TEDS Site Phosphorylation of the Yeast Myosins I Is Required for Ligand-induced but Not for Constitutive Endocytosis of the G Protein-coupled Receptor Ste2p*

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The yeast myosins I Myo3p and Myo5p have well established functions in the polarization of the actin cytoskeleton and in the endocytic uptake of the G protein-coupled receptor Ste2p. A number of results suggest that phosphorylation of the conserved TEDS serine of the myosin I motor head by the Cdc42p activated p21-activated kinases Ste20p and Cla4p is required for the organization of the actin cytoskeleton. However, the role of this signaling cascade in the endocytic uptake has not been investigated. Interestingly, we find that Myo5p TEDS site phosphorylation is not required for slow, constitutive endocytosis of Ste2p, but it is essential for rapid, ligand-induced internalization of the receptor. Our results strongly suggest that a kinase activates the myosins I to sustain fast endocytic uptake. Surprisingly, however, despite the fact that only p21-activated kinases are known to phosphorylate the conserved TEDS site, we find that these kinases are not essential for ligand-induced internalization of Ste2p. Our observations indicate that a different signaling cascade, involving the yeast homologues of the mammalian PDK1 (3-phosphoinositide-dependent-protein kinase-1), Phk1p and Phk2p, and serum and glucocorticoid-induced kinase, Ypk1p and Ypk2p, activate Myo3p and Myo5p for their endocytic function.

Myosins I constitute a well characterized and ubiquitous group of unconventional myosins, which participate in a variety of cellular processes, including endocytosis, phagocytosis, cell motility, secretion, and cell polarity (1, 2). As other myosins, myosins I bear an N-terminal actin-activated ATPase, which can translocate actin filaments \textit{in vitro} (3). A short positively charged tail that binds acidic phospholipids and targets the myosin to the appropriate cellular membranes follows the conserved motor domain (1, 2, 4, 5). The funnel and the protozoal myosins I bear an additional C-terminal extension that participates in the activation of Arp2/3-dependent actin polymerization (6–10). In the yeast \textit{Saccharomyces cerevisiae}, two highly homologous genes encode myosins I: \textit{MYO3} and \textit{MYO5}. Deletion of either gene does not result in any obvious phenotype, whereas, depending on the strain background, a double knock-out is lethal or very sick, suggesting functional redundancy (11, 12). Genetic analysis indicates that the yeast myosins I participate in the polarization of the actin cytoskeleton and in endocytosis. Cortical actin patches, which concentrate in the daughter cells in wild type budding yeast, partially redistribute to mother cells in a double \textit{myo3}Δ\textit{myo5}Δ null mutant generated in a permissive strain background (11, 12). Besides, Myo3p and Myo5p have a well established function in the ligand-induced internalization of the \textalpha-factor pheromone receptor Ste2p. Ste2p is a G protein-coupled receptor that triggers the mating response in the presence of \textalpha-factor (13). Binding of the pheromone to its receptor accelerates its internalization and degradation rate as part of a pheromone desensitization program (14). Temperature-sensitive myosin I mutants are blocked in their capacity to internalize radioactively labeled \textalpha-factor immediately upon a shift to restrictive temperature (11, 12). The endocytic \textalpha-factor uptake requires the most C-terminal domain of the myosins I, which participates in the induction of Arp2/3-dependent actin polymerization (8), and it might also require its motor activity (12).

A number of results suggest that the actin-activated ATPase of the protozoal and the fungal myosins I is induced by phosphorylation of a conserved serine or threonine positioned on a surface loop that contacts the actin filament (15). Phosphorylation of this residue induces a conformational change that accelerates phosphate release during ATP hydrolysis \textit{in vitro} (16). The unconventional mammalian myosins VI also bear a serine or a threonine at this position, and therefore, they might also be regulated by phosphorylation (2). Other myosins have a glutamate or an aspartate at this site, suggesting that a negative charge is required there for full myosin ATPase activity (17). Because only thr-3, glt-1, asp-1, and ser is present at this position in the myosin motor domain, this site is named the TEDS site (17).

A number of results illustrate the physiological relevance of the myosin I TEDS site phosphorylation. \textit{In vivo} phosphorylation of this residue has been demonstrated for the protozoal myosins I (18, 19), and mutation of the TEDS site to alanine of the \textit{Dictostelium myoB} results in a protein unable to complement a double \textit{myoA}/\textit{myoB} null mutant (20, 21). Consistently, the yeast \textit{MYO3} TEDS site serine to alanine mutant (\textit{myo3}-S357A) fails to complement the synthetic lethal phenotype of a double \textit{myo3}Δ\textit{myo5}Δ null strain (20, 21). Interestingly, however, not all fungal myosin I functions require phosphorylation of this residue and/or full ATPase activity, since a TEDS site to alanine mutation in the \textit{Aspergillus nidulans} MYOA only causes slight defects when compared with the null mutation (22, 23).
myosins I are targets of the Cdc42p-activated PAKs controlling actin
still unknown (36). Numerous results demonstrate that the yeast
same cell is synthetically lethal, suggesting that they share an essen-
STE20 genes encode PAKs expressed during vegetative growth in yeast:
cytoskeleton and in the development of cell polarity (31). Three
of a
ATPase can control the endocytic uptake rate in yeast and that a kinase
site phosphorylated state significantly accelerates constitutive Ste2p
receptor. Further, we show that a Myo5p mutant that mimics the TEDS
site phosphorylation in the endocytic uptake. Interestingly, we
Purification of the protozoal myosin I TEDS site kinase identified
In the present work, we have investigated the role of the yeast myosin
I TEDS Site Phosphorylation of Myo5p

