A New Genetic Method for Isolating Functionally Interacting Genes: High *plo1*$^+$-Dependent Mutants and Their Suppressors Define Genes in Mitotic and Septation Pathways in Fission Yeast

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ABSTRACT

We describe a general genetic method to identify genes encoding proteins that functionally interact with and/or are good candidates for downstream targets of a particular gene product. The screen identifies mutants whose growth depends on high levels of expression of that gene. We apply this to the *plo1*$^+$ gene that encodes a fission yeast homologue of the polo-like kinases. *plo1*$^+$ regulates both spindle formation and septation. We have isolated 17 high *plo1*$^+$-dependent (*pld*) mutants that show defects in mitosis or septation. Three mutants show a mitotic arrest phenotype. Among the 14 *pld* mutants with septation defects, 12 mapped to known loci: *cdc7*, *cdc15*, *cdc11 spg1*, and *sid2*. One of the *pld* mutants, *cdc7-PD1*, was selected for suppressor analysis. As multicopy suppressors, we isolated four known genes involved in septation in fission yeast: *spg1*$^+$, *sce3*$^+$, *cdc8*$^+$, and *rho1*$^+$, and two previously uncharacterized genes, *mpd1*$^+$ and *mpd2*$^+$. *mpd1*$^+$ exhibits high homology to phosphatidylinositol 4-phosphate 5-kinase, while *mpd2*$^+$ resembles *Saccharomyces cerevisiae SMY2*; both proteins are involved in the regulation of actin-mediated processes. As chromosomal suppressors of *cdc7-PD1*, we isolated mutations of *cdc16* that resulted in multisepta-tion without nuclear division. *cdc16*$^+$, *dma1*$^+$, byr3$^+$, *byr4*$^+$ and a truncated form of the *cdc7* gene were isolated by complementation of one of these *cdc16* mutations. These results demonstrate that screening for high dose-dependent mutants and their suppressors is an effective approach to identify functionally interacting genes.

THE family of polo-like kinases (plks) is conserved from yeast to humans. With the exception of a small subfamily of mammalian homologues that may function early in the cell cycle, polo kinase function is required at various steps in mitosis such as G2/M transition, bipolar spindle formation, APC (anaphase promoting complex)-mediated proteolysis, and cytokinesis in various eukaryotic systems (Glover *et al.* 1996, 1998; Nigg 1998). Mutations in the Drosophila gene *polo*, encoding the founding member of this family, result in the formation of abnormal bipolar spindles and monopolar spindles (Llamazares *et al.* 1991). The requirement for bipolar spindle formation has also been shown for plks in other organisms, including mammalian plk1, Xenopus plx, and *Schizosaccharomyces pombe* Plo1 (Ohkura *et al.* 1995; Lane and Nigg 1996; Qian *et al.* 1998).

In contrast, the existing mutants of the *Saccharomyces cerevisiae* plk gene, *cdc5*, arrest at a late stage of mitosis with separated chromosomes and an elongated spindle (Byers and Goetsch 1974; Kitada *et al.* 1993). CDC5 is thought to be a substrate-specific regulator of the APC as *cdc5* mutants are defective in APC-mediated proteolysis of cyclin B but not of PDS1 (Cohen-Fix *et al.* 1996; Shirayama *et al.* 1998). In the Xenopus cell-free system, plx is required for APC-mediated proteolysis of mitotic proteins but no substrate specificity is observed (Descombes and Nigg 1998). In addition, mammalian plk1 is capable of phosphorylating subunits of the APC and can activate the APC *in vitro* (Kotani *et al.* 1998).

There is also evidence that human plk1 and Xenopus plx function even earlier at the onset of mitosis/meiosis (Lane and Nigg 1996; Qian *et al.* 1998). Xenopus plx was originally identified as a kinase capable of phosphorylating and activating the maturation promoting factor-activating phosphatase, cdc25 (Kumagai and Dunphy 1996). In addition there is evidence that *S. cerevisiae cdc5* has an involvement in the onset of DNA replication and adaptation to the DNA damage checkpoint (Hardy and Pautz 1996; Toczyski *et al.* 1997).

The activity and localization of plks undergo major changes during the cell cycle. The abundance of mammalian and *S. cerevisiae* polo-like kinases peaks during mitosis (Golsteyn *et al.* 1994; Charles *et al.* 1998; Shirayama *et al.* 1998). In Xenopus cleavage cycles, on the other hand, the level of plx appears constant (Des-

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combes and Nigg 1998). The polo-like kinases are enzymatically activated during mitosis (Fenton and Glover 1993; Abrieu *et al.* 1998; Qian *et al.* 1998). Furthermore, the subcellular localization of polo-like kinases changes during the cell cycle. In mammalian cells plks localize on centrosomes during early mitosis and then relocate onto the central spindle region (Golsteyn *et al.* 1995). In *S. cerevisiae* CDC5 is localized on the spindle pole bodies (SPBs) in a cell cycle-dependent manner (Cheng *et al.* 1998; Shirayama *et al.* 1998).

The multifunctional nature of an individual polo kinase was first demonstrated by work on the fission yeast polo kinase encoded by *plo1*$^+$ (Ohkura *et al.* 1995). Gene disruption experiments indicated that plo1 was required for both mitotic progression and cytokinesis. Cells without plo1 kinase entered mitosis but arrested mitotic progression with a monopolar spindle. This arrest resembles certain consequences of Plk defects in Drosophila, Xenopus, and humans. When cells have sufficient residual gene product to support nuclear division, cytokinesis defects are revealed, in which cells fail to form a medial actin ring and initiate septum formation. Overexpression experiments consolidate this view of an involvement of *plo1*$^+$ in both mitosis and septation. Overexpression of *plo1*$^+$ disrupts bipolar spindle formation in wild-type cells and can induce septum formation even in interphase cells that are unable to commit to mitosis. Recently, temperature-sensitive alleles of *plo1* were isolated that revealed a new aspect of Plo1 function during septation/cytokinesis (Bähler *et al.* 1998). These conditional mutants are defective in septation site determination and show a phenotype that resembles that of the *dmf1/mid1* mutants (Chang *et al.* 1996; Sohrmann *et al.* 1996). Bähler *et al.* (1998) showed that *plo1*$^+$ is required for the relocation of Dmf1/Mid1 from the nucleus to cytoplasm. In addition to Dmf1/Mid1, several potential substrates have been proposed in various systems but actual phosphorylation sites and the *in vivo* roles of such phosphorylations are not yet identified.

Although the abundance of plo1 kinase appears constant through the fission yeast cell cycle, plo1 kinase localizes to SPBs in a cell cycle-dependent manner (Bähler *et al.* 1998; Mulvihill *et al.* 1999). The accumulation of plo1 kinase on the SPB is the earliest observable mitotic event in fission yeast. This localization persists until metaphase and gradually fades away as anaphase progresses. The kinase activity of Plo1 is also regulated during the cell cycle (K. Tanaka, J. Petersen, D. P. Mulvihill and I. M. Hagan, unpublished results). However, little is known about the mechanisms of regulation of polo-like kinases in fission yeast or in any other system.

To identify potential regulators or downstream targets of plo1 kinase we have devised a genetic screen to identify functionally interacting genes. Because plo1 kinase has more than one essential function, a suppressor screen that relies on restoring the viability of a mutant may not be ideal for this purpose unless alleles affecting one specific function are used. In our screen we isolate mutants that are dependent upon high levels of Plo1 protein for viability. The rationale behind this approach is that the lethality caused by the partial loss of function of regulators or effectors may be rescued by elevated plo1 kinase activity. We describe the results of such a screen that has identified mutations in genes required for both mitosis and septation.

MATERIALS AND METHODS

Handling of fission yeast and cytological methods: Basic handling of fission yeast was carried out as described in Moreno *et al.* (1991), Alfa *et al.* (1993), and Ohkura *et al.* (1995). Yeast extract (YE) and Edinburgh minimal medium (EMM) were used as rich and minimal media, respectively. Transformation of fission yeast was carried out by the high-efficiency lithium method (Okazaki *et al.* 1990). DNA and septal materials were stained with DAPI (4′,6-diamidino-2-phenylindole) and Calcofluor, respectively, as described in Ohkura *et al.* (1995). Samples were observed using an Axioskop or Axioplan2 (Zeiss) microscope and images were captured using CCD cameras (Photometrics or Hamamatsu). Captured images were processed using Quips (Vysis) or Openlab (Improvision) and Photoshop (Adobe).

