Fine Structure Analysis of the Yeast Centrin, Cdc31p, Identifies Residues Specific for Cell Morphology and Spindle Pole Body Duplication

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ABSTRACT

Centrin/Cdc31p is a Ca$^{2+}$-binding protein related to calmodulin found in the MTOC of diverse organisms. In yeast, Cdc31p localizes to the SPB where it interacts with Kar1p and is required for SPB duplication. Recent findings suggest that centrin also functions elsewhere in the cell. To dissect the functions of Cdc31p, we generated $cdc31$ mutations chosen only for temperature sensitivity, but otherwise unbiased as to phenotype. Three phenotypes of the $cdc31$ mutants, temperature sensitivity, G2/M arrest, and cell lysis, were not well correlated, indicating that the mutations may differentially affect Cdc31p’s interactions with other proteins. Alleles near the C-terminal region exhibited high G2/M arrest and genetic interactions with kar1- $D_{17}$, suggesting that this region modulates an SPB-related function. Alleles causing high lysis and reduced Kic1p kinase activity mapped to the middle of the gene, suggesting disruption of a KIC1-like function and defects in activating Kic1p. A third region conferred temperature sensitivity without affecting cell lysis or G2/M arrest, suggesting that it defines a third function. Mutations in the C-terminal region were also defective for interaction with Kic1p. Mapping the alleles onto a predicted structure of Cdc31p, we have identified surfaces likely to be important for interacting with both Kar1p and Kic1p.

MICROTUBULE organizing centers (MTOCs) are nearly ubiquitous eukaryotic organelles that nucleate microtubules and regulate their dynamics. Although functionally conserved, MTOCs show vast morphological diversity as exemplified by the mammalian centrosome, the Chlamydomonas basal body, and the yeast spindle pole body (SPB). During interphase, the MTOC and the microtubules direct intracellular trafficking and organelle positioning (for review see Balcázar 1996; Reinsch and Gonczy 1998). Just prior to mitosis, the MTOC duplicates and the two MTOCs establish the poles of the mitotic spindle. Subsequent attachment of the spindle microtubules to the chromosomes ensures equal segregation of the genetic material, resulting in two daughter cells each having a full set of chromosomes and one MTOC. Clearly, proper timing and execution of MTOC duplication is essential for mitosis. In the absence of MTOC duplication, a bipolar spindle cannot be formed and all subsequent steps of mitosis cannot be executed. Bipolar spindle defects result in activation of the spindle assembly checkpoint that causes a cell cycle arrest at G2/M (Wells 1996). Hyperamplification of the centrosome, the mammalian MTOC, also occurs during cellular transformation and cancer (Salisbury et al. 1999). Therefore, the elucidation of MTOC duplication is important for understanding both the normal cell cycle and the defective cell cycle during cancer.

In general, the MTOCs can be thought of as cytoplasmic organelles that play a central role in the nuclear division cycle. Because of this duality, MTOCs are ideally positioned to coordinate the nuclear and cytoplasmic divisions, as originally suggested by Boveri in 1903 (Moritz and Sauer 1996). Centrin is a centrosomal protein with significant cytoplasmic roles and has been suggested to provide this coordinating function (Paolelli et al. 1996).

Studies in yeast have defined several steps of SPB duplication as well as key regulatory and structural protein components. The SPB is a disc-shaped structure embedded in the nuclear envelope (Byers and Goetsch 1974). The half-bridge is a specialized region of the nuclear envelope found adjacent to the SPB. The nascent SPB forms from a satellite structure that appears on the cytoplasmic side of the half-bridge during G1 (Byers and Goetsch 1974). The satellite grows into a duplication plaque by addition of SPB components and is eventually inserted into the nuclear envelope, where duplication is completed. It has been hypothesized that insertion occurs by contraction of the half-bridge mediated by Cdc31p (Adams and Kilmartin 1999).

Proteins involved in the earliest steps of SPB duplication include Cdc31p, Kar1p, and the ubiquitin-related proteins Dsk2p and Rad23p. Mutations in KAR1, CDC31, or DSK2 and RAD23 cause cells to arrest at G2/M, with large buds and duplicated DNA, but unduplicated SPBs. The unduplicated SPBs lack a satellite, suggesting that
these proteins are required for satellite formation. Certain alleles of KAR1 and CDC31 also lack a half-bridge, suggesting that Kar1p and Cdc31p may also play a role in half-bridge formation or maintenance. Kar1p is a nuclear membrane protein, found on the cytoplasmic side of the half-bridge (Vallen et al. 1992; Spang et al. 1995). Kar1p helps localize Cdc31p to the half-bridge, and a kar1ΔA17 mutant fails in SPB duplication due to mislocalization of Cdc31p. Cdc31p binds to a small region of Kar1p, which is partially deleted in kar1ΔA17 (Biggins and Rose 1994; Spang et al. 1995). Dominant mutations in CDC31 and DSK2 suppress kar1ΔA17 by relocating Cdc31p to the SPB. In addition, genetic interactions implicate the Pck1p pathway as playing a positive role in Cdc31p’s function in SPB duplication (Khalfan et al. 2000). Despite the well-established requirement for Cdc31p at the SPB, the specific role of Cdc31p and its downstream targets are not known.

Centrin/Cdc31p shares homology with calmodulin/Cmd1p (Baum et al. 1986; Huang et al. 1988). Cdc31p and Cmd1p are members of a protein superfamily characterized by four EF-hand Ca²⁺-binding domains (Moncier et al. 1990) contained in two lobes connected by a flexible tether (Head 1992). Calmodulin binds to an amphipathic α-helix in ligands through hydrophobic domains on each lobe. The flexible tether allows the two lobes to come together on either side of the ligand (Persechini and Krebsinger 1988). Cdc31p and Cmd1p are 42% identical (Baum et al. 1986), suggesting conservation of structure and ligand-binding properties. In support of this view, the Cdc31p-binding site in Kar1p is closely related to the IQ calmodulin-binding site (Geier et al. 1996).

Centrin is found in centrosomal structures across the eukaryotic phyla. Mammals have three centrin genes that express distinct isoforms. Cen1p is expressed only in the testis of adult mice, at the time of spermatogenesis, suggesting a meiosis-specific role (Hart et al. 1999). HsCen2p is restricted to ciliated cells and is upregulated during ciliogenesis (LeDizet et al. 1998). HsCen2p localizes to the connecting cilium and may play a role in cellular motility and microtubule severing (Wolfrum 1995; Wolfrum and Salisbury 1998). However, >90% of HsCen2p is found in cytosolic fractions and may play a role in coordinating the nuclear and cytoplasmic division cycles (Paoletti et al. 1996). HsCen3p is the closest human homologue of Cdc31p and appears to play a role in centrosomal duplication (Middendorp et al. 2000) similar to Cdc31p. Although expression of HsCen3p does not suppress cdc31 mutants, it does block SPB duplication by competing with Cdc31p (Middendorp et al. 2000).

