Cdc1 Is Required for Growth and Mn^{2+} Regulation in *Saccharomyces cerevisiae*

Madan Paidhungat*[,1] and Stephen Garrett[†]

**Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710 and [†]Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, University Heights, Newark, New Jersey 07103-2714*

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ABSTRACT

Cdc1 function was initially implicated in bud formation and nuclear division because *cdc1*(Ts) cells arrested with a small bud, duplicated DNA, and undivided nucleus. Our studies show that Cdc1 is necessary for cell growth at several stages of the cell cycle, as well as in pheromone-treated cells. Thus, Cdc1 depletion might affect bud formation and nuclear division, as well as other cellular processes, by blocking a process involved in general cell growth. Cells depleted of intracellular Mn^{2+} also exhibit a *cdc1*-like phenotype and recent results suggested Cdc1 might be a Mn^{2+}-dependent protein. We show that all of the conditional *cdc1*(Ts) alleles tested cause cells to become sensitive to Mn^{2+} depletion. In addition, Cdc1 overproduction alleviates the chelator sensitivity of several Mn^{2+} homeostasis mutants. These findings are compatible with a model in which Cdc1 regulates intracellular, and in particular cytosolic, Mn^{2+} levels which, in turn, are necessary for cell growth.

CELLS of the yeast *Saccharomyces cerevisiae* divide by budding. Bud growth requires processes that expand cell-surface area and increase cell volume, including macromolecular synthesis, cell-wall biosynthesis, and ion homeostasis (Pringle and Hartwell 1981). Although these processes are probably required by all growing cells, mutants with defects in the first two processes arrest in G1 (Pringle and Hartwell 1981), whereas mutants with defects in cell-wall biosynthesis exhibit a small-bud terminal arrest (Levin and Bartlett-Heubusch 1992).

Cell-wall biosynthesis is regulated by the yeast protein kinase C homolog, Pkc1 (Kamada *et al.* 1996), as well as the yeast GTPase, Rho1 (Drgonova *et al.* 1996). Cells lacking either of these activities exhibit a cell-wall integrity defect that can result in lysis throughout the cell cycle (Kamada *et al.* 1995; Levin and Bartlett-Heubusch 1992; Yamochi *et al.* 1994) and during pheromone-induced shmoo formation (Errede *et al.* 1995). Nevertheless, cells depleted of Pkc1 or Rho1 arrest with small buds, 2N DNA, and undivided nuclei (Levin *et al.* 1990; Yamochi *et al.* 1994). Because cell-wall biosynthesis is required for general cell growth, the small-bud arrest displayed by both mutants suggests cell-wall biosynthesis or integrity is limiting during bud growth (Levin and Bartlett-Heubusch 1992).

The *cdc1-1*(Ts) mutant was originally described as exhibiting a small-bud arrest, completing DNA replication but arresting with undivided nuclei (Hartwell *et al.* 1970; Hartwell 1971). Although later studies suggested most *cdc1-1*(Ts) cells arrested without an apparent bud (Hartwell 1971), that anomaly was addressed by a model in which Cdc1 was required for bud emergence as well as bud growth (Hartwell 1971). However, mutations in *CDC1* have been associated with a wide spectrum of phenotypes so it is "difficult to attribute to this gene product a role in one known cell cycle event" (Hartwell 1974). For example, in contrast to most "*cdc*" mutant cells, which continue to enlarge after arrest, *cdc1*(Ts) cells arrest growth and division simultaneously (Hartwell 1971). In addition, *cdc1*(Ts) mutants exhibit defects in macromolecular synthesis, cell viability (Hartwell 1971), mating (Reid and Hartwell 1977), spindle-pole body duplication (Byers and Goetsch 1974), and intrachromosomal recombination (Halbrook and Hoekstra 1994). Molecular analysis has shown that *CDC1* gene is essential (Halbrook and Hoekstra 1994; Supek *et al.* 1996) but has been uninformative about the biochemical activity or function of Cdc1.

Recent studies provide a link between Cdc1 function and intracellular Mn^{2+}. A majority of cells within a Mn^{2+}-depleted culture arrest with a phenotype (small bud, duplicated DNA, and undivided nucleus; Loukin and Kung 1995) that is similar to the prototypic *cdc1*(Ts) arrest. As with *cdc1*(Ts) mutants (Hartwell 1971), Mn^{2+}-depleted cells lose viability only after arresting growth (Loukin and Kung 1995). Moreover, the conditional growth defect of two *cdc1*(Ts) mutants was rescued by Mn^{2+} supplement (Loukin and Kung 1995). Finally, an allele of *cdc1* (*cdc1-200*) was recently isolated in a screen for chelator-sensitive mutants and shown to be rescued by overproduction of the high-affinity,

Corresponding author: Stephen Garrett, Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical Center, 185 South Orange Ave., University Heights, Newark, NJ 07103-2714. E-mail: garretst@umdnj.edu

[1]*Present address:* Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06030.

plasma membrane Mn^{2+} transporter, Smf1 (Supek *et al.* 1996). These results prompted Supek and colleagues to propose that Cdc1 might be a Mn^{2+}-dependent, cell-division cycle protein (Supek *et al.* 1996).

The studies described here show that Cdc1 is required for cell growth in several stages of the cell cycle, as well as in pheromone-treated cells. These results suggest Cdc1 plays a role in general cell growth and expansion and support the notion that Cdc1 does not play a direct role in cell-cycle progression. The role in general cell growth may account for the pleiotropic effects of Cdc1 depletion on bud formation, spindle-pole body duplication, mating, and cell viability. We also show that Cdc1 overproduction ameliorates the chelator sensitivity of several Mn^{2+} homeostasis mutants, and that conditional *cdc1*(Ts) mutants are sensitive to the depletion of intracellular Mn^{2+}. These findings suggest Cdc1 regulates intracellular, probably cytosolic, Mn^{2+}, which is necessary for cell growth.

MATERIALS AND METHODS

Media: Standard yeast media were prepared as described (Kaiser *et al.* 1994). Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and NaCl were added to autoclaved YEPD medium, whereas sorbitol was added prior to autoclaving. MnCl2 was added to YEPD medium adjusted to pH 5.5 with HCl. Resistance to EGTA varied quantitatively with the agar [Difco, Detroit, or BBL (Beckton Dickinson and Co., Catonsville, MD)], presumably as a result of contaminating ions. Results described were obtained using granulated agar from BBL.

Yeast growth conditions and manipulations: Yeast growth was scored after incubating plates for 3–5 days at 23° and 2–4 days at 30° and 36°. Procedures for genetic manipulation of yeast strains have been previously described (Kaiser *et al.* 1994).

