Expression of FLR1 Transporter Requires Phospholipase C and Is Repressed by Mediator*

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In budding yeast, phosphoinositide-specific phospholipase C (Plc1p encoded by PLC1 gene) is important for function of kinetochores. Deletion of PLC1 results in benomyl sensitivity, alterations in chromatin structure of centromeres, mitotic delay, and a higher frequency of chromosome loss. Here we intended to utilize benomyl sensitivity as a phenotype that would allow us to identify genes that are important for kinetochore function and are downstream of Plc1p. However, our screen identified SIN4, encoding a component of the Mediator complex of RNA polymerase II. Deletion of SIN4 gene (sin4Δ) does not suppress benomyl sensitivity of plc1Δ cells by improving the function of kinetochores. Instead, benomyl sensitivity of plc1Δ cells is caused by a defect in expression of FLR1, and the suppression of benomyl sensitivity in plc1Δ sin4Δ cells occurs by derepression of FLR1 transcription. FLR1 encodes a plasma membrane transporter that mediates resistance to benomyl. Several other mutations in the Mediator complex also result in significant derepression of FLR1 and greatly increased resistance to benomyl. Thus, benomyl sensitivity is not a phenotype exclusively associated with mitotic spindle defect. These results demonstrate that in addition to promoter-specific transcription factors that are components of the pleiotropic drug resistance network, expression of the membrane transporters can be regulated by Plc1p, a component of a signal transduction pathway, and by Mediator, a general transcription factor. The results thus suggest another layer of complexity in regulation of pleiotropic drug resistance.

The hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) yields two prominent eukaryotic second messengers, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP3). In higher eukaryotes, the hydrophilic IP3 triggers the release of calcium from internal stores and thus modulates Ca2+ signaling (1–3). The hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) yields two prominent eukaryotic second messengers, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP3). In higher eukaryotes, the hydrophilic IP3 triggers the release of calcium from internal stores and thus modulates Ca2+ signaling (1–3).

EXPERIMENTAL PROCEDURES

Strains and Media—All yeast strains used in this study are isogenic to W303 and are listed in Table 1. Standard genetic techniques were used of chromatin remodeling complexes in vivo and in vitro (6, 7). The induction of the phosphate-responsive PHOS gene, chromatin remodeling of its promoter, and recruitment of Swi/Snf and Ino80 chromatin remodeling complexes are impaired in the ipk2/arg82 mutant strain (7). In vitro, nucleosome mobilization by the yeast Swi/Snf complex is stimulated by IP3, whereas IP2 inhibits nucleosome mobilization by yeast Isw2 and Ino80 complexes and Drosophila SNUC complex (6). A possible mechanism by which InsPs affect chromatin remodeling may involve effects on protein conformation of the chromatin remodeling complexes (7). Alternatively, IP3, IP4, or IP6 might affect the interaction between chromatin remodeling complexes and chromatin, as has been shown for phosphatidylinositol 4,5-bisphosphate and the Swi/Snf complex (8).

In Saccharomyces cerevisiae, the gene coding for phosphatidylinositol-specific PLC (PLC1) is not essential; however, its deletion results in a number of phenotypes (9). We found that Plc1p associates with kinetochores and appears to regulate binding of microtubules to kinetochores (10, 11). Kinetochores are specialized protein complexes that assemble at centromeric DNA and bind to spindle microtubules. This attachment is essential for proper chromosome segregation and cell cycle progression. We have found that cells with deletion of the PLC1 gene (plc1Δ) display a higher frequency of chromosome loss, noccadole sensitivity, and mitotic delay. In addition, chromatin extracts from plc1Δ cells exhibit reduced microtubule binding to binucleosomes. PLC1 displays strong genetic interactions with components of the inner kinetochore, and plc1Δ cells display alterations in core centromeric chromatin structure. Chromatin immunoprecipitation experiments indicate that Plc1p localizes to the centromeric loci independently of microtubules (11). These results are consistent with the view that Plc1p affects kinetochore function, possibly by modulating centromeric chromatin structure.