### Table 1

| Yeast strains | Genotype | Source/Reference |
|---------------|----------|------------------|
| SC1G50        | Mata ade2 his3 leu2 trp1 ura3 bar1 | M. I. Geli |
| RH976         | Mata ade2 his3 myo5::TRP1 leu2 trp1 ura3 bar1 | Ref. 44 |
| RH977         | Mata his3 myo33::HIS2 leu2 trp1 ura3 bar1 | Ref. 44 |
| SC1G275       | Mata ade2 lys2 his3 myo5::TRP1 leu2 trp1 ura3 bar1 | This study |
| SC1G276       | Mata his3 leu2 trp1 ura3 bar1 myo5::TRP1 myo33::HIS MYO5::URA3 | This study |
| SC1G568       | Mata ade6 his3 leu2 trp1 ura3 bar1 myo5::TRP1 myo33::HIS MYO5::URA3 | This study |
| SC1G569       | Mata ade2 his3 trp1 ura3 bar1 myo5::TRP1 myo33::HIS MYO5::URA3 | This study |
| RH987         | Mata ura3 trp1 ade2 his3 leu2 bar1 can1 ste20::URA3 | H. Riezman |
| CY123         | Mata CDC42::LEU2 leu2 his3 lys2 | D. Drubin |
| DDY1300       | Mata cdc42–1::LEU2 ura3 his3 lys2 | D. Drubin |
| SC1G618       | Mata CDC42::LEU2 ura3 leu2 his3 lys2 bar1::URA3 | This study |
| SC1G619       | Mata cdc42–1::LEU2 ura3 his3 lys2 bar1::URA3 | This study |
| SC1G623       | Mata cdc42–133::LEU2 ura3 leu2 his3 lys2 bar1::URA3 | This study |
| SC1G624       | Mata cdc42–129::LEU2 ura3 leu2 his3 lys2 bar1::URA3 | This study |
| SC1G625       | Mata cdc42–118::LEU2 ura3 leu2 his3 lys2 bar1::URA3 | This study |
| SC1G588       | Mata ade2 his3 leu2 trp1 ura3 bar1::ste20::kanMX cla4::LEU2 skm1::HIS3 pcla4–as3 (CEN TRP1) | M. Peter |
| SC1G586       | Mata ade2 his3 leu2 trp1 ura3 bar1::ste20::kanMX cla4::LEU2 skm1::HIS3 pcla4–as3 (CEN TRP1) | M. Peter |
| SC1G587       | Mata ade2 his3 leu2 trp1 ura3 bar1::ste20::kanMX cla4::LEU2 skm1::HIS3 pcla4–as3 (CEN TRP1) | M. Peter |
| SC1G588       | Mata ade2 his3 leu2 trp1 ura3 bar1::ste20::kanMX cla4::LEU2 skm1::HIS3 pCLA4 (CEN TRP1) | M. Peter |
| EGY48         | Mata ade2 his3 lys2 trp1 | Ref. 40 |
| SC1G604       | Mata ade2 his3 lys2 trp1 bar1::URA3 pklh2–2::LEU2 pklh1–ts | This study |
| SC1G605       | Mata ade17 ade2 trp1 ura3 bar1 myo5::TRP1 pklh2–2::LEU2 pklh1–ts | This study |
| SC1G609       | Mata his3 leu2 lys2 trp1 ura3 bar1 myo5::TRP1 ykl1–ts | This study |
| SC1G700       | Mata his3 leu2 lys2 trp1 ura3 bar1 myo5::TRP1 ykl1–ts | This study |
| SC1G617       | Mata ade2 trp1 leu2 his3 ura3 bar1 (can1) pklh2::LEU2 myo5::TRP1 ppk1–2–2 (CEN URA3 ts) | This study |
| SC1G676       | Mata ade2 trp1 leu2 his3 ura3 bar1 (can1) pklh2::LEU2 myo5::TRP1 ppk1–2–2 (CEN URA3 ts) | This study |
| SC1G546       | Mata ade2 trp1 leu2 his3 ura3 bar1 (can1) pklh2::LEU2 myo5::TRP1 ppk1–2–2 (CEN URA3 ts) | This study |
| SC1G545       | Mata his3 leu2 lys2 trp1 ura3 bar1 sla2::HIS3 myo3–357A::URA3 | Ref. 57 |
| SC1G546       | Mata his3 leu2 lys2 trp1 ura3 bar1 sla2::HIS3 myo3–357A::URA3 | Ref. 57 |
| SC1G546       | Mata his3 leu2 lys2 trp1 ura3 bar1 sla2::HIS3 myo3–357A::URA3 | Ref. 57 |
| SC1G546       | Mata his3 leu2 lys2 trp1 ura3 bar1 sla2::HIS3 myo3–357A::URA3 | Ref. 57 |
| SC1G546       | Mata his3 leu2 lys2 trp1 ura3 bar1 sla2::HIS3 myo3–357A::URA3 | Ref. 57 |