Centrin plays a role in microtubule severing in the flagellated green alga Chlamydomonas reinhardtii (Sanders and Salisbury 1989, 1994). In these cells, centrin localizes to three different fibrous structures in the nucleobasal body apparatus (Huang et al. 1988) and is thought to function during their calcium-mediated contraction. Mutations in zfh2, the gene for Chlamydomonas centrin, lead to defects in basal body localization and/or segregation (Tailon et al. 1992). In higher plants, a centrin-related protein localizes to microsomes and the plasmodesmata at the cell plate, suggesting that it may function during cytokinesis or in intercellular transport (Blackman et al. 1999; Stoppin-Mellet et al. 1999).

In yeast, Cdc31p also physically interacts with a protein kinase, Kic1p. The kinase activity of Kic1p is defective in cdc31 mutants, indicating that Cdc31p mediates Kic1p function (Sullivan et al. 1998). Strikingly, mutations in KIC1 and certain cdc31 alleles result in defects in bud morphology and cell integrity defects, suggesting that Cdc31p and Kic1p may also play a role in these processes (Lussier et al. 1997; Sullivan et al. 1998).

Taken together, the results from diverse systems suggest multiple cellular roles for centrin. Lack of well-defined alleles of cdc31 that affect a single function has precluded a systematic analysis of these functions. All previous cdc31 alleles were obtained in genetic screens that were biased toward its role in SPB duplication. Three temperature-sensitive alleles, cdc31-1, cdc31-2, and cdc31-5, were isolated on the basis of their uniform cell cycle arrest at G2/M (Byers 1981). However, the cdc31-1 mutant required multiple cell cycles at the nonpermissive temperature before it arrested at G2/M (Byers 1981). Such multiple cycle mutants are thought to be defective for the synthesis or assembly of the protein (Hartwell 1974). Such alleles may be defective for multiple functions, but may preferentially affect the most sensitive function first. After prolonged incubation, the existing cdc31 alleles were found to exhibit allele-specific cell lysis and bud morphology defects (Sullivan et al. 1998). The fourth allele, CDC31-16, was isolated as a dominant suppressor of kar1ΔA17 and has a recessive loss-of-function defect in SPB duplication (Vallen et al. 1994). Because of the requirements of their acquisition, mutations that specifically affect the additional functions of CDC31 would not have been identified from these screens. The existence of multiple functions for Cdc31p has prevented its further genetic analysis by the acquisition of suppressor mutations.

To dissect the multiple functions of Cdc31p, we set out to isolate multiple alleles solely on the basis of the criterion of temperature sensitivity. This relatively unbiased screen has identified mutations in the different functions of the protein. We have identified clusters of alleles that appear to be specific for each function. Mutations in the central part of the protein are defective in Kic1p kinase activity, whereas mutations in the carboxy-terminal region are defective in Kic1p binding. The carboxy-terminal region also mediates the Kar1p-related function of Cdc31p, because mutations in this region led to a high G2/M arrest and failed to localize to the SPB. Certain mutations with a high G2/M arrest...
Microbial techniques and yeast strain construction: Yeast media and microbial techniques were essentially as described (Rose et al. 1990). Bacterial media were as described (Sambrook et al. 1989). and bacterial strain XL-Blue was used for all bacterial manipulations. All restriction enzymes were from New England Biolabs (Beverly, MA) and were used according to the supplier’s specifications. Primers were from the Princeton University synthesis and sequencing facility or from Gibco BRL (Gaithersburg, MD). DNA sequencing was performed at the Princeton University synthesis and sequencing facility. Yeast strains were constructed using standard genetic techniques and are congenic to S288c.

Site-directed mutagenesis of the phenylalanine residues of Cdc31p was performed using the *duet*-ung method (Bio-Rad Laboratories, Hercules, CA). For random hydroxylamine mutagenesis, plasmid MR3523 (CDC31, HIS3, CEN/ARS) was mutagenized in vitro (Rose et al. 1990) and transformed into strain MS2584 (cdc31Δ::LEU2 MATα ura3-52 leu2-3, 112 his3-Δ200 MR2225 [CDC31 URA3 CEN/ARS]). We screened 8000 colonies and identified temperature-sensitive transformants after selecting against the wild-type plasmid on 5-fluoroorotic acid (5-FOA). We identified nine temperature-sensitive transformants. One of the mutant plasmids contained two different base pair changes and was not studied further. Two plasmids had the same mutation, and only one of them was studied further. The remaining six alleles (cdc31-6, -21, -30, -49, -54, and -65) were further analyzed along with the PCR-generated mutants.

For PCR-mediated mutagenesis (Leung et al. 1989; Muhlrad et al. 1992), plasmid MR3523 was gapped using SnuB1 and Cta1 and the resulting linear plasmid was transformed into MY2584 along with a mutagenized PCR fragment overlapping the gap on the plasmid. The mutagenized PCR fragment was amplified using primers PR287 (5'-CTG CAC GTT GTA AAA CG-3'), and PR288 (5'-ATT TAA GCT CGA AAT GGC-3'), plasmid MR3523 as a template, and mutagenic PCR conditions with 0.9 mM MnCl2. Homologous recombination in yeast recovered circular plasmids. We screened 6282 colonies and identified 40 temperature-sensitive transformants. Of these, 14 had single base pair changes, 19 had double base pair changes, and 7 had triple base pair changes. To ensure that only a single function of Cdc31p is defective in each mutant, only the single mutants were studied further. All single mutants and their amino acid substitutions are listed in Table 1.

The mutations were integrated at the endogenous CDC31 locus in strains MS1554 (MATα ura3-52 leu2-3, 112 ade2-101 his3-Δ300) and MS2290 (MATα ura3-52 leu2-3, 112 ade2-101 his3-Δ300) by a PCR-based method that uses two sets of adaptomers, or chimeric oligomers complementary to two different DNA sequences (Erdemiz et al. 1997). In the first round of PCR, each allele was amplified with primers PR301 (5'-GAT CCC CGG GAA TTG TTA TAA CTA TGG TCT GGT TAG GAS-3') and PR302 (AAT TCC AGC TGA CCA TCA TGA GTA AGA ACA GAT CAT G-3'), resulting in molecules tagged with adaptomers A and B. In parallel, two truncated overlapping fragments of the *Kluuyveromyces lactis URA3* gene (a generous gift of N. Erdeniz) were amplified with primers PR299 (5'-TCT AAG ATT ATT ATT CGT GAA CAC AAA AAG AAA AAG GCA AGA AAG CTG TCC AAA TTA TTA CGA TAA ATC ATC GTT TTT AAG AGC TGG TGT-3') and PR303 (5'-CAT GGT GGT CAG CTT GGA TTA CAT GTA GAT GTA TCT GTT GGT T-3') and PR298 (5'-GAG CAA TGA ACC CAA TGA CAA AAT CAT C-3') and PR304 (5'-CAT GCC AAT TCC CGG GGA TCG TGA TTC GGA GAA CAT CG-3'), resulting in molecules tagged with the adaptomers a or b, respectively. In the second round of PCR, the matching tags on the molecules (A with a and B with b) allowed fusion of each allele to either of the tagged URA3 fragments. To integrate each allele into the genome, the fusion molecules were cotransformed into yeast (Gietz and Schiestl 1995) and transformants were selected on synthetic complete (SC) medium lacking uracil. Homologous recombination between the two fusion molecules and the genome resulted in integration of the URA3 marker flanked by the mutant alleles on both sides. A single altered copy of the mutant alleles was obtained by selecting for loss of the URA3 gene on 5-FOA (Boeke et al. 1987). Integration of the mutant alleles was scored by temperature sensitivity and by sequencing the genomic *CDC31* locus that had been amplified by PCR.