In this study, we planned to utilize benomyl sensitivity of plc1Δ cells as a phenotype that would allow us to identify genes that function downstream of PLC1 in a pathway important for kinetochore activity, spindle function, and chromosome transmission. However, we found that mutations in the Mediator complex suppress benomyl sensitivity of plc1Δ cells by a mechanism that is independent of the kinetochore and spindle. Benomyl sensitivity of plc1Δ cells is caused by a defect in FLR1 expression. FLR1 encodes a multidrug membrane transporter that belongs to a major facilitator superfamily (12–14). FLR1 overexpression confers resistance to benomyl and several other drugs (15–17). Mutations in the Mediator complex result in derepression of FLR1 expression and increased resistance to benomyl. The results thus demonstrate that mutations that affect transcriptional regulation may result in an altered expression pattern of multidrug permeases and dramatic changes in cellular drug resistance.

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11 The abbreviations used are: PLC, phosphoinositide-specific phospholipase C; InsPs, inositol polyphosphates; IP2, inositol trisphosphate; IP3, inositol tetrakisphosphate; IP4, inositol pentakisphosphate; IP5, inositol hexakisphosphate; MTs, microtubules; PDR, pleiotropic drug resistance; PP-IP, inositol pyrophosphates; 4-NQO, 4-nitroquinoline 1-oxide; WT, wild type.
to manipulate yeast strains (18). Cells were grown in rich medium (YPD; 1% yeast extract, 2% Bacto-peptone, 2% glucose) or under selection in synthetic complete medium (SC) containing 2% glucose and, when appropriate, lacking specific nutrients to select for a plasmid or strain with a particular genotype. Meiosis was induced in diploid cells by incubation in 1% potassium acetate.

Screen for Suppressors of Benomyl Sensitivity of plc1Δ Cells—To isolate pbr mutants (plc1Δ benomyl-resistant), we spread plc1Δ cells onto YPD plates, and we replica-plated ~50,000 resulting colonies onto YPD plates containing 10 μg/ml benomyl. After resting on the same plates, we isolated 13 plc1Δ strains with second site spontaneous mutations that suppress benomyl sensitivity (plc1ΔBen⁸). Because plc1Δ/plc1Δ homozygous diploids do not sporulate, we backcrossed the mutants three times to the wild-type strain W303-1a to purify the genetic background and to eliminate mutants in which the benomyl resistance is caused by mutations in multiple genes. To assess the dominance/recessivity, the resulting 10 mutants were mated with the parental plc1Δ strain. Only three of the resulting diploids displayed benomyl resistance; therefore, the corresponding mutations were considered dominant. However, seven diploids were benomyl-sensitive, and thus the mutants were considered recessive. The complementation grouping was accomplished by crossing the individual mutants and determining whether the resulting plc1Δ/plc1Δ diploids were sensitive or resistant to benomyl. When the diploid strain behaved like the original haploid strains (Ben⁸), then the mutation was concluded to be in the same gene, and the original haploid strains belonged to the same complementation group. When the diploid strain, unlike the original haploid strains, was sensitive to benomyl, then the mutation was concluded to be in two different genes, and the corresponding haploids belonged to different complementation groups. The seven plc1ΔBen⁸ mutants belong to three complementation groups, designated pbr1 (four mutants), pbr2 (two mutants), and pbr3 (one mutant). Because some tub2 alleles (TUB2 encodes β-tubulin) result in recessive benomyl resistance, it was possible that one of the complementation groups represents mutations in the TUB2 gene. To test this possibility, we performed linkage analysis. Representatives from the three complementation groups were mated with a strain with a marked TUB2 gene (haploid segregant of strain CUY409; Ref. 19). In this strain, the HIS3 marker was inserted just downstream of the TUB2 gene. When the resulting diploids were sporulated and dissected, the resistance to benomyl segregated randomly with respect to TUB2-HIS3, demonstrating that none of the complementation groups represented mutations in the TUB2 gene. Cloning of the wild-type gene responsible for benomyl resistance of the plc1Δ mutant was accomplished by screening one representative from the pbr1 complementation group (pbr1-1) with a yeast genomic library on a plasmid carrying CEN and LEU2 (American Type Culture Collection, Manassas, VA). About 5,000 transformants were allowed to grow on SC-Leu and were subsequently replica-plated onto SC-Leu containing 10 μg/ml benomyl. Plasmids were recovered from the transformants that grew well on SC-Leu but failed to grow on SC-Leu containing 10 μg/ml benomyl and were reintroduced back into the pbr1-1 mutant to confirm the phenotype. The inserts in six plasmids isolated in this way were identified by sequencing. The only gene that was not truncated and was present on all six inserts was SIN4. To determine whether SIN4 is allelic to the pbr1-1 mutation, the pbr1-1 mutant was crossed with strain DY1702 (sine1::TRP1; Ref. 20). The resulting diploid was benomyl-resistant, and upon transformation with the SIN4 plasmid (sine4 homologous diploid does not sporulate), sporulation, and dissection, all plc1Δ haploids cured of the SIN4 plasmid were also benomyl-resistant. Thus, no recombination between the pbr1-1 locus and sine1::TRP1 was detected, and we conclude that pbr1-1 is allelic to SIN4.