DNA manipulation: General DNA manipulations were carried out according to Sambrook *et al.* (1989). Plasmid DNAs from *Escherichia coli* and DNA fragments were purified using kits (Promega, Madison, WI, and QIAGEN, Valencia, CA). DNA sequencing was carried out using the dye termination kit and an automatic sequencer (ABI). The *spg1* gene from *pld* mutants was amplified by PCR (polymerase chain reaction), using flanking primers, from mutant cells incubated in 0.02N NaOH at 100°. The PCR products were directly sequenced using internal primers (sequences available on request).

Isolation and genetic characterization of *pld* mutants: *h*$^-$ *leu1 int[nmt1-plo1*$^+$*, LEU2]* was selected as a stable transformant of *h*$^-$ *leu1* with pHN204 (Ohkura *et al.* 1995) containing *nmt1-plo1*$^+$ and *LEU2*. Immunoblotting showed that, in the presence of thiamine, the level of Plo1 is comparable to wild type. The strain was mutagenized by ethyl methylsulfonate (EMS, Sigma, St. Louis) or nitrosoguanidine (NTG, Sigma) and stored for up to 2 mo at −70° in 20% glycerine in YE. The mutation rates were roughly 1/90,000 per gene for EMS and 1/5,000 for NTG judged by the mutation rates for auxotrophic loci. Mutagenized cells were plated on EMM (200–500 colonies per plate) and incubated at 30° for 2 or 3 days. Colonies were replica plated onto EMM with phloxine B (20 mg/liter, Sigma) and EMM with phloxine B and 4 μm thiamine to repress the *nmt1* promotor. After 2 days at 30°, colonies on both plates were replica plated again onto similar plates. After another 2 days the colonies on each plate were compared, and those that showed poorer growth or a darker red color on the plate containing thiamine were streaked out on both EMM and EMM containing thiamine at 30° to compare colony size. Only strains that showed better growth on EMM were selected as high *plo1*$^+$-dependent (*pld*) mutants for further analysis. These *pld* mutants were streaked on EMM and EMM containing thiamine and incubated at 22°, 30°, and 35° to test for growth. The 14 *pld* strains showing early septation defects were first tested for allelism with representative alleles of the early septation mutants listed in Table 1 by random spore analysis. The

TABLE 1

Strains used for the study

Strains	Genotype	Source
HOH01J	*h⁻ cdc7-24*	Nurse *et al.* (1976)
HOH02A	*h⁻ cdc11-119*	Nurse *et al.* (1976)
HOH02B	*h⁻ cdc14-118*	Nurse *et al.* (1976)
HOH02C	*h⁻ cdc15-136*	Nurse *et al.* (1976)
CFC135	*h⁺ leu1-32 cdc16-116*	Minet *et al.* (1979)
IH1469	*h⁺ ade6 leu1-32 ura4-d18 sid1-239*	Balasubramanian *et al.* (1998)
IH1470	*h⁺ ade6-M216 leu1-32 ura4-d18 sid2-250*	Balasubramanian *et al.* (1998)
IH1471	*h⁺ ade6 leu1-32 ura4-d18 sid4-A1*	Balasubramanian *et al.* (1998)
HOH02D	*h⁻ leu1-32*	Our stock
CFC149	*h⁺ leu1-32*	Our stock
CFC013	*h⁻ leu1-32 ura4-d18*	Our stock
CFC014	*h⁺ his2-245 leu1-32ura4-d18*	Our stock
CFC010	*h⁻ leu1 int[nmt1-plo1⁺, LEU2]*	This study

five mutants that were not allelic to these septation mutants were tested for allelism to each other in the same way. Strains used for further analysis were backcrossed at least twice with wild type.

Isolation and characterization of the genomic fragments that complement *cdc7-PD1*: Temperature-sensitive (ts⁻) *h⁻ leu1-32 ura4-D18 cdc7-PD1* was transformed with a genomic library in the *ura4⁺* marked multicopy vector pUR19 (Barbet *et al.* 1992) and plated out on EMM containing leucine at 35°. Transformants that grew under these conditions were picked and retested for the Ts⁺ Ura⁺ phenotype. Cosegregation of Ts⁺ and Ura⁺ phenotypes was tested to ensure that the suppression of temperature sensitivity was due to the presence of the plasmid. Ts⁺ Ura⁺ transformants were incubated under nonselective conditions overnight and plated out onto both EMM containing uracil and 2 g/liter fluoroorotic acid (Melford Laboratories) and EMM for 3–4 days at 25°. Ura⁻ (without plasmids) and Ura⁺ (with plasmids) derivatives were streaked out side by side on an EMM plate containing uracil at 35° to compare colony sizes. Plasmids were isolated from the transformants as described (Hoffman and Winston 1987) and amplified in *E. coli* (*XL1-Blue*; Stratagene, La Jolla, CA). Purified plasmids were reintroduced into *h⁻ leu1-32 ura4-D18 cdc7-PD1* cells and the resulting Ura⁺ transformants were streaked out at 35° for confirmation. Both ends of the genomic fragment in each plasmid were sequenced using M13 forward and reverse primers. The sequences were compared with known *S. pombe* genomic sequences in the database (Sanger Centre) using BLAST (Altschul *et al.* 1990). To determine the gene responsible for complementation, plasmids were subcloned and tested for complementation of *cdc7-PD1* temperature sensitivity.

Isolation and characterization of chromosomal suppressors of *cdc7-PD1*: To isolate chromosomal suppressors, single colonies of temperature-sensitive *cdc7-PD1* grown at 25° were plated out on YE and incubated at 35°. Ts⁺ revertants from each plate were tested for cold sensitivity (Cs⁻) at 22°. Three independent Cs⁻ revertants were studied further. Temperature-sensitive progeny were recovered from all three revertants after crossing with wild type, indicating that extragenic mutations caused the suppression. To determine whether these suppressor mutations were allelic, the three revertants were crossed with each other. Recombination between suppressor mutations would give progeny with the *cdc7-PD1* single mutation. None of the crosses produced temperature-sensitive progeny, indicating that the three suppressor mutations were allelic to each other. Given the similarity of the phenotype to *byr4* and *cdc16*, we tested allelism to these loci. A strain carrying the suppressor mutation and *cdc7-PD1* were crossed with a *ura4⁺* integrant at the *byr4* locus or *cdc16-116*. Recombinants (Ura⁻ Ts⁺ or Ura⁺ Cs⁻ progenies) between *byr4* and the suppressor mutation were isolated. No recombinants (wild-type or *cdc7-PD1* progeny) between the suppressor mutation and *cdc16* were isolated, indicating the suppressor mutation is allelic to *cdc16*.

We also found that in the absence of the *cdc7-PD1* mutation all of the suppressor mutations were lethal, even at 35°. To confirm this, the *ura4⁺* gene was integrated at the *cdc7* locus, and the integrant was crossed with *h⁻ leu1-32 ura4-D18 cdc7-PD1 cdc16-sp1*. All Cs⁻ progenies were Ura⁻ (and therefore have *cdc7-PD1*), indicating that the *cdc7-PD1* mutation is required for survival of the suppressor mutation. *h⁻ leu1-32 ura4-d18 cdc7-PD1 cdc16-sp1* was transformed with a *S. pombe* genomic library (Barbet *et al.* 1992) at the permissive temperature, 35°, and then plated out on selective media. After incubating at 35° overnight to allow expression of plasmid genes, plates were transferred to 22° to select Cs⁺ transformants. Once the plasmid dependency of the Cs⁺ transformants was determined, the plasmids were purified, and they were reintroduced into the original strain for confirmation. Inserts were sequenced and the resulting sequences compared with the database as described earlier.