Yeast and bacterial strains: Yeast strains and sources are listed in Table 1. Crosses between the original *cdc1*(Ts) isolates, 369, 342, 131, 296, 456, and E1-6 (Hartwell *et al.* 1973), and a standard laboratory strain, Y294 (Fedor-Chaiken *et al.* 1990), resulted in the segregation of more than one mutation that conferred a temperature-sensitive growth defect. To separate *cdc1*(Ts) alleles from background mutations, *cdc1*(Ts) segregants from the first set of crosses were backcrossed at least three times to Y294 or FY71. In those crosses, the *cdc1-1*(Ts) allele was identified by the small-bud arrest phenotype it conferred, whereas other *cdc1*(Ts) alleles were followed by their inability to complement the *cdc1-1*(Ts) growth defect. The *cdc1-4*(Ts) and *cdc1-5*(Ts) mutants were not studied further because they did not display a tight temperature-sensitive growth defect. Finally, the *cdc1-1*, *cdc1-2*, *cdc1-6*, and *cdc1-7* alleles were placed in congenic strains (FY11, FY388, FY416, and FY434, respectively) through three consecutive backcrosses to the wild-type *CDC1* strain FY70. Thus, each *cdc1*(Ts) allele was backcrossed at least seven times with related wild-type laboratory strains and at least the last three of those crosses were with a *CDC1* strain (FY70) that is isogenic with the *cdc1-1*(Ts) strain FY11.

Bacterial strains MC1066 and DH5α were used for plasmid manipulations and have been described (Casadaban *et al.* 1983; Woodcock *et al.* 1989).

DNA manipulations: A 5.6-kb *Bgl*II fragment containing *CDC1* was cloned into the unique *Bam*HI site of the low-copy *URA3* vector YCp50 to generate plasmid pGS257. The 3.5-kb *Hin*dIII fragment containing *CDC1* was cloned into the unique *Hin*dIII site of YCp50 to generate plasmid pFB1, and into the unique *Hin*dIII site of the low-copy *HIS3* vector pRS313 (Sikorski and Hieter 1989) to generate plasmid pFB383. Three different high-copy *CDC1* plasmids were generated by cloning the *Hin*dIII fragment carrying *CDC1* into YEp13, pRS202 or pRS305-2μ (Ward *et al.* 1995) to generate YEp13-*CDC1* (pFB28), pRS202-*CDC1* (pFB565), and pRS305-2μ-*CDC1* (pFB569), respectively.

The *smf1Δ::URA3* and YEp24-*SMF1* constructs were obtained from V. Culotta (Johns Hopkins University, Baltimore). The *pmr1Δ::HIS3* plasmid (pAL47) carries the *HIS3* marker inserted at the *Bam*HI site in *PMR1* (Hartley *et al.* 1996). To generate the high-copy *PMR1* plasmid, pFB428, a

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
Y294	*MATα trp1-289 leu2-3,112 his3Δ1 ura3-52*	Fedor-Chaiken *et al.* 1990
SGY386	Y294 *CDC1:URA3:CDC1*	This study
SGY392	*MAT***a** *ade1 trp1 leu2 his3 ura3 cdc1-1(Ts)*	This study
FY11[a]	*MAT***a** *ade1 trp1 leu2 his3 ura3 cdc1-1(Ts)*	This study
FY12	*MATα ade8 trp1 leu2 his3 ura3 cdc1-1(Ts)*	This study
FY70	FY11 *CDC1*	This study
FY71	*MAT***a** *ade8 trp1 leu2 his3 ura3*	This study
FY388	*MAT***a** *ade1 trp1 leu2 his3 ura3 cdc1-2(Ts)*	This study
FY416[a]	*MAT***a** *ade1 trp1 leu2 his3 ura3 cdc1-6(Ts)*	This study
FY434	*MAT***a** *ade1 trp1 leu2 his3 ura3 cdc1-7(Ts)*	This study
FY451	FY11 *bar1Δ::LEU2*	This study
FY453	FY70 *bar1Δ::LEU2*	This study
FY454	FY388 *bar1Δ::LEU2*	This study
FY523	FY70 *pmr1Δ::HIS3*	This study
FY598	FY70 *smf1Δ::URA3*	This study
FY599	*MATα ade8 trp1 leu2 his3 ura3 smf1Δ::URA3*	This study

[a] Strains FY11 and FY416 were previously referred to as 2-12A and 373-14C, respectively (Loukin and Kung 1995).

6.7-kb *Pvu*II genomic fragment containing *PMR1* was cloned into the *Pvu*II sites of pRS202. Plasmid pFB430 (pRS202-*pmr1*::4bp), which was used as the vector control for pFB428, was generated by linearizing pFB428 at the unique *Eco*RI site in the *PMR1* coding region, filling in the staggered ends with Klenow, and religating.

Growth curves, cell counts, and analyses of cellular DNA content: Exponentially growing cultures (OD_{600} = 0.5 to 1.0/ml) were diluted into YEPD medium to an OD_{600} of 0.05/ml. After 1 hr incubation at 23° (time zero), 15-ml aliquots were shifted to either 30° or 36°. Subsequent OD_{600} readings were taken at 1.5-hr intervals. To determine cell number and cell-cycle distribution, 0.9-ml samples were fixed overnight at 4° with formaldehyde (3.7% v/v), sonicated briefly (Branson probe sonicator, 20 pulses at 25 W; Branson Ultrasonics Corp., Danbury, CT), and examined under 1000× magnification. Buds that had an apparent diameter of less than one-fourth the diameter of the mother cell were classified as small, and all other buds were considered large. Cellular DNA content was determined by fluorescence-activated cell sorting (FACS) after staining with propidium iodide as described (Nash *et al.* 1988).

Shmoo formation: Log-phase cultures of *bar1* mutants were diluted into YEPD pH 5.5 medium to an OD_{600} of 0.05/ml and incubated at 23° in the presence of 30–40 nM α factor (Sigma Chemical Co., St. Louis). At various times after pheromone addition, cells were collected, fixed with formaldehyde (3.7% v/v), and examined for the formation of shmoos.

Viability studies using nutrient-deprived and α factor-treated cells: Exponentially growing cultures were harvested, washed in water, and incubated in starvation medium (water, minimal medium lacking uracil or leucine, or rich medium lacking a carbon source) for 24 hr at 23°. After starvation, typically >85% of cells were unbudded. Starved cells were inoculated into rich or starvation medium and incubated at 23° or 36°. Cell viability was determined at 0- and 24-hr postinoculation. An identical protocol was used for viability studies with α factor-treated cells, except that YEPD pH 5.5 medium with, or without, 40 nM α factor was substituted for starvation medium and the cells were washed to remove pheromone prior to temperature shift.