S1 Nuclease Analysis—Oligonucleotides complementary to the genes assayed by S1 nuclease analysis are as follows: FLR1, 5’-CGGTA-
GAGGATTCAGACGAGCTACTTATAGGACGCAGTATTATAT-
CATAGTGAGGAAGATG-3’; YOR1, 5’-GGTGGGGTTGTATT-
CTGTTCCACATATATTTACGTCGAGGACCAAAGATTTTTG-
CTAGGGGT-3’; YCF1, 5’-CGGTATCTCACAACAGACTGATGCC-
ATCCTAGAGACAAATTCATTCCGGCCTATATTGTAGGCC-
TCTCAC-3’; PDR5, 5’-GGAGTTTTGTGACGCTGCGGTCA-
GAGTCTTGCAGCTTTTGAGGACGTCTCAC-3’; SNQ2, 5’-GAGACTCGCGCGCTTTCCAGCAGTGGCACA-
ACTGCGTCTCGAGTTGTCTTGCTGCCAG-3’; ACT1, 5’-GC-
TTAGTAAAAGGACCGGTTTGTCTTCGGCGACCTCCTCAA-
TTGTGTGAGAGTACTA-3’.
Total RNA was isolated from cultures grown in YPD medium to A600 nm ~1.0 by the hot phenol method as described previously (21). S1 probes were end-labeled in a 25-μl reaction mixture (5 pmol of oligonucleotide, 125 μCi of [γ-32P]ATP, 6,000 Ci/mmol (PerkinElmer Life Sciences), 1 X T4 polynucleotide kinase buffer and 20 U T4 polynucleotide kinase (New England Biolabs)) at 37 °C for 1 h. The reaction mixture was diluted with 25 μl of water; T4 polynucleotide kinase was inactivated at 65 °C for 20 min, and the labeled oligonucleotides were purified using MicroSpin G-25 columns (Amersham Biosciences). The labeled oligonucleotides (0.5 pmol) were hybridized with 20 – 40 μg of total RNA in a 50-μl reaction mixture (0.3 mM NaCl, 1 mM EDTA, 40 mM HEPES, pH 7.0, and 0.1% Triton X-100) for 12 h at 55 °C and treated with S1 nuclease (PerkinElmer Life Sciences) as described previously (21). The samples were analyzed on 20% denaturing polyacrylamide gels, and quantification was performed using a PhosphorImager (PerkinElmer Life Sciences).

Minichromosome Stability Assay—Mitotic minichromosome stability was measured as a fraction of cells that retained the plasmid after growth in nonselective medium (22, 23). Briefly, wild-type, plc1Δ, sin4Δ, and plc1Δsin4Δ cells were transformed with pRS413 plasmid (CEN, HIS3). For each transformant, five single colonies were inoculated separately into medium nonselective for pRS413 plasmid (YPD medium containing benomyl at 2 μg/ml) and grown for about 24 h at 28 °C. At the end of the growth, the cultures were still in the exponential phase, as determined by counting the cells with a hemocytometer and by measuring A600 nm. For each culture, the frequency of His+ cells was determined at the time of inoculation (F0) and at the end of nonselective growth (Fend) by plating appropriately diluted cultures on YPD plates and subsequent replica plating onto synthetic complete medium lacking histidine (SC-His). The rate of plasmid loss per generation was determined as described (22, 23), according to the following equation: Fend = F0 (F0/G)G, where F0 is the fraction of cells in each generation that retain the minichromosome, and G is the number of generations. The fraction of cells that lose the minichromosome per generation is 1 – F0. The number of cell doublings was calculated by counting the total cell number at the beginning and at the end of nonselective growth.