RESULTS

Isolation of high *plo1⁺*-dependent mutants: The wild-type *plo1* gene was placed under the control of the conditional *nmt1⁺* promotor (Maundrell 1993) and integrated into the genome of an otherwise wild-type *S. pombe* strain (Figure 1). In the absence of thiamine (the inductive condition for the *nmt1⁺* promotor) this strain expressed Plo1 protein at levels roughly one order of magnitude higher than wild type. Under such conditions growth was marginally slower than in cells that do not carry the *nmt1-plo1⁺* construct. Cytological analysis indicated a low frequency (~10%) of the mitotic and septation defects that are typically associated with *plo1⁺* overexpression from a multicopy vector (Ohkura *et al.* 1995). In the presence of thiamine the levels of Plo1

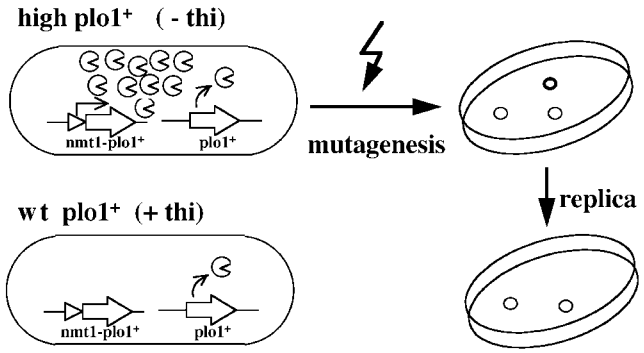

Figure 1.—Schematic diagram of the screen for high *plo1*$^+$-dependent (*pld*) mutants. A strain that has *plo1*$^+$ under the control of the *nmt1* promotor in addition to wild-type *plo1*$^+$ gene expresses elevated levels of Plo1 protein in the absence of thiamine (top half) and normal level of Plo1 protein on media containing thiamine (bottom half). The strain is mutagenized and colonies are allowed to grow without thiamine. The colonies are replicated onto the plate containing thiamine to select high *plo1*$^+$-dependent mutants whose growth is dependent on high level of Plo1.

were similar to wild type and no cytological abnormalities were observed.

This strain was mutagenized with EMS or NTG. Mutagenized cells were then plated onto selective media without thiamine to induce high levels of *plo1*$^+$ expression and incubated at 30° for 2 or 3 days. The resulting colonies were replicated twice onto media containing thiamine to switch off gene expression from the *nmt1*$^+$ promotor and onto media without thiamine as a control. To facilitate the identification of mutants dependent upon high-level expression of *plo1*$^+$ (high *plo1*$^+$-dependent mutants), these plates also contained Phloxine B, which stains dead cells dark red. Colonies that grew well in the absence of thiamine but grew poorly or not at all or stained red in the presence of thiamine were selected. These mutants were tested further by streaking to give single colonies in the presence or absence of thiamine, and the size of colonies was compared. Strains showing better growth in the absence of thiamine at 30° were selected as high *plo1*$^+$-dependent (*pld*) mutants for further analysis. Under these conditions the original strain without mutagenesis shows slightly slower growth on EMM without thiamine.

Basic characterization of *pld* mutations: From a total of 600,000 colonies screened, 19 high *plo1*$^+$-dependent mutants that showed better growth on plates containing thiamine (the condition where cells produced high levels of Plo1) than on plates with thiamine (the condition where cells produced normal levels of Plo1) at 30° were identified. All 19 strains were tested for growth at various temperatures (22°–35°) both in the presence and absence of thiamine (Table 2). The degree of dependency on elevated levels of Plo1 varied in different mutants and at different temperatures. At normal levels of *plo1*$^+$ expression most of the mutants showed temperature-sensitive growth that could be completely or partially rescued by high levels of expression of *plo1*$^+$. One example is shown in Figure 2A. Two mutants, PD2 and PD26, were not temperature sensitive but were dependent on high *plo1*$^+$ expression at all temperatures tested.

TABLE 2

Growth profile of *pld* mutants

		+ thiamine			− thiamine		
		35°	30°	22°	35°	30°	22°
		I. Septation mutants					
cdc7(pld1)	*PD1*	−	±	++	++	++	++
	PD3	±	±	++	++	++	++
	PD9	−	−	++	++	++	++
	PD17	−	±	++	±	++	++
	PD23	±	±	++	+	++	++
	PD32	+	+	++	++	++	++
cdc15(pld2)	*PD2*	−	−	−	++	++	++
	PD12	−	+	+	++	++	+
cdc11(pld3)	*PD21*	±	±	++	+	++	++
spg1 (pld4)	*PD19*	−	±	++	++	++	++
	PD20	−	±	++	+	++	++
sid2 (pld5)	*PD11*	±	+	++	+	++	++
pld6	*PD10*	−	±	++	+	++	++
—	*PD37*	−	−	++	−	+	++
		II. Mitotic mutants					
pld8	*PD24*	−	+	++	−	++	++
pld9	*PD26*	−	±	±	++	++	++
pld10	*PD36*	−	−	±	−	+	+
		III. Others					
pld11	*PD8*	−	−	++	++	++	++
pld12	*PD16*	−	++	++	+	++	++

Original mutants isolated from the screen were streaked to single colonies on medium containing thiamine (wild-type level of Plo1) and lacking thiamine (elevated level of Plo1) and incubated at 35°, 30°, or 22°. ++, good growth comparable to the parental strain; +, poor growth; ±, microcolony formation; −, no growth. ++ > + > ± > −.

To eliminate the possibility that the *nmt1-plo1*$^+$ construct was essential for the apparent high *plo1*$^+$-dependent phenotype, we crossed out *nmt1-plo1*$^+$ from these strains to obtain temperature-sensitive mutants whenever possible. The wild-type *plo1*$^+$ gene, under the control of its own promoter on a multicopy vector, was then introduced into the resulting temperature-sensitive mutants. In all cases examined, the temperature-sensitive lethality of these mutants could be fully or partially rescued by the introduction of the wild-type *plo1*$^+$ gene on a multicopy vector, but not by the introduction of an empty vector (Figure 2B). In other words *plo1*$^+$ acted as a multicopy suppressor of *pld* mutations. This confirmed that the differential growth in the presence or absence of thiamine observed in the original strains was due to suppression of the *pld* mutations by a high level of *plo1*$^+$ expression.

The cytological phenotype of each of the *pld* mutants was determined to identify the particular pathways on

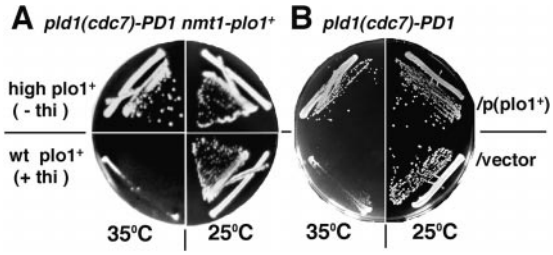

Figure 2.—The *pld1-PD1* mutant, a new allele of *cdc7*, showing high *plo1*$^+$ dependency. (A) *pld1(cdc7)-PD1 nmt1-plo1*$^+$ cells were grown on a plate without thiamine at 25°. The strain is streaked out on media containing (bottom) or lacking (top) thiamine and incubated at 25° (right) or 35° (left). The plates were photographed after 3 days at 35° or 5 days at 25°. In the absence of thiamine, cells express elevated levels of Plo1. In the presence of thiamine the cells expressed a wild-type level of Plo1. At 35° growth of *cdc7-PD1* depends on high expression of *plo1*$^+$ (in the absence of thiamine), while the growth at 25° does not depend on high expression of *plo1*$^+$. (B) The temperature-sensitive *cdc7-PD1* single mutant was transformed with wild-type *plo1*$^+$ gene under its own promoter (pHN191) on a multicopy vector, and the vector without an insert as a control. Transformants were grown at the permissive temperature, 25°, and streaked out on minimal media at 25° and 35° to test for temperature sensitivity. Introduction of multicopy *plo1*$^+$ can complement the temperature sensitivity of *cdc7-PD1*.