Invertase assays: Exponentially growing cells were harvested, washed, and inoculated to an OD_{600} of 0.6/ml into 20 ml YEP medium supplemented with 0.05% glucose. After incubation for either 4.5 hr at 23° or 1.5 hr at 23° followed by 3 hr at 30°, cells were harvested, washed twice in 50 mm Tris-PO_4^{3-} pH 6.8, 1 mm EDTA, 10 mm NaN_3, suspended in 0.1 ml 50 mM Tris-PO_4^{3-} pH 6.8, 1 mm EDTA, 1 mm EGTA, 10% glycerol, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin, 1 μg/ml Pepstatin A, 0.2 mm PMSF, and vortexed with 0.2 ml glass beads (0.45μ mesh, Sigma Chemical Co.). The lysate was cleared at 14 krpm in a microfuge for 20 sec and assayed for invertase activity and protein content (Bio Rad Bradford assay with BSA standards; Bio-Rad Labs., Hercules, CA). Lysates (100 μg protein) were adjusted to 0.2% SDS, warmed at 37° for 5 min, and separated by 7% sodium dodecyl sulfate (SDS)-PAGE (10–20 mA, 10–12 hr, 23°). Invertase activity was detected by washing the gel in 0.1 m sodium acetate pH 5.1, 0.1 m sucrose (<0.05% invert sugar, EM Science, Gibbstown, NJ) for 10 min at 4°, 10 min at 37°, and 5 min at 37°. Fresh buffer was used at each step. The gel was rinsed in water and stained for glucose by heating in 0.5 N NaOH, 1 mg/ml 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co.) until color developed.

RESULTS

***cdc1*(Ts) mutants arrest with a mixture of small-budded and unbudded cells:** The *cdc1-1*(Ts) mutant has been described as alternately exhibiting a small-bud (Hartwell *et al.* 1970) or unbudded (Hartwell 1971) arrest. To examine this discrepancy, we characterized the terminal arrest phenotype conferred by several independent *cdc1*(Ts) alleles (Hartwell *et al.* 1973) after placing them in closely related strains (materials and methods). At 30°, *cdc1-1*(Ts) cells accumulated predominantly (65%) with a small bud (Table 2), consistent with the original studies implicating Cdc1 in bud growth (Hartwell *et al.* 1970). At 36°, most (55%) of the cells arrested without a bud, although a significant percentage (40%) arrested with a small bud (Table 2). Interestingly, other *cdc1*(Ts) alleles conferred similar arrest phenotypes (Table 2), suggesting that the variation was not

TABLE 2

Distribution of cells with respect to bud size

		Percent of cells with:[a]		
CDC1 allele	Temperature	No bud	Small bud	Large bud
CDC1	23°	44.5	18.0	36.4
	30°	44.7	15.5	39.9
	36°	44.2	17.1	38.7
cdc1-1	23°	35.8	27.9	36.0
	30°	23.7	65.4	10.6
	36°	55.5	40.2	5.3
cdc1-2	23°	38.7	20.1	42.2
	36°	52.4	43.8	3.8
cdc1-6	23°	39.7	21.1	40.2
	36°	23.7	62.8[b]	13.5
cdc1-7	23°	49.5	20.3	30.2
	36°	47.8	44.9	7.3

[a] Percentages were calculated from a sample of 200 cells.

[b] Bud sizes of arrested *cdc1-6*(Ts) cells were larger than those of the *cdc1-1*(Ts) cells shown in Figure 2.

specific to the *cdc1-1*(Ts) mutant. Because the proportion of unbudded cells varied with the *cdc1*(Ts) allele, the temperature (Table 2), and the ploidy of the cell (data not shown and Paidhungat and Garrett 1998), the terminal phenotype may be influenced by the severity of the *cdc1* defect. This phenomenon could account for the variation observed previously (Hartwell 1971).

To determine the cell-cycle distribution of the unbudded cells (Hartwell *et al.* 1970), we examined cortical actin localization in *cdc1-1*(Ts) and *CDC1* cells after 3 hr at 36°. In the *cdc1-1*(Ts) mutant, most (>70%) of the unbudded cells displayed cortical actin patches over the entire cell surface (data not shown), whereas actin patches were localized at the bud tip in cells with small buds (data not shown). Similar results were observed in the *CDC1* control (data not shown). Thus, the majority of unbudded *cdc1-1*(Ts) cells had not initiated an early step in bud emergence (Lew and Reed 1993).

DNA content of arrested *cdc1*(Ts) cells: Bud emergence mutants complete DNA synthesis, whereas strains blocked in G1 initiate neither bud development nor DNA synthesis (Hartwell 1974). The *cdc1-1*(Ts) mutant was previously shown to arrest after DNA replication (Hartwell 1971). However, those studies measured DNA synthesis by incorporation of labeled precursors and might not have detected a small population of cells with unreplicated DNA. Accordingly, we estimated DNA content of individual cells by propidium iodide fluorescence-activated cell sorting. Whereas log phase cultures contained approximately equal proportions of cells with 1N (unreplicated) and 2N (replicated) DNA content (Figure 1), >85% of the *cdc1-1*(Ts) cells arrested at 30° displayed 2N DNA content (Figure 1), and thus had progressed through G1. By contrast, a significant percentage of the *cdc1-1*(Ts) (20%) (Figure 1) and *cdc1-2*(Ts) (15%) (data not shown) cells arrested at 36° contained 1N DNA content. Thus, at least some of the *cdc1-1*(Ts) cells exhibit defects in bud formation, actin patching, and DNA replication, consistent with a G1 arrest.

***cdc1-1*(Ts) mutants exhibit a cell growth defect during shmoo formation:** *cdc1-1*(Ts) cells fail to enlarge upon arrest (Hartwell 1971; and Figure 1). Because *cdc1-1*(Ts) cells also arrest in G1 (Figure 1, Table 1), Cdc1 may be required for cell (and bud) growth in more than one stage of the cell cycle. Cell growth is also required for pheromone-induced mating projection (or shmoo) formation (Cid *et al.* 1995). To determine if Cdc1 was necessary for shmoo formation, we treated a *cdc1-1*(Ts) *bar1* strain with mating pheromone under conditions (23°) where *cdc1-1*(Ts) cells are viable but exhibit a growth defect. Whereas *CDC1 bar1* cells formed shmoos within 4 hr of α-factor addition, *cdc1-1*(Ts) *bar1* cells did not form mating projections or change in size after 9 hr of treatment (Figure 2). These results show that Cdc1 is required for shmoo formation and, because shmoo formation occurs in G1 (Pringle and Hartwell 1981), support the notion that Cdc1 is required for cell growth in more than one stage of the cell cycle.

Cdc1 is required for viability in growing cells: In contrast to what has been observed with *pkc1*(Ts) mutants, *cdc1*(Ts) cell growth arrest precedes cell death (Hartwell 1971; data not shown). If *cdc1*(Ts) viability loss results from a primary defect in an essential cell growth process, nongrowing cells should be impervious to Cdc1 depletion. Mutant *cdc1-2*(Ts) cells were arrested for growth by starvation in water for 24 hr at 23°, shifted to 36° with or without the addition of rich medium, and assayed for viability. Whereas *cdc1-2*(Ts) cells shifted to rich medium suffered a 100-fold viability loss within 24 hr, almost all of the starved cells remained viable (Figure 3). The protective effect of water was due to nutrient starvation because identical results were obtained with starvation media lacking either a single auxotrophic requirement or a carbon source. Nutrient starvation also prevented viability loss of *cdc1-1*(Ts) cells (data not shown). Thus, inhibition of growth prevents viability loss upon Cdc1 depletion.