Minichromosome-Microtubule Binding Assay—Preparation of yeast lysates containing minichromosomes and the minichromosome-microtubule binding assay were done as described previously (24, 25). Briefly, yeast cultures were grown to an A600 nm of ~0.6 after maintaining the cells in exponential growth for several generations. Nocodazole was added to the cultures to 15 μg/ml for 6 h, and nocodazole was also present during preparation of spheroplasts (24). Cells were spheroplasted by glusulase and osmotically lysed in EBB buffer (10 mM Tris-Cl, pH 7.4, 10 mM MgCl2, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol). Minichromosomes were eluted from nuclei by adding 0.3 mM NaCl. After 5 min of incubation, the extracts were 3-fold diluted by adding EBB buffer and subjected to two subsequent centrifugations, each at 15,000 × g for 20 min. The clear supernatant was removed, supplemented with 10 μM Taxol, and used for the microtubule-binding assay. Purified tubulin was polymerized in vitro into microtubules (MTs) of an average length of 2 μm as directed by manufacturer (ICN Biochemicals, Inc), and stabilized by addition of the MTs stabilizing drug Taxol (10 μM final concentration). Different amounts of stabilized MTs were added to 500-μl aliquots of the cleared extracts to initiate the microtubule-binding assay. After 15 min of incubation at room temperature, the reaction mixtures were centrifuged at 15,000 × g for 8 min. The supernatant and pellet fractions were separated, and the amount of minichromosomes in each fraction was determined by Southern blot using an Amp’ probe. The calibration of the band intensities was performed by loading different amounts of the Amp’ DNA fragment on the gels, and the band intensities of the scanned images were quantified using UN-SCAN-IT software (Silk Scientific).

Fluorescence Microscopy—Cells in 1 ml of media were fixed for 1 h by the addition of formaldehyde to 3.7% concentration. Cells were centrifuged, washed two times with phosphate-buffered saline, resuspended in 1 ml of 70% ethanol, and kept at 23 °C for 30 min. After centrifugation, washing, and sonication for 10 s, cells were stained with 0.5 μg/ml 4’,6-diamidino-2-phenylindole and observed using Nikon Eclipse 800 microscope equipped with SPOT RT CCD camera, UV filter set, and ×100/1.4 n.a. oil immersion objective.

RESULTS

Isolation of pbr Mutants—Hypersensitivity to the microtubule-destabilizing drugs benomyl and nocodazole is a phenotype shared by mutants with defects in kinetochore, mitotic spindle, and mitotic checkpoint (26–30). Because deletion of the PLC1 gene, encoding phospholipase C, causes hypersensitivity to nocodazole and benomyl (Ref. 10; plc1Δ cells cannot grow in the presence of 5 μg/ml nocodazole or 10 μg/ml benomyl), we used this phenotype to identify genes functioning downstream of PLC1. We speculated that chromosomal suppressors of the benomyl sensitivity of the plc1Δ strain may arise because of the mutations in genes functioning in a pathway that either regulates or affects the function of the mitotic spindle. We isolated seven recessive mutations that include three complementation groups as follows: pbr1 (four mutants), pbr2 (two mutants), and pbr3 (one mutant). In this study, we described the identification of SIN4, a gene that corresponds to the pbr1 complementation group (see “Experimental Procedures”). Subsequent experiments demonstrated that pbr2 is allelic to MED2 (see “Results”; mutations in several components of the Mediator cause increased expression of FLR1 and resistance to benomyl). Because the plc1Δpbr1-1 mutant was phenotypically identical to the plc1Δ sin4Δ strain, we used the latter strain in further experiments aimed at elucidating the mechanism of suppression. SIN4 encodes a component of the Mediator complex of RNA polymerase II. However, mutations in SIN4 exhibit pleiotropic phenotypes reminiscent of mutations of histones, suggesting a role for Sin4p in the regulation of chromatin structure (20, 31, 32).