All kar1Δ17 cdc31 double mutants were constructed by transforming strain MS6286 (kar1Δ17 cdc31Δ::LEU2 MATα ura3-52 leu2-3, 112 ade2-101 his3-Δ300 MR2018 [CDC31, URA3, CEN/ARS]) with a HIS3 CEN/ARS plasmid containing each allele. Loss of the wild-type *CDC31* plasmid was selected on 5-FOA at 23°. Strains that did not grow on 5-FOA after repeated attempts and extended time were deemed synthetically lethal. Strains that grew on 5-FOA at 23° were further analyzed at 30°, 35°, and 37° using a 10-fold dilution plate assay on SC medium.

Microscopic analysis: To examine the G2/M arrest phenotype of the various *cdc31* alleles, strains were grown in synthetic medium at 23° to early logarithmic phase and one-half of the cultures were shifted to 37° for 2, 4, 6, or 8 hr. To examine the nuclear morphology, cells were harvested by centrifugation and fixed with methanol/acetic acid (3:1 ratio) for 0.5 hr on ice and stained with 4',6-diamino-2-phenylindole (DAPI) for 0.5 hr on ice. DAPI was obtained from Accurate Biochemicals and Scientific Corp. (Westbury, NY).

Previously described indirect immunofluorescence methods were used to visualize tubulin (Rose et al. 1990). The *cdc31* mutant strains were grown to early logarithmic phase at 23° and were shifted to 37° for 4 hr. Cells were harvested and fixed with 4% formaldehyde for 1.5 hr. Rat anti-tubulin antibody (YOL 1/34; Accurate Biochemicals and Scientific Corp.) was used at a 1:2 dilution, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG secondary antibody (Boehringer Mannheim Biochemicals, Indianapolis) was used at a 1:1000 dilution.

Cell viability was assayed with the cell-permeable two-color fluorescent probe, FUN-1 (Millard et al. 1997). Metabolically active cells convert FUN-1 from a diffuse pool of green fluorescent stain to orange-red intensely fluorescent intravacuolar structures. Conversion of FUN-1 to the vacuolar structures requires both plasma membrane integrity and metabolic capability. Metabolically inactive cells with intact plasma membranes do not form the intravacuolar structures and retain diffuse green cytoplasmic fluorescence. In contrast, dead cells lacking an intact plasma membrane exhibit intense yellow cytoplasmic fluorescence. FUN-1 was added to 1 ml of cell culture to a final concentration of 10 μM (Molecular Probes, Eugene, OR). The cultures were incubated at room temperature in the dark for 0.5 hr. Cells were examined by differential interference and fluorescence microscopy using a FITC filter. 
set (Axioptot; Carl Zeiss, Thornwood, NY). Greater than 100 cells were counted for each sample.

Cdc31p was localized in strains containing each allele integrated at the endogenous cdc31 locus. Cultures were grown in synthetic complete medium until logarithmic phase and were shifted to 37°C for 4 hr. Cells were harvested and prepared for immunostaining as previously described (Rout and Kilman 1990) using rabbit anti-Cdc31p polyclonal antibody at a dilution of 1:300 (Biggin and Rose 1994) and a FITC-conjugated goat anti-rabbit secondary antibody at a dilution of 1:1000 (Boehringer Mannheim). We counted >100 DAPI-stained nuclei for each strain. Nuclei that contained one or two dots of FITC signal were included in the “Cdc31p localization” data and nuclei with no detectable FITC dots were counted as “Cdc31p mislocalization.”

**Two-hybrid interactions:** For two-hybrid interaction analysis, the cdc31 alleles were cloned into plasmid pGBT9 [P-gal-GAL4 BD, TRP1, 2µ] and were assayed against a library isolate of KIC1 (Sullivan et al. 1998) in plasmid pAD424 [P-gal-GAL4 AD, LEU2, 2µ] (Fields and Song 1989). The two-hybrid reporter strain PJ69-4A was used in all instances (James et al. 1996). Assays of β-galactosidase activity were performed using a crude yeast extract and activity was measured as previously described (Rose et al. 1990).

**Protein techniques and kinase assays:** Protein extracts were prepared from strains containing each cdc31 allele integrated into the genome. Cultures were grown in SC medium at 23°C until logarithmic phase and one-half of each culture was shifted to 37°C for 4 hr. To assay Cdc31p mutant protein levels, 85 µg of total yeast protein extracts were loaded on 15% SDS-polyacrylamide gels and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Affinity-purified rabbit anti-Cdc31p antibody was used at 1:300 dilution (Biggin and Rose 1994) and horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1000 (Boehringer Mannheim). We counted >100 DAPI-stained nuclei for each strain. Nuclei that contained one or two dots of FITC signal were included in the “Cdc31p localization” data and nuclei with no detectable FITC dots were counted as “Cdc31p mislocalization.”

**Protein modeling:** The Cdc31p three-dimensional structure and Cdc31p. Accordingly, we mutated the Phe residues described (<i>Green et al.</i> 1996). Assays of β-galactosidase activity were performed using ECL Western blotting reagents (Amersham Life Science). For Kic1p kinase assays, plasmids pEGKT [P-gal-GST URA3 2µ] (Mitchell et al. 1993) and MR3041 [P-gal-GST-KIC1 URA3 2µ] (Sullivan et al. 1998) were transformed into strains containing each allele integrated into the genome. Cultures were grown at 23°C until logarithmic phase in SC medium lacking uracil and containing rifampinic as the sole carbon source. The cultures were induced by the addition of galactose to a final concentration of 2% for 4 hr at 23°C and protein extracts were prepared and kinase assays were performed as previously described (Laube et al. 1995) with slight modifications (Sullivan et al. 1998).

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**Protein modeling:** The Cdc31p three-dimensional structure was predicted by the SWISS-MODEL protein modeling server to predict the three-dimensional structure of Cdc31p. Cdc31p and Cdc31p are 42% identical (Baum et al. 1986) and therefore are likely to have similar three-dimensional structure. The resulting prediction for the structure of Cdc31p was based upon the average of two different NMR structures of calmodulin. Like calmodulin, Cdc31p was predicted to fold into a dumbbell-shaped structure with amino- and carboxy-terminal lobes connected by an α-helical loop. Each lobe contains two EF-hand Ca²⁺-binding domains. The dumbbell is predicted to curve around to form a doughnut; in calmodulin the central channel is the site for interaction with a peptide ligand containing the IQ site (Ikura et al. 1992; Meador et al. 1992). In calmodulin, the central α-helix is flexible and adopts a more extended structure in the absence of protein ligand. Figure 1C shows three different views of the predicted structure. For Cdc31p, the amino-terminal region is drawn as extended. Colored residues reflect different functional groups on the basis of our phenotypic analysis described below.