Starvation might prevent viability loss by arresting cells in G1 rather than by inhibiting cell growth. Thus, we determined if Cdc1 was required for viability of pheromone-treated cells, which grow but arrest division in G1 (Pringle and Hartwell 1981). A *cdc1-2*(Ts) *bar1* strain was arrested with α factor for 4 hr at 23°, shifted to 36° in the presence, or absence, of pheromone, and then assayed for viability (Figure 4). In contrast to nutrient starvation, pheromone treatment neither enhanced nor compromised *cdc1-2*(Ts) viability (Figure 4). Moreover, only 20–30% of control cells (*CDC1 bar1* at 23° and 36°; *cdc1-2*(Ts) *bar1* at 23°) adapted to α factor within 24 hr (Figure 4), so adaptation, and the resumption of cell-cycle progression, could not account for viability loss. Similar observations were made with a *cdc1-1*(Ts) *bar1* strain (data not shown). These results show *cdc1*(Ts) cells lose viability only during periods of active growth, and suggest cell death is a consequence of a cell's attempt to grow in the absence of Cdc1 function. These results also support the notion that Cdc1 function is required in more than one stage of the cell cycle.

Cdc1 depletion does not affect cell-wall integrity: Pkc1-deficient cells lyse during growth and exhibit defects in bud development as well as shmoo formation. The *pkc1*Δ mutant can proliferate in medium of high osmotic strength, presumably because osmotic stabilization prevents cell lysis (Levin and Bartlett-Heubusch 1992). Although the growth defects of *cdc1-1*(Ts), *cdc1-6*(Ts), and *cdc1-7*(Ts) strains were completely alleviated by 1 m sorbitol or 0.5 m NaCl at 30° (Figure 5), only the *cdc1-6*(Ts) mutant was even partially remediated at 36° (Figure 5). Moreover, *cdc1*Δ mutants did not grow on sorbitol-supplemented medium (data not shown). Thus, osmotic stabilization rescued the *cdc1*(Ts) growth defect only under a limited set of conditions.

Although Rho1-depleted strains exhibit defects com-

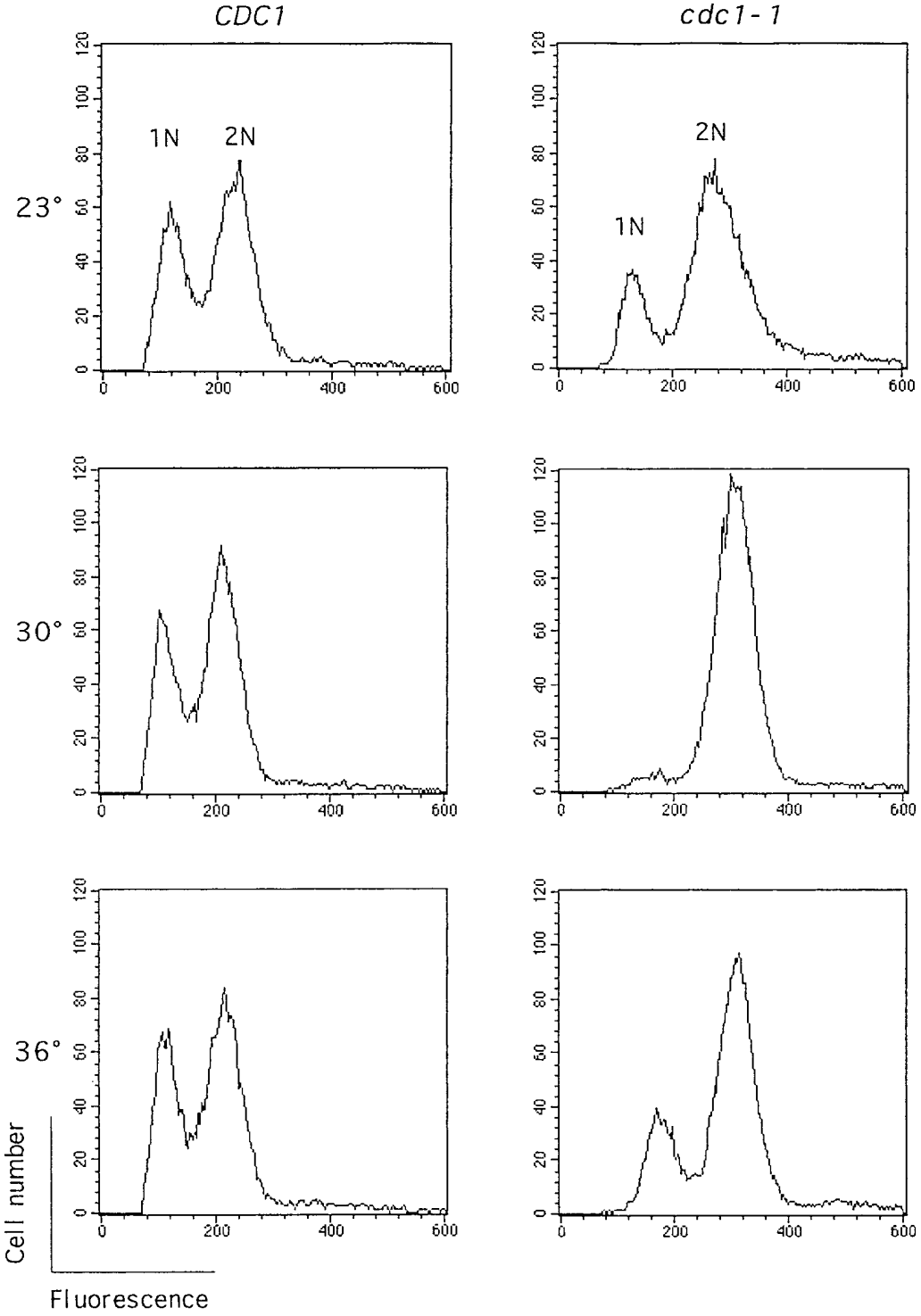

Figure 1.—DNA content of arrested *cdc1-1*(Ts) cells. Exponential cultures of strain FY11 (*cdc1-1*) or FY70 (*CDC1*) were incubated for 4.5 hr at 23°, 30° or 36° and subjected to FACS as described (materials and methods).

mon to cell-wall integrity mutants (Yamochi *et al.* 1994), a *rho1*Δ mutant is not rescued by osmotic stabilization. Accordingly, we determined if *cdc1-1*(Ts) cells became prone to lysis under conditions in which they were osmotically stabilized. *cdc1-1*(Ts) cells were grown for 13 hr at 30° in sorbitol-supplemented YEPD medium, rapidly diluted into hypotonic medium, and assayed for viability. Sorbitol-protected cells resumed growth normally (data not shown), suggesting that preincubation at 30° had not made them susceptible to lysis in hypotonic medium. By contrast, mutants with defects in the Pkc1 pathway lose 70% viability within 3 min of dilution into hypotonic solution (Lee and Levin 1992). Thus, osmotic rescue of the *cdc1-1*(Ts) growth defect does not result from stabilization against cell lysis. These studies do not support a role for Cdc1 in cell-wall integrity. This conclusion is consistent with the temporal relation between cell death and arrest (data not shown) (Hart-