### MATERIALS AND METHODS

**Strains, Genetic Techniques, and Two-hybrid Analysis**—Yeast strains used in this study are listed in Table 1. Unless otherwise mentioned, strains without plasmids were grown in complete YPD medium and strains with plasmids were selected on SDC minimal medium (14). Sporulation, tetrad dissection, and scoring of genetic markers were performed as described (38). Transformation of yeast was accomplished by the lithium acetate method (39). Detailed information about yeast strain generation is available by request. Dot spots were prepared from fresh saturated cultures. Cells were diluted to 10⁷ cells/ml, and 5 μl of 4 × 1–10 serial dilutions were spotted on plates with the adequate solid medium. Cells were grown for 2 days at the indicated temperature. The interaction trap two-hybrid system was used (40). Plasmids pEG202, pG4–5, pRFHM–1, and pSH18–34 and the strain EGY48 were obtained from R. Brent (Harvard Medical School, Boston, MA). To measure β-galactosidase activity, EGY48 bearing the lexAop-lacZ reporter plasmid pSH18–34 was co-transformed with the appropriate pEG202- and pG4–5-derived plasmids and streaked out on X-gal-containing SGC–His–Trp–Ura plates. Pictures were taken after 2–3 days at 30°C.

**DNA Techniques and Plasmid Construction**—Plasmids used in this study and their relevant features are listed in Table 2. DNA manipulations were performed as described (41). Details for plasmid construction are available under request.

**Ste2p Internalization Assays and Carboxypeptidase Y (CPY) Pulse and Chase**—³5S-α-factor uptake assays to analyze ligand-induced internalization of Ste2p were performed as described (14). For experiments with temperature-sensitive mutants, cells were preincubated in 37°C prewarmed YPD for 30 min before the addition of ³5S-α-factor. For the experiments with the analogue-sensitive mutant, cells were preincu-
### TABLE 2

Plasmids

All plasmids listed in this table carry an ori and an AmpR and bear a yeast centromeric origin of replication except for the pJG4-5 and pEG202 series that bear a yeast 2 μ origin of replication and the pGST series that do not bear a yeast origin of replication. aa, amino acids.

| Plasmid | Yeast marker | Insert | Source/Reference |
|---------|--------------|--------|-----------------|
| YCplac33 | URA3 | | Ref. 68 |
| P33MYOS | URA3 | MYOS | Ref. 12 |
| P33myo5-S357A | URA3 | myo5-S357A | This study |
| P33myo5-S357E | URA3 | myo5-S357E | This study |
| plNY54 | URA3 | MYOS | This study |
| plNmyo5-S357A | URA3 | myo5-S357A | This study |
| plNmyo5-S357E | URA3 | myo5-S357E | This study |
| p3GFPMYOS | URA3 | GFP MYOS | This study |
| p3GFpmyo5-S357A | URA3 | GFP pmyo5-S357A | This study |
| p3GFpmyo5-S357E | URA3 | GFP pmyo5-S357E | This study |
| p3MYOSHA | URA3 | MYOSHA | This study |
| p33myo5-S357AHA | URA3 | myo5-S357AHA | This study |
| p33ProtAMYO5 | URA3 | ProtAMYO5 | This study |
| p33ProtAMyo5-S357A | URA3 | ProtAMyo5-S357A | This study |
| p33ProtAMYO5-HHA | URA3 | ProtAMYO5-HHA (aa 1–700, Myo5p Head) | This study |
| p33ProtAMyo5-H-S357AHA | URA3 | ProtAMyo5-H-S357AHA (aa 1–700, Myo5p ApHead) | This study |
| p111da2-n | LEU2 | slat2-n (aa 1–320) | This study |
| pcla4-H His3 | HIS3 | Cla4-H (aa 521–1090) This study |
| pcla4-as3 | TRP1 | Cla4-as3 | Ref. 42 |
| pCLA4 | TRP1 | CLA4 | This study |
| pEG202myo5-Hn | HIS3 | Lexa-myo5Hn (aa 1–733) | This study |
| pEG202myo5-T | HIS3 | Lexa-myo5T (aa 757–1219) | This study |
| pEG202BICOID | HIS3 | Lexa-BICOID | Ref. 40 |
| pG4–5PKH1 | TRP1 | B42-PKH1 | This study |
| pG4–5PKH2 | TRP1 | B42-PKH2 | This study |
| pG4–5PRK1 | TRP1 | B42-PRK1 | This study |
| pG4–5YCK1 | TRP1 | B42-YCK1 | This study |
| pG4–5PKC1 | TRP1 | B42-PKC1 | This study |
| pG4–5YPK1 | TRP1 | B42-YPK1 | This study |
| pG4–5YPK2 | TRP1 | B42-YPK2 | This study |
| pG4–5 | TRP1 | B42 | Ref. 40 |
| pGEX-5X-3 | | GST | | |
| pGST-myo5-TEDS | | GST-myo5 (aa 322–391) | This study |
| pGST-myo5-TEDS-SA | | GST-myo5-S357A (aa 322–391) | This study |
| pGST-myo5-TEDS-SE | | GST-myo5-S357A (aa 322–391) | This study |
| pEG(KG)-YPK1 | URA3 leu2-d | GST-YPK1 | Ref. 45 |
| pEG(KG)-YPK2 | URA3 leu2-d | GST-YPK2 | Ref. 45 |
| pEG(KT)-ypk2-H65S | URA3 leu2-d | GST-ypk2-H65S | This study |
| pEG(KT)-ypk2-K374A | URA3 leu2-d | GST-ypk2-K374A | This study |