**Generation of multiple cdc31 alleles:** To generate multiple alleles of cdc31, we performed in vitro mutagenesis and isolated temperature-sensitive alleles, without bias for cell cycle phenotype. We used three different mutagenesis protocols to maximize saturation of the screen and broaden the spectrum of possible base pair substitutions. First, we used a site-directed mutagenesis protocol similar to that used to define the multiple functions of calmodulin (Ohya and Botstein 1994b). In that study, Ohya and Botstein mutated single or multiple phenylalanine (Phe) residues of CMD1 to alanine. The Phe residues in calmodulin are evolutionarily conserved and interact with a peptide ligand in the crystal structure (Ikura et al. 1992). In calmodulin, single and multiple Phe → Ala mutations generated 14 temperature-sensitive alleles that could be grouped into four complementation groups with distinct phenotypes (Ohya and Botstein 1994a). Cdc31p has nine Phe residues, six of which are conserved between Cmd1p and Cdc31p. Accordingly, we mutated the Phe residues in Cdc31p and assayed their phenotypic consequences. Unlike CMD1, all but one of these mutations resulted in a lethal phenotype. The basis of this difference between Cdc31p and Cmd1p is unclear. The single temperature-sensitive allele, cdc31-F54A, was in a Phe residue unique to Cdc31p and arrested with a large-budded G2/M arrest phenotype similar to the previously identified cdc31-I allele.

Next, to isolate random temperature-sensitive mutations, we used both hydroxylamine and PCR mutagenesis of the gene in vitro and identified 21 mutations with single base pair changes. Twenty-six additional mutants had two or more base pair changes and were not studied further. Because the mutants were isolated on a centromere-based plasmid, we next integrated them into the genome at the endogenous CDC31 locus. We were un-
able to integrate three alleles (cdc31-6, -57, and -89), suggesting that these alleles might be lethal when present in a single copy. We therefore characterized these alleles on a plasmid. All random mutations and their amino acid substitutions are listed in Table 1.

The distribution of the alleles in the protein showed a number of interesting trends. First, 85% of the mutations clustered in the carboxy-terminal half of the protein (Figure 1, A and B), suggesting that either the amino-terminal region does not perform any essential functions or mutations in the amino-terminal region often lead to lethality. It is interesting that, among the centrinis, the amino-terminal region is the most variable portion of the protein (BHATTACHARYA et al. 1993). It is two to three times longer than the amino-terminal region of Cmd1p and it has been proposed to confer specificity and/or functional diversity (BHATTACHARYA et al. 1998). Therefore, the few new alleles in the amino-terminal half may be particularly valuable in elucidating the function of this part of the protein.

Second, the majority of random mutations (65%) were on the surface of the predicted protein structure (cdc31-30, -159, -152, -134, -57, -122, -6, -98, -89, -97, -54, and -65), suggesting that they do not interfere with protein folding and/or stability, but may affect binding of interacting proteins (Figure 1C). Strikingly, all except cdc31-30 were on the same side of the protein. The remainder of the alleles (cdc31-115, -138, -145, -49, -158, -168, and -113) were on the inside of the protein (Figure 1D). Of these, cdc31-113 was buried in the hydrophobic core of the carboxy-terminal lobe, whereas the rest line an internal cavity analogous to the hydrophobic domains of the calmodulin ligand-binding site. In contrast, the majority of the site-directed Phe → Ala mutations lined the internal cavity of Cdc31p (Figure 1E). The only exception, Phe54, mapped to the surface and resulted in a temperature-sensitive phenotype.

**Microscopic analysis of phenotypes:** To ascertain the basis for the temperature sensitivity of the new cdc31 alleles and identify the function(s) affected by each allele, we first examined their cellular morphology. In particular, we investigated whether the new mutants had phenotypes similar to the existing cdc31 alleles, including G2/M cell cycle arrest, cell lysis, and actin cytoskeleton defects. Cdc31p’s role in SPB duplication is most easily examined and 3G). These results indicate that the central region of the core of the carboxy-terminal lobe, whereas the rest line that requires cells to have intact plasma membranes and be metabolically active to convert diffuse cytoplasmic green fluorescence into intense orange-red vacuolar structures (see MATERIALS AND METHODS). Cells that have lost plasma membrane integrity exhibit bright yellow cytoplasmic fluorescence. Wild-type cultures showed >95% with a monopolar spindle. The separation of the buds may reflect an additional bud neck defect or the completion of cytokinesis in these mutants.

Cdc31p was observed to play a role in cell integrity and cell wall morphogenesis via interaction with Fks1p (SULLIVAN et al. 1998). We examined cell viability and lysis in the new cdc31 mutants using FUN-1, a fluorescent stain that requires cells to have intact plasma membranes and be metabolically active to convert diffuse cytoplasmic green fluorescence into intense orange-red vacuolar structures. Therefore, for these mutants, digestion of the cell walls caused a subset of large-budded cells to separate into two un budded cells, one un budded and one with a monopolar spindle. The separation of the buds may reflect an additional bud neck defect or the completion of cytokinesis in these mutants.
ruption of the actin cytoskeleton may be a secondary consequence of the G2/M arrest, and not a specific defect of a subset of cdc31 alleles.

Localization of mutant Cdc31 proteins: Depending on the fixation conditions for immunofluorescence, Cdc31p shows either diffuse, uniform cytoplasmic staining (Biggins and Rose 1994; Levy et al. 1998) or localizes as a single dot at the edge of the nucleus, coincident with the SPB (Biggins and Rose 1994; Spang et al. 1995). To investigate whether the new alleles affect Cdc31p localization, we quantified the extent to which each mutant protein localizes to the SPB (Figure 2C). There was a wide variation in the extent to which the mutations disrupted Cdc31p localization to the SPB. Although most alleles caused a defect in localization, a subset of alleles (cdc31-21, -30, -49, -89, and -115) was essentially like wild type with <10% mislocalization. These may represent a novel class of Cdc31p mutants. Certain mutants showed significant SPB localization (e.g., 50% for cdc31-113) although the Cdc31-113p was not detectable by Western blot analysis (see below and Figure 5B). This result may reflect the relative difference in sensitivity of the two assays (indirect immunofluorescence vs. Western blot analysis). The amount of Cdc31-113p undetectable by Western blot analysis may be easily observed by indirect immunofluorescence when concentrated at the SPB.