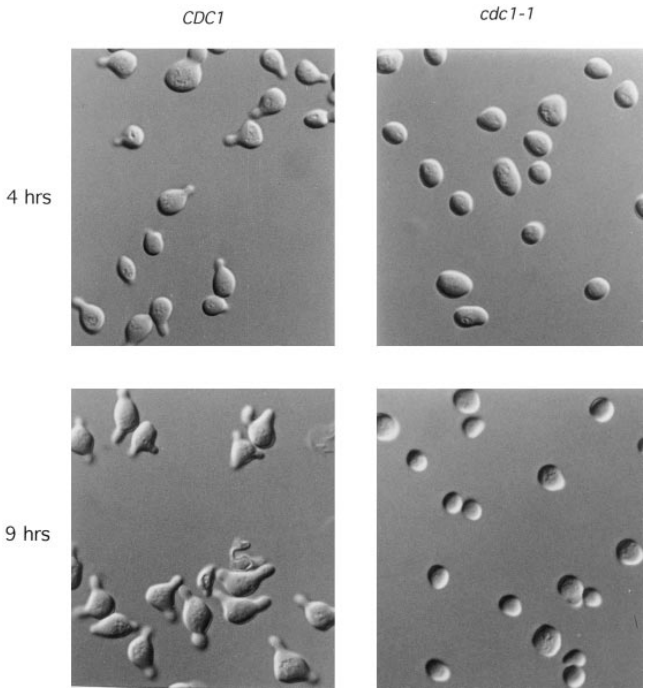

Figure 2.—Shmoo formation in *cdc1-1*(Ts) cells. Exponential cultures of *cdc1-1 bar1* (FY451) or *CDC1 bar1* (FY453) strains were treated with 40 nm α factor and incubated at 23° for 4 hr and 9 hr.

well 1971), as well as the absence of a genetic interaction between *cdc1-1*(Ts) and mutations [*pkc1-2*(Ts) and *BCK1-20*] in the Pkc1 pathway (data not shown) or *cdc1-1*(Ts) and *RHO1* overexpression (Yamochi *et al.* 1994).

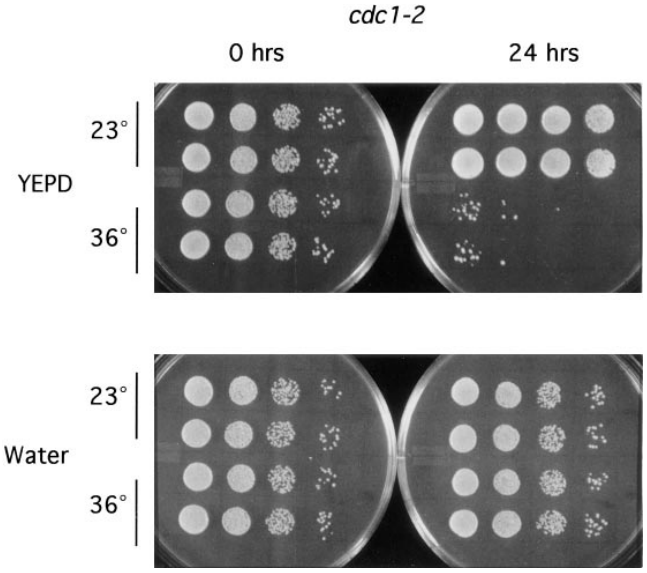

Figure 3.—Nutrient starvation protects *cdc1-2*(Ts) cells from viability loss. A nutrient-starved culture of strain FY388 [*cdc1-2*(Ts)] was transferred to water or YEPD medium at 23° and 36°. At 0 hr and 24 hr after transfer, 10 μl of 10-fold serial dilutions (left to right) were spotted on YEPD agar and incubated at 23°.

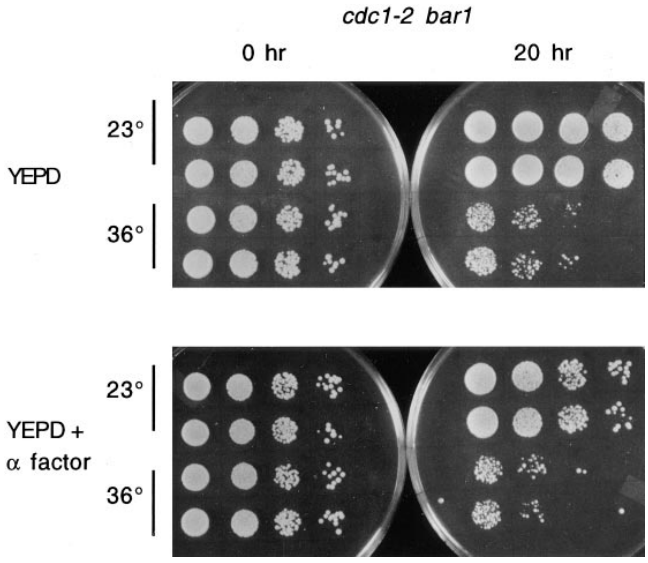

Figure 4.—α factor-arrested *cdc1-2*(Ts) cells lose viability at 36°. The *cdc1-2*(Ts) *bar1* strain, FY454, was treated with α factor for 4 hr at 23°, washed, and then transferred to YEPD medium at 23° and 36° with, or without, α factor. Viability was tested 0 hr and 20 hr after temperature shift.

***cdc1*(Ts) mutants are sensitive to depletion of intracellular Mn^{2+}:** The *cdc1-200* mutant is sensitive to chelator treatment and can be rescued by overexpression of the Mn^{2+} transporter gene, *SMF1* (Supek *et al.* 1996). To test if chelator sensitivity was a general reflection of Cdc1 function, we measured the sensitivity of the *cdc1*(Ts) mutants to Mn^{2+} depletion. Even at the "permissive" temperature, all of the *cdc1*(Ts) mutants tested exhibited a severe growth defect on medium supplemented with EGTA (Figure 6). The chelator sensitivity could be complemented by *CDC1* (Figure 7) or alleviated by overproduction of the plasma membrane Mn^{2+} transporter, Smf1 (data not shown).

Most chelators deplete several divalent cations from the medium. Thus, the EGTA sensitivity of *cdc1*(Ts) mutants might result from the depletion of cations other than Mn^{2+}. Loss of Smf1 function significantly reduces Mn^{2+} uptake in medium containing ≤ 5 μm Mn^{2+} (Supek *et al.* 1996). Accordingly, we determined if deleting *SMF1* affected *cdc1-1*(Ts) growth in YEPD (0.3 μm Mn^{2+}) or minimal medium (3 μm Mn^{2+}). In a cross between *cdc1-1 SMF1* and *CDC1 smf1Δ::URA3* haploid strains, only 1 of 42 expected *cdc1-1 smf1Δ::URA3* segregants formed a viable colony, and that colony grew extremely slowly at 23°. Progeny of the other three genotypes were recovered at expected frequencies. Thus, Smf1-dependent Mn^{2+} uptake, which is dispensable to a wild-type *CDC1* strain (Supek *et al.* 1996), is essential to growth of a Cdc1-compromised strain. These results support the notion that *cdc1*(Ts) mutants are specifically sensitive to Mn^{2+} depletion.