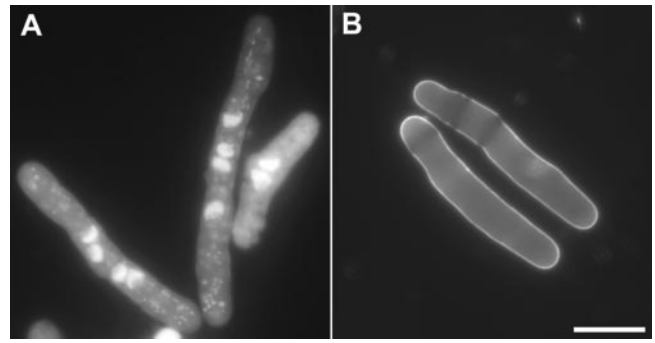

Figure 3.—Septation defects in *pld1(cdc7)-PD1* mutants. Temperature-sensitive *pld1(cdc7)-PD1* was cultured in YE at 25° and incubated at 35° for 4 hr. Cells were fixed and stained with DAPI (A) to observe DNA and Calcofluor (B) to label cell wall and septal materials. The strain shows abnormalities typical of early septation mutants: long multinucleate cells with no deposition of septal materials.

which each of the *pld* genes functioned. The mutants that showed temperature sensitivity without high *plo1*$^+$ expression were grown in liquid culture at the permissive temperature and then shifted to the restrictive temperature. Cells were fixed at different time points following the temperature shift and stained with the DNA dye, DAPI. To examine the defective phenotype of those mutants that did not show temperature sensitivity, cells were first cultured in media lacking thiamine and the high level expression of *plo1*$^+$ was switched off by addition of thiamine to the culture. This approach was straightforward, but as plo1 kinase is not degraded in a cell cycle-specific fashion (Mulvihill *et al.* 1999), three or more generations are required to reduce Plo1 protein to wild-type levels after the addition of thiamine. Cells were therefore fixed and stained with DAPI between 18 and 26 hr after the addition of thiamine. Mutants examined by both methods showed the same cytological defects but there was a tendency for defects to be seen at a higher frequency where it was possible to use the temperature shift approach. This is most probably because temperature shift provides a direct and synchronized inactivation of the gene product, compared with the "switch-off" method in which high levels of Plo1 protein are gradually diluted out.

This cytological analysis identified three distinct phenotypic classes. The first class consisting of 14 mutants showed only septation defects. The second class (3 mutants) had mitotic defects. The final class consisted of 2 mutants that showed neither septation nor mitotic or other notable defects. The mutations were not linked to each other. In summary, 17 out of 19 *pld* mutants had defects in the pathways in which plo1 kinase has been shown to be involved, thus suggesting that this screen was effective in identifying interacting genes.

***pld* mutants defective in septation:** All of the 14 *pld* mutants that had a septation defect produced elongated multinucleate cells that failed to form septa under the conditions inhibiting their growth. Mitosis was not affected in any of these *pld* mutants. Nurse *et al.* (1976) classified septation mutants into two categories, early and late, depending upon whether any attempt was made at septation. Early septation mutants are defined as mutants that fail to make a septum and late septation mutants are defined as mutants that deposit septal material but in a disorganized manner. Late septation mutants are now known to be defective in actin ring formation, which takes place at an early stage of mitosis and is essential but not sufficient for septum formation (Balasubramanian *et al.* 1992, 1994; McCollum *et al.* 1995). Calcofluor staining of *pld* mutants indicated that no septal material was deposited in any of the cytokinesis-defective *pld* mutants (Figure 3 provides an example), indicating that they fall into the early class of septation mutants.

Linkage analysis revealed that these early septation *pld* mutants fell into six complementation groups, *pld1–pld6* (Table 2). *pld1* (six alleles: *PD1*, *PD3*, *PD9*, *PD17*, *PD23*, and *PD32*) was allelic to *cdc7*, *pld2* (two alleles: *PD2* and *PD12*) was allelic to *cdc15*, and *pld3* (1 allele: *PD21*) was allelic to *cdc11*. None were linked to *cdc14*. PD37 was found to contain more than one mutation; therefore allelism could not be determined. In addition to the original mutants identified by Nurse *et al.* (1976) four more loci, *spg1*, *sid1*, *sid2*, and *sid4*, have been subsequently identified (Schmidt *et al.* 1997b; Balasubramanian *et al.* 1998).

To determine whether any of the remaining strains—*pld4-PD19*, *-PD20*, *pld5-PD10*, *pld6-PD11*, and *PD37*—

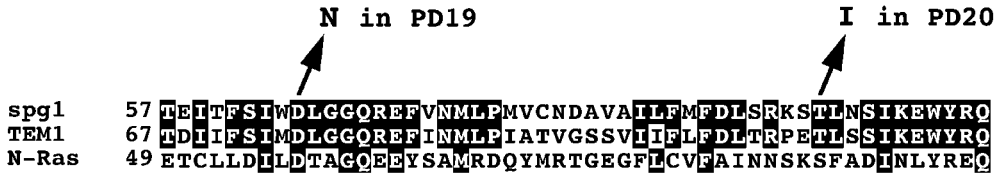

Figure 4.—Mutations in *spg1* that confer high *plo1*$^+$ dependency. Part of the wild-type Spg1 protein sequence (SPTREMBL accession no. P87027) is shown, together with *S. cerevisiae* TEM1p (SWISSPROT P38987) SWISSPROT and human N-Ras (SWISSPROT P01111) sequences. Identical residues are marked. The amino acid substitutions predicted for the mutations in *spg1-PD19* and *-PD20* are shown by arrows. The substitutions are aspartate (D; GAC) to aspardine (N; AAC) in PD19 and threonine (T; ACA) to isoleucine (I; ATA) in PD20.

contain mutations in *spg1*, PCR was used to amplify a genomic fragment containing the *spg1* gene from all five strains. The nucleotide sequences of the amplified *spg1* genes were determined. The two allelic mutants of *pld4* (*-PD19* and *-PD20*) contained different single-point mutations in the coding sequence of the *spg1* gene (Figure 4), while the other three mutants had a sequence that was identical to the reported wild-type *spg1*. The mutations in both *pld4-PD19* and *pld4-PD20* caused a conversion from G to A (or C to T), which is expected from alkylating mutagens, such as the EMS or NTG we used in this screen. The mutation in *PD19* changed the 65th amino acid residue from D (Asp) to N (Asn), and the *PD20* mutation changed the 97th amino acid residue from T (Thr) to I (Ile). The aspartate residue is conserved through all known G-proteins and is implicated in GTP binding. The threonine is also conserved among all G-proteins, with some exceptions that have a serine residue at the equivalent position. These results indicated that the two mutations *pld4-PD19* and *-PD20*, which confer high *plo1*$^+$ dependency, were allelic to *spg1* and are likely to affect general properties shared by all G-proteins rather than Spg1-specific function.

pld5-PD10 and *pld6-PD11* were crossed with *sid1*, *sid2*, and *sid4*. A cross between *pld5* and *sid2* did not produce recombinant progeny, indicating that *pld5* is alleleic to *sid2*. *pld6* is not allelic to any of the tested early septation mutants.

***pld* mutants defective in mitosis:** Three *pld* mutants, *PD24*, *PD26*, and *PD36*, showed defects in chromosome segregation. Genetic analysis indicated that they were not allelic to each other and therefore defined three independent loci, *pld8*, *pld9*, and *pld10*. *pld8-PD24* was temperature sensitive in the presence of thiamine, but removal of thiamine to allow high expression of the *plo1*$^+$ gene improved growth at the semirestrictive temperature (Table 2). The *nmt1-plo1*$^+$ construct was therefore crossed out to obtain a temperature-sensitive mutant. To examine the cytological defects, *pld8-PD24* cells were cultured at the permissive temperature (25°), then shifted to the restrictive temperature (35°), and then processed for DAPI staining (Figure 5A). Although the culture was asynchronous in terms of cell cycle progression prior to the temperature shift, cells with condensed chromosomes temporarily accumulated after the shift (Figure 5B). In such cells chromosomes were condensed so that three individual chromosomes were sometimes clearly visible, indicating that chromosome separation was prevented in this mutant. After the frequency of the cells with condensed chromosomes had peaked, the frequency of "cut" cells increased, and cell separation occurred in the absence of nuclear division. The cut phenotype occurs when the septum bisects the nucleus and cells complete separation (Hirano *et al.* 1986). The *pld8-PD24* mutant is therefore specifically defective in chromosome separation but behaves normally in the cytokinesis and septation pathway.

pld9-PD26 and *pld10-PD36* were inviable at all temperatures tested unless *plo1*$^+$ was expressed at an elevated level. Therefore, we examined the defective phenotype by switching off *plo1*$^+$ gene expression from the *nmt1* promoter by the addition of thiamine to the culture. Microscopic analysis of cells fixed and stained with DAPI 18 to 26 hr after the addition of thiamine revealed that both mutants become considerably elongated and accumulated overcondensed chromosomes; however, they rarely showed a cut phenotype (Figure 5, C and D). In summary the three mitotic *pld* mutants we isolated are defective in chromosome separation.

A *pld* mutant *cdc7(pld1)-PD1* is a useful tool to isolate genes that positively regulate septation: The septation class of *pld* mutants contained six new alleles of *cdc7*, two new alleles of *cdc15*, and one new *cdc11* allele. While each of these new mutations conferred dependency upon elevated Plo1 levels, we found that the original temperature-sensitive alleles (*cdc7-24*, *cdc11-119*, and *cdc15-136*; Nurse *et al.* 1976) could not be rescued by introduction of the wild-type *plo1* gene on a multicopy vector, even at the semirestrictive temperature. Therefore, the new alleles isolated in this screen have properties that are distinct from those of the original mutants. We therefore reasoned that these new alleles would be useful for the identification of genes that positively regulate septation and/or the actin dynamics accompanying division.

Two different genetic approaches were taken to use the *cdc7(pld1)-PD1*^{ts} mutant to screen for suppressors. In the first case multicopy suppressors were isolated from an *S. pombe* genomic library based on a multicopy vector (pUR19; Barbet *et al.* 1992). Fourteen plasmids that rescued the temperature sensitivity of the *cdc7-PD1* mutant were isolated. Through a combination of restriction

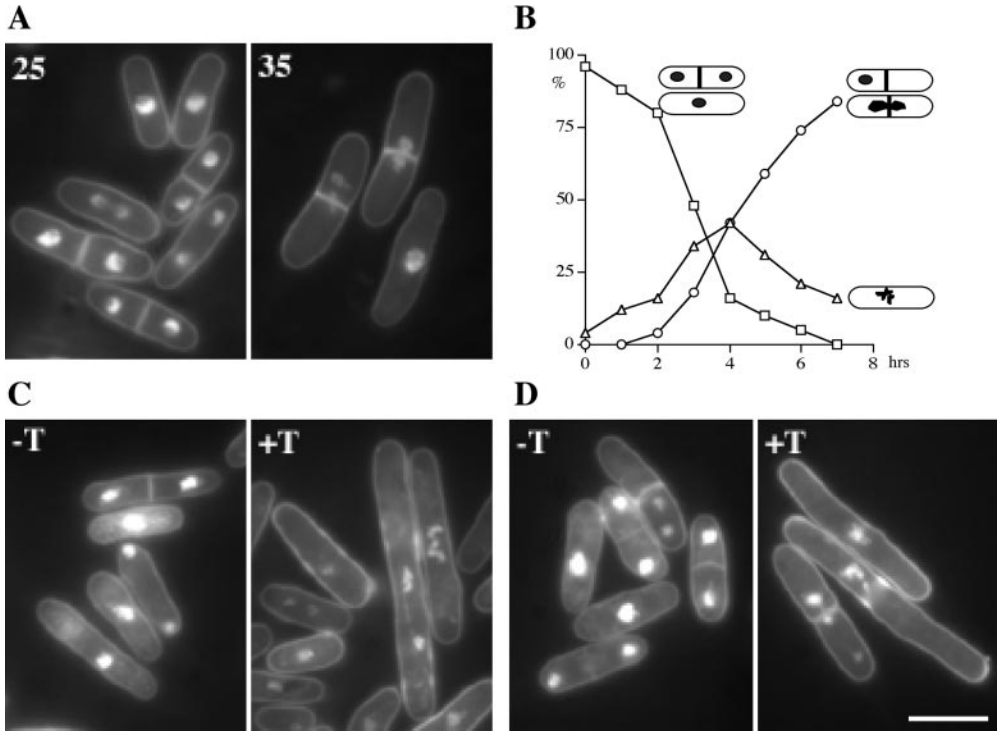

Figure 5.—Mitotic defects in *pld* mutants. (A) DAPI staining of the *pld8-PD24* mutant grown at the permissive (25°) and restrictive (35°) temperatures. (B) Time course of *pld8-PD24* grown at 25° and then shifted to the restrictive temperature (35°). Samples were taken every hour and the phenoype examined by DAPI staining. (□) Interphase and mitotic cells with normal appearance, (△) cells with condensed nuclei, (○) cells showing a cut phenotype. *pld9-PD26* (C) and *pld10-PD36* (D) grown in the presence (+T) and absence (−T) of thiamine and stained with DAPI, which visualizes cell outlines and septa as well as DNA.

mapping, hybridization, and partial sequencing, we found that the 14 plasmids represented seven nonoverlapping genomic sequences. Subcloning the original inserts defined the genes responsible for the rescue of *cdc7-PD1* temperature sensitivity.

The *cdc7*$^+$ and *plo1*$^+$ genes could rescue the temperature sensitivity of *cdc7-PD1* (data not shown). The *cdc7*$^+$ gene was isolated twice in this screen, but the *plo1*$^+$ gene was not isolated, indicating that the screen was not saturated. Among the remaining six multicopy suppressors four were known genes that have been implicated in septation in fission yeast—*spg1*$^+$, *sce3*$^+$, *cdc8*$^+$, and *rho1*$^+$ (Table 3). *spg1*$^+$ (septation promoting G-protein) encodes a small G-protein that associates with Cdc7 (Schmidt *et al.* 1997b). *sce3*$^+$ (suppressor of cdc11) was originally identified as a multicopy suppressor of the septation mutant *cdc11-136*, but was also found to suppress all three known alleles of *cdc11* and the *cdc14-118* mutation (Schmidt *et al.* 1997a). It encodes a nonessential protein with a putative RNA binding domain. It is not yet clear how it rescues the septation defects of *cdc11* and *cdc14*. *cdc8*$^+$ encodes a tropomyosin, which has been found to associate with the medial actin ring (Balasubramanian *et al.* 1992). Formation of the actin ring and subsequent septum formation are disorganized in *cdc8* mutants (Nurse *et al.* 1976; Balasubramanian *et al.* 1992). *rho1*$^+$ is one of two genes that encode a conserved small G-protein, Rho (Nakano *et al.* 1997). Rho protein is implicated in the regulation of actin-mediated processes in various systems (see Van Aelst and D'Souza-Schorey 1997 for review). In fission yeast Rho1 protein is localized along the medial actin ring and at the growing ends of the cell. The *rho1* disruptant is defective in cell shape control and septation (Nakano *et al.* 1997).

Two genes, which we have called *mpd1*$^+$ and *mpd2*$^+$ (multicopy suppressor of *pld1*), were previously uncharacterized in fission yeast; however, the amino acid sequences of their predicted gene products showed that similar proteins have been identified in other organisms. *mpd1*$^+$ encodes a protein that shares significant similarity (31 and 53% identity in 426 residues) with mammalian PIP kinase and *S. cerevisiae* MSS4p (Yoshida *et al.* 1994; Figure 6, A–C). As part of the signal transduction pathway, PIP kinase is thought to play a key role in the regulation of actin-mediated processes in response to external signals. In mammalian cells it catalyzes the production of phosphatidylinositol 4,5 bisphosphate (4,5 PIP_2), which binds to and regulates the activity of several actin-binding proteins, such as vinculin and alpha-actinin (Fukami *et al.* 1992; Gilmore and Burridge 1996). The involvement of PIP kinase or 4,5 PIP_2 in cytokinesis has not yet been examined in any organism. *mpd2*$^+$ encoded a protein that has significant similarity (38 and 37% identity in 201 residues) to a protein encoded by the *S. cerevisiae SMY2* gene and an uncharacterized *S. cerevisiae* gene (YPL105c; Figure 6, D–F). *SMY2* was identified as a multicopy suppressor of a mutation in a type V myosin (*myo2-66*), which is involved in vesicular transport during cell growth (Johnston *et al.* 1991; Govindan *et al.* 1995). As no mutations in *smy2* have been identified, the function of the protein remains unclear.

Plasmids carrying suppressor genes were introduced into the other temperature-sensitive *pld* mutants to test

TABLE 3

Summary of genes that complement *cdc7-PD1* on a multicopy vector

Plasmid (no. of isolates)	Gene	Product	Localization	Loss of function	Overproduction
pRES62 (2)	*cdc7*	Protein kinase	SPB	No septa	Multisepta
pRES1 (4)	*spg1*	G-protein	SPB	No septa	Unregulated septation
pRES24 (4)	*sce3*	RNA binding protein?	Cytoplasm	No defects	Complement *cdc11* and *cdc14*
pRES45 (2)	*rho1*	G-protein	Medial ring, growth tip	Cell separation, round cell	Thick wall
pRES147(1)	*cdc8*	Tropomyosin	Medial ring	No medial ring	?
pRES110(1)	*msd1*	PIP kinase	?	?	?
pRES154(1)	*msd2*	SMY2p homologue	?	?	? (*Sc SMY2* complements *myo2*)
pHN191(0)	*plo1*	Protein kinase	SPB	No septa, no medial ring, monopolar spindles	Unregulated septation, monopolar spindles

Figures in parentheses after the names of representative plasmids indicate the number of plasmids isolated in the screen. For references see Balasubramanian *et al.* (1992), Fankhauser and Simanis (1994), Nakano *et al.* (1997), Schmidt *et al.* (1997a,b).

whether the multicopy suppressors of *cdc7-PD1* could suppress other *pld* mutants (Table 4). Some of the multicopy suppressors could rescue more than one mutant, suggesting that the suppression of *cdc7-PD1* represented a functional interaction between the genes rather than an indirect consequence of changes in general cell physiology, such as nonspecific stabilization of protein structure. In contrast, the original *cdc7-24* allele that does not show high *plo1*$^+$ dependency was not rescued by any of these multicopy suppressors.

A chromosomal suppressor of *cdc7-PD1* induces hyperactivation of septum formation: The multicopy suppressor screen successfully identified genes positively involved in septation. As a complementary approach, chromosomal revertant mutants of *cdc7-PD1* were isolated in the hope that mutations that promote septation and counteract the septation defect of *cdc7-PD1* would be identified. We isolated a number of Ts$^+$ revertants, which grew well at 35°, from the temperature-sensitive *cdc7-PD1* mutant. For ease of subsequent analysis we limited our analysis to the studies of three independent cold-sensitive revertants that do not grow at 22°. Genetic analysis indicated that all three suppressor mutations were extragenic and mapped to the *cdc16* locus (we call them *cdc16-sp1*, *sp2*, and *sp3*). In the absence of the *cdc7-PD1* mutation these individual *cdc16* mutations were lethal at all temperatures tested. In other words, *cdc7-PD1*^{ts} can suppress the lethality of these *cdc16* mutations (and vice versa) at 35° but fails to do so at 22°. For this reason we examined the phenotype of the *cdc16* mutations in the presence of *cdc7-PD1*^{ts}.

At 35°, cells containing both *cdc7-PD1* and the *cdc16* mutations grew at nearly the same rate as wild type and looked relatively normal (Figure 7A). After shifting down to the restrictive temperature (22°), the frequency of septated cells gradually increased, reaching 70% 4 hr after the shift to 22° (Figure 7, B and C). (The generation time of wild-type cells was about 4 hr under these conditions.) The majority of septated cells had two nuclei separated by a septum, but a significant number of septated cells had only one interphase nucleus that was bisected by the septum or located to one side of the septum. Such cells did not complete cell separation, but rather accumulated more than one septum after longer incubation (Figure 7, B and C). As this phenotype is unlike that of the *cdc7-PD1* single mutant, this septation phenotype is most probably due to the presence of the *cdc16* mutation. Similar phenotype has been observed in the original *cdc16-116* allele and the gene deletion (Minet *et al.* 1979; Fankhauser *et al.* 1993; Cerutti and Simanis 1999).

From an *S. pombe* genomic library (Barbet *et al.* 1992) we have isolated genomic clones that complement the lethality of one of the *cdc16* mutations. *cdc16*$^+$, *dma1*$^+$, *byr3*$^+$, *byr4*$^+$, and *cdc7*$^+$ were all isolated in this way. *cdc16*$^+$ and *dma1*$^+$ were able to complement *cdc16-116* as already reported (Fankhauser *et al.* 1993; Murone and Simanis 1996) while others were not.

cdc16$^+$ is required to limit septation to once per cell cycle (Fankhauser *et al.* 1993). Cdc16 is thought to inactivate the septation promoting G-protein Spg1 by acting in a complex with Byr4 to generate its GTPase activating protein (GAP) complex (Furge *et al.* 1998). Cdc16 is also a component of the spindle assembly checkpoint. *dma1*$^+$ was identified as a multicopy suppressor of *cdc16-116* (Murone and Simanis 1996) and is also required for the spindle assembly checkpoint, but has no essential function in the regulation of septation. *byr3*$^+$ is not an essential gene nor is it implicated in septation (Xu *et al.* 1992). It is interesting, however, that both *byr3*$^+$ and *byr4*$^+$ were originally isolated on the basis of their ability to suppress the mating defects

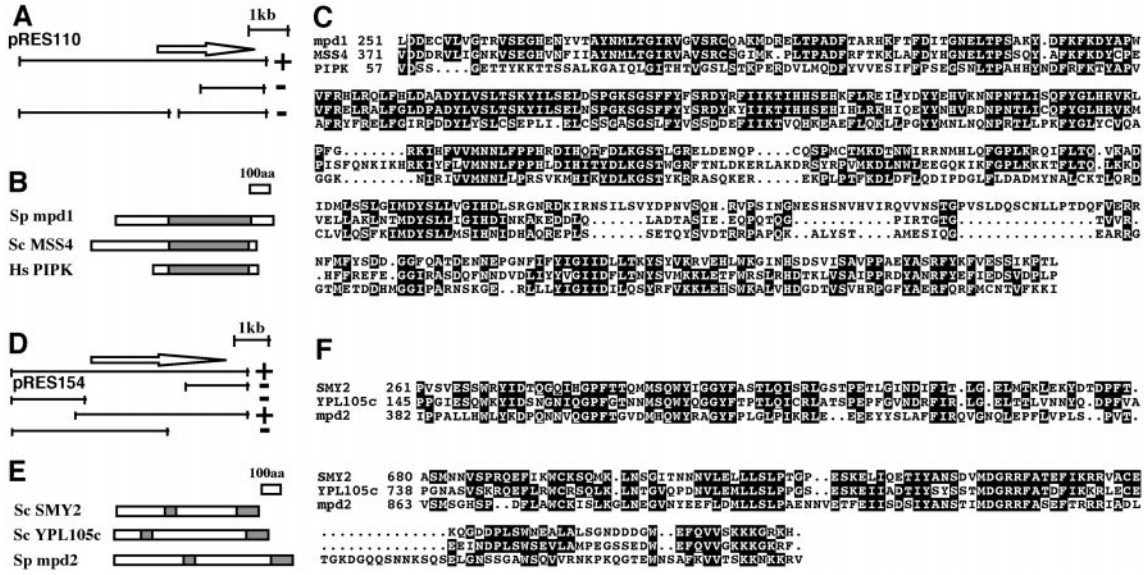

Figure 6.—Multicopy suppressors of *cdc7-PD1* encode proteins similar to phophatidylinositol 4-phosphate 5-kinase and *S. cerevisiae* SMY2p. (A, B, C), *mpd1*$^+$. (C, D, E), *mpd2*$^+$. (A, D) Genomic regions from the two plasmids (pRES110 and pRES154) that complement the temperature sensitivity of *cdc7-PD1* are shown on the top. Subclones represented by solid bars were tested for complementation activity. (+) or (−) on the right indicates presence or absence of the complementing activity. The arrow indicates the coding region responsible for complementation. (B, E) Predicted amino acid sequence from the coding sequence is compared with proteins from other organisms. Shaded boxes represent regions with significant similarity. Sc and Hs represent *S. cerevisiae* and *Homo sapiens*, respectively. Mpd1 (SPAC19G12.14, TrEMBL accession no. O39853); Mpd2 (SPAC4F10.13c, TrEMBL accession no. O36025); Sc MSS4p (multicopy suppressor of *sst4*, SWISSPROT accession no. P38994); Hs PIPK (phosphatidylinositol 4-phosphate 5-kinase type I alpha, SWISSPROT accession no. Q99755); Sc SMY2p (suppressor of *myo2*, accession no. P32909); Sc YPL105c (open reading frame with unknown function, accession no. Q12215). (C, F) Amino acid sequence comparisons of the regions represented in shaded box in B and E are shown. Only identical residues are marked.

caused by deletion of *ras1* (Xu *et al.* 1992; Song *et al.* 1996). It will be of future interest to determine the relationship between *byr3*$^+$ and *byr4*$^+$ and the involvement of *byr3*$^+$ in septation.

Unexpectedly, the *cdc7* gene, a septation inducer, was also isolated in the screen. However, close examination revealed that the genomic fragment responsible for *cdc16-sp1* suppression lacked the first exon of the *cdc7*$^+$ gene. This would produce an amino-terminal truncated protein, lacking an essential subdomain required for protein kinase activity. It is likely that this truncated Cdc7 protein acts as a dominant-negative protein inhibiting septum formation, as a kinase-null *cdc7* mutant has been shown to act in this way (Fankhauser and Simanis 1994). This would be consistent with our observation that the lethality of the *cdc16* mutations is complemented by a *cdc7* mutation.

DISCUSSION

Here we have described the use of a novel genetic approach to identify functionally interacting genes and

TABLE 4

Complementation of *pld* mutants by multicopy suppressors of *cdc7-PD1*

Plasmid	Gene	*cdc7-PD1*	*cdc7-PD23*	*cdc15-PD2*	*cdc11-PD21*	*pld5-PD10*	*cdc7-24*
pRES62	*cdc7*	++	++	++	±	+	++
pRES1	*spg1*	++	++	++		+	−
pRES24	*sce3*	+	++	−	±	+	−
pRES45	*rho1*	+	±	−	±	+	−
pRES147	*cdc8*	+	+	−	±	+	−
pRES110	*mpd1*	++	±	−	±	±	−
pRES154	*mpd2*	+	+	−	±	±	−
pHN191	*plo1*	++	+	++	+	+	−

Each *pld* mutant, together with *cdc7-24* (no *pld* phenotype), was transformed with plasmids carrying multicopy suppressors of *cdc7-PD1* or the *cdc7*$^+$ gene. Growth of transformants was tested on medium with thiamine and appropriate supplements at 35°, except for *cdc15-PD2* at 30°. ++, good complementation; −, no complementation; ++ > +> ± > −.

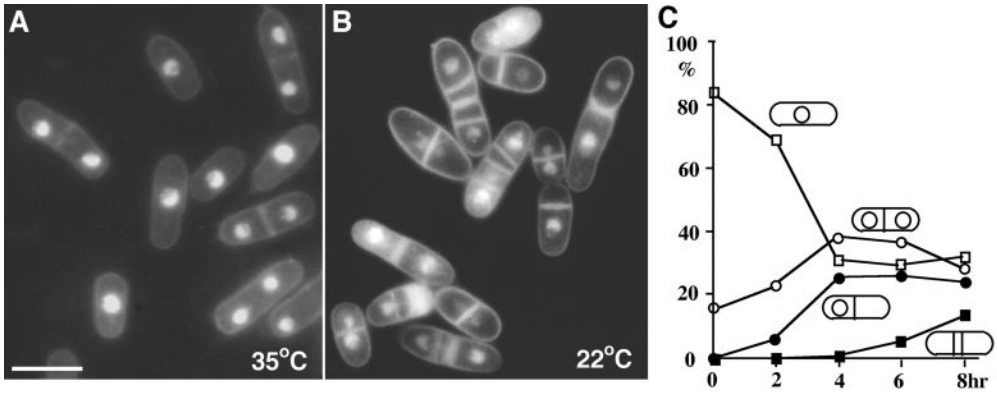

Figure 7.—Unregulated septation in a chromosomal suppressor of *cdc7-PD1*, *cdc16-sp1*. As *cdc16-sp1* mutation is lethal at all temperatures tested, we examined the phenotype in the presence of the *cdc7-PD1*^{ts} mutation. (A) *cdc16-sp1 cdc7-PD1*^{ts} cells were cultured at 35° (the restrictive temperature of *cdc7-PD1*^{ts}) and stained with DAPI. No or few abnormalities are observed, indicating good suppression. Bar, 10 μm. (B) The same strain was incubated for 8 hr at 22° (the permissive temperature of *cdc7-PD1*^{ts}). DAPI staining shows high frequency of septation, multiple septation, and septation without nuclear division. No mitotic defects were observed. (C) After the culture was transferred to 22° (time 0), samples were taken every 2 hr and examined by DAPI staining. (□) Cells without septation, (○) binucleate cells with one septum, (●) mononucleate cells with one septum. The nucleus is bisected by the septum or displaced in one side of the septum. (■) Cells with multiple septa.

have demonstrated the effectiveness of the method using the *plo1*$^+$ gene in fission yeast. A screen was carried out for mutants dependent upon a high level of expression of *plo1*$^+$ for growth. Nineteen high *plo1*$^+$-dependent mutants were isolated. Seventeen of these had defects in either mitotic progression or septation, both pathways in which *plo1*$^+$ gene function is implicated. We have also shown that *pld* mutants have potential as tools for identifying other interacting genes in these pathways. We identified a number of genes involved in septation, starting with one of the *pld* mutants, *cdc7-PD1*, using screens for both chromosomal and multicopy suppressors. By combining these screens, we have isolated most of the known regulators of septum formation, demonstrating that this is a powerful means to identify a group of genes functioning in a related process.

***plo1* and septation:** The isolation and characterization of high *plo1*$^+$-dependent mutants revealed a close genetic interaction between *plo1*$^+$ and the early septation genes. Gene disruption indicated that *plo1*$^+$ is required for both medial actin ring formation and septation, as well as formation of the bipolar spindle (Ohkura *et al.* 1995). Overexpression of the *plo1*$^+$ gene in interphase induced both actin ring and septum formation and activated the Spg1 early septation pathway (Mulvihill *et al.* 1999). Subsequently, three temperature-sensitive *plo1* alleles have been identified that are all defective in determining the site of septation (Bähler *et al.* 1998). These observations suggest that *plo1*$^+$ is involved in multiple aspects of septation. Our results provide the first genetic link between *plo1*$^+$ and the early septation genes. Mutations in the early septation genes result in failure to initiate septum formation (Nurse *et al.* 1976; Fankhauser and Simanis 1993, 1994; Fankhauser *et al.* 1995). Conversely, inhibitory genes that prevent the initiation of more than one septum per cell cycle have also been identified (Fankhauser *et al.* 1993; Song *et al.* 1996). Genetic and molecular studies suggest that early septation genes and inhibitory genes closely interact to regulate the timing of septation (Marks *et al.* 1992; Song *et al.* 1996).

Many of the genes identified in this screen, *cdc7*, *cdc11*, *cdc15*, *spg1*, and *sid2*, are known to be involved in the regulation of septation. The *cdc7*$^+$ gene encodes a protein kinase that is required to initiate septum formation (Fankhauser and Simanis 1994). The GTP-bound form of a G-protein encoded by *spg1*$^+$ associates with and recruits cdc7 kinase onto the SPB (Sohrmann *et al.* 1998). Remarkably, the timing of the disappearance of these proteins from the two sister SPBs is asymmetric, although the meaning of this is not fully understood (Sohrmann *et al.* 1998). The kinetics of hydrolysis by Spg1 appears to be regulated by Cdc16/Byr4 and Cdc11. Cdc16 and Byr4 act as a GAP for Spg1 (Furge *et al.* 1998, 1999). The *cdc11*$^+$ gene has not been cloned, but genetic evidence suggests that it may act as a GDP/GTP exchange protein for *spg1*$^+$ (Schmidt *et al.* 1997b). *sid2*$^+$ encodes a protein kinase that is a component of the SPB throughout the cell cycle and localizes transiently to the cell division site during septation. This transient localization depends on other early septation genes, suggesting that Sid2 may act downstream of the Spg1 cascade (Sparks *et al.* 1999). The localization of components of the septum formation regulatory network on the SPBs is consistent with a close functional relationship with the plo1 protein kinase, which is also localized on the SPBs during mitosis (Bähler *et al.* 1998; Mulvihill *et al.* 1999).

As a reflection of the close functional interactions *in vivo*, it has been reported that changes in dosage or activity of some of these genes can suppress mutations in others (Murone and Simanis 1996; Sohrmann *et al.* 1996; Schmidt *et al.* 1997b). Therefore, it is not surprising that we observed genetic interactions between *plo1*$^+$ and more than one of the early septation genes. Although our results clearly suggest a close functional interaction between *plo1*$^+$ and the early septation genes, they do not allow us to determine which genes

are specifically interacting with *plo1*$^+$. It is possible that some of these proteins are substrates or regulators of plo1 kinase. Consistent with this we have observed that overproduction of plo1 kinase can activate the Spg1 cascade and, in turn, recruitment of the Plo1 protein to the SPB is affected by mutations in the *cdc7* gene (Mulvihill *et al.* 1999). Further studies will be required to establish exactly how plo1 kinase cooperates with these genes to induce septum formation.

In *S. cerevisiae* a parallel genetic interaction is reported among the late mitotic genes including *CDC5*, *CDC15*, and *TEM1* (Kitada *et al.* 1993; Shirayama *et al.* 1994), but the exact modes of the interaction at the molecular level are not known. In the budding yeast, late mitotic genes are required for the degradation of mitotic cyclins, disassembly of the elongated mitotic spindle, and subsequent events. *CDC5*, *CDC15*, and *TEM1* are sequence homologues of *S. pombe plo1*$^+$, *cdc7*$^+$, and *spg1*$^+$ (Fankhauser and Simanis 1994; Ohkura *et al.* 1995; Schmidt *et al.* 1997b). High expression of *CDC5* can suppress mutations of *cdc15* and *tem1* (Kitada *et al.* 1993; Jaspersen *et al.* 1998). Thus, molecular interactions involving *plo1*$^+$/*CDC5* may be conserved in the two yeasts.

Suppressors of *cdc7-PD1* mutant and regulation of actin dynamics: A screen for suppressors of one *pld* mutant, *cdc7-PD1*, was highly fruitful. Four known genes, *spg1*$^+$, *sce3*$^+$, *cdc8*$^+$, and *rho1*$^+$, were isolated as multicopy suppressors, all of which have been shown to be involved in septation in *S. pombe* (Balasubramanian *et al.* 1992; Nakano *et al.* 1997; Schmidt *et al.* 1997a,b). In addition, we isolated two previously uncharacterized genes that encode proteins showing high homology to PIP kinase and *S. cerevisiae* SMY2p. Both are thought to be involved in the regulation of actin-mediated processes in other organisms (Fukami *et al.* 1992; Gilmore and Burridge 1996), but their involvement in cytokinesis has not been studied and their function in *S. pombe* was undetermined.

Cdc7 kinase is not required for medial actin ring formation but is essential for subsequent septum formation (Fankhauser and Simanis 1994). As discussed earlier, the regulation of cdc7 kinase has been well documented (Fankhauser and Simanis 1994; Schmidt *et al.* 1997b; Sohrmann *et al.* 1998). On the other hand, how cdc7 kinase triggers septum formation at the site of the medial actin ring is not known, and no substrates of cdc7 kinase have been identified. Genetic interactions between cdc7 kinase and tropomyosin (encoded by *cdc8*), Rho G-protein (*rho1*), PIP kinase, or SMY2p homologues may represent a link between this kinase and the medial actin ring. It will be of future interest to examine the interaction between these genes and with *cdc7*$^+$. There is evidence that Rho protein activates PIP kinase in mammalian cells (Chong *et al.* 1994). Active PIP kinase produces phosphatidylinositol 4,5-bisphosphate, which is known to regulate the function of actin-binding proteins, such as gelsolin, p39CapZ, alpha-actinin, profilin, and vinculin *in vitro* (Lassing and Lindberg 1985; Janmey and Stossel 1987; Yu *et al.* 1990; Fukami *et al.* 1992; Gilmore and Burridge 1996). The advanced genetics of *S. pombe* will assist examination of these interactions *in vivo*.

The chromosomal suppressors of *cdc7-PD1* identified one locus, *cdc16*. *cdc16*$^+$ is required for limiting septum formation to once per cycle by inhibiting the Spg1 cascade. Multicopy suppressors of the mutation include another known gene involved in the inhibition of septum formation, *byr4*$^+$, together with a dominant-negative *cdc7* gene encoding a truncated protein lacking part of the kinase domain. In addition two known genes, *dma1*$^+$ and *byr3*$^+$, have been isolated, neither of which is essential for the regulation of septation. However, there are some observations that link these genes with those that inhibit septum formation. High-level expression of *dma1*$^+$ can complement the multiple septation phenotype of *cdc16-116* (Murone and Simanis 1996). Both *byr3*$^+$ and *byr4*$^+$ genes were originally isolated in a screen for multicopy suppressors of mating defects in the *ras1* deletion (Xu *et al.* 1992; Song *et al.* 1996). Therefore, both genes share at least two properties when present on multicopy vectors: complementation of the *ras1* deletion and the ability to suppress *cdc7-PD1*.

Advantages of high dose-dependent mutant screening: The methodology used for the high *plo1*$^+$-dependent mutant screen can be applied to any genes. It is of interest to compare this method to which we assign the generic term "high dose-dependent mutant screen" to multicopy suppressor screens and other conventional methods, such as a chromosome suppressor screen or synthetic lethal screen. We see that it offers three main advantages, each derived from the fact that a cloned gene is used as the starting point for the screen. First, because the complete genomes of many organisms have been or are in the process of being sequenced, it is increasingly common to initiate a study from cloned genes. Second, it has been estimated that two-thirds of genes are dispensable for growth in yeast, and a significant proportion of those are probably due to functional redundancy (Goffeau 1996; Fairhead *et al.* 1998). Nonessential genes can be studied using this method if they are involved in an essential pathway. Third, because many multicopy suppressors cannot complement a null mutation, the starting mutation used in the screen determines the outcome in many cases. In contrast, this method will eventually identify interacting genes even if only some specific alleles of the genes show a genetic interaction.

The end product of this approach is a bank of mutants. This provides two advantages. The mutant phenotype can be used in deciding which of those should be studied further. In addition, high dose-dependent mutants should be ideal for suppressor screening, because they are, by definition, suppressed by at least one

other gene. Our results suggest that the *pld* allele of *cdc7* is superior for suppressor screening.

Finally, multifunctional genes can be studied. If genes are required for two or more essential pathways, suppression of one pathway cannot suppress the lethality of the mutation. Therefore suppressor screening would be powerless for these genes, unless mutant alleles that affect only one essential pathway are used. In contrast, high dose-dependent mutant screens provide a powerful alternative method for investigating multifunctional genes. This was illustrated recently when an attempt was made to isolate genomic DNAs that suppress temperature-sensitive alleles of *plo1* mutants (Bähler *et al.* 1998). A total of 18 clones was obtained from two different alleles. Although the intention of the screen was, in this case, to isolate the gene defined by the mutations, it was striking that all 18 clones carried the *plo1*$^+$ gene.

Application to other systems: We believe high dose-dependent mutant screening can be adapted to any genetic system, with minor modification, because the only requirements are a conditional high-expression system and effective mutant isolation methods. In the budding yeast *S. cerevisiae*, there are well-developed conditional expression systems (Johnston and Davis 1984). As multicopy suppressor screens have been successful and are commonly used in *S. cerevisiae*, the high dose-dependent mutant screen should be successfully applied to this organism. Drosophila is one of the most important genetic model systems for higher eukaryotes. Its genetics is highly advanced and a conditional high expression system (Brand and Perrimon 1993) and efficient mutant screening methods have been established. Recently, a systematic screen for genes that upon overexpression suppress a mutation (equivalent to multicopy suppressor screening in yeast) was described and proven to be successful (Rorth *et al.* 1998). Therefore, it should be equally productive to screen for mutations that are suppressed by high expression of a gene of interest, *i.e.*, carry out a high dose-dependent mutant screen in Drosophila. The resulting mutation could also be used for modifier screening to identify more interacting genes. In mammalian systems, some examples of large-scale mutant screens have been reported using cultured cell lines (Nishimoto and Basilico 1978). Therefore, high dose-dependent mutant screens should be possible if combined with the conditional high expression systems that are already established (Resnitzky *et al.* 1994).

We hope that this report will trigger the application of this new method in a wide variety of genes and organisms and that this method will be proven to be an effective genetic method in various systems.

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