Genetic interactions between kar1Δ17 and cdc31 alleles: Genetic interactions, such as synthetic lethality or suppression, are important probes of function and physical contacts. We sought to explore the relationship between KAR1 and the new cdc31 alleles by assessing genetic interactions in double mutants with kar1Δ17. The cdc31 alleles fell into four groups with respect to genetic interactions with kar1Δ17 (Figure 4). In the first group of alleles, cdc31-6,
-115, and -152, the temperature spectrum was not altered by the presence of kar1Δ17 (Figure 4A); these alleles did not show genetic interactions with karΔ17. The second group of alleles, cdc31-21, -49, -57, -65, -89, -125, -145, -158, -159, and -168 were synthetically lethal with kar1Δ17; these double mutants failed to grow at any temperature (data not shown). A third group of alleles, cdc31-138, -122, and -54, showed a marked enhancement of growth defect in combination with kar1Δ17 and failed to grow at temperatures permissive for either single mutant (Figure 4B). Most alleles in the carboxy-terminal region of Cdc31p were synthetically lethal or showed enhanced growth defects. Strikingly, dominant non-temperature-sensitive cdc31 alleles isolated as kar1Δ17 suppressors also mapped to this region (Vallen et al. 1994; see also Figure 4). A fourth group of alleles, cdc31-30, -113, -134, and -98, showed a suppression phenotype where the double mutant grew at temperatures restrictive for either single mutant (Figure 4C). Remarkably, cdc31-30 and kar1Δ17 behaved as dominant cosuppressors of each other because the double mutant grew in the presence of either wild-type plasmid. This behavior is similar to the interaction between kar1Δ17 and CDC31-16 (Vallen et al. 1994). In contrast, cosuppression between cdc31-113 and kar1Δ17 was dominant to KAR1, but recessive to CDC31 (Figure 4C and data not shown). The cosuppression in the cdc31-
134 kar1-D17 double mutant was recessive to both KAR1 and CDC31. Finally, kar1-D17 is a recessive suppressor of cdc31-98. The recessivity of some of the cosuppressors suggests that they act by a mechanism other than by increasing the affinity of binding to Kar1p or a downstream effector.

**Microscopic analysis of cdc31 kar1-D17 double mutants:** To further analyze the basis of the genetic interactions between kar1-D17 and the new cdc31 alleles, we examined the cell morphology of the double mutants. The synthetically lethal combinations could not be analyzed because they did not grow under any conditions. We therefore analyzed the alleles that showed enhanced growth defects (Table 2A). We found that the double mutants containing cdc31-54 and cdc31-122 had a higher level of large-budded cells at both the permissive (25°) and the semipermissive (35°) temperatures. The cdc31-138 kar1-D17 mutant had a higher level of large-budded cells (60%) compared to either single mutant [kar1-D17 (22%) and cdc31-138 (18%)] at 35°.

We also analyzed the alleles that did not show any growth phenotypes in combination with kar1-D17. Double mutants between alleles cdc31-6, -115, and -152 and kar1-D17 arrested at G2/M to the same degree as the single mutants alone (Table 2B). Finally, the cosuppressing alleles, cdc31-30, -113, -134, and -98, showed a reduction in G2/M arrest phenotype as compared to kar1-D17 (Table 2C), suggesting that they suppress the growth defect of kar1-D17 by suppressing its SPB duplication defect.

**The central domain of Cdc31p is required for Kic1p kinase activity:** Cdc31p has been implicated to function with Kic1p in cell wall morphogenesis and cell integrity (Sullivan et al. 1998). Cdc31p and Kic1p interact directly and Kic1p kinase activity was reduced in the two cdc31 alleles that were previously examined. Therefore, we tested the new cdc31 alleles for their effect on Kic1p kinase activity. To ensure similar levels of Cdc31p, the kinase assays were performed on cultures grown at the permissive temperature. Figure 5A shows that the amino-terminal mutants (cdc31-115, -138, and -30) contained wild-type levels of Kic1p kinase activity. These results correlate with the lack of cell lysis in these alleles, suggesting that the amino-terminal region of the protein does not have a specific cell wall-related function. Alternatively, the temperature sensitivity of these mutants may be due to reduced protein Kic1p-complex stability at the nonpermissive temperature, rather than to loss of a specific function. To test this we measured Kic1p kinase activity in cultures grown at 37° and found that it was comparable to wild-type levels (data not shown), indicating that the temperature sensitivity in cdc31-115, -138, and -30 is not due to a defect in Kic1p kinase activity.

Alleles in the central part of the protein showed drastically reduced levels of Kic1p kinase activity. Five alleles (cdc31-152, -159, -49, -134, and -145) had a defect at both permissive and nonpermissive temperatures, whereas two alleles (cdc31-158 and -168) had a temperature-sensitive defect. A mutation that maps to the region between the third and the fourth EF hands had wild-type levels of Kic1p kinase activity, suggesting that this part of the protein does not affect Kic1p function. Certain alleles in the carboxy-terminal region also showed a temperature-sensitive reduction in Kic1p kinase activity, most notably cdc31-54 and -21. In the case of cdc31-54, the temperature-sensitive kinase defect correlates with a temperature-sensitive loss of Cdc31p. However, this is not the case in cdc31-21, suggesting that this region may also contribute to Kic1p function.

Temperature-sensitive alleles may arise from mutations that disrupt the protein structure, leading to protein misfolding and degradation at the nonpermissive temperature. Therefore, one trivial explanation for lack of Kic1p kinase activity is absence of Cdc31p. Although we performed the kinase assays on cultures grown at the permissive temperature, we also directly examined the levels of Cdc31p at both the permissive and nonpermissive temperatures. The mutants behaved in three different patterns with respect to cdc31 protein levels (Figure 5B). First, some mutants had wild-type protein levels at both temperatures. Other mutants showed temperature-dependent reduction in protein levels. Finally, some mutants had low protein levels at both temperatures. Of particular concern, the mutants that showed severe reduction in Kic1p kinase activity did express wild-type levels of Cdc31p at the permissive temperature. Therefore, because the protein extracts were prepared from cells at the permissive temperature, the lack of Kic1p kinase activity was not due to absence of Cdc31p. In summary,
Figure 2.—G2/M arrest and cell lysis phenotypes are not correlated. Each part is a separate linear representation of Cdc31p. Amino acids 45–147 are depicted, because mutations in the first 44 and the last 14 amino acids were not recovered. The vertical bars depict the position of each mutation and the numbers underneath each bar represent the allele number. The height of the bars represents the percentage of each phenotype for each allele. The numbers above A depict the approximate amino acid positions. Greater than 100 cells were counted for each allele for each phenotype. (A) Percentage of large-budded cells with a single nucleus after 4 hr at 37°C. Alleles in the carboxy-terminal region (gray shading) had a higher G2/M arrest phenotype as compared to alleles in the amino-terminus. (B) Percentage of cell lysis after 4 hr at 37°C. Alleles in the center of the protein (gray shading) had higher cell lysis then alleles in the amino- and carboxy-termini. (C) Percentage of cells showing Cdc31p mislocalization.

we identified the central part of the protein, encompassing the region between the second and the third EF hands, as being important for activating the Kic1p kinase, with a minor contribution from the carboxy-terminal region of Cdc31p.

