

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-Δ17*, 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 BALCZON 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 understand-

ing 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 (PAOLETTI *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

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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 *kar1-Δ17* mutant fails in SPB duplication due to mislocalization of Cdc31p. Cdc31p binds to a small region of Kar1p, which is partially deleted in *kar1-Δ17* (BIGGINS and ROSE 1994; SPANG *et al.* 1995). Dominant mutations in *CDC31* and *DSK2* suppress *kar1-Δ17* by relocalizing Cdc31p to the SPB. In addition, genetic interactions implicate the Pkc1p 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 Ca^{2+} -binding domains (MONCRIEF *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 KRETSINGER 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 *vfl2*, the gene for Chlamydomonas centrin, lead to defects in basal body localization and/or segregation (TAILLON *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 *kar1-Δ17* 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

did not have a localization defect, suggesting that they can bind Kar1p but fail to execute a downstream step. Mutations in the amino-terminal region led to neither SPB- nor Kic1p-related defects, providing candidates for future exploration of novel functions. Our findings represent significant progress in the dissection of the functions of Cdc31p and may provide insight into the function of centrin in other organisms.

MATERIALS AND METHODS

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 XL1-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 congeneric to S288c.

Site-directed mutagenesis of the phenylalanine residues of Cdc31p was performed using the *dut-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 *Sna*BI and *Cla*I 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'-CAC GAC 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 6345 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 (ERDENIZ *et al.* 1997). In the first round of PCR, each allele was amplified with primers PR301 (5'-GAT CCC CGG GAA TTG CCA TGT TAA CTA TCG GTG CAA ATA G-3') and PR302 (AAT TCC AGC TGA CCA CCA TGA TGA GTA AGA ACA GGT CAT C-3'), resulting in molecules tagged with adaptomers **A** and **B**. In parallel, two trun-

cated, overlapping fragments of the *Kluyveromyces lactis URA3* gene (a generous gift of N. Erdeniz) were amplified with primers PR299 (5'-TCT AAG AAG ATT AAA ATT CGT GAA CAC AAA AAG AAA AAG GCA AGA AAG CTG TCC AAA TTA TTA CGA TAA ATC AAT ATC CGT TTT AAG AGC TTG GTG-3')/PR303 (5'-CAT GGT GGT CAG CTG GAA TTC GAT GAT GTA GTT TCT GGT T-3') and PR298 (5'-GAG CAA TGA ACC CAA TAA CGA AAT C-3')/PR304 (5'-CAT GGC AAT TCC CGG GGA TCG TGA TTC TGG GTA GAA GAT 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 *kar1-Δ17 cdc31* double mutants were constructed by transforming strain MS6286 (*kar1-Δ17 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- α -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 μ M (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 (Axiophot; 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° for 4 hr. Cells were harvested and prepared for immunostaining as previously described (ROUT and KILMARTIN 1990) using rabbit anti-Cdc31p polyclonal antibody at a dilution of 1:300 (BIGGINS 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_{ADHI}-GAL4* BD, *TRP1*, 2 μ] and were assayed against a library isolate of *KIC1* (SULLIVAN *et al.* 1998) in plasmid pGAD424 [*P_{ADHI}-GAL4* AD, *LEU2*, 2 μ] (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° until logarithmic phase and one-half of each culture was shifted to 37° for 4 hr. To assay Cdc31p mutant protein levels, 85 μ g 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 (BIGGINS and ROSE 1994) and horseradish peroxidase-conjugated secondary donkey anti-rabbit IgG was used at 1:5000 dilution (Amersham Life Science, Arlington Heights, IL). Signal was detected using ECL Western blotting reagents (Amersham Life Science).

For Kic1p kinase assays, plasmids pEGKT [*P_{GAL}-GST URA3* 2 μ] (MITCHELL *et al.* 1993) and MR3041 [*P_{GAL}-GST-KIC1 URA3* 2 μ] (SULLIVAN *et al.* 1998) were transformed into strains containing each allele integrated into the genome. Cultures were grown at 23° until logarithmic phase in SC medium lacking uracil and containing raffinose 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° and protein extracts were prepared and kinase assays were performed as previously described (LAUZE *et al.* 1995) with slight modifications (SULLIVAN *et al.* 1998).

Protein modeling: The Cdc31p three-dimensional structure was predicted by the SWISS-MODEL protein modeling server (PEITSCH 1995, 1996; GUEX and PEITSCH 1997) and manipulated using the RasMol 2.0 program (SAYLE and MILNER-WHITE 1995). The SWISS-PROT protein database was searched by a fast alignment program (BLASTP) using the protein sequence of Cdc31p. Amino acids 11–161 of Cdc31p were used in the model structure. The templates used to generate the model were the NMR structures for *Xenopus laevis* calmodulin in the absence of calcium (1CFC; KUBONIWA *et al.* 1995) and *Drosophila melanogaster* calmodulin complexed with the calmodulin-binding domain of rabbit skeletal myosin light chain kinase in the presence of calcium (2BBMA and 2BBNA; IKURA *et al.* 1992).

RESULTS

Prediction of the structure of Cdc31p: To aid in the analysis of the mutant alleles of *CDC31*, we used the

SWISS-MODEL protein modeling server to predict the three-dimensional structure of Cdc31p. Cmd1p 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 Ca^{2+} -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 \rightarrow 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 *cdc31-1* 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 centrins, 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.* 1993). 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 by determining the percentage of cells arrested in G2/M with a large bud and a single nucleus. Wild-type cultures showed <5% of cells in this category. The new alleles showed a wide range of G2/M arrest (Figure 2A). On average, alleles in the carboxy-terminal half (amino acids 95–161) showed a high G2/M arrest ($\bar{x} = 60\%$), whereas alleles in the amino-terminal region showed a lower G2/M cell cycle arrest ($\bar{x} = 35\%$). The amino- and carboxy-terminal regions of Cdc31p fold into separate lobes and the carboxy-terminal region had been previously suggested to play a role in SPB duplication because of the position of dominant suppressors of *kar1-Δ17* (VALLEN *et al.*

1994; see also Figure 4). Our results corroborate these observations.

The previously identified *cdc31* mutants arrested at G2/M with monopolar spindles due to the failure in SPB duplication. To confirm that the G2/M arrest of the new mutants was also due to a failure in SPB duplication, we examined their spindles by indirect immunofluorescence of α -tubulin. In all of the new *cdc31* mutants, >95% of the large-budded cells had monopolar spindles, consistent with a failure in SPB duplication (Figure 3, A and B).

Interestingly, for a subset of mutants (*cdc31-134*, *-57*, *-6*, *-89*, *-97*, and *-65*) the number of large-budded cells observed by immunofluorescent staining of tubulin was consistently lower than when identical cultures were observed by DAPI staining. The total number of large-budded cells was reduced by 23–56% ($\bar{x} = 40\%$). Immunofluorescence of these mutants also revealed corresponding increases in the numbers of anucleate cells and unbudded cells with monopolar spindles. The two techniques differ in that the immunofluorescence protocol uses Zymolyase to remove the cell walls. Thus, for these mutants, digestion of the cell walls caused a subset of large-budded cells to separate into two unbudded cells, one anucleate 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.

Cdc31p was observed to play a role in cell integrity and cell wall morphogenesis via interaction with Kic1p (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 (see MATERIALS AND METHODS). Cells that have lost plasma membrane integrity exhibit bright yellow cytoplasmic fluorescence. Wild-type cultures showed >95% cells with the orange-red vacuolar structures indicative of metabolically active cells with intact plasma membranes (Figure 3E). For alleles toward the amino- and carboxy-terminal regions, most cells were like the wