Cdc50p, a Conserved Endosomal Membrane Protein, Controls Polarized Growth in *Saccharomyces cerevisiae*

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During the cell cycle of the yeast *Saccharomyces cerevisiae*, the actin cytoskeleton and the growth of cell surface are polarized, mediating bud emergence, bud growth, and cytokinesis. We identified *CDC50* as a multicopy suppressor of the *myo3 myo5-360* temperature-sensitive mutant, which is defective in organization of cortical actin patches. The *cdc50* null mutant showed cold-sensitive cell cycle arrest with a small bud as reported previously. Cortical actin patches and Myo5p, which are normally localized to polarization sites, were depolarized in the *cdc50* mutant. Furthermore, actin cables disappeared, and Bni1p and Gic1p, effectors of the Cdc42p small GTPase, were mislocalized in the *cdc50* mutant. As predicted by its amino acid sequence, Cdc50p appears to be a transmembrane protein because it was solubilized from the membranes by detergent treatment. Cdc50p colocalized with Vps21p in endosomal compartments and was also localized to the class E compartment in the *vps27* mutant. The *cdc50* mutant showed defects in a late stage of endocytosis but not in the internalization step. It showed, however, only modest defects in vacuolar protein sorting. Our results indicate that Cdc50p is a novel endosomal protein that regulates polarized cell growth.

INTRODUCTION

The establishment of cell polarity is the culmination of a set of processes by which cells create specialized cortical domains. This asymmetric organization of the cytoskeleton, secretory system, and plasma membrane components, along an appropriate axis, is regulated by a number of proteins that lead to the assembly of a polarized and specialized cortical actin cytoskeleton (Drubin and Nelson, 1996). These polarized actin networks then mediate the sorting and delivery of factors required to execute and maintain cell polarity.

The budding yeast *Saccharomyces cerevisiae* is an excellent model system for studying the regulation of cell and cytoskeletal polarity (Pringle *et al.*, 1995; for reviews, see Drubin and Nelson, 1996). *S. cerevisiae* cells grow by budding, a process in which the rigid cell wall is expanded locally as a result of polarized secretion. Cell surface extension is preceded by the polarized organization of two actin filament-containing structures: actin cortical patches and ac-

tin cables. Late in G1, actin cables orient along the mother-bud axis, usually terminating at actin patches clustered at a site for bud emergence (Karpova *et al.*, 1998). As secretory vesicles are delivered to this site, the bud emerges. As the bud grows, actin patches and cables polarize to the bud tip, allowing apical growth of the bud.

Budding is initiated by the activation and action of Cdc24p and Cdc42p at the incipient bud site (Sloat *et al.*, 1981; Johnson and Pringle, 1990): Cdc24p is a guanine nucleotide exchange factor for Cdc42p, a Rho family small GTPase (Zheng *et al.*, 1994). The activated, GTP-bound form of Cdc42p binds to its effectors, Cla4p and Ste20p (PAK family protein kinases; Cvrckova *et al.*, 1995), Bni1p (a formin family protein; Kohno *et al.*, 1996; Evangelista *et al.*, 1997), and Gic1/2p (Brown *et al.*, 1997; Chen *et al.*, 1997), to transmit signals to downstream components that regulate reorganization of the actin cytoskeleton. Bni1p is a component of the 12S polarisome, which comprises Spa2p, Bud6p/Aip3p, and Pea2p (Sheu *et al.*, 1998). These proteins are localized to a polarized site and are involved in various aspects of cell polarity (for a review, see Pruyne and Bretscher, 2000). Bni1p and its related protein, Bnr1p, physically interact with an actin-binding protein, profilin, which is implicated in actin polymerization (Kohno *et al.*, 1996; Evangelista *et al.*, 1997) and also are required for formation

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of actin cables (Evangelista *et al.*, 2002; Sagot *et al.*, 2002). Gic2p physically interacts with Bud6p and thus may link Cdc42p with the polarisome complex (Jaquenoud and Peter, 2000). The formation or reorganization of cortical actin patches is regulated by cortical patch-like protein structures that include Myo3p, Myo5p, Vrp1p, Bee1p/Las17p, Sla1p, Sla2p, and proteins of the Arp2/3 complex (Pruyne and Bretscher, 2000). These proteins are involved in the uptake step of endocytosis through actin cytoskeleton regulation (Wendland *et al.*, 1998). Cortical actin patches are localized to polarized sites and are depolarized in the *cdc42* mutant, suggesting that Cdc42p may regulate polarization of cortical actin patches. For a more complete description of the regulators of yeast cell polarity, see Pruyn and Bretscher (2000).

Many of the proteins that regulate cell polarity are localized to the cortical region in polarized sites, including the growing tip of a bud or a cytokinesis site. The mechanism by which protein transport to a polarized site occur is unknown for all these proteins except for Bud6p, which is localized to the bud site through the secretory pathway (Jin and Amberg, 2000). Mutations in early- or late-acting components of the secretory apparatus lead to Bud6p mislocalization. The endosomal system may also function to transport proteins to a specialized region of the plasma membrane. For example, transport of Chs3p, the catalytic subunit of Chitin synthase III, to the site of incipient chitin ring formation may be mediated by a branch of the secretory pathway involving vesicular traffic from the endosome to the plasma membrane (Chuang and Schekman, 1996; DeMarini *et al.*, 1997; Santos and Snyder, 1997; Valdivia *et al.*, 2002).

In this article, we have characterized *CDC50*, which was previously identified as a cell-cycle mutation (Moir *et al.*, 1982). The *cdc50* mutant cells are arrested with a small bud and display depolarization of cortical actin patches at low temperatures. The mutant phenotype may be caused by mislocalization of Bni1p and Gic1p. Cdc50p is a conserved integral membrane protein localized to the late endosomal/prevacuolar compartment. Our results indicate that Cdc50p is a novel protein that regulates cell polarization.

MATERIALS AND METHODS

Media and Genetic Techniques

Unless otherwise specified, strains were grown in rich medium YPDA (1% yeast extract [Difco Laboratories, Detroit, MI], 2% bacto-peptone [Difco], 2% glucose, and 0.01% adenine). Strains carrying plasmids were selected in synthetic medium (SD) containing the required nutritional supplements (Rose *et al.*, 1990). Standard genetic manipulations of yeast were performed as described (Guthrie and Fink, 1991). Yeast transformations were performed by the lithium acetate method (Elble, 1992). *Escherichia coli* strains DH5 α and XL1-Blue were used for construction and amplification of plasmids.

Strains and Plasmids

Yeast strains used in this study are listed in Table 1. Yeast strains carrying the complete deletion of *CDC50* (*cdc50 Δ*), HA-tagged *CDC50* (*CDC50-HA*), GFP-tagged *CDC50* (*CDC50-EGFP*), GFP-tagged *MYO5* (*MYO5-EGFP*), GFP-tagged *BNR1* (*BNR1-EGFP*), and GFP-tagged *GIC1* (*GIC1-EGFP*) were constructed by the PCR-based procedure as described previously (Longtine *et al.*, 1998). All constructs made by the PCR-based procedure were verified by colony-PCR amplification to confirm that replacement had occurred at the expected locus. To generate *ups27 Δ ::HIS3* strains, the 3.6-kb DNA

fragment containing *ups27 Δ ::HIS3* derived from pKU65 (a gift from K. Umehayashi) was used to transform appropriate strains to His⁺. *SPA2-GFP* strains were constructed by integrating the linearized plasmid p406S2G (Arkowitz and Lowe, 1997) at *URA3* locus. *BNR1-EGFP* strains were constructed as follows. The 3' region of the genomic *BNR1* was replaced with the ~3.0-kb DNA fragment containing the 3' 256-base pair fragment of *BNR1* coding region fused to EGFP gene, selectable marker gene, and 3'-flanking region of *BNR1*. *MYO2-ARG-GFP* strains were constructed by a cross of YJC1431 (*MYO2-ARG-GFP::HIS3*) with YKT249 (*cdc50 Δ ::HIS3MX6*) followed by tetrad dissection.

Plasmids used in this study are listed in Table 2. Schemes for the construction of plasmids are available on request.

Isolation of Multicopy Suppressors of the *myo5-360* Mutant

The *myo3 myo5-360* strain (YKT111) was transformed with a yeast genomic DNA library constructed in the multicopy plasmid YEpl3 (kindly provided by Y. Ohya). After transformation, cells were incubated at 30°C for 24 h to allow recovery and then were incubated at 35°C for 3 d. Approximately 18,000 transformants were screened, and 56 transformants that reproducibly grew at 35°C were obtained. The transformants that contained *MYO3* or *MYO5* on the plasmid were identified by colony PCR and were eliminated. From each of the remaining 11 transformants, plasmids were recovered for further analysis. All of these 11 plasmids conferred the temperature-resistant growth on YKT111. The genes present in the 11 plasmids were identified by sequencing both ends of the inserts.

Antibodies

The mouse anti-HA (HA.11) mAb was purchased from BABCO (Richmond, CA). The mouse anti-myc (9E10) mAb was from Sigma Chemical (St. Louis, MO). The rabbit polyclonal anti-Anp1p antibodies and the mouse anti-Pep12p mAb were gifts from S. Munro and Y. Ohsumi, respectively. The rabbit anti-GFP antiserum was obtained from Molecular Probes Inc. (Eugene, OR). Horseradish peroxidase (HRP)-conjugated secondary antibodies (sheep anti-mouse IgG and donkey anti-rabbit IgG) used for immunoblotting were purchased from Amersham Biosciences (Piscataway, NJ). The Cy2-conjugated secondary antibodies (donkey anti-mouse IgG) and the Cy3-conjugated secondary antibodies (donkey anti-rabbit IgG) used for immunofluorescence were purchased from Jackson ImmunoResearch (West Grove, PA).

Immunoblotting Analysis

Proteins in SDS sample buffer were separated by SDS-PAGE and electroblotted to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% milk in TBST (50 mM Tris, pH 7.5, 200 mM NaCl, and 0.05% Tween 20) for 30 min at room temperature and incubated with primary antibody (anti-HA antibody diluted 1:2000, anti-Anp1p antibodies diluted 1:8000, and anti-Pep12p antibody diluted 1:10000 in 5% milk in TBST) at 4°C overnight. After three washes with 5% milk in TBST, the membrane was incubated with secondary antibody (anti-mouse-HRP or anti-rabbit-HRP diluted 1:1000 in TBST) for 1 h at room temperature. After three washes with TBST, the membrane was developed by chemiluminescence (ECL, Amersham Biosciences).

Western blotting of Ste3p-myc was performed essentially as described previously (Spelbrink and Nothwehr, 1999). Briefly, cells were propagated for several generations at 30°C in synthetic medium containing 2% galactose. Cultures with an optical density of 0.2 were then shifted to 18°C, and glucose was added to a 2% final concentration in medium. Two OD₆₀₀ units of the culture were taken at 1.5-h intervals to 12 h after the addition of glucose, and cells were immediately collected by centrifugation at 4°C. Denatured protein extracts were prepared as described by Mochida *et al.* (2002). The Ste3p-myc protein was detected using the mouse anti-c-myc mAb 9E10.

Table 1. *S. cerevisiae* strains used in this study

Strain ^a	Genotype	Reference or source
YEF473	<i>MATa/α lys2-801/lys2-801 ura3-52/ura3-52 his3Δ-200/his3Δ-200 trp1Δ-63/trp1Δ-63 leu2Δ-1/leu2Δ-1</i>	Longtine <i>et al.</i> (1998)
YJC1431	<i>MATα ura3 his3Δ-200 leu2 MYO2-ARG-GFP::HIS3</i>	A gift from J. Cooper
YKT38	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1</i>	This study
YKT111	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 myo3Δ::TRP1 myo5-360::KanMX6</i>	This study
YKT249	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6</i>	This study
YKT269	<i>MATa/α lys2-801/lys2-801 ura3-52/ura3-52 his3Δ-200/his3Δ-200 trp1Δ-63/trp1Δ-63 leu2Δ-1/leu2Δ-1 CDC50-EGFP::KanMX6/CDC50</i>	This study
YKT271	<i>MATa/α lys2-801/lys2-801 ura3-52/ura3-52 his3Δ-200/his3Δ-200 trp1Δ-63/trp1Δ-63 leu2Δ-1/leu2Δ-1 CDC50-EGFP::KanMX6/CDC50-EGFP::KanMX6</i>	This study
YKT282	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 CDC50-HA::HIS3MX6</i>	This study
YKT569	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 vps27Δ::HIS3 CDC50-EGFP::KanMX6</i>	This study
YKT442	<i>MATa/α lys2-801/lys2-801 ura3-52/ura3-52 his3Δ-200/his3Δ-200 trp1 Δ-63/trp1Δ-63 leu2Δ-1/leu2Δ-1 cdc50Δ::HIS3MX6/cdc 50Δ::HIS3MX6</i>	This study
YKT455	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 BNI1-EGFP::KanMX6</i>	This study
YKT495	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 BNI1-EGFP::KanMX6</i>	This study
YKT570	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 URA3::SPA2-EGFP</i>	This study
YKT571	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 URA3::SPA2-EGFP</i>	This study
YKT579	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 GIC1-EGFP::KanMX6</i>	This study
YKT580	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 GIC1-EGFP::KanMX6</i>	This study
YKT512	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 MYO2-ARG-GFP::HIS3</i>	This study
YKT514	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 MYO2-ARG-GFP::HIS3</i>	This study
YKT526	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 BNR1-EGFP::KanMX6</i>	This study
YKT527	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 BNR1-EGFP::KanMX6</i>	This study
YKT159	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 myo3Δ::TRP1 MYO5-EGFP::KanMX6</i>	This study
YKT547	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 myo3Δ::TKP1 MYO5-EGFP::KanMX6</i>	This study
YKM14	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 [P_{ACT1}-GFP-BUD6 CEN4 URA3]</i>	Transformant of YKT38 with pDAb204
YKM15	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 [P_{ACT1}-GFP-BUD6 CEN4 URA3]</i>	Transformant of YKT249 with pDAb204
YKM34	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 [SEC3-GFP CEN6 URA3]</i>	Transformant of YKT38 with SEC3-GFP 3-1
YKM35	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 [SEC3-GFP CEN6 URA3]</i>	Transformant of YKT249 with SEC3-GFP 3-1
YKM22	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 [P_{ACT1}-BNR1-EGFP CEN URA3]</i>	Transformant of YKT38 with pAGX1-BNR1
YKM23	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 [P_{ACT1}-BNR1-EGFP CEN URA3]</i>	Transformant of YKT249 with pAGX1-BNR1
YKM42	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 [P_{GALI}-STE3-myc CEN6 URA3]</i>	Transformant of YKT38 with pSL2099
YKM43	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 [P_{GALI}-STE3-myc CEN6 URA3]</i>	Transformant of YKT249 with pSL2099
YKM59	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 [CDC3-GFP TRP1 CEN]</i>	Transformant of YKT38 with pRS314-CDC3G
YKM60	<i>MATa lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1 cdc50Δ::HIS3MX6 [CDC3-GFP TRP1 CEN]</i>	Transformant of YKT249 with pRS314-CDC3G

^a YKT and YKM strains are isogenic derivatives of YEF473 except for YKT512 and YKT514 (see MATERIALS AND METHODS).

Subcellular Fractionation and Extraction of Cdc50p-HA

Crude extracts for the cell fractionation and extraction experiments were prepared from midlog cells expressing Cdc50p-HA (YKT282). Harvested cells were lysed by vigorous agitation with 0.4-mm glass

beads in lysis buffer (0.3 M sorbitol, 10 mM Tris, pH 7.5, 0.1 M NaCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [PMSF], 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Extracts were precleared by centrifugation at 500 × *g* for 5 min to discard unbro-

Table 2. Plasmids used in this study

Plasmid	Characteristics	Reference or source
pAGX1	<i>P_{ACT1}-EGFP-T_{TDH3} URA3 CEN6</i>	Ozaki-Kuroda <i>et al.</i> (2001)
pAGX1-BNR1	<i>P_{ACT1}-EGFP-BNR1-T_{TDH3} URA3 CEN6</i>	A gift from K. Ozaki-Kuroda
pKU65	<i>ups27Δ::HIS3</i>	A gift from K. Umeyashashi
p406S2G	<i>SPA2-GFP URA3</i>	Arkowitz and Lowe (1997)
pSRG92	<i>myc-VPS21 URA3 2μm</i>	Gerrard <i>et al.</i> (2000)
pSL2099	<i>P_{GAL1}-STE3::myc LEU2 CEN6</i>	Davis <i>et al.</i> (1993)
SEC3-GFP 3-1	<i>SEC3-GFP URA3 CEN6</i>	Finger <i>et al.</i> (1998)
pDAB204	<i>P_{ACT1}-GFP-BUD6 URA3 CEN4</i>	Amberg <i>et al.</i> (1997)
YEplac181	<i>LEU2 2μm</i>	Gietz and Sugino (1988)
pRS314-CDC3G	<i>CDC3-GFP TRP1 CEN</i>	A gift from M. Iwase
pKT1029	<i>MYO5 LEU2 2μm</i>	This study
pKT1257	<i>LAS17 LEU2 2μm</i>	This study
pKT1241	<i>BNI1-EGFP-KanMX6</i>	This study
pKT1258	<i>UBP5 LEU2 2μm</i>	This study
pKT1259	<i>CDC50 LEU2 2μm</i>	This study
pKT1265	<i>CDC50-EGFP URA3 CEN</i>	This study
pKT1266	<i>CDC50-EGFP LEU2 2μm</i>	This study

ken cells and debris. For subcellular fractionation experiments, precleared cell extracts were separated into pellet (P13) and supernatant (S13) fractions by centrifugation at $13,000 \times g$ for 15 min. The S13 fraction was further separated into pellet (P100) and supernatant (S100) fractions by centrifugation at $100,000 \times g$ for 1 h. Equal portion of cell extracts from each fraction was subjected to SDS-PAGE followed by immunoblotting. For extraction experiments, precleared cell extracts were treated with 0.5 M NaCl, 2.5 M urea, 0.1 M sodium carbonate, pH 11, 1% Triton X-100, or lysis buffer. After 1-h incubation on ice, the samples were centrifuged at $100,000 \times g$ for 1 h to separate soluble and particulate fractions. Proteins of soluble fractions were once precipitated by addition of trichloroacetic acid. Protein pellets from both fractions were solubilized in the same volume of sample buffer and analyzed by immunoblotting.

Sucrose Gradient Fractionation

The fractionation of subcellular organelles was based on sedimentation through a sucrose step gradient (Antebi and Fink, 1992). Briefly, 500 OD₆₀₀ units of midlog cells (YKT282) were harvested by centrifugation, washed twice with water, and resuspended in 10 ml of 100 mM Tris, pH 9.4, and 10 mM DTT. After 10-min incubation at 30°C, the cells were harvested by centrifugation and resuspended in 20 ml of Spheroplast medium (1 M sorbitol in SD medium). Zymolyase 100T (Seikagaku Corp., Tokyo, Japan) was then added to 0.5 unit/OD₆₀₀ units, and the cell suspension was incubated at 30°C for 10 min. After confirmation of spheroplasting by microscopic observation, the cell suspension was transferred on ice to stop digestion. The spheroplasts were then harvested through a 1.4 M cushion of sorbitol, resuspended in 2.5 ml of lysis buffer (0.2 M sorbitol, 50 mM potassium acetate, 1 mM DTT, 2 mM EDTA, 20 mM HEPES-KOH, pH 6.8) containing protease inhibitors (aprotinin, leupeptin, and PMSF) and homogenized (30 strokes) with a 7-ml glass Dounce homogenizer (Wheaton Science Products, Millville, NJ). The homogenates were cleared of intact cells and debris by centrifugation for 10 min ($1000 \times g$) at 4°C; this step was repeated to ensure the complete removal of cellular debris. The cleared homogenate (1 ml) was loaded on an 11-step sucrose gradient poured into a thin-walled ultracentrifuge tube (Hitachi, Tokyo, Japan). The gradient was composed of 1-ml layers (18–54% wt/wt in 4% increments) of sucrose layered over a 65% (wt/wt) sucrose pad (1.0 ml) with each step prepared in 10 mM HEPES-KOH, pH 7.5, and 1 mM MgCl₂. The gradients were centrifuged at $150,000 \times g$ for 3 h at 4°C in

P40ST rotor (Hitachi). Equivalent fractions (1 ml) were collected manually from the top of the gradient. All fractions were assayed for the relevant distribution of marker proteins by immunoblotting (Anp1p, Pep12p, and Cdc50p-HA) or enzymatic activity (α -mannosidase). When not in use, fractions were stored at -80°C .

α -Mannosidase Assay

Vacuolar α -mannosidase activity was assessed essentially as described previously (Roberts *et al.*, 1991). Briefly, 0.1 ml of each fraction was mixed with 0.3 ml of 0.1 M MES-NaOH, pH 6.5, 0.2% Triton X-100, and 0.1 ml of 10 mM *p*-nitrophenyl- α -D-mannopyranoside (Sigma). After 3-h incubation at 37°C, the reaction was stopped by adding 0.5 ml of 0.5 M glycine-Na₂CO₃, pH 10.0, and the absorbance at 400 nm was measured.

Cell Labeling and Immunoprecipitation

Vacuolar sorting of carboxypeptidase Y (CPY) was examined by pulse-chase and immunoprecipitation experiments essentially as described previously (Seaman *et al.*, 1997). A brief description is as follows. Yeast cells were grown to midlog phase in SD media supplemented by appropriate amino acids at 18 or 30°C for 11 h. OD equivalent of cells were collected, resuspended in SD-Met media, and grown at 18 or 30°C for 30 min. The cells were labeled with 20–30 μCi of Tran³⁵S-label (ICN Radiochemicals, Irvine, CA) at 18 or 30°C for 30 min or 10 min, respectively. During the chase period, unlabeled methionine and cysteine were added to concentrations of 5 and 1 mM, respectively. The chase was terminated by adding samples to an equal volume of cold 2 $\times$ spheroplast stop solution (2 M sorbitol, 50 mM Tris, pH 7.5, 40 mM Na₂S₂O₃, and 20 mM DTT). The samples were incubated on ice for 10 min and then treated with 10 μg of zymolyase 100T (Seikagaku). The cells and media solutions were then separated by centrifugation for 1 min. Both fractions were precipitated with 5–10% trichloroacetic acid and subjected to immunoprecipitation using rabbit antibodies against CPY (a gift from Y. Ohsumi). Quantification of CPY of each fraction was performed by SDS-PAGE followed by an analysis using a Phosphorimager system (Fuji Photo Film, Tokyo, Japan).

Microscopic Observations

Visualization of the actin cytoskeleton and in vivo observation of GFP fusion proteins were performed as described previously (Mo-

chida *et al.*, 2002). In some cases where images of GFP-fusion proteins in fixed cells were same as those in living cells, cells were fixed with 5% formaldehyde followed by three washes with phosphate-buffered saline before observation.

For a lipophilic styryl dye FM4-64 (*N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)hexatrienyl) pyridinium dibromide) staining, cells were grown in YPDA to late log growth at 18 or 30°C. Four OD₆₀₀ units of cells were incubated at 18 or 30°C for 15 min and labeled with 32 μ M FM4-64 dye (Molecular Probes) in 100 μ l of YPDA. Cells were harvested by centrifugation, resuspended in 200 μ l of fresh YPDA, and chased at 18 or 30°C for indicated time period.

Quinacrine staining of vacuole was carried out as previously described (Rothman *et al.*, 1989). Cells were incubated in YPDA at 30 or 18°C to OD₆₀₀ $\approx$ 1.0. One OD₆₀₀ unit of cells was collected by centrifugation, resuspended in YPDA buffered to pH 7.6 containing 200 μ M quinacrine (Sigma), and incubated at 30°C for 30 min or 18°C for 1 h. After the incubation, cells were washed with the same medium without the dye and observed immediately by differential interference contrast (DIC) optics and fluorescence microscopy. The vacuole lumen was visualized using CellTracker Blue CMAC (Molecular Probes) as suggested in the manufacturer's protocol.

For Cdc50p-GFP immunofluorescence, cells were fixed, permeabilized, and incubated with rabbit polyclonal antiserum against GFP (Molecular Probes). Secondary antibodies were Cy3-conjugated anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratory). For myc-Vps21p immunofluorescence, mouse monoclonal anti-c-myc antibody (9E10) was used as a primary antibody, and secondary antibodies were Cy2-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratory). Cells were observed with a Nikon ECLIPSE E800 microscope (Nikon Instec, Tokyo, Japan) with HB-10103AF super high-pressure mercury lamp and 1.4NA 100 $\times$ Plan Apo oil immersion objective (Nikon Instec) with appropriate fluorescence-filter sets (Nikon Instec) or DIC optics. Images presented in this article were acquired using digital cooled CCD camera (C4742-95-12NR; Hamamatsu photonics K.K., Hamamatsu, Japan) and the AQUACOSMOS software (Hamamatsu photonics). Observations are based on the examination of at least 100 cells.

Fluid-Phase Endocytosis

Lucifer yellow-carbohydrazide (Sigma) accumulation experiment was performed as described previously (Dulic *et al.*, 1991). Lucifer yellow uptake was carried out for 2, 4, and 8 h at 18°C. Samples were observed with fluorescence microscopy using a B-2E/C fluorescein isothiocyanate bandpass filter set (excitation, 465–495 nm; dichroic mirror, 505 nm; emission, 515–555 nm) and DIC optics as described above.

Electron Microscopy

Samples for transmission electron microscopy were prepared as previously described (Sun *et al.*, 1992). Wild-type or *cdc50* mutant cells were precultured in YPD at 30°C and shifted to 18°C for 12 h. After centrifugation, pellets of cells were mounted on copper meshes to form a thin layer and plunged into liquid propane. Frozen cells were transferred to 4% OsO₄ in anhydrous acetone that had been precooled in a dry ice/acetone bath and kept at –80°C for 48 h. Samples were held at –20°C for 2 h, at 4°C for 2 h, and then at room temperature for 2 h. After a wash with anhydrous acetone, samples were embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate and observed under a JEM-2000FXII electron microscope (JEOL, Tokyo, Japan).

RESULTS

Isolation of CDC50 as a Multicopy Suppressor of the *myo3 myo5-360* Mutation

MYO3 and MYO5 encode type I myosins in *S. cerevisiae*. Myo3p and Myo5p are components of the cortical patch-like structures that regulate formation of cortical actin patches. To identify genes involved in the regulation or function of the actin cytoskeleton, we isolated multicopy suppressors of the temperature-sensitive growth phenotype of the *myo3 myo5-360* mutant (isolation of *myo5-360* will be described elsewhere). Eleven plasmids that did not contain MYO3 or MYO5 and reproducibly suppressed the temperature-sensitive growth were identified. Eight of 11 plasmids contained nonoverlapping inserts that were distinct from each other; therefore, each of them represents a different suppressor gene. The suppressor gene in three plasmids was identified by testing individual subcloned fragments for suppressing activity. These genes were *LAS17*, *CDC50*, and *UBP5* (Figure 1A). Las17p is the yeast counterpart of mammalian Wiskott-Aldrich syndrome protein and has been shown to interact with Myo3p and Myo5p to regulate actin polymerization (Evangelista *et al.*, 2000; Lechler *et al.*, 2000). Ubp5p is a putative ubiquitin-specific protease whose function remains unknown (Papa *et al.*, 1999). The *CDC50* gene was first identified in a mutant exhibiting cell cycle arrest at low temperatures (Moir *et al.*, 1982). It was subsequently cloned and disrupted, but its function in the cell cycle has not been characterized thoroughly (Radji *et al.*, 2001). In this article, we will focus on the characterization of *CDC50*; other genes will be described elsewhere. *CDC50* encodes a 391-amino acid protein, Cdc50p, that has homologues in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens* but does not contain any known functional amino acid sequence motifs (Figure 1B). The amino acid sequence predicts that the Cdc50p family contains two transmembrane regions near the NH₂- and COOH-termini, suggesting that Cdc50p is a membrane-spanning protein.

The *cdc50* Mutant Shows Defects in Polarized Growth

The first isolated *cdc50-1* mutant exhibited a cold-sensitive cell cycle arrest with a small bud and an undivided nucleus (Moir *et al.*, 1982). To further explore the function of Cdc50p, a null mutation of *CDC50* was constructed. *cdc50* mutant cells grew normally at 30, 35, and 37°C but grew very slowly at 25°C and did not grow at 18°C, as reported for the original *cdc50* point mutant. We examined the morphological phenotype of the *cdc50* mutant cells after 12-h incubation at 18°C. The doubling time of our wild-type strain at 18°C was $\sim$ 4 h (unpublished data). At 30°C, no morphological alterations were seen in the mutant, but at 18°C, there was a significant increase in cells with small buds in the *cdc50* mutant cell culture (Figure 2). When incubated at 30°C, the *cdc50* mutant cell culture contained 25% (*n* = 653) cells with small buds; this percentage increased to 40% (*n* = 1076) after incubation at 18°C for 12 h. In contrast, the fraction of small-budded cells in wild-type cell culture decreased from 28% (*n* = 503) at 30°C to 23% (*n* = 1072) after incubation at 18°C for 12 h. Another morphological alteration was noted: >90% of mother cells of *cdc50* mutant were large and round,

Figure 1. Suppression of *myo3Δ myo5-360* mutation by multicopy *CDC50* and amino acid sequence of Cdc50p. (A) Suppression of *myo3Δ myo5-360* mutation by multicopy *CDC50*, *UBP5*, or *LAS17*. The *myo3Δ myo5-360* strain (YKT111) was transformed with pKT1029 (YEplac181-MYO5), pKT1258 (YEplac181-UBP5), pKT1257 (YEplac181-LAS17), pKT1259 (YEplac181-CDC50), or YEplac181. Three independent transformants from each were streaked onto an SD-Leu plate and incubated at 35°C for 3 d. (B) Sequence alignment of Cdc50p and its homologous proteins from different organisms. Full-length amino acid sequences from the database were initially aligned using the CLUSTAL W program and the alignment was then optimized by BOXSHADE program. Black boxes indicate identical amino acids. Gray boxes indicate similar amino acids. The bars above the sequences indicate the possible transmembrane domains. The GenBank accession numbers for the given sequences are as follows: *S. cerevisiae* (CAA42249.1), *S. pombe* (CAA21916.1), *C. elegans* (AAC48073.1), *D. melanogaster* (AAF48613.1), and *H. sapiens* (NP060717.1).

in contrast to the ellipsoidal morphology of wild-type mother cells (Figure 2). These morphological phenotypes were similarly observed in diploid cells homozygous for the *cdc50* mutation (unpublished data). Our results suggest that Cdc50p is required for polarized growth after a bud has

emerged. Staining of filamentous actin with phalloidin revealed that cortical actin patches, which are normally polarized to the growing sites, were depolarized in >90% of the small-budded *cdc50* mutant cells (Figure 2, Actin). Myo5p is a component of cortical patch structures that assemble

Figure 2. Organization of the actin cytoskeleton and subcellular localization of Myo5p-GFP in wild-type and *cdc50Δ* cells. Wild-type (YKT159) and *cdc50Δ* (YKT547) strains that contain the *MYO5-EGFP* construct were grown for 12 h at 18 or 30°C, fixed, stained with rhodamine-phalloidin, and visualized either by DIC or by epifluorescence (actin and Myo5p-GFP). Bar, 5 μ m.

actin and that often colocalize with actin patches (Anderson *et al.*, 1998; Evangelista *et al.*, 2000; Lechler *et al.*, 2000). The Myo5p-GFP patches, which were polarized in wild-type cells, also lost this polarization in >90% of the small-budded *cdc50* mutant cells (Figure 2, Myo5p-GFP). However, colocalization of Myo5p-GFP with cortical actin patches was still seen in the *cdc50* mutant. Actin cables, which play an essential role in polarized secretion (Pruyne *et al.*, 1998), are observed along the mother-bud axis in wild-type cells. Actin cables, however, were not observed in >95% of the *cdc50* mutant cells, consistent with the observed defect in polarized growth in the *cdc50* mutant (Figure 2, Actin).