bated in 24 °C prewarmed YPD containing 100 μM 1NM-PP1 (kindly provided by E. Weiss and D. Drubin (42)) or mock-treated. Uptake assays were performed at least three times, and the mean and S.D. values were calculated per time point. The S.D. values were less than 10% of the value. For the analysis of constitutive endocytosis, surface-exposed Ste2p was measured with 35S-radiolabeled α-factor as described (14). CPY pulse and chase was performed as described (43) using 35S-labeling CPY (12) for immunoprecipitation. Protein concentration of samples was resolved on 10% SDS-polyacrylamide gels. Coomassie Blue-stained gels were analyzed by autoradiography. GST-Ypk1p or wild type or mutant GST-Ypk2p (100 ng (for experiments with [γ-32P]ATP) or 300 ng (for experiments with [γ-35P]ATP)) were added to 10 μl of GST-myo5-TEDS- or GST-myo5-TEDS-SA-coated beads in a final 20-μl volume in the presence of 1 mM ATP and 4 μCi of [γ-32P]ATP or [γ-35P]ATP. After a 30-min incubation at 30 °C, the reaction was stopped by the addition of SDS sample buffer to a final concentration of 0.8% and ATP to a final concentration of 10 mM. The resulting samples were resolved on 10% SDS-polyacrylamide gels. Coomassie Blue-stained gels were analyzed by autoradiography. GST-Ypk1p, GST-
Ypk2p, GST-Ypk2-H459Yp, and GST-Ypk2-K373Ap were purified from yeast YC123 as described (45, 46).

**Fluorescence Microscopy Techniques**—Phalloidin staining was performed on 4% formaldehyde-fixed cells in the presence 300 mM TRITC-phalloidin (Sigma) as described (47). Samples were visualized using a Zeiss Axiosvert 35 fluorescence microscope. For the experiments with the analogue-sensitive mutants, cells were incubated for 1 h in the presence of 100 μM 2′-NPO-PP1 or mock-treated before fixation. The percentage of unpolarized cells was calculated by counting the number of small budded cells exhibiting more than two actin patches in the mother cell. More than 100 cells were examined per experiment. For staining of cells with FM4-64, SCMIG275 transformed with plasmids encoding the green fluorescent protein-tagged wild type and mutant Myo5p were grown to 10⁷ cells/ml, harvested, resuspended in 25 μl of YPD, and incubated on ice for 1 min. FM4-64 (Molecular Probes, Inc., Eugene, OR) was added to 8 μM, and cells were further incubated on ice for 15 min. Cells were harvested at 4 °C and resuspended in 50 μl of ice-cold 2% aglinate in 50 mM glycine, pH 6.2, containing 8 μM FM4–64. 2 μl of sample were placed on an ice-cold slide and carefully covered with a coverslip. 2 μl of 50 mM CaCl₂ was added to the sample from every side of the coverslip to solidify the aglinate. Cells were immediately visualized using a Leica TCS SP confocal microscope equipped with Ne-He and argon lasers.

**ProtA-Myo5p Purification and MALDI-TOF Analysis of TESD Site Phosphorylation**—N-terminal Protein A-tagged Myo5p was purified from SCMIG275 transformed with p33ProtAMYOS. 10¹¹ yeast log phase cells were harvested and resuspended in 100 μl of IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml bestatin, 1 μg/ml leupeptin, 1 μg/ml antipain) and glass bead-lysed. 1 ml of IP, 1% Triton X-100 (IPT) containing protease inhibitors was added/g of wet pellet, mixed, and incubated for 10 min on ice. Unbroken cells and debris were eliminated by centrifugation at 3,600 rpm at 4 °C for 10 min. The supernatant was recovered and incubated for 2 h at 4 °C in the presence of IgG-Sepharose (Amersham Biosciences). Beads were washed with IPT and IP, and Myo5p was released with 10 units/ml TEV protease (Invitrogen) in 50 mM Tris-HCl, pH 8, 0.5 mM EDTA for 2 h at 16 °C. Myo5p was eluted from the beads in the presence of 0.5 M NaCl. Coomassie Blue staining of an SDS-polyacrylamide gel of IgG-Sepharose precipitates from yeast expressing ProtA-Myo5p demonstrated the presence of two polypeptides of ~130 and 150 kDa (Fig. 1C) that could be decorated with rabbit anti-Myo5p antibody. To further investigate if the Myo5p TESD site was phosphorylated in vivo, we purified it from yeast expressing a Protein A N-terminal tagged version of MYO5 (PROTA-MYO5) (Fig. 1C and “Materials and Methods”). ProtA-Myo5p was trypsin-digested in gel, and phosphopeptides were enriched by IMAC. MALDI-TOF analysis of the IMAC eluate revealed peptides with masses of 1022.48, 1178.68, 1917.93, and 2074.2 that correlated with the masses of the tryptic monophosphorylated Myo5p peptides 775–782, 774–782, 356–372, and 355–372, respectively (Fig. 1D). Fragmentation by PSD confirmed the identity of these peptides and revealed Ser₃⁵⁷ and Ser₇⁷⁻ as the sites of phosphorylation (Fig. 1E and data not shown).