Binding of mutant Cdc31 proteins and Kic1p: We next tested whether lack of Kic1p kinase activity was due to a defect in Cdc31p-Kic1p binding. For this purpose, we used the yeast two-hybrid system with the Cdc31p mutants fused to the Gal4p DNA-binding domain and Kic1p fused to the Gal4p activation domain (Fields and Song 1989; James et al. 1996). Figure 6 shows the β-galactosidase activity levels for the different combinations of Kic1p and Cdc31 mutant fusion proteins for cultures grown at 37°C. Results obtained from cultures grown at 30°C were comparable, except for one instance, discussed below. Alleles in the amino-terminal region (cdc31-115, -138, and -30) exhibited wild-type or higher levels of β-galactosidase activity, suggesting that they were not impaired for binding to Kic1p. Alleles in the central part of the protein that caused severe defects in Kic1p kinase activity exhibited variable phenotypes with respect to the Kic1p two-hybrid interaction. Alleles cdc31-152, -159, and -158 showed wild-type Kic1p binding. The cdc31-49 allele showed a temperature-sensitive defect in β-galactosidase activity, whereas cdc31-134 and -145 showed moderate defects at both temperatures (~25% β-galactosidase activity as compared to the wild type). Most alleles in the carboxy-terminal region of Cdc31p (cdc31-57, -122, -6, -98, -113, -54, -97, -65, and -21) showed severely reduced β-galactosidase levels comparable to the vector control. Some of these alleles showed temperature-sensitive defects in Kic1p kinase activity (cdc31-21 and cdc31-54). We were surprised to find that the other alleles had wild-type Kic1p kinase activity (cdc31-6 and cdc31-113). Lack of β-galactosidase
activity was not due to lack of two-hybrid fusion proteins because immunoblot analysis showed the same level of hybrid proteins for all alleles from cultures grown at both 30º and 37º (data not shown). In conclusion, we found that mutations in the carboxy-terminal region of Cdc31p strongly disrupted binding to Kic1p, whereas mutations toward the central part of the protein had a minor effect.

**DISCUSSION**

Phenotypic studies on temperature-sensitive mutants of *cdc31* revealed that different mutants exhibited distinct phenotypes. Table 3 summarizes the findings and groups the mutants on the basis of their phenotypes. Analysis of the results suggested that different regions of Cdc31p mediate distinct functions, described in detail below.

**Uncoupling of Cdc31p localization and G2/M arrest:**

Cdc31p localizes to the SPB where it activates SPB duplication (Spang et al. 1993; Biggins and Rose 1994; Vallen et al. 1994). Interestingly, some of the *cdc31* mutations dissociate these two functions. Three alleles (*cdc31*-21, -49, and -89) showed normal localization to the SPB and a relatively high degree of G2/M arrest (49–59%; lines 2, 5, and 6 in Table 3), indicating that they uncouple SPB duplication from Cdc31p localization. We propose that these alleles affect interactions with additional components downstream in the SPB duplication pathway. The *cdc31*-49 and *cdc31*-21 alleles had additional Kic1p-related defects, suggesting that they affect multiple functions, similarly to previously identified *cdc31*-1, -2, -5, and -16 (Biggins and Rose 1994). The *cdc31*-89 allele (shown in yellow in Figure 1) did not have obvious additional defects, suggesting that it may be specifically defective for SPB duplication.

**Distinct regions of Cdc31p bind to and activate Kic1p:**

Mutations in the middle region of Cdc31p caused severe defects in Kic1p kinase activity. Most notably, mutations *cdc31*-152, -159, -166, and -158 (line 4 in Table 3, shown in blue in Figure 1) caused specific defects in activating Kic1p because the proteins did bind to Kic1p and the mutants did not have a pronounced G2/M arrest defect. Although Cdc31p was not present at the SPB in these alleles, the lack of G2/M arrest suggests that adequate levels of Cdc31p have localized to the SPB but were not detected by immunofluorescence. These alleles may provide a means for elucidating the mechanism by which Cdc31p activates Kic1p kinase activity.

Three additional mutations in the middle of Cdc31p, *cdc31*-49, -134, and -145 (lines 6 and 7 in Table 3, shown in red in Figure 1), exhibited strong Kic1p activation defects and Kic1p-binding defects. However, they also exhibited moderate to high G2/M arrest defects, suggesting that they are defective for multiple functions.

Alleles in the carboxy-terminal region of Cdc31p

**Figure 3—Characteristic *cdc31* phenotypes.** (A and B) Mutations in the carboxy-terminal region of Cdc31p caused cells to arrest in G2/M as large-budded cells with one nucleus and a monopolar spindle. Representative *cdc31*-6 cells are shown (A) DAPI; (B) tubulin. (C and D) Localization of Cdc31p to the SPB was detected by immunofluorescence. Shown is a *cdc31*-49 mutant, which does not disrupt localization. (C) DAPI; (D) Cdc31p. (E–G) The FUN-1 stain was used to measure membrane integrity and cell viability. In metabolically active cells with intact membranes, FUN-1 accumulates in the vacuole and forms characteristic red structures. In cells with disrupted plasma membranes, FUN-1 stains the cytoplasm bright yellow. (E) wild-type cells; (F) *cdc31*-113 mutant with few inviable cells; (G) *cdc31*-134 mutant with many inviable cells.
CDC31 Alleles Separate Its Functions

Figure 4.—Genetic interactions between kar1-Δ17 and cdc31 alleles. The genotypes of each strain are cdc31Δ::LEU2 kar1-Δ17 [cdc31* HIS3 CEN/ARS], where * represents the different CDC31 alleles. The column on the right shows the effective genotypes, where strains that are kar1-Δ17 contain a URA3 vector plasmid, while strains that are KAR1 contain a KAR1 URA3 CEN/ARS plasmid. Three 10-fold serial dilutions are shown for each strain at each temperature. (A) Alleles cdc31-115, -6, and -152 had the same temperature spectrum alone or in combination with kar1-Δ17, suggesting that they do not interact genetically. (B) Alleles cdc31-138, -54, and -122 showed an enhanced growth defect when combined with kar1-Δ17, with the double mutant having a lower nonpermissive temperature than either single mutant. (C) Alleles cdc31-30, -113, -98, and -134 showed cosuppression phenotype with kar1-Δ17, where the double mutant grew at temperatures permissive for either single mutant. (D) Schematic representation of the genetic interaction on the linear sequence of Cdc31p. Amino acids 45–147 are depicted, because the first 44 and the last 14 amino acids were not mutated in any of the alleles. Vertical bars represent mutations of amino acids, and the color of the bar represents the nature of the genetic interaction as depicted on the left. The numbers under each bar represent the allele number and the numbers at the top depict the approximate amino acid positions.