Characterization of plc1Δ sin4Δ Mutant—Deletion of PLC1 results in alterations in the structure of centromeric chromatin (11), higher frequency of minichromosome loss, mitotic delay, and reduced binding of minichromosomes to microtubules (10). Because Sin4p is implicated in regulation of chromatin structure (20, 32), it was possible that suppression of benomyl sensitivity in plc1Δ cells by sin4Δ mutation (Fig. 1A) occurs at the level of chromatin structure and activity of the kinetochore. To test this possibility, we performed three assays aimed at assessing the function of the kinetochore in wild-type, plc1Δ, sin4Δ, and plc1Δsin4Δ strains. Because the mitotic stability of minichromosomes depends on the function of kinetochores, we determined the stability of the pRS413 minichromosome in wild-type, plc1Δ, sin4Δ, and plc1Δsin4Δ strains in the absence of benomyl and in the presence of a low concentration of benomyl (Fig. 1B). The stability of the pRS413 minichromosome was measured as a fraction of cells that retained the minichromosome after growth in nonselective medium (22). As we reported previously, plc1Δ cells displayed about 5-fold higher minichromosome loss rate than wild-type cells (10). If Sin4Δ suppresses benomyl sensitivity of plc1Δ cells by improving the function of the kinetochore, then we would expect that plc1Δsin4Δ cells would have a lower rate of minichromo-
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To gain more insight into the role of SIN4 in the regulation of kinetochore activity, we used an assay developed by Kingsbury and Koshland (24, 25) to measure the ability of minichromosomes formed in vivo to bind microtubules in vitro. Wild-type, plc1Δ, sin4Δ, and plc1Δsin4Δ strains were transformed with centromeric plasmid (minichromosome) pRS414, which was then assayed in clarified extracts of these cultures for binding to Taxol-stabilized microtubules. To improve the sensitivity of the assay and to exclude the possibility that any difference in microtubule binding activity between the strains is merely the result of different cell cycle profiles, the assay was performed with lysates prepared from nocodazole-arrested cells (Fig. 1C). The G2/M transition is the phase when the kinetochore is required for progression of the cell cycle through mitosis. It is also when the kinetochore is in its most active state (24, 25). Kinetochores of cells arrested with nocodazole at the G2/M transition exhibit the highest microtubule binding activity (10). Our data for the wild-type strain correspond to these previous results. The saturating concentration of microtubules pelleted about 48 and 50% of the plasmid in the wild-type and sin4Δ strains, respectively, but only 27% in the plc1Δ strain. Again, sin4Δ mutation did not improve the activity of kinetochores in plc1Δ cells, and microtubules pelleted about 28% of minichromosomes from plc1Δsin4Δ lysates (Fig. 1C).

We have shown previously that Plc1p is important for high fidelity chromosome segregation and activity of kinetochores. Consequently, plc1Δ cells experience delay at the G2/M stage of the cell cycle because the kinetochore-based mitotic checkpoint control system detects defects in kinetochore-microtubule interaction in plc1Δ cells and mediates G2/M delay. To assess whether this G2/M delay is eliminated in plc1Δsin4Δ cells, we examined cell and nuclear morphology of wild-type and plc1Δ cells during exponential growth at 30 °C (Table 2). The frequency of large budded cells (diameter of the bud is at least 75% of the diameter of the mother cell) with a single nucleus within 50% of the mother cell proximal to the neck (33, 34) was 11 and 13% for the wild-type and sin4Δ strains, respectively. The frequency of large budded cells for plc1Δ strain was 30% and for plc1Δsin4Δ strain was 29%.

Thus, the above results suggest that the sin4Δ mutation does not suppress the mitotic phenotypes of plc1Δ cells by increasing the kinetochore activity or by improving some other aspect of spindle function. However, sin4Δ suppresses benomyl sensitivity of plc1Δ cells (Fig. 1A) and significantly enhances the stability of minichromosomes in plc1Δ cells in the presence of benomyl (Fig. 1B).

FIGURE 1. Benomyl sensitivity of plc1Δ cells is suppressed by sin4Δ mutation independently of kinetochore function. A, benomyl sensitivity of plc1Δ cells is suppressed by sin4Δ mutation. Cells of the indicated strains were streaked on three YPD plates containing 10 μg/ml benomyl and were incubated for 3 days at 30 °C. A typical plate is shown. B, mitotic stability of minichromosomes in WT, plc1Δ, sin4Δ, and plc1Δ sin4Δ cells. The minichromosome stability was measured for at least five independent transformants as a fraction of cells that retained the minichromosome after growth in nonselective YPD medium (containing or not containing benomyl) as described under “Experimental Procedures.” The results are reported as the percentage of minichromosome loss per generation at 30 °C (standard deviations are indicated). C, minichromosome binding assay in wild type, plc1Δ, sin4Δ, and plc1Δ sin4Δ cells. Cleared lysates were prepared from the indicated strains transformed with centromeric plasmid pRS414. Cells were arrested at the G2/M stage of cell cycle by incubating for 6 h in the presence of 15 μg/ml nocodazole. Under these conditions, 90% of the cells of all four strains arrested as large budded cells. Different amounts of bovine microtubules were added to the lysates and incubated for 15 min at 20 °C. Microtubules were pelleted, and the percentage of minichromosomes that co-sedimented with microtubules was determined. The results represent means of three independent experiments, which agreed to within 15%.