Cdc1 is not necessary for glycosylation of secreted invertase: Secreted proteins undergo Ca^{2+} and Mn^{2+}-

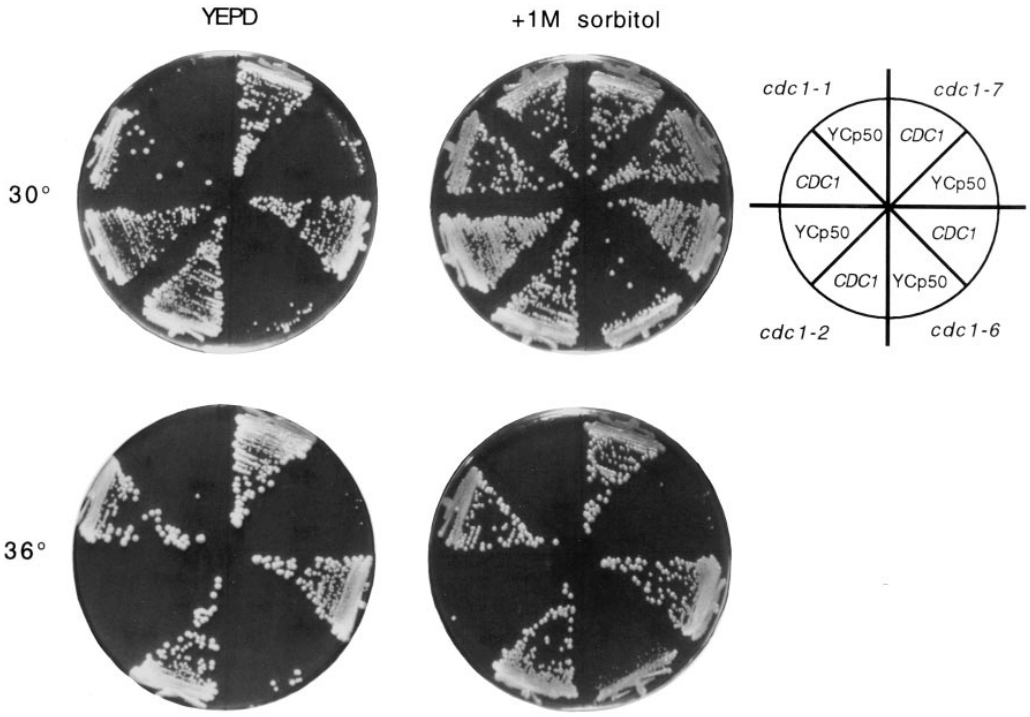

Figure 5.—Sorbitol partially rescues the *cdc1*(Ts) growth defect. Strains containing plasmids YCp50 and *CDC1* (pFB1) were streaked onto YEPD agar or YEPD agar containing 1 m sorbitol, and incubated at 23° (data not shown), 30° or 36°. Strains were *cdc1-1* (FY11), *cdc1-2* (FY388), *cdc1-6* (FY416), and *cdc1-7* (FY434).

dependent glycosylation while traversing the Golgi apparatus. Some aspect of this process may be required for bud growth because *och1*Δ mutants, which lack a Mn^{2+}-dependent mannosyl transferase, exhibit a conditional bud-growth defect (Nagasu *et al.* 1992). Secreted invertase (Suc2) isolated from wild-type strains migrates as a broad band on SDS-PAGE as a result of heterogeneous glycosylation. By contrast, invertase from a mutant that lacks the Golgi Ca^{2+}/Mn^{2+} transporter Pmr1 (Antebi and Fink 1992; Lapinskas *et al.* 1995), migrates as a discrete band of faster mobility that is characteristic of underglycosylated forms. The altered mobility is due, in part, to a defect in Pmr1-dependent Ca^{2+}/Mn^{2+} transport because it can be partially reversed by addition of 0.2 mm Mn^{2+} (data not shown) or 1 mm Ca^{2+} (data not shown) (Antebi and Fink 1992). Interestingly, invertase isolated from the *cdc1-1*(Ts) strain migrated with a pattern identical to that of invertase from wild-type strains (data not shown). The absence of faster migrating invertase forms could not be attributed to a lack of *de novo* protein synthesis at 30° because invertase activity of the *cdc1-1*(Ts) mutant was comparable (>80%) to that of the wild-type strain (data not shown). Thus, the growth defect of the *cdc1-1*(Ts) mutant cannot be ascribed to a defect in Mn^{2+}-dependent protein glycosylation.

Mn^{2+} sequestration into the Golgi antagonizes *cdc1*(Ts) growth: Cdc1 might mediate another, essential, Mn^{2+}-dependent Golgi function. According to that scenario, the *cdc1*(Ts) growth defect would be alleviated by manipulations that raise Golgi Mn^{2+} levels. The transporter Pmr1 pumps Mn^{2+} and Ca^{2+} into the lumen of the Golgi (Antebi and Fink 1992; Lapinskas *et al.* 1995). Surprisingly, the *cdc1-1*(Ts) mutant containing a high-copy *PMR1* plasmid exhibited a severe growth defect on minimal medium containing 3 μm Mn^{2+} (Figure 7). By contrast, an isogenic *CDC1* strain was unaffected by the same *PMR1* plasmid. The effect of Pmr1 overproduction on the *cdc1-1*(Ts) mutant could be attributed to Mn^{2+} depletion because the growth defect was reversed by supplementing the medium with 1 mm Mn^{2+} (Figure 7). Thus, the *cdc1*(Ts) growth defect was exacerbated, not ameliorated, by raising Golgi Mn^{2+} sequestration. Because Pmr1 overproduction also reduces cytosolic Mn^{2+} (Lapinskas *et al.* 1996), these results are consistent with *cdc1*(Ts) mutants being sensitive to cytosolic Mn^{2+} depletion.