**Phosphorylation of the Yeast Myosin I TESD Site Is Required for the Organization of the Actin Cytoskeleton**—Experimental evidence from Wu and co-workers (21) demonstrated that mutation of the Myo3p TESD serine to alanine resulted in loss of Myo3p function in yeast. However, instability or mislocalization of the protein might have caused the loss of function in these experiments. On the other hand, the contribution of the TESD site phosphorylation to any particular myosin I function could not be examined under these circumstances. Interestingly, we found that the analogous mutation in MYOS (MYOS-S357A) could indeed complement the lethality of a double myo3Δ myo5Δ knock-out, indicating that phosphorylation of the TESD site might be required for at least the essential myosin I function in yeast. However, instability or mislocalization of the protein might have caused the loss of function in these experiments. On the other hand, the contribution of the TESD site phosphorylation to any particular myosin I function could not be examined under these circumstances. Interestingly, we found that the analogous mutation in MYOS (MYOS-S357A) could indeed complement the lethality of the myo3Δ myo5Δ double knock (Fig. 2A), although it caused a temperature-sensitive growth defect (Fig. 2B). This result is consistent with our previous observations suggesting that Myo5p plays a predominant role in the cell when compared with Myo3p (12). No growth defect could be detected when the serine 357 was mutated to a negative charged amino acid (myo5-S357E) (Fig. 2, A and B). This result demonstrated that the unphosphorylated myosin I is sufficient to sustain growth, at least under optimal conditions.

To investigate if TESD site phosphorylation was required for the polarization of the actin cytoskeleton, myo3Δ myo5-S357A cells were grown at permissive temperature (23 °C), fixed, and stained with

**RESULTS**

The Myo5p TESD Site Is Phosphorylated in Vivo—Despite all data supporting the physiological relevance of the myosins I TESD site phosphorylation, phosphorylation at this position in vivo has only been demonstrated for the protozoal myosins I (18, 19). Since some of the fungal myosins I do not require phosphorylation to fulfill their cellular tasks (22, 23), we decided to first investigate if the S. cerevisiae Myo5p TESD site was phosphorylated in vivo. In order to address this matter, the wild type Myo5p or a mutant bearing a TESD serine to alanine substitution (Myo5-S357Ap) was immunoprecipitated from ³²P₂O₄ radiolabeled yeast cells. An equivalent phosphorylation signal could be detected for samples expressing the wild type and mutant Myo5p. No signal could be detected when the anti-Myo5p antibody was omitted in the immunoprecipitation or when the proteins were immunoprecipitated from cells lacking MYOS (Fig. 1A). Interestingly, C-terminal truncation of Myo5p resulted in loss of the radioactive signal (Fig. 1B), suggesting that if the Myo5p TESD site was phosphorylated, phosphorylation requires an intact Myo5p tail.
TRITC-phalloidin to visualize filamentous actin. Almost 80% of the myo3/H9004 myo5-S357A small budded yeast cells exhibited a clearly depolarized actin cytoskeleton with more than two actin patches present at the mother cells (Fig. 2, C and D). Under the same conditions, only 1% of small budded yeast expressing the wild type Myo5p presented a depolarized cytoskeleton (Fig. 2, C and D). These results suggested that phosphorylation of the myosin I TEDS site is required to sustain a properly polarized actin cytoskeleton in yeast. Interestingly, nearly 30% of the small budded myo3/H9004 myo5-S357E cells also exhibited a depolarized cytoskeleton (Fig. 2, C and D), suggesting that dephosphorylation of the TEDS site is also required for an accurate cytoskeletal organization.

**Phosphorylation of the Myosin I TEDS Site Is Required for Ligand-induced but Not for Constitutive Internalization of Ste2p**—To investigate if the myosin I endocytic function also requires TEDS site phosphorylation, we examined the kinetics of constitutive and ligand-induced endocytosis of Ste2p (14) in cells expressing the Myo5p TEDS site mutants as the only source of myosin I (myo3/H9004 myo5-S357E and myo3/H9004 myo5-S357A). As shown in Fig. 3A, the myo3/H9004 myo5-S357A strain exhibited a strong defect in its capacity to internalize Ste2p in the presence of α-factor when compared with cells expressing the wild type Myo5p. Strikingly, however, constitutive internalization of the receptor appeared unaffected in these cells (Fig. 3B). The prominent α-factor uptake defect installed in the myo3/H9004 myo5-S357A strain was not the result of cell sickness, since biosynthetic transport and maturation of the vacuolar protease carboxypeptidase Y (43) was unaffected (Fig. 3C). Further, immunoblot analysis of the wild type and the mutant Myo5p (Fig. 4A) and confocal fluorescence microscopy of strains expressing green fluorescent protein-tagged versions of these proteins demon-
strated that the observed phenotypes were not due to instability or mislocalization (Fig. 4B). These data indicated that TEDS site phosphorylation of the myosin I is required in yeast for fast, ligand-induced internalization of Ste2p but not for its slow, constitutive uptake. Interestingly, we also found that cells expressing the Myo5p mutant mimicking the phosphorylated state (myo3Δ myo5-S357E) displayed a slight but significant acceleration of the constitutive Ste2p uptake kinetics when compared with cells expressing the wild type Myo5p (Fig. 3B). This result suggested that phosphorylation of the myosin I TEDS site can control the endocytic uptake rate, possibly through activation of the myosin I ATPase. No acceleration could be detected, however, when the internalization assay was done in the presence of the pheromone (Fig. 3A). Other endocytic factors might limit the fast ligand-induced Ste2p internalization rate. Actually, although the initial uptake kinetics of the myo3Δ myo5-S357E strain was nearly identical to that of the wild type, mutant cells only managed to internalize 70% of the total cell-bound α-factor as opposed to 100% for cells expressing wild type Myo5p (Fig. 3A). Depletion of the available endocytic machinery in cells with constitutively accelerated endocytosis or excessive recycling of Ste2p to the plasma membrane could account for the observed phenotype in the myo3Δ myo5-S357E cells (49).