some loss than plc1Δ cells. However, sin4Δ mutation did not suppress the minichromosome loss rate in plc1Δ cells (Fig. 1B). Benomyl (2 μg/ml) increased the minichromosome loss rate less than 2-fold in the wild-type strain but almost 5-fold in plc1Δ cells. Most interestingly, the minichromosome loss rate was almost unaffected in plc1Δsin4Δ cells by the presence of benomyl. This result suggests that sin4Δ mutation does not improve performance of kinetochores in plc1Δ cells in the absence of benomyl but, somehow, protects plc1Δ cells from the effects of benomyl.
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TABLE 2

*Denotes asynchronous cultures of WT (W303-1a), plc1Δ (HL1-1), sin4Δ (DY1702), and plc1Δsin4Δ (ND96) growing in YPD at 30 °C were stained with 4',6-diamidino-2-phenylindole, and nuclear and bud morphologies were scored. At least 300 separated cells were scored for each sample in each experiment. The experiment was performed three times, and standard deviations are indicated. UB means un budded cells; SB, small budded cells (diameter of the bud less than 75% of the diameter of the mother cell); LB, large budded cells with single nuclei (cells with nuclei within 50% of the mother cell proximal to the neck and with diameter of the bud at least 75% of the diameter of the mother cell); LB (single nucleus) DB, cells with two buds (occasionally cells with more than two buds were observed and were also scored as DB).

| Strain     | Cell morphology* |
|------------|------------------|
|            | UB (single nucleus) | LB (single nucleus) | LB (two separate nuclei) | DB |
| WT 38 ± 3.9 | 33 ± 3.1         | 11 ± 2.1           | 18 ± 2.6                | 0  |
| plc1Δ 25 ± 2.8 | 18 ± 2.0       | 30 ± 3.6           | 21 ± 2.7                | 6 ± 1.5 |
| sin4Δ 38 ± 4.0 | 30 ± 3.1       | 13 ± 2.0           | 19 ± 2.5                | 0  |
| plc1Δsin4Δ 26 ± 2.4 | 20 ± 2.3       | 29 ± 3.2           | 23 ± 2.8                | 2 ± 0.5 |

A Logarithmic asynchronous cultures of WT (W303-1a), plc1Δ (HL1-1), sin4Δ (DY1702), and plc1Δsin4Δ (ND96) growing in YPD at 30 °C were stained with 4',6-diamidino-2-phenylindole, and nuclear and bud morphologies were scored. At least 300 separated cells were scored for each sample in each experiment. The experiment was performed three times, and standard deviations are indicated. UB means un budded cells; SB, small budded cells (diameter of the bud less than 75% of the diameter of the mother cell); LB, large budded cells with single nuclei (cells with nuclei within 50% of the mother cell proximal to the neck and with diameter of the bud at least 75% of the diameter of the mother cell); LB (single nucleus) DB, cells with two buds (occasionally cells with more than two buds were observed and were also scored as DB).

FIGURE 2: sin4Δ mutation suppresses defect in FLR1 expression in plc1Δ cells. A, the indicated strains were grown in YPD medium at 30 °C to 

A Logarithmic asynchronous cultures of WT (W303-1a), plc1Δ (HL1-1), sin4Δ (DY1702), and plc1Δsin4Δ (ND96) growing in YPD at 30 °C were stained with 4',6-diamidino-2-phenylindole, and nuclear and bud morphologies were scored. At least 300 separated cells were scored for each sample in each experiment. The experiment was performed three times, and standard deviations are indicated. UB means un budded cells; SB, small budded cells (diameter of the bud less than 75% of the diameter of the mother cell); LB, large budded cells with single nuclei (cells with nuclei within 50% of the mother cell proximal to the neck and with diameter of the bud at least 75% of the diameter of the mother cell); LB (single nucleus) DB, cells with two buds (occasionally cells with more than two buds were observed and were also scored as DB).

reduced in plc1Δ cells, although sin4Δ and plc1Δsin4Δ cells express significant amounts of FLR1 even without induction with benomyl, and expression of FLR1 in these two strains appears to be more persistent (Fig. 2). These results thus provide a mechanism for benomyl sensitivity of plc1Δ cells and its suppression by sin4Δ mutation.

IP4 and/or IP5 Are Required for FLR1 Induction and Benomyl Resistance—The Plc1p-dependent pathway produces multiple InsP5s with diverse functions. IP4, produced by Plc1p, is converted into IP4 and IP5 by Ipk1p (4, 5). IP5 was implicated in chromatin remodeling (6, 7). IP5 produced from IP3 by Ipk1p, regulates mRNA export from the nucleus (5). Kcs1p produces inositol pyrophosphates, PP-IP4 and PP-IP5, that are involved in homologous DNA recombination (38) and regulation of telomere length (39, 40). In addition, inositol pyrophosphates are able to phosphorylate proteins in vivo by a nonenzymatic mechanism (41). Because plc1Δ cells are completely devoid of all InsPs (4, 5), we wanted to identify the specific inositol polyphosphate that is required for expression of FLR1 and benomyl resistance. We compared the ability of wild-type, plc1Δ, ipk2Δ, ipk1Δ, and kcs1Δ strains to grow in the presence of benomyl (Fig. 3A). Although the ipk1Δ strain was almost as resistant to benomyl as the wild-type strain, the kcs1Δ strain was noticeably less resistant, and the ipk2Δ strain was as sensitive to benomyl as the plc1Δ strain. To determine whether benomyl resistance correlates with the ability to activate the FLR1 gene, we treated wild-type, plc1Δ, ipk2Δ, ipk1Δ, and kcs1Δ cells with benomyl and determined FLR1 expression (Fig. 3B). Similarly to plc1Δ cells, ipk2Δ cells fail to induce FLR1, although ipk1Δ and kcs1Δ cells express intermediate levels of FLR1. Thus, the ability to induce expression of FLR1 in the presence of benomyl requires synthesis of IP4 and/or IP5 and correlates with the ability to grow in the presence of benomyl (Fig. 3). Lack of IP6 in ipk1Δ cells and PP-IP4 and PP-IP5 in kcs1Δ cells results in a somewhat decreased level of FLR1 expression. However, it appears that the level of FLR1 expression in ipk1Δ cells is sufficient to provide wild-type levels of benomyl resistance.
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Mutations in Several Components of Mediator Cause Increased Expression of FLR1 and Resistance to Benomyl—Sin4p is a component of the Mediator complex of RNA polymerase II. The Mediator plays an essential role in both basal and activated transcription. In addition to the essential role of Mediator complex in transcriptional activation, several lines of evidence indicate the involvement of Mediator also in transcriptional repression. Sin4p, Rgr1p, and Gal11p are required for repression of the HO, SUC2, and IME1 genes (20, 42, 43), and yeast cells lacking the MED1 gene, which encodes a subunit of the Rgr1 module, display defects in both repression and induction of the GAL gene expression (44).

To determine whether in addition to sin4Δ mutations in other components of the Mediator suppress benomyl sensitivity of plc1Δ cells, we constructed corresponding double mutants and tested their ability to grow in the presence of benomyl (Fig. 4). In addition to sin4Δ, med1Δ, med2Δ, med1Δplc1Δ, and med2Δplc1Δ displayed greatly increased levels of resistance to benomyl, although srp8Δ, srp10Δ, srp11Δ, and tup1Δ are only somewhat more resistant to benomyl than the wild-type cells (Fig. 4).

To test whether any of these mutations is allelic to our pbr2 or pbr3 mutations, we crossed pbr2-1 and pbr3-1 mutants (see “Experimental Procedures”) to med1Δ, med2Δ, sin4Δ, srp8Δ, srp10Δ, and srp11Δ strains. The resulting diploids were sporulated and dissected, and the benomyl sensitivity of both the diploids and haploid segregants was determined. Except for the benomyl-resistant diploid carrying pbr2-1 and med2Δ mutations, all other diploids were benomyl-sensitive. Upon dissection, the diploid carrying pbr2-1 and med2Δ mutations consistently yielded four benomyl-resistant haploids. Thus, no recombination between pbr2 locus and med2Δ was detected, and we conclude that pbr2 is allelic to MED2.

Because the sin4Δ mutation caused derepression of FLR1 transcription even in the absence of benomyl (Fig. 2), we determined FLR1 expression in a series of strains with mutations in other components of the Mediator. As expected, med2Δ, med1Δ, sin4Δ, and also srp11Δ and srp10Δ display increased expression of FLR1 (Fig. 5). Because the multidrug membrane transporters involved in drug resistance, such as Pdr5p, Snq2p, Ycf1p, and Yor1p display overlapping substrate specificity and are regulated by the same transcriptional factors (13, 14, 36), we wanted to determine whether their expression is also affected by mutations in components of the Mediator (Fig. 5). It appears that mutations in the Mediator complex (med2Δ, med1Δ, sin4Δ, srp11Δ, and srp10Δ) cause most significant derepression of FLR1. Expression of the other transporters is affected by Mediator mutations to a much smaller extent (Fig. 5). Benomyl induces expression of FLR1 significantly more than expression of the other transporters (Fig. 6). In addition, FLR1 is the only transporter that shows in the presence of benomyl significantly higher expression in wild-type and plc1Δ sin4Δ strain than in plc1Δ strain. Thus, it appears that the expression of FLR1 and not YOR1, YCF1, PDR5, and SNQ2 correlates with the resistance to benomyl. This is in agreement with the finding that flr1Δ cells are sensitive to benomyl, and overexpression of FLR1 confers resistance to benomyl (15).