Regulators of Polarized Growth are Mislocalized in the *cdc50* Mutant

Numerous proteins that regulate the establishment of cell polarity have been identified (reviewed in Pruyne and

Bretscher, 2000). These proteins are localized to the growing sites, such as a bud tip or a cell division site. We examined whether these proteins were properly localized to the bud of the *cdc50* mutant. Proteins that were examined include Cdc24p, Bni1p, Bnr1p, Spa2p, Bud6p, Gic1p, Sec3p, Myo2p, and Cdc3p (reviewed in Pruyne and Bretscher, 2000). Cdc24p is a guanine nucleotide exchange factor for the Cdc42p small GTPase (Zheng *et al.*, 1994), which is required for initiation of budding (Sloat *et al.*, 1981; Johnson and Pringle, 1990). Bni1p is a member of the formin family, and Bnr1p is a homolog of Bni1p (Evangelista *et al.*, 1997; Imamura *et al.*, 1997). Bni1p and Bnr1p are required for the formation of actin cables (Evangelista *et al.*, 2002; Sagot *et al.*, 2002). Bni1p, Spa2p, and Bud6p are components of the 12S polarisome complex, which play an important role in polarized growth (Sheu *et al.*, 1998). Gic1p is an effector of Cdc42p, but its function remains unknown (Brown *et al.*, 1997; Chen *et al.*, 1997). Sec3p is a landmark for polarized

Figure 3. Localization of various GFP-tagged proteins involved in cell polarity in *cdc50Δ* mutant. Cells were incubated for 12 h at 18°C and observed by microscopy immediately (Spa2p, Cdc3p, and Sec3p) or after fixation with 5% formaldehyde (Bni1p, Bnr1p, Gic1p, Bud6p, and Myo2p). GFP-tagged proteins were visualized using a GFP band-pass filter. The strains used were as follows: YKT455 (Bni1p in WT), YKT495 (Bni1p in *cdc50Δ*), YKT526 (Bnr1p in WT), YKT527 (Bnr1p in *cdc50Δ*), YKM22 (Bnr1p overexpressed from the *ACT1*-promotor in WT), YKM23 (Bnr1p overexpressed from the *ACT1*-promotor in *cdc50Δ*), YKT579 (Gic1p in WT), YKT580 (Gic1p in *cdc50Δ*), YKT570 (Spa2p in WT), YKT571 (Spa2p in *cdc50Δ*), YKM59 (Cdc3p in WT), YKM60 (Cdc3p in *cdc50Δ*), YKT512 (Myo2p in WT), YKT514 (Myo2p in *cdc50Δ*), YKM14 (Bud6p overexpressed from the *ACT1*-promotor in WT), YKM15 (Bud6p overexpressed from the *ACT1*-promotor in *cdc50Δ*), YKM34 (Sec3p in WT), and YKM35 (Sec3p in *cdc50Δ*). O/E indicates overexpression under the control of the *ACT1* promoter. Bar, 5 μm.

secretion (Finger *et al.*, 1998), and it has recently been shown to interact with Cdc42p (Zhang *et al.*, 2001). Myo2p is a type V myosin motor, which is required for polarized transport of secretory vesicles (Johnston *et al.*, 1991). Cdc3p is a septin, which plays an important role in cytokinesis and is localized to the bud neck region (Kim *et al.*, 1991). GFP-Cdc24p was normally localized to a tip of small buds in *cdc50* mutant cells arrested at 18°C (unpublished data), being consistent with the results that *cdc50* cells are arrested after a bud has emerged. In contrast, in >95% of small-budded *cdc50* mutant cells arrested at 18°C, Bni1p-GFP was not localized to the bud tip, and it was instead localized to tubular membranous structures that were mainly present around cortical regions (Figure 3). Similarly, Gic1p-GFP was also localized to tubular structures in >80% of small-budded *cdc50* mutant cells (Figure 3). These tubular structures may be reminiscent of mitochondria, but staining of mitochondria in the *cdc50* mutant with the mitochondria-specific dye DASPMI suggested that the tubular structures containing Bni1p-GFP were distinct from mitochondria (unpublished data). Spa2p-GFP was localized normally to the bud but was also detected as a few cortical spots in ~20% of the mother cells of small-budded *cdc50* cells (Figure 3). Bud6p-GFP, Sec3p-GFP, and Myo2p-GFP were localized normally to the bud in >80% of small-budded *cdc50* mutant cells (Figure 3). Bnr1p-GFP and Cdc3p-GFP were normally localized to the bud neck in *cdc50* cells as in wild-type cells (Figure 3). When GFP-Bnr1p was overexpressed in the *cdc50* mutant under the control of the *ACT1* promoter, however, it was localized to short bar-like structures around the bud neck in ~30% of *cdc50* cells. More interestingly, ~5% of the *cdc50* cells had a ring structure containing GFP-Bnr1p at a site distal from the bud neck (Figure 3). This ring structure may represent the previous cell division site, which was not seen in wild-type cells overexpressing GFP-Bnr1p. Our results suggest that defects in polarized growth of the *cdc50* mutant may be partly due to improper localization of regulators of polarized growth.

Cdc50p Localizes to Endosomal Membranes

To determine intracellular localization of Cdc50p, a C-terminal GFP-tagged allele was generated and integrated into the genome as the sole copy of *CDC50*. This strain grew normally at 18°C and did not show morphological alterations (unpublished data); thus, the tagged *CDC50* allele appeared fully functional. To facilitate observation of the cellular localization of Cdc50p-GFP, heterozygous diploids were constructed and observed. Cdc50p-GFP appeared as scattered or perivacuolar dots reminiscent of endosomal membranes (Figure 4A). Vps21p was shown to be colocalized with Pep12p in the endosomal/prevacuolar compartments (Gerrard *et al.*, 2000). In this experiment, *VPS21* was overexpressed, resulting in collapse of the Vps21p and Pep12p punctate staining pattern into one to three larger staining structures per cell (Singer-Kruger *et al.*, 1995; Gerrard *et al.*, 2000). These observations established Vps21p as a useful tool for the analysis of endosomal markers by immunofluorescence. We examined, by indirect immunofluorescence microscopy, whether Cdc50p-GFP colocalized with Vps21p. Overexpression of *VPS21* altered the distribution of Cdc50p-GFP such that Cdc50p-GFP clearly colocalized with Vps21p in fewer, but larger, structures (Figure 4B).

To examine whether Cdc50p-GFP is localized to endosomal compartments in wild-type cells, we used the fluorescent lipophilic dye, FM4-64. FM4-64 is endocytosed in living cells, travels through the endocytic pathway, and accumulates at the vacuole (Vida and Emr, 1995). Therefore, FM4-64 can be used to trace endocytic intermediates. We examined whether Cdc50p-GFP colocalized with FM4-64 during the chase period of endocytic delivery of FM4-64 to vacuoles at 25°C. In an early stage of endocytosis (2 min), only 4% (*n* = 450) of FM4-64-positive structures showed colocalization with Cdc50p-GFP structures, but in a later stage (12 min), 84% (*n* = 931) of FM4-64 structures showed colocalization with Cdc50p-GFP structures (Figure 4C). In 17 min, FM4-64 signals were seen at vacuolar membranes, but 79% (*n* = 492) of punctate FM4-64 structures still colocalized with Cdc50p-GFP (unpublished data). These results suggest that Cdc50p-GFP is localized to late endosomal/prevacuolar compartments, rather than to early endosomes.

VPS27 is characterized as a class E *VPS* gene, and cells lacking Vps27p have a large, aberrant prevacuole structure (the class E compartment) next to the vacuole (Raymond *et al.*, 1992; Piper *et al.*, 1995). Proteins that normally reside in or travel through the endosomal/prevacuolar compartment accumulate in the class E compartment of *vps27* cells (Piper *et al.*, 1995). FM4-64 also accumulates at the class E compartment of *vps27* cells (Vida and Emr, 1995). Cells of the *vps27* mutant expressing Cdc50p-GFP were loaded with FM4-64 dye, followed by a 30-min chase in fresh medium. Both Cdc50p-GFP and FM4-64 accumulated in the class E compartment (Figure 4D). A compartment that possesses Cdc50p-GFP, but not FM4-64 dye, was also detected in some of the *vps27* cells, suggesting that Cdc50p is also localized to a compartment other than endosomal compartments. Taken together, our results suggest that Cdc50p-GFP is mainly localized to late endosomal/prevacuolar compartments.

Biochemical Analysis of Cdc50p

The localization of Cdc50p was further characterized through biochemical fractionation. A C-terminal, HA-tagged allele was generated and integrated into the genome as the sole copy of *CDC50*. This strain grew normally at 18°C and did not show morphological alteration, indicating that the *CDC50-HA* allele is fully functional (unpublished data). Western blot analysis of protein extracts from *CDC50-HA* cells showed a single major protein band at ~70 kDa, which is larger than the expected molecular weight of Cdc50p-HA, 49.8 kDa. This difference may be due to N-glycosylation of Cdc50p: there are five putative N-glycosylation sites between the first and second transmembrane regions and one such site between the second transmembrane region and the C terminus. After clearing centrifugation at $300 \times g$ to remove unbroken cells, the lysate was sequentially centrifuged to generate $13,000 \times g$ pellet (P13), $100,000 \times g$ pellet (P100), and $100,000 \times g$ supernatant (S100) fractions. When wild-type cell lysates are fractionated under these conditions, the P13 fraction primarily contains large membrane structures, such as vacuolar membranes, plasma membrane, endoplasmic reticulum, mitochondria, and nuclei, whereas the P100 fraction contains Golgi membranes and transport vesicles (Marcusson *et al.*, 1994). Endosomal membranes containing the syntaxin homologue Pep12p distribute be-

Figure 4. Microscopic observation of GFP-tagged Cdc50p. (A) Punctate localization of Cdc50p-GFP. DIC and GFP images of exponentially growing YKT269 cells containing the *CDC50-GFP* allele. (B) Colocalization of Cdc50p-GFP with myc-Vps21p. Indirect immunofluorescence was performed as described in MATERIALS AND METHODS. Wild-type cells (YKT38) overexpressing both myc-Vps21p (pSRG92) and Cdc50p-GFP (pKT1266) were grown to midlog phase before fixation. Cdc50p-GFP was visualized by using rabbit polyclonal anti-GFP antibodies and Cy3-conjugated secondary antibodies. The same cells were also stained for myc-Vps21p by using mouse monoclonal anti-c-myc antibody and Cy2-conjugated secondary antibodies. Cy2 and Cy3 images were collected and merged to show coincidence of the two staining patterns. Bar, 5 μ m. (C) Colocalization of Cdc50p-GFP with FM4-64. Wild-type cells containing the *CDC50-GFP* allele (YKT271) were grown to midlog phase, labeled in 32 μ M FM4-64 for 30 min at 0°C and then chased in fresh medium for 2 min or 12 min at 25°C. Cells were fixed with 1% formaldehyde for 15 min at 25°C. FM4-64 and Cdc50p-GFP images were acquired under the red and green fluorescence channels, respectively. A merged image of these two channels is also shown. Bar, 5 μ m. (D) Accumulation of FM4-64 and Cdc50p-GFP in the class E compartment of *vps27Δ* cells. *vps27Δ* cells containing the *CDC50-GFP* allele (YKT569) were grown to midlog phase, labeled in 32 μ M FM4-64 for 15 min at 30°C, and then chased in fresh medium for 30 min at 30°C. DIC images of the same cells were collected to visualize the vacuoles. Bar, 5 μ m.

tween the P13 and P100 pellets (Becherer *et al.*, 1996). Soluble proteins found in the cytosol or within the lumen of osmotically sensitive organelles are found in the S100 fraction. Cdc50p-HA was found in the P13 and S100 fractions (Figure 5A). There was no difference in mobility between the P13 Cdc50p-HA and S100 Cdc50p-HA. We attempted to solubilize Cdc50p-HA in the P13 fraction by pretreating cell lysates with various chemicals before the $100,000 \times g$ centrifugation. Cdc50p-HA was solubilized by treatment with 1% Triton X-100 but not with either of 0.5 M NaCl, 2.5 M urea, or 0.1 M Na_2CO_3 (Figure 5B), indicating that Cdc50p-HA associates with the P13 fraction as an integral membrane protein, as suggested by its amino acid sequence (Figure 1B). We do not know at present whether the Cdc50p-HA present in the S100 fraction is associated with small vesicles that do not sediment at $100,000 \times g$ or is present in the cytosol unassociated with lipid-containing structures.

To further characterize the complex with which Cdc50p associates, the $1000 \times g$ supernatant cellular fraction from Cdc50p-HA-expressing cells was subjected to equilibrium sedimentation through a sucrose gradient. Fractions were collected followed by Western blot analysis and assay for enzymatic activity. Anti-HA antibody detected Cdc50p-HA in fractions 3–7 with a single peak (fraction 5). Pep12p was present in fractions 4–6 with the same single peak as Cdc50p-HA (Figure 5C). Anp1p is a Golgi protein that is involved in retention of several Golgi enzymes (Jungmann and Munro, 1998). Anp1p was detected in fractions 5–7 with a peak (fraction 6) that partially overlapped the Cdc50p-HA fractions. Vacuolar marker enzyme α -mannosidase activity was distributed to fraction 1 away from the Cdc50p-HA peak. These results, together with our cytological localization results of Cdc50p-GFP, strongly suggest that Cdc50p resides mainly in the endosomal/prevacuolar compartment.

cdc50 Mutant Cells Exhibit Defects in Vacuolar Functions

Localization of Cdc50p in an endosomal compartment suggests that *CDC50* may be involved in vacuolar function. In wild-type cells in late log phase, numerous small vacuoles coalesce into one larger vacuole. When viewed under DIC microscopy, *cdc50* cells did not have a visible vacuole. Instead, many small vesicular bodies were seen dispersed throughout the cell (unpublished data). To further analyze vacuole function in *cdc50* cells, we examined vacuolar acidification in this mutant. A fluorescent dye, quinacrine can be used as a marker for this; it is only accumulated in the vacuolar lumen upon acidification by the vacuolar H^+ -ATPase (Weisman *et al.*, 1987; Rothman *et al.*, 1989). *cdc50* cells were incubated with quinacrine and viewed under fluorescence microscopy. Several fragmented vacuoles were stained with quinacrine in $>80\%$ of *cdc50* cells, indicating that vacuoles are acidified but morphologically abnormal in *cdc50* mutant (Figure 6A). The fragmented vacuoles were also visualized in $>80\%$ of *cdc50* cells with CellTracker Blue CMAC, a dye that stains the vacuole lumen (Figure 6A). Class B and C vacuolar protein-sorting (*vps*) mutants show moderate and severe vacuole fragmentation, respectively. In these mutants, carboxypeptidase Y (CPY) is missorted and, as a consequence, is secreted into the growth medium (Raymond *et al.*, 1992). We examined whether Cdc50p is required for vacuolar sorting of CPY. After a 10-min pulse with

Tran ^{35}S label, wild-type cells display two precursor forms of ^{35}S -labeled CPY, a 67-kDa species (p1) in the endoplasmic reticulum (ER) and a fully glycosylated 69-kDa form (p2) in the Golgi. On delivery to the vacuole, precursors are cleaved

Figure 5. Association of Cdc50p-HA with endosomal/prevacuolar membrane. (A) Subcellular fractionation of Cdc50p-HA. Precleared extracts from yeast strain YKT282 containing the *CDC50-HA* allele were subjected to sequential differential centrifugation as described in MATERIALS AND METHODS. Equal portions of the fractions (0.25 OD₆₀₀ unit equivalents) were separated by SDS-PAGE, transferred to PVDF membrane, and probed with an anti-HA antibody. (B) Differential solubilization of Cdc50p-HA. Aliquots of precleared extracts from yeast strain YKT282 were treated for 1 h on ice with the various indicated reagents. The samples were then centrifuged to separate the supernatant (S) and pellet (P) fractions as described in MATERIALS AND METHODS. The presence of Cdc50p-HA in the P and S fractions was determined by Western blot using an anti-HA antibody. (C) Sucrose step gradient centrifugation analysis of Cdc50p-HA. Cell lysates were prepared from yeast strain YKT282 and fractionated in 18–65% sucrose step density gradients by centrifugation for 3 h. Equivalent volumes from each fraction were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with antibodies against HA, Pep12p, and Anp1p. The activity of the vacuolar marker enzyme, α -mannosidase, was assayed as described in MATERIALS AND METHODS.

Figure 6. Vacuolar functions and morphology of the *cdc50Δ* mutant. (A) Vacuolar quinacrine accumulation and vacuolar morphology in wild-type and *cdc50Δ* mutant. Cells grown at 18°C for 11 h were incubated with quinacrine or CellTracker Blue CMAC. Fluorescence and DIC images of wild-type (YKT38) and *cdc50Δ* (YKT249) strains are shown. Bar, 5 μm. (B) CPY secretion in wild-type and *cdc50Δ* strains. Wild-type (YKT38) and *cdc50Δ* (YKT249) strains were grown for 11.5 h at 30 or 18°C, pulse-labeled with Tran³⁵S-label for 10 or 30 min, and chased for another 30 or 90 min, at 30°C (lanes 1–4) or 18°C (lanes 5–8), respectively. CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions, resolved by SDS-PAGE, and visualized using a phosphorimager system.

to the 65-kDa mature form (m) by proteinase A (Stevens *et al.*, 1982). The *cdc50* mutant processed CPY normally during a 30-min chase period when grown and assayed at 30°C (Figure 6B, lanes 3 and 4). At 18°C, slight accumulations of p1 CPY and p2 CPY were observed after a 90-min chase, indicating that the CPY processing is slowed somewhat in both the ER-to-Golgi and Golgi-to-vacuole steps in the *cdc50* mutant. The *cdc50* mutant secreted a small fraction of p2 CPY (3.5% of total CPY) into the extracellular medium (Figure 6B, lane 8). Taken together, our results suggest that CDC50 is involved in vacuolar function. However, the *cdc50* mutant is distinct from the >40 *S. cerevisiae vps* mutants: *vps* mutants do not display defects in the processing step from p1 CPY to p2 CPY, and *vps* mutants secrete a large fraction of CPY (>30%) as p2 CPY into the extracellular medium (Raymond *et al.*, 1992).

To assess more closely the structure of the *cdc50* mutant, we performed electron microscopy (EM) on the *cdc50* mutant. In *cdc50* cells grown at low temperature (18°C), EM revealed aggregations of small 30- to 40-nm vesicles (indicated by small arrows in Figure 7, B and C) and abnormal large membranous structures (indicated by arrowheads in Figure 7, D and E); both of these structures were observed in ~10% of EM sections of *cdc50* cells but in <1% of EM sections of wild-type cells (Figure 7A). These abnormal structures, whose identity is unknown, may represent the vesicular bodies that were observed by DIC optics. In addition, in several thin sections of *cdc50* cells, vacuolar morphology was aberrant (Figure 7D), which may reflect fragmented

vacuoles seen by quinacrine and CellTracker Blue CMAC stainings.

Possible Involvement of Cdc50p in Endocytic Transport

Certain cell surface receptors, their ligands, and other plasma membrane components destined for degradation are delivered to the vacuole by the endocytic pathway, which converges with the biosynthetic pathway at a prevacuolar endosome-like compartment (Singer and Riezman, 1990; Vida *et al.*, 1993). To examine the direct consequences of the loss of Cdc50p function on the endocytic pathway, *cdc50* cells were tested for their ability to internalize and deliver endocytic markers to the vacuole. Wild-type and *cdc50* cells grown at 30 or 18°C were stained with FM4-64 and chased in YPDA at either 30 or 18°C, respectively (Figure 8A). At 30°C, both wild-type and *cdc50* cells internalized and delivered FM4-64 to the vacuoles after a 1-h chase. At 18°C, wild-type cells delivered FM4-64 to the vacuoles after a 3-h chase. In contrast, although *cdc50* cells internalized FM4-64, >90% of cells exhibited a punctate staining pattern even after a 3-h chase. These punctate structures in *cdc50* cells were similar to those at 0 h, which represent presumptive endocytic intermediates (Vida and Emr, 1995). These punctate structures were much smaller than the fragmented vacuoles observed in Figure 6. This result suggests that Cdc50p is required for endocytic transport.

Figure 7. Electron microscopic observation of the *cdc50Δ* mutant. Wild-type (YKT38; A) and *cdc50Δ* mutant cells (YKT249; B–E) were grown to an early log phase at 30°C, incubated at 18°C for 12 h, prepared for electron microscopy by the freeze-substitution fixation method, and observed under an electron microscope. The boxed regions in panels B and D are enlarged in panels C and E, respectively. Arrowheads in D and E indicate abnormal structures enclosed by membrane. N, the nucleus; V, the vacuoles. Bars, 0.2 μ m.

Cortical patch-like protein structures are necessary for the assembly of cortical actin patches and for the internalization step of both fluid phase and receptor-mediated endocytosis (Wendland *et al.*, 1998). Mutants containing alterations in genes encoding these cortical actin patch-assembly proteins, including Myo3/5p, Sla2p (End4p), Vrp1p, and Las17p, show defects in the internalization step. Because the actin cytoskeleton is perturbed in the *cdc50* mutant, *CDC50* may be required for the internalization step of endocytosis. FM4-64, however, can enter the cell independent of *SLA2* (*END4*) gene functions (Vida and Emr, 1995). Therefore, we used another endocytic marker for fluid phase endocytosis, Lucifer yellow (LY; Riezman, 1985), which cannot enter cortical actin patch-assembly mutants. As shown in Figure 8B, LY was delivered to wild-type vacuoles after a 2-h chase at 18°C. In the *cdc50* mutant, LY was internalized, but it remained in punctate structures in >90% of *cdc50* cells even after an 8-h chase, as seen for the endocytosis of FM4-64. These results indicate that cortical patch-like protein structures in the *cdc50* mutant have normal endocytic machinery, even though they lack polarization.

The involvement of *CDC50* in endocytosis was also assessed for the α -factor pheromone receptor Ste3p, which is internalized and delivered to the vacuole, where it is de-

graded in a *PEP4*-dependent manner. The rapid constitutive turnover of Ste3p is mediated by a signal in the cytoplasmic domain of Ste3p and is dependent on genes that effect trafficking through the endocytic pathway (Davis *et al.*, 1993). To assess the half-life of Ste3p, wild-type and *cdc50* strains carrying *P_{GAL1}-STE3-myc* were propagated at 30°C in media containing galactose. Glucose was added to shut off expression of *STE3-myc*, and immediately the cultures were shifted to 18°C. The amount of Ste3p-myc present at various time points after addition of glucose was assessed by Western blot analysis using an anti-myc antibody. In the wild-type strain the majority of Ste3p-myc was degraded within 3 h. In contrast, Ste3p-myc was turned over more slowly in the *cdc50* mutant (Figure 8C). Combined with our observation that LY was internalized in the *cdc50* mutant, these results suggest a role for Cdc50p in mediating transport of Ste3p after internalization through the endocytic pathway.

