CB213[*sit4-102*])	this study
RH4325	*leu2 ura3 his4 lys2 trp1 pkc1::LEU2* (YCp50[*pkc1-2*])	this study
RH4329	*leu2 ura3 his4 lys2 trp1 lcb1-100 pkc1::LEU2* (YCp50[*pkc1-2*])	this study
RH4336	*leu2 ura3 trp1 yck1Δ yck2-2*	this study
RH4337	*leu2 ura3 his3 ade2 trp1 lcb1-100 yck1Δ yck2-2*	this study
RH4598	*leu2 ura3 his3 lys2 ade2 pkc1::LEU2* (YCp50[*pkc1-2*]) *yck1Δ yck2-2*	this study
RH4742	*leu2 ura3 his3 ade2 lys2 yck1Δ yck2-2*	this study

All strains listed in this table are *MAT***a** and *bar1*.

molecules, for instance sphingoid bases, may regulate the relative phosphorylation/dephosphorylation activity of the complex.

In summary, our results are the first example of sphingoid base synthesis being used to control protein phosphorylation regulating a step of membrane trafficking. Many details remain to be discovered, including the demonstration and identification of a sphingoid base-activated protein kinase and the mechanism whereby sphingoid base controls the relative activities of protein kinases and phosphatases. The ease of genetic and molecular studies in yeast should help to understand these questions.

Materials and methods

Plasmids, strains, media and genetic manipulations

Plasmids and yeast strains used in this study are listed in Tables I and II, respectively. Yeast strain RH3745 was constructed by crossing AHY86 (*MAT***a** *cdc55::LEU2 leu2 ura3 his3*; Healy *et al*., 1991) twice into our genetic background. All other disruption mutants were created by integrative transformation using standard techniques. To construct the temperature-sensitive protein phosphatase strains, heterozygous disruptions were made in a diploid strain, and after introduction of the relevant temperature-sensitive allele on a plasmid the diploids were sporulated and dissected to generate the *MAT***a** strains used in this study. Disruption plasmids pMC101 (*pph21::HIS3*), pMC104 (*pph22::LEU2*), pMC89 (*pph3::URA3*) for the PPH genes were from H.Ronne (Uppsala) and the *sit4::HIS3* disruption cassette was amplified by PCR from strain Y1361 (Nickels and Broach, 1996). Plasmids with temperature-sensitive protein phosphatase alleles were CB3138 (*pph21-102/TRP1/CEN*; Lin and Arndt, 1995) and CB213 (*sit4-102/LEU2/CEN*; Sutton *et al*., 1991). Yeast cell cultures and genetic manipulations were carried out essentially as described by Sherman *et al*. (1983). Yeast cells were transformed by the lithium acetate method using single-stranded carrier DNA and dimethyl sulfoxide (DMSO) (Schiestl and Gietz, 1989; Hill *et al*., 1991). Rich YPUAD medium and synthetic minimal media (SD) complemented with the appropriate nutrients for plasmid maintenance were prepared as described (Munn *et al*., 1995).

Endocytosis assays

LY (Fluka, Buchs, Switzerland) assays were performed as described (Dulic *et al*., 1991; Munn and Riezman, 1994). Yeast pre-cultures were grown at 24°C in SD selective media in order to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD to mid-log phase, shifted to 37°C for 15 min and incubated for 1 h at 37°C with LY. [^{35}S]α-factor uptake assays were performed on mid-log phase cells using the continuous presence protocol as described (Dulic *et al*., 1991). Pre-cultures were performed at 24°C in SD selective media in order to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD medium; the α-factor uptake assays were carried out at 24 or 37°C after 15 min pre-incubation at the respective temperature. All uptake assays were performed at least twice, the results shown are from one of the independent experiments that gave nearly identical results.

Rhodamine–phalloidin staining of actin

Yeast cell pre-cultures were grown at 24°C in SD selective media in order to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD medium to early log phase. Cells at 1×10^7 cells/ml were then incubated for 2 h at 37°C, fixed in formaldehyde and stained with TRITC–phalloidin (Sigma, St Louis, MO) to visualize F-actin essentially as described previously (Benedetti *et al*., 1994).

Acknowledgements

We thank L.Robinson, K.Arndt, J.Pringle, H.Ronne, M.Stark, T.Beck, T.Schmelzle and S.Desrivières for strains and plasmids, Jeannette Holenstein and Thomas Aust for technical assistance, C.Sütterlin for communicating unpublished results, and Antje Heese-Peck and Pierre Morsomme for helpful comments on the manuscript. This work was supported by the Canton of Basel-Stadt, EMBO and HFSP long-term fellowships (to S.F.) and a grant from the Swiss National Science Foundation (to H.R.).

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Received February 25, 2000; revised and accepted May 2, 2000