In addition to derepression of FLR1, several Mediator mutations also caused mild derepression of YOR1, YCF1, PDR5, and SNQ2 (Fig. 5). To determine whether this derepression caused a multidrug resistance phenotype, we spotted corresponding Mediator mutants on plates containing model substrates 4-nitroquinoline 1-oxide (4-NQO), cycloheximide, and oligomycin (Fig. 7). Snq2p is responsible for detoxification of 4-NQO, although cycloheximide is a substrate for Pdr5p. Pdr5p and Snq2p transport many structurally and functionally unrelated drugs with significant overlap in substrate specificity. Oligomycin is a substrate for Yor1p, and Ycf1p transports glutathione conjugates into the vacuole (for review see Ref. 13). It appears that Mediator mutations do not cause resistance to any of the tested compounds (Fig. 7). Although the med1Δ strain does not display increased sensitivity to any of the tested compounds, the sin4Δ strain is slightly more sensitive to cycloheximide than the wild-type strain, and the med2Δ mutant has increased sensitivity to both 4-NQO and cycloheximide. Double mutants plc1Δ sin4Δ, plc1Δ med1Δ, and plc1Δmed2Δ are more sensitive to the tested
compounds than plc1Δ or single Mediator mutations. Thus, despite the fact that med1Δ, med2Δ, or sin4Δ mutations dramatically elevate resistance to benomyl, they do not confer a multidrug resistance phenotype.

**DISCUSSION**

We have shown previously that in budding yeast, Plc1p and InsPs produced by the Plc1p-dependent pathway affect function of kinetochores, probably by modulating centromeric chromatin structure (10, 11). Because plc1Δ cells are sensitive to benomyl and nocodazole, we speculated that this phenotype is a consequence of the partially compromised kinetochore function. In this study, we intended to utilize benomyl sensitivity as a phenotype that would allow us to identify genes that are important for the function of the kinetochore or spindle and that are downstream of Plc1p and InsPs. However, our screen identified SIN4, encoding a component of the Mediator complex. Because Sin4p was implicated in the regulation of chromatin structure (20), we speculated that the suppression of benomyl sensitivity of plc1Δ cells by sin4Δ mutation is because of the altered chromatin structure of the centromeres in plc1Δsin4Δ cells that results in an improved kinetochore function. However, our characterization of the plc1Δsin4Δ mutant showed that sin4Δ mutation does not suppress benomyl sensitivity of plc1Δ cells by improving the function of kinetochores (Fig. 1 and Table 2). In the absence of benomyl, the minichromosome loss rate, binding of
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minichromosomes to microtubules in vitro (Fig. 1), and mitotic delay (Table 2) are indistinguishable in plc1Δ and plc1Δsin4Δ strains. The minichromosome loss rate, however, is significantly lower in plc1Δsin4Δ cells than in plc1Δ cells in the presence of benomyl. This result thus suggested that sin4Δ improves kinetochore function of plc1Δ cells only in the presence of benomyl. Subsequent experiments demonstrated that sin4Δ mutation causes derepression of the membranetransporter FLR1 that mediates resistance to benomyl (15).

Benomyl is an antimitotic drug that destabilizes microtubules and inhibits microtubule-mediated processes, including nuclear division, migration, and fusion (30). Mutations in components of the mitotic spindle result in benomyl sensitivity or resistance (28, 29). Benomyl was also used with great success as a tool that allowed identification of genes that affect expression of Yap1p also suppresses benomyl sensitivity of FLR1 promoter. In addition, we have demonstrated that in addition to these promoter-specific transcription factors, expression of the transporters can be regulated by component(s) of signal transduction pathway(s) (Plc1p) and by general transcription factor(s) (Mediator). The results thus suggest another layer of complexity in regulation of the PDR network.

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