Cdc1 overproduction suppresses the EGTA sensitivity of *pmr1*Δ and *smf1*Δ mutants: The EGTA sensitivity of a *pmr1*Δ mutant (Antebi and Fink 1992; Lapinskas *et al.* 1995; and Figure 8), was partially alleviated by Smf1 overproduction (Figure 8). Thus, increasing Mn^{2+} influx into the cytosol compensated for the lack of active Mn^{2+} transport into the Golgi. Cdc1 overproduction also restored EGTA resistance to *pmr1*Δ mutants (Figure 8), consistent with the notion that Cdc1 regulates intracellular (and possibly cytosolic) Mn^{2+} levels. To ask if suppression by *CDC1* overexpression was dependent upon a functional *SMF1* gene, we attempted to construct a *smf1*Δ*pmr1*Δ double mutant. However, *smf1*Δ and *pmr1*Δ are synthetically lethal (data not shown). As an alternative test of Smf1 dependence, we determined if *CDC1* overexpression relieved the EGTA sensitivity of the *smf1*Δ*::URA3* strain (Supek *et al.* 1996). Only the *smf1*Δ*::URA3* mutant containing the high-copy *CDC1* plasmid grew on medium containing 4 mm EGTA (Fig-

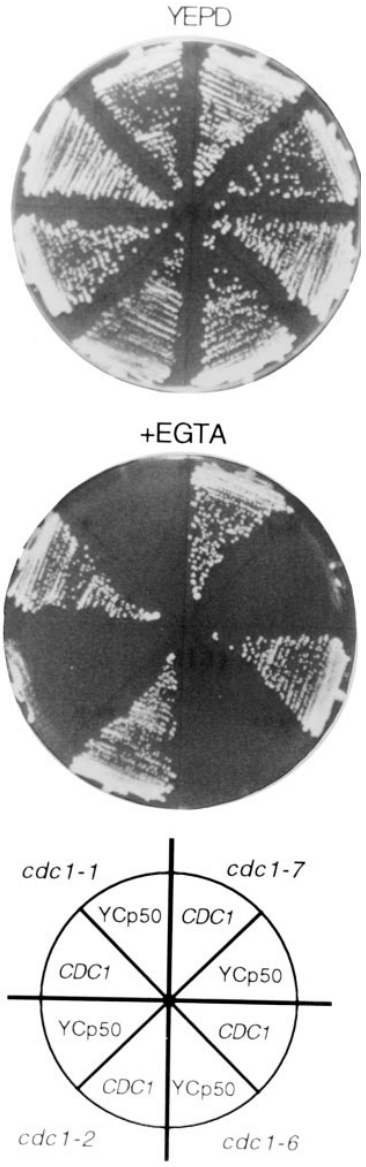

Figure 6.—Mutations in *CDC1* confer sensitivity to 2 mm EGTA. Strains containing plasmids YCp50 or *CDC1* (pFB1) were streaked onto YEPD agar supplemented with EGTA and incubated at 23°. Strains were *cdc1-1* (FY11), *cdc1-2* (FY388), *cdc1-6* (FY416), and *cdc1-7* (FY434).

ure 9). These results support the notion that Cdc1 regulates intracellular Mn^{2+} levels and suggest that it does so through a Smf1-independent mechanism.

DISCUSSION

Cdc1 and cell growth: Previous studies suggested that *cdc1-1*(Ts) cells stopped growing after arrest (Hartwell 1971). Our observations extend those findings by showing that the *cdc1-1*(Ts) growth defect is not restricted to cells in a single stage of the cell cycle (Figure 1; Table 2) and by implicating Cdc1 function in cell growth during shmoo formation (Figure 2). Thus, Cdc1 is essential for general cell growth.

What is the cell growth process in which Cdc1 is involved? Mutants with defects in protein synthesis and energy activation accumulate as small, unbudded cells in G1 (Pringle and Hartwell 1981), whereas most *cdc1*(Ts) cells arrest after exiting G1 and initiating DNA synthesis (Figure 1; Table 2). Moreover, the phenotypic similarities between Cdc1-depleted cells and cells with defects in cell-wall biosynthesis (Levin and Bartlett-Heubusch 1992; Yamochi *et al.* 1994) cannot be reconciled with the incomplete osmotic rescue (Figure 5), lack of cell lysis (data not shown), and delayed cell death of *cdc1*(Ts) cells. These results, along with the lack of genetic interaction between *cdc1*(Ts) mutations and mutations in *PKC1* or *RHO1* (data not shown; Yamochi *et al.* 1994), suggest Cdc1 is not involved in cell-wall integrity. Finally, in contrast to the Golgi mannosyl-transferase Och1 (Nagasu *et al.* 1992), Cdc1 is not required for protein glycosylation (data not shown).

Although the specific Cdc1-dependent process has not been identified, a cell growth process could account for the prototypic arrest (small bud, 2N DNA, undivided nucleus), heterogeneous arrest, and pleiotropic defects of the *cdc1*(Ts) mutants. Strains with cell-wall biosynthesis defects exhibit a small-bud terminal phenotype (Levin and Bartlett-Heubusch 1992; Yamochi *et al.* 1994), presumably because cell-wall expansion is most prominent during growth of the bud. By analogy, a similar demand upon a Cdc1-dependent growth process would

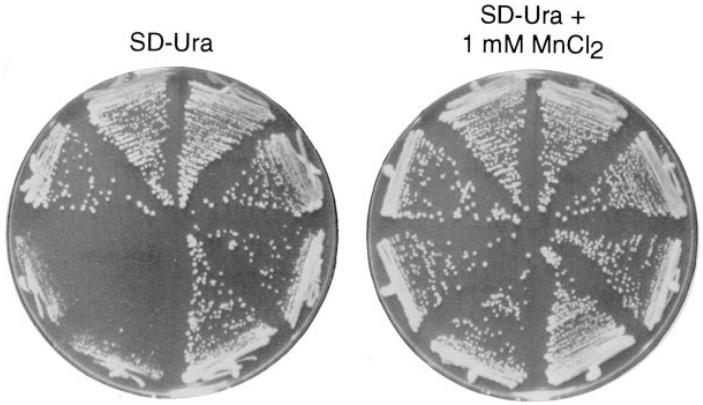

Figure 7.—*PMR1* overexpression exacerbates *cdc1-1* (Ts) growth. Strains FY11 (*cdc1-1*) and FY70 (*CDC1*) containing a high-copy *PMR1* (pRS202-*PMR1*) or control (pRS202-*pmr1::4bp*) plasmid were streaked onto minimal medium (SD-URA) agar with, or without, Mn^{2+} supplement.