(cdc31-57, -122, -6, -98, -54, -97, -65, -113, and -21; lines 5 and 8 in Table 3; shown in red in Figure 1) caused a strong defect for Kic1p-binding in the two-hybrid assay. On the basis of the previous analysis, we proposed that these alleles also affect Kar1p binding, suggesting that the Kic1p- and Kar1p-binding domains overlap on the carboxy-terminal surface of Cdc31p. This conclusion is not unprecedented for two reasons. First, calmodulin binds most of its substrates on the same surface, in the central region of the protein. Second, Kic1p and Kar1p share a 15-residue motif that is similar to calmodulin-binding domains that may mediate binding to the same region of Cdc31p for both proteins (Geier et al. 1996; Sullivan et al. 1998).

Strikingly, some alleles that showed a strong defect in Kic1p binding were not defective in Kic1p kinase activity (cdc31-54, -113, and -6). We offer four possible interpretations of these data. One possibility is that when Kic1p cannot bind Cdc31p, then Kic1p is free to interact with and be activated by other proteins. By this model, the alleles in the middle of Cdc31p interact with Kic1p, resulting in a dead-end complex, and therefore Kic1p is no longer free to interact with other proteins. One candidate for such a surrogate activator is calmodulin, because it shares sequence homology with Cdc31p and is known to interact with and activate kinases. Although a two-hybrid interaction between Kic1p and Cm1p was not detected (Sullivan et al. 1998), this was done in the presence of Cdc31p. Second, the two assays may have different levels of sensitivity. For example, the kinase assay may be sensitive enough to detect activation of Kic1p kinase activity even when binding to Cdc31p is greatly impaired. The alleles that caused moderate defects in Kic1p binding and strong defects in kinase activity suggest that Kic1p requires Cdc31p for activity. Presumably, alleles exhibiting wild-type kinase activity but low levels of β-galactosidase activity still bind Kic1p but at levels undetectable by the two-hybrid assay.
TABLE 2

G2/M cell cycle arrest in kar1-Δ17 cdc31: Double mutant combinations (A) that showed enhanced growth defects, (B) that did not show any genetic interactions, and (C) that showed suppression

<table>
<thead>
<tr>
<th>Genotype</th>
<th>8 hr</th>
<th>6 hr</th>
<th>8 hr</th>
</tr>
</thead>
<tbody>
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</tr>
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</tr>
<tr>
<td>cdc31-98 kar1-Δ17</td>
<td>23</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

The percentage of large-budded cells for each culture is shown, representing the G2/M arrest phenotype. (A) All strains are cdc31Δ:LEU2 kar1-Δ17 [cdc31 Δ HIS3 CEN/AR5], where * represents the different CDC31 alleles. Strains designated as kar1-Δ17 contain a URA3 vector, while the other strains contain a [KARI URA3 CEN/ARS] plasmid. The strain designated wild type contains a (CDC31 HIS3) and a [KARI URA3] plasmid. The strain designated kar1-Δ17 contains a [CDC31 HIS3] plasmid and a URA3 plasmid. Strains were grown in synthetic medium lacking uracil and histidine to midlogarithmic phase at 23° and one-half of each culture was shifted to 35° for 8 hr. The relatively low G2/M arrest of kar1-Δ17 may be because the experiment was performed at the intermediate temperature of 35°. (B) All strains are cdc31Δ:LEU2 kar1-Δ17 [cdc31 Δ HIS3 CEN/ARS], where * represents the different CDC31 alleles. The strain designated kar1-Δ17 contains a [CDC31 HIS3] plasmid. Strains were grown in synthetic medium lacking histidine to midlogarithmic phase at 23° and one-half of each culture was shifted to 37° for 6 hr. (C) All strains are cdc31Δ:LEU2 kar1-Δ17 [cdc31 Δ HIS3 CEN/ARS], where * represents the different CDC31 alleles. The strain designated kar1-Δ17 contains a [CDC31 HIS3] plasmid. Strains were grown in synthetic medium lacking histidine to midlogarithmic phase at 23° and one-half of each culture was shifted to 37° for 8 hr.

Third, the fusion proteins in the two-hybrid assay may have different properties from the wild-type proteins. Fourth, Cdc31p may regulate Kic1p both positively and negatively. By this model, Kic1p has constitutive activity when not bound to Cdc31p, regulated activity when bound to Cdc31p, and is not active when bound to mutant Cdc31p defective for activation. Mutations cdc31-115 and cdc31-6 may be defective in negatively regulating Kic1p because they have higher than wild-type kinase activities. Interestingly, whereas cdc31-115 exhibited a higher than wild-type β-galactosidase activity in the two-hybrid assay, cdc31-6 was defective in Kic1p binding. Because these two mutations map to distinct regions of Cdc31p, they may affect Kic1p kinase activity by distinct mechanisms.

A surface on the carboxy-terminal region of Cdc31p may be important for binding Kic1p and Kar1p: Eleven alleles (cdc31-138, cdc31-145, cdc31-21, cdc31-30, cdc31-98), the cells exhibited a higher than wild-type β-galactosidase activity in the two-hybrid assay, cdc31-6 was defective in Kic1p binding. Because these two mutations map to distinct regions of Cdc31p, they may affect Kic1p kinase activity by distinct mechanisms.
to the genetic interactions with the Pkc1p pathway (Khalfan et al. 2000).

**Insights from the phenotypes of cdc31 kar1Δ17 double mutants:** The new cdc31 alleles showed a variety of genetic interactions when combined with kar1Δ17. In principle, we imagine three nonexclusive ways to explain the synthetic lethality and growth defects between kar1Δ17 and the different cdc31 alleles. One type of allele may cause a partial defect in binding to Kar1p. Deleting the Cdc31p-interaction domain on Kar1p with ble mutants: The new cdc31 alleles showed a variety of genetic interactions when combined with kar1Δ17. In the kar1Δ17 mutation may further compromise the Kar1p-Cdc31p interaction, resulting in enhanced growth...
and G2/M defects. Because cdc31-54 and cdc31-122 map to the carboxy-terminal region, we propose that they may be partially defective in binding Kar1p. The second type of allele may cause a defect in binding to an additional SPB component but may allow localization to the SPB by interacting with Kar1p. The kar1Δ17 mutation may sever this residual localization, resulting in the enhanced defect. On the basis of the distant position of cdc31-138, we propose that it may be defective in binding additional SPB components. Finally, other alleles may severely reduce the level or stability of Cdc31p to the extent that there is insufficient protein to interact with Kar1p.