DISCUSSION

In this study, we have characterized a conserved membrane protein, Cdc50p, identifying its role in the formation of cell polarity and membrane trafficking. Our biochemical frac-

Figure 8. Endocytic transport in the *cdc50Δ* mutant. (A) Internalization of the fluorescent lipophilic dye FM4-64 in wild-type and *cdc50Δ* strains. Wild-type (YKT38) and *cdc50Δ* mutant (YKT249) cells were labeled with FM4-64 dye for 15 min at 30 or 18°C, and then chased in fresh medium for 1 h or 3 h, respectively. (B) Fluid phase endocytosis in wild-type strain and *cdc50Δ* mutant. Wild-type (YKT38) and the *cdc50Δ* mutant (YKT249) cells were incubated for 12 h at 18°C, stained with Lucifer yellow for 2, 4, and 8 h at 18°C. Bar, 5 μm (C) Endocytic delivery and degradation of the Ste3p-myc protein. Wild-type (YKM42) and *cdc50Δ* mutant (YKM43) cells, both harboring plasmids pSL2099 encoding the *STE3-myc* gene under the control of the galactose-inducible *GAL1* promoter, were grown at 30°C in minimal media containing galactose. The cultures were then shifted to 18°C, and glucose was added to shut off expression of *STE3-myc*. Aliquots of cells were removed at the indicated time points, and protein extracts were prepared immediately from the cells. Samples were separated by SDS-PAGE and analyzed by Western blotting using a mouse anti-myc antibody.

tionation and cell biological experiments strongly suggest that Cdc50p is a membrane-spanning protein that is localized to the endosomal/prevacuolar compartment. Consistent with this, we found that *CDC50* is required for the endocytic delivery of FM4-64 and Lucifer yellow to the vacuole at 18°C. However, sorting of CPY to the vacuole was affected minimally in *cdc50*, distinguishing the *cdc50* mutant from conventional vacuolar protein sorting (*vps*) mutants. Most *vps* mutants do not display growth defects; Cdc50p possesses a unique set of cellular functions compared with other endosomal proteins.

We isolated *CDC50* as a multicopy suppressor of the temperature-sensitive growth defect of the *myo3 myo5-360* mutant. Myo3p and Myo5p are components of cortical patch-like protein structures, which regulate the assembly of cortical actin patches (Pruyne and Bretscher, 2000). Cortical patch-like protein structures are required for the internalization step in both fluid phase and receptor-mediated endocytosis (Wendland *et al.*, 1998). Overexpression of *CDC50* did not suppress the defect in Lucifer yellow uptake and delocalization of cortical actin patches of the *myo3 myo5-360* mutant (unpublished data), suggesting that type I myosins in yeast may possess another function that is related to that of *CDC50*. The *cdc50* mutant displayed defects in organization of the actin cytoskeleton: delocalization of cortical actin patches and Myo5p and an absence of apparent actin cables. The internalization step of endocytosis is normal in *cdc50* mutant, however, as indicated by successful internalization of FM4-64 and Lucifer yellow in the *cdc50* mutant at 18°C. Therefore, the polarized localization, but not the function, of cortical patch-like protein structures is impaired in the *cdc50* mutant. This defect may be due to mislocalization of Bni1p, which is implicated in polarization of cortical actin patches (Evangelista *et al.*, 1997; Imamura *et al.*, 1997).

Many (40%) of the *cdc50* mutant cells were arrested with a small bud at 18°C, suggesting that *cdc50* is defective in bud growth but not in initiation of budding. The *rho1* mutant also shows cell-cycle arrest with a small bud, due to defects in synthesis of cell wall glucan at the growing tip (Yamochi *et al.*, 1994; Drgonova *et al.*, 1996; Qadota *et al.*, 1996). However, the cell wall defects do not seem to be a cause for the small-bud phenotype of the *cdc50* mutant, because 1) the cell wall of a bud in the *cdc50* mutant arrested at 18°C shows normal thickness and morphology by electron microscopy (unpublished data); and 2) the growth defect of the *cdc50* mutant is not suppressed by the presence of 1 M sorbitol in the medium (unpublished data). Another possibility is that the small-bud phenotype of *cdc50* mutant is due to a failure to maintain Bni1p and Gic1p at the bud tip. Mislocalization of Bni1p, Gic1p, and Gic2p may account for the growth defect of the *cdc50* mutant because the *bni1* mutation is synthetically lethal with the *gic1 gic2* mutation (Bi *et al.*, 2000). In the *cdc50* mutant, Bni1p and Gic1p are mislocalized, whereas Cdc24p, Myo2p, Sec3p, and Bud6p are properly localized, suggesting that Cdc50p is specifically involved in localizing or maintaining Bni1p and Gic1p at the growing sites. Bni1p and Bnr1p are required for the formation of actin cables (Evangelista *et al.*, 2002; Sagot *et al.*, 2002). Loss of Bni1p localization at the bud tip may account for the observed loss of apparent actin cables in *cdc50* cells. However, *cdc50* cells seem to possess at least some actin cables that are not detected with standard fluorescence microsc-

copy, because, in *cdc50* cells, 1) Bnr1p is normally localized to the bud neck; and 2) a type V myosin Myo2p, whose polarized localization is dependent on actin cables (Pruyne *et al.*, 1998), is normally localized to the bud. Therefore, loss of actin cables is probably not the major cause of the defects in polarized growth of *cdc50* mutant. Bni1p, Gic1p, and Gic2p may possess another function in polarized growth, or a defect, which remains to be found in *cdc50* mutant, may be also responsible for the defects in polarized growth in *cdc50* mutant.

One possible role for Cdc50p is direct involvement in the transport of Bni1p and Gic1p to sites of polarized growth. The secretory pathway mediates transport of Bud6p to polarized sites (Jin and Amberg, 2000), but it is unknown how this process occurs for Bni1p and Gic1p. How is Cdc50p, which resides in the endosomal compartment, involved in the localization of Bni1p and Gic1p? The localization of Cdc50p to the endosomal compartment is reminiscent of the dynamic localization of Chs3p, the catalytic subunit of chitin synthase III, which is found at the incipient bud site in unbudded cells and at the bud neck in small-budded cells. Chs3p at the plasma membrane is endocytosed and delivered to an endosome-like compartment, the chitosome, and is recycled back to growth sites (Ziman *et al.*, 1998; Valdivia *et al.*, 2002). Bni1p and Gic1p may be localized to a polarized site in a similar manner. There is no evidence, however, that Bni1p and Gic1p are found in the endosomal compartment in wild-type cells. Moreover, the tubular structures which were positive for Bni1p and Gic1p in the *cdc50* mutant did not look like the punctate pattern of presumptive endosomal compartments in *cdc50* cells, which were visualized with FM4-64 (Figure 8A). We also stained ER and Golgi in *cdc50* cells with respective marker proteins, Sec63p-GFP (Prinz *et al.*, 2000) and Sec7p-GFP (Seron *et al.*, 1998), but neither staining was similar to the Bni1p-GFP- and Gic1p-GFP-positive structures (unpublished data). In our EM study, *cdc50* cells displayed small vesicles and large membranous structures, which might contain the mislocalized Bni1p or Gic1p. Further studies are required to elucidate how Cdc50p regulates the localization of Bni1p and Gic1p.

A gene in *S. cerevisiae*, *ROS3*, has been found recently to be required for internalization of fluorescent-labeled phosphatidylcholine and phosphatidylethanolamine, but not phosphatidylserine, by transbilayer translocation across the plasma membrane (Kato *et al.*, 2002). Molecular cloning of *ROS3* revealed that *ROS3* is allelic to *LEM3/YNL323w*, which is homologous to *CDC50*. Ros3p displays high overall homology to Cdc50p (41% identity), including two putative transmembrane domains. The results suggest that Ros3p regulates transbilayer movement ("flip-flop") of phospholipids, which is implicated in the asymmetric organization of phospholipids. This phenomenon, in which phosphatidylethanolamine and phosphatidylserine are enriched in the inner leaflet facing the cytoplasm, whereas phosphatidylcholine, sphingomyelin, and glycolipids are predominantly located on the outer leaflet, has been well documented for the plasma membranes of numerous cell types (Williamson and Schlegel, 1994; Diaz and Schroit, 1996). Cdc50p may also regulate the asymmetric organization of phospholipids, which may in turn play an important role in the cell polarization and membrane trafficking.

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