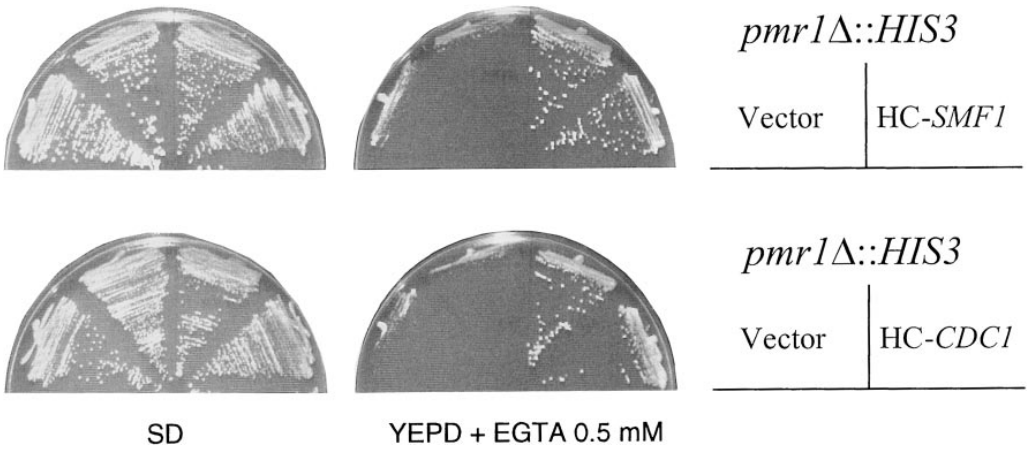

Figure 8.—Overexpression of *SMF1* or *CDC1* alleviates the *pmr1*Δ EGTA sensitivity. Transformants of strain FY523 (*pmr1*Δ) were streaked onto selective agar, or YEPD agar containing 0.5 mm EGTA. Plasmids were YEp24, YEp13, YEp24-*SMF1* or YEp13-*CDC1*.

readily explain the small-bud arrest of the *cdc1*(Ts) mutant. In turn, the defect in bud growth might engage the morphogenesis checkpoint at the G2/M border (Lew and Reed 1995), thereby delaying nuclear division. According to that model, Cdc1 depletion should not affect cell-cycle progression in large-budded cells that have completed bud growth. Consistent with that idea, *cdc1*(Ts) populations contain fewer large-budded cells than exponentially growing cultures (Table 2). On the other hand, the heterogeneous arrest of the *cdc1*(Ts) mutant can be accommodated by the fact that bud emergence, DNA replication, and spindle-pole body duplication initiate after cells pass the growth-dependent point in G1 known as START (Pringle and Hartwell 1981). Thus, most of the *cdc1*(Ts) phenotypes could be accommodated by a model in which the Cdc1-dependent growth process was limiting during bud formation but also required for progression through START. Consistent with this idea, only unbudded *cdc1*(Ts) cells exhibit the spindle-duplication defect (Byers and Goetsch 1974). Finally, a cell-growth defect would account for the mating and viability problems of *cdc1*(Ts) mutants. The defect in recombination repair (Halbrook and Hoekstra 1994), by contrast, is harder to reconcile with a defect in cell growth.

Cdc1 and intracellular Mn^{2+} distribution: Previous studies suggested Cdc1 might be a "Mn^{2+}-dependent" protein (Supek *et al.* 1996). That proposal was based on the observation that the *cdc1-200* (Gly_{149} to Arg) mutation conferred sensitivity to EGTA, presumably by reducing the affinity of the mutant Cdc1 protein to Mn^{2+}. However, our studies show that Mn^{2+} depletion is associated with general defects in Cdc1 function (Figures 6 and 7) and is not unique to the *cdc1-200* allele. In addition, Cdc1 overproduction ameliorates the chelator sensitivity of mutants (*pmr1, smf1*) with defects in Mn^{2+} homeostasis (Figures 8 and 9). We suggest, therefore, that Cdc1 influences cellular tolerance to Mn^{2+} depletion by regulating intracellular Mn^{2+}. According to this scenario, Cdc1 might either directly catalyze Mn^{2+} transport or regulate Mn^{2+} transporters. We favor the latter possibility because the sequence of the Cdc1 protein (Halbrook and Hoekstra 1994) does not predict the presence of membrane-spanning domains.

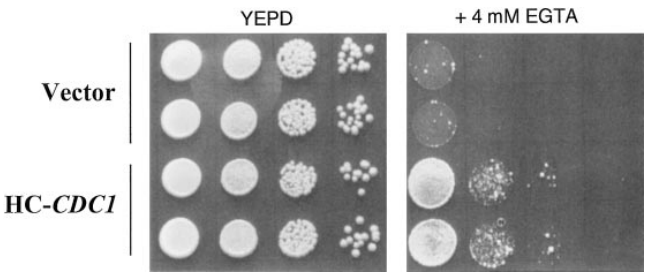

Figure 9.—*CDC1* overexpression alleviates *smf1*Δ EGTA sensitivity. Serial 10-fold dilutions of two independent transformants of strain FY598 (*smf1*Δ) were spotted onto YEPD agar with, or without, 4 mm EGTA. Plasmids were vector (pRS305-2μ) or *CDC1* (pRS305-2μ-*CDC1*).

Could depletion of intracellular Mn^{2+} account for the terminal arrest of *cdc1*(Ts) mutants? Cells depleted of intracellular Mn^{2+} arrest with a small bud, duplicated DNA, and undivided nucleus (referred to as "2N minibudded arrest" in Loukin and Kung 1995), phenotypes identical to those displayed by *cdc1*(Ts) mutant cells under some conditions (Table 2; Figure 1). Under the same conditions, Mn^{2+} supplement rescues the *cdc1*(Ts) growth defect (Loukin and Kung 1995). These results suggest a model in which loss of Cdc1 function results in the depletion of intracellular Mn^{2+}, which in turn debilitates a process that is limiting during bud growth. Although an obvious location for such a Mn^{2+}-dependent process is the Golgi (Antebi and Fink 1992; Lapinskas *et al.* 1995; Nagasu *et al.* 1992), the Mn^{2+} requirement of the *cdc1-1*(Ts) mutant is exacerbated by overexpression of the Golgi Mn^{2+} transporter gene, *PMR1* (Figure 9). Because Pmr1 overproduction also reduces cytosolic [Mn^{2+}] (Lapinskas *et al.* 1996), *cdc1*(Ts) mutants may instead be sensitive to depletion of cytosolic Mn^{2+}. Together, these studies are at least consistent with Cdc1 functioning in the maintenance of cytosolic Mn^{2+} levels.

At first blush, it would appear that Mn^{2+} depletion

alone cannot account for the *cdc1*(Ts) growth defect. For example, *cdc1*(Ts) cells exhibit the small-bud arrest only under some conditions (Table 2; Figure 1), and Mn^{2+} supplement does not completely remedy the *cdc1*(Ts) growth defect (Paidhungat and Garrett 1998). However, the different phenotypes displayed by *cdc1*(Ts) mutants and Mn^{2+}-depleted cells could reflect differences in either the severity or rapidity of Mn^{2+} depletion. For example, Mn^{2+} levels might drop gradually upon chelator treatment or in *cdc1*(Ts) cells at intermediate temperatures, but drop precipitously under more severe conditions. Consistent with this idea, chelator-mediated Mn^{2+} depletion arrests cells after a considerable lag (Loukin and Kung 1995). Similar arguments have been made to explain the variations in terminal arrest that are observed between strains gradually (*pkc1*Δ/*GAL-PKC1*) or quickly [*pkc1*(Ts)] depleted of Pkc1 activity (Levin and Bartlett-Heubusch 1992). Moreover, we recently showed that Cdc1 function can be completely bypassed by genetic manipulation of intracellular Mn^{2+} (Paidhungat and Garrett 1998). Thus, Mn^{2+} depletion may indeed be the sole cause of the *cdc1*(Ts) growth defect.

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