The suppressing alleles represent a particularly interesting class because they mapped to an unexpected region of the protein. At first glance, suppressor mutations might be expected to suppress kar1Δ17 by restoring binding to Kar1p, and as such they are predicted to be rare and to cluster to the Kar1p binding region. However, a relatively high proportion of the temperature-sensitive mutations (19%) suppressed kar1Δ17 and most of them did not map to the putative Kar1p-interacting region. Instead, they mapped to the second EF hand (cdc31-30), the central loop (cdc31-134), the third EF hand (cdc31-98), and the region between the third and the fourth EF hands (cdc31-113). This is in contrast to the more carboxy-terminal location of the earlier set of suppressors (Figure 4 and Vallen et al. 1994). The modes of suppression were also different, with cdc31-30 and cdc31-113 being dominant and cdc31-98 and cdc31-134 being recessive. This is also in contrast to the original set of non-temperature-sensitive suppressors that were all dominant (Figure 4 and Vallen et al. 1994). Three of the four new suppressors showed a Cdc31p mislocalization defect, suggesting that they do not suppress by increasing the affinity to Kar1p. One possible explanation for suppression is that these alleles cause a defect in binding to other ligands and are therefore free to bind Kar1p.

**Are cell lysis and Kic1-kinase activity correlated?** The cluster of alleles causing the highest cell lysis defect coincided with the cluster of alleles exhibiting defects in Kic1p kinase activity in the middle of the protein. However, this correlation was not absolute. Whereas cdc31-134 showed the highest lysis defect (55%) and a strong kinase activity defect, cdc31-159 had a similar kinase defect but negligible lysis. One possibility is that these phenotypes are not related and that cdc31-134 is defective for two different interactions. This would suggest that Cdc31p has Kic1p-unrelated roles in cell wall morphogenesis. In support of this model, the preexisting CDC31 alleles have Kic1p-independent cell morphogenesis defects (Sullivan et al. 1998). Another prediction of this model is that the lysis and Kic1p kinase defects should be separable phenotypes. Whereas we found alleles that are defective in Kic1p kinase activity only, we have not found alleles that lyse but exhibit a wild-type Kic1p kinase activity. Therefore, a second interpretation of our results is that the two phenotypes are related, but that the ability to activate Kic1p is compromised to different degrees in the different cdc31 alleles, in a way that is not measured by the kinase assay. By this model, cdc31-134 would have the most severe defect in activating Kic1p, resulting in appreciable cell lysis, whereas the other alleles are only partially defective. A third possibility is that lysis is a secondary consequence of multiple defects. Therefore, cdc31-134, which has high G2/M arrest and a Kic1p kinase defect, may be defective for multiple functions that in combination lead to lysis, whereas the neighboring alleles are defective only in Kic1p kinase activity.

**Cdc31p and calcium:** Although Cdc31p contains four EF-hand domains, only the first and the fourth EF hands bind calcium (Geier et al. 1996). Mutations in conserved

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**TABLE 3**

Summary of defects of temperature-sensitive cdc31 alleles and the predicted function that they disrupt

<table>
<thead>
<tr>
<th>Allele</th>
<th>G2/M localization</th>
<th>Cdc31p localization</th>
<th>Kic1p kinase activity</th>
<th>Kic1p binding</th>
<th>Putative defective function</th>
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<tr>
<td>1</td>
<td>115, 30</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Novel</td>
</tr>
<tr>
<td>2</td>
<td>89</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>SPB duplication</td>
</tr>
<tr>
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<tr>
<td>7</td>
<td>134, 145</td>
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<td>−</td>
<td>−</td>
<td>−/+</td>
</tr>
<tr>
<td>8</td>
<td>57, 122, 6, 98, 54, 97, 65, 113</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Kic1p and Kar1p binding</td>
</tr>
</tbody>
</table>

+ indicates that the mutants behaved like the wild type with respect to that phenotype. − in the G2/M column indicates that the mutants exhibited >50% large-budded arrest. − in the Cdc31p localization column indicates that the mutant exhibited >50% mislocalization. Ts, a temperature-sensitive defect; ND, not determined.
aspartate residues in the first and fourth EF-hand domains that severely reduced the affinity for calcium were lethal at 23° or 30°, whereas the analogous mutations in the second or third EF hand had no growth defects (Geier et al. 1996). Interestingly, we recovered temperature-sensitive mutations in the second, third, and fourth EF hands. Mutations in the aspartate residues critical for binding calcium were not recovered. We observed extensive clustering of alleles to the third and fourth EF hands, indicating that both of these regions perform important functions. Most mutations in both the third and fourth EF-hand clusters caused severe defects in Kic1p binding. Thus, calcium binding by an EF hand domain, per se (e.g., third EF hand), is not essential for function. This is consistent with the finding that Kic1p kinase activity is not affected by the presence of calcium (Sullivan et al. 1998).

Comparisons between Cdc31p and calmodulin: Cdc31p and Cmd1p share 42% sequence identity (Baum et al. 1986), and the structure of Cdc31p was predicted to be similar to that of calmodulin. In addition, the two proteins may be similar in that they can both bind multiple substrates. The substrate specificity of calmodulin seems to be determined by specific Phe residues in the binding site (Okano et al. 1998). By analogy, it may be possible to identify residues in the carboxy-terminal surface or the internal cavity of Cdc31p that do not lead to temperature sensitivity but that are important for binding either Kar1p or Kic1p.

One major difference between Cdc31p and calmodulin is the importance of the conserved Phe residues. Whereas mutating single Phe residues in calmodulin did not lead to any observable phenotypes, the analogous mutations in edc31 led primarily to lethality. In contrast, single mutations in residues adjacent to the Phe in the internal cavity and on the surface of Cdc31p caused a temperature-sensitive phenotype. Temperature-sensitive point mutations were extremely difficult to isolate in CMD1 (Davis et al. 1986). The greater severity of the Phe → Ala alleles in CDC31 may reflect the greater ease of acquisition of conditional mutants in CDC31 relative to CMD1.

Interestingly, although Phe105 → Ala substitution in Cdc31p resulted in lethality, Phe105 → Leu (edc31-158) or Phe105 → Tyr (edc31-168) substitutions resulted in temperature sensitivity. Phe105 in Cdc31p corresponds to Phe92 in Cmd1p that is mutated in cmd1-226 and results in abnormal actin organization. Cmd1-226p is defective in binding the Ca\(^{2+}\)/calmodulin-dependent protein kinase, but not calcineurin (Okano et al. 1998). Strikingly, the analogous mutations in CDC31, edc31-158 and edc31-168, are also defective in activating a kinase, Kic1p.

Intragenic complementation is often used as a means to define alleles defective in nonoverlapping functions. Calmodulin alleles that affect distinct functions were readily identifiable by their mutual intragenic suppression (Ohya and Botstein 1994a). In contrast, in repeated trials we failed to detect any intragenic complementation between the different CDC31 alleles. However, Cdc31p may oligomerize, precluding simple intragenic complementation. Alternatively, all of the mutants may exhibit sufficient overlap in their defects such that no mutant combination is restored to wild-type function.

In conclusion, fine structure analysis of Cdc31p has identified regions of the protein important for binding its known ligands, as well as regions that potentially mediate interactions with novel targets. Future analysis of the new alleles may identify the novel targets of Cdc31p and elucidate the specific roles of Cdc31p in SPB duplication and cell wall biogenesis.

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LITERATURE CITED


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