Sustained Cdc42p Signaling through the PAKs Is Not Essential for Ligand-induced Ste2p Internalization—Our data demonstrated that TEDS site phosphorylation was necessary not only for the polarization of the actin cytoskeleton but also for ligand-induced Ste2p internalization. Since only PAKs are known to phosphorylate the conserved myo-

FIGURE 2. Myosin I TEDS site phosphorylation is required for the polarization of the actin cytoskeleton in S. cerevisiae. A, tetrad analysis of spores derived from heterozygous diploids created by crossing a myo3Δ mutant (RH3977) with either a myo5Δ strain (RH3976) or strains bearing a wild type MYO5 or myo5-S357A and myo5-S357E mutant alleles integrated at the MYOS locus. The filled circles indicate predicted myo3Δ myo3Δ, MYO5 myo3Δ, myo5-S357A myo3Δ, and myo5-S357E myo3Δ haploid cells. B, dot spots grown at the indicated temperatures of MYO5 myo3Δ (SCMIG567), myo5-S357A myo3Δ (SCMIG568), and myo5-S357E myo3Δ (SCMIG569) strains. C, fluorescence micrographs showing small budded cells of a wild-type (SCMIG50) strain or the strains described in B, fixed and stained with TRITC-phalloidin to visualize filamentous actin. The arrows point to actin patches. Bar, 1 μm. D, graph showing the percentage of budded cells exhibiting more than two actin patches per mother cell for the experiment described in C.

FIGURE 3. Myosin I TEDS site phosphorylation is required for ligand-induced but not for constitutive endocytosis of the G protein-coupled receptor Ste2p. A, ligand-induced Ste2p internalization is defective in a myo3Δ myo5-S357A mutant. Shown is 35S-labeled α-factor internalization kinetics of wild-type (SCMIG50), MYO5 myo3Δ (SCMIG567), myo5-S357A myo3Δ (SCMIG568), and myo5-S357E myo3Δ (SCMIG569) strains. The graphs plot the percentage of cell-associated counts internalized per time point. B, constitutive Ste2p internalization kinetics of strains described in A. The graph plots the percentage of cell surface-exposed Ste2p per time point with respect to time point 0 (10 min after the addition of cycloheximide). C, Cpy maturation in the strains described in A. Cells were pulsed for 5 min with [35S]methionine and [35S]cysteine and chased for the indicated times. Carboxypeptidase Y was immunoprecipitated and analyzed by autoradiography. p1, endoplasmic reticulum form; p2, Golgi form; m, mature vacuolar form.
sin TEDS site, we decided to investigate if the Cdc42p/PAK/myosin I signaling pathway, which is required in yeast to polarize the actin cytoskeleton, also functioned to activate Myo3p and Myo5p for their endocytic function. For that purpose, we first created a temperature-sensitive kinase-dead PAK mutant by deleting the chromosomal genes encoding SKM1 and STE20 and by introducing a H685Y mutation in a highly conserved residue of the CLA4 core kinase domain (50). As expected, the cla4-H685Y ste20Δ skm1Δ strain (pak-ts) was temperature-sensitive for growth (Fig. 5B). At permissive temperature (24 °C), the growth rate of pak-ts cells was comparable with the wild type (Fig. 5B), although some of the mutant cells presented elongated buds similar to those described in the cla4-75 ste20Δ mutant (Fig. 5C) (33). Upon a 30-min shift to 37 °C, pak-ts cells stopped dividing and adopted a rounded morphology analogous to that described for other cla4 ste20Δ mutants at this temperature (Fig. 5C) (35, 42). Also, consistent with previous publications (35, 42), our pak-ts mutant was unable to repolarize its actin cytoskeleton at elevated temperature (Fig. 5, D and E). In wild type yeast, heat stress causes rapid depolarization of the actin cytoskeleton, which only recovers 2–3 h after prolonged incubation at elevated temperature (51). Our results demonstrated that a temperature shift to 37 °C tightly inactivated the Cla4-H685Yp in vivo.

To investigate if PAKs were required to sustain ligand-induced Ste2p internalization, we performed an α-factor uptake assay using the cla4-H685Y ste20Δ skm1Δ mutant upon a shift to restrictive temperature. Surprisingly, upon 30-min preincubation at 37 °C, the α-factor internalization kinetics of the mutant cells was identical to that of the wild type (Fig. 5F). In addition, we failed to detect any α-factor uptake defect in yeast strains bearing four different cdc42 mutations (cdc42-1, cdc42-118, cdc42-123, and cdc42-129) (Fig. 6) that are known to specifically interfere with Cdc42p signaling to the PAKs (52). Most strikingly, the cdc42-1 mutation that specifically hinders the signaling pathway to the myosin I (9) did not alter the α-factor uptake kinetics at restrictive temperature. Additional preincubation at restrictive temperature of the pak-ts and cdc42 mutants resulted in very low α-factor binding to the cells, maybe reflecting a defect in Ste2p traffic to the plasma membrane (53, 54).

To further inspect the endocytic uptake phenotype of a conditional pak mutant under the very same experimental circumstances that clearly installed an actin polarity defect, we decided to use a chemically sensitive CLA4 allele, cla4-as3 (42). The Cla4-as3p bears two mutations that enlarge the ATP binding pocket of the kinase without significantly affecting its activity in vivo (42). These mutations allow binding of the membrane-permeable pyridimidine analogue 1NM-PP1, which can then efficiently and specifically inactivate the Cla4-as3p kinase (42). It was previously shown that the in vivo activity of Cla4-as3p is inhibited upon 1-h incubation in the presence of 25 μM 1NM-PP1 (42). Under the same conditions, no uptake defect was observed in the ste20Δ skm1Δ cla4-as3 (pak-as3) mutant when compared with a wild type or an isogenic ste20Δ skm1Δ CLA4 strain (data not shown). Further, increasing the concentration of 1NM-PP1 to 100 μM did not alter the internalization kinetics of the conditional pak-as3 mutant (Fig. 7A). In contrast to the uptake kinetics, the actin distribution in the 100 μM 1NM-PP1-treated pak-as3 cells was similar to that observed in the myo3Δ myo5S337A strain.
(Figs. 2C and 7B). Upon 1-h incubation in the presence of the drug, pak-as3 cells appeared big and round, and more than 80% of the small budded yeast exhibited a depolarized actin cytoskeleton (Fig. 7, B and C). This terminal phenotype was strikingly different from that previously described for the ste20Δ cl4Δ-as3 double mutant (42). Deletion of SKM1 and/or treatment of the cells with a higher 1NM-PP1 concentration may account for the observed differences. The actin and morphology defects installed in the pak-as3 cells were dependent on the presence of the cl4Δ-as3 mutation and the drug, since they did not appear in the mock-treated pak-as3 strain or in the 1NM-PP1-treated ste20Δ skm1Δ CLA4 cells (Fig. 7, B and C).

The data strongly indicated that ligand-induced endocytosis of Ste2p was not abolished under conditions that tightly block the Cdc42p-activated PAK signaling pathway, and the data suggested that kinases other than PAKs can also activate the myosins I for their endocytic function.

A Signaling Cascade Involving the Yeast Homologues of the Mammalian PDK1 and SGK Might Activate the Myosins I for Their Endocytic Function—Searching for proteins required for ligand-induced Ste2p internalization that might physically and genetically interact with the myosins I, we found the essential and functionally redundant Pkh1p and Pkh2p, the yeast homologues of the mammalian PDK1 (55). Sphingoid base-activated Pkh1p and Pkh2p are thought to initiate a signaling cascade that modulates the endocytic uptake in yeast (55). Interestingly, we found that deletion of the chromosomal copy of MYO5 caused a synthetic growth defect in the temperature-sensitive mutant strain or in the 1NM-PP1-treated ste20Δ skm1Δ CLA4 cells (Fig. 7, B and C).

The yeast Pkh1p and Pkh2p and the mammalian PDK1 are known to activate a number of downstream kinases that bear a consensus motif (Thr-Phe-Cys-Gly-Thr-X-Glu-Tyr, where Thr is the phosphorylatable residue and X is any amino acid). Such kinases include members of the protein kinase C, protein kinase A, and SGK families (58–61). Pkh1p and Pkh2p are also able to phosphorylate in vitro the yeast Pkc1p (protein kinase C) and the functionally redundant highly similar SGK homologues Ypk1p and Ypk2p (Fig. 8C) (55, 62–64). The Myo3p and Myo5p TESD site does not match the PDK1 consensus motif. Therefore, we suspected that these molecular motors might not be direct targets of Pkh1p and Pkh2p. These kinases might rather work to recruit and to activate the actual TESD site kinase. To identify kinases that might mediate between Pkh1/2p and the myosins I, we searched for synthetic interactions between the myos5Δ null mutation and mutations in genes encoding the known Pkh1/2p targets Pkc1p, Ypk1p, and Ypk2p (pck1-ts, myo5Δ, and ypk2Δ). Interestingly, although we failed to observe synthetic growth defects in the double mutants, we detected a strong and a mild synthetic α-factor uptake defect when MYO5 was deleted in the ypk2Δ and ypk1Δ backgrounds, respectively. The synthetic uptake defect observed in the ypk2Δ myo5Δ mutants could still be observed when the myo5Δ null mutation was substituted by the myo5-S357A point mutation, thus suggesting that the lack of Ypk2p interfered with the Myo5p ATPase function. Only additive uptake
TEDS Site Phosphorylation of Myo5p

defects were observed when the myo5Δ mutation was combined with a pck1-ts mutation or with a partial loss of function mutation in another gene required for early events in the endocytic uptake (sla2-n) (Fig. 9). These results suggested that the yeast homologues of the mammalian SGK and Myo5p functionally cooperate in the endocytic uptake of Ste2p. Consistent with this view, we found that Ypk2p and, to a lesser extent, Ypk1p interacted with Myo5p in the two-hybrid assay (Fig. 10A). No significant activation of the reporter was triggered when Pkc1p was used in the assay (Fig. 10A).

To test if Ypk2p or Ypk1p directly and specifically phosphorylate the Myo5p TESD site, an in vitro kinase assay was performed using purified components. For that purpose, a recombinant purified Myo5p fragment fused to GST bearing the TESD site (amino acids 322–391) (GST-TESD) was incubated in the presence of [γ-32P]ATP and purified Ypk2p or Ypk1p fused to GST. In the presence of Ypk2p, the TESD construct was covalently labeled with radioactive phosphate (Fig. 10B). Phosphorylation occurred in the TESD serine, since phosphorylated GSVYHVPLNIVQADAVR (1917,64) and RGSVYHVLNIQVADA (2073,72), could be detected in samples treated with GST-Ypk2p but not in untreated samples (Fig. 10C). An extra band probably corresponding to the autophosphorylated Ypk2p could also be observed on the autoradiography of all samples containing this kinase. Neither autophosphorylation nor TESD phosphorylation could be detected in the samples containing purified Ypk1p (Fig. 10B), suggesting that this kinase might not be active under the conditions assayed. TESD site phosphorylation in the presence of purified GST-Ypk2p required an active enzyme, since mutation of conserved residues in the kinase domain (His459 and Lys773) (50, 65) completely abolished the phosphorylation of the Myo5p TESD site (Fig. 10B).

All of these data strongly indicated that kinases other than PAKs can also phosphorylate the conserved myosin I TESD site. Further, the results suggested that the yeast myosins I are targets of the endocytic uptake of Ste2p. Since the mutant was stable and it was properly localized, the data indicated that TESD site phosphorylation is required for at least two myosin I functions in budding yeast. These observations are in striking agreement with the results from Novak and Titus (20) showing that substitution of the Dictyostelium discoideum myoB TESD
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Serine by alanine generates a protein that is properly localized but cannot complement the endocytic and actin organization defects of the myoA−/myoB− double mutant. However, in agreement with what was demonstrated for the A. nidulans MYOA (22, 23), we also found that phosphorylation of the myosins was not essential to sustain slow constitutive endocytosis. Also in agreement with the results in A. nidulans (66), we found that the basal ATPase activity of the unphosphorylated myosin I might be rate-limiting in this process, since mutants that mimicked the phosphorylated state significantly accelerated constitutive endocytosis. Since Myo5p TES site phosphorylation is essential for rapid α-factor-induced internalization of Ste2p, our results suggest that fungi can use TES site phosphorylation and dephosphorylation to control the endocytic uptake rate in response to intracellular or extracellular signals.

Two simple models might explain the observation that myosin I TES site phosphorylation is required for ligand-induced but not for constitutive internalization of Ste2p. In the first model, a slow and a fast endocytic pathway would co-exist, only the last one being sustained by the phosphorylated myosins I. Ligand binding might then simply target Ste2p to the fast endocytic pathway. A second model to explain the observed results would propose that certain intracellular or extracellular signals might be able to modulate the endocytic uptake rate by inducing signaling cascades that phosphorylate and activate Myo3p and Myo5p. Interestingly, modulation of TES site phosphorylation by extracellular signals has been described in D. discoideum. In this organism, treatment with the chemottractant cAMP causes a transient 3-fold increase in the amount of myoB phosphorylated at the TES site (19). Analysis of this possibility will now require careful inspection of the Myo5p TES site phosphorylation state under conditions that induce endocytosis. Unfortunately, this is not a trivial experiment, given that, in contrast to the D. discoideum myoB (19), Myo5p is phosphorylated at other sites besides serine 357 (Fig. 1), and the expected increase in the amount of the phosphorylated isoform might be subtle and transient.

**Different Signaling Pathways Lead to Phosphorylation and Activation of the Yeast Myosins I for Distinct Cellular Functions—Mutations in genes encoding myosins I cause multiple defects in eukaryotic cells. Although the primary cause leading to such phenotypes is not always fully understood, it is reasonable to think that myosins I participate in different cellular tasks, which might be regulated by distinct intracellular and/or extracellular signals. To date, however, only PAKs have been identified as TES site kinases, and only the Cdc42p/PAK1/myosins I signaling pathway required for the polarization of the actin cytoskeleton in yeast was described (see Introduction). Reinforcing the physiological relevance of this signaling cascade, we showed that Myo5p was phosphorylated in vivo and that a MYOS TES site serine to alanine mutant (myo5-S357A) failed to complement the actin polarization defect of a myosin I null strain. On the other hand, our results clearly showed that even though myosin I TES site phosphorylation was required for ligand-induced Ste2p internalization, tight in vivo inactivation of the Cdc42p/PAK activities signaling pathway did not result in any significant uptake defect, not even under experimental conditions that clearly installed severe actin defects. These data demonstrated that sustained Cdc42p/PAK activity is not essential to support fast endocytic uptake in yeast, and they suggested that a different signaling cascade can activate Myo3p and Myo5p for their endocytic function.

Consistent with this view, we found that Myo5p physically and functionally interacts with different components of a signaling pathway that is known to control endocytosis in yeast (55, 63); the yeast homologues of the mammalian PDK1, PkhiP, and Pkh2p and their downstream
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targets Ypk2p and Ypk1p. Further, we demonstrated that purified Ypk2p specifically phosphorlates the Myo5p TEDS site in vitro.

The mammalian PKD1 stands at a pivotal point in cell signaling, and it mediates a multitude of cellular responses following extracellular stimulation by peptide growth factors and hormones. Besides cell death and survival, deregulation of PKD1 influences a wide variety of processes, including cell growth, motility, and differentiation (67). In yeast, Pkh1/2p have been shown to influence cell wall biogenesis (64) and endocytosis (55, 63). Our data provide now evidence for the first molecular mechanism whereby Pkh1/2p and Ypk1/2p can modulate the endocytic uptake rate by controlling the myosin I TEDS site phosphorylation in vivo. In particular, we demonstrate here that Pkh1/2p and Ypk1/2p phosphorylate the Myo5p TEDS site in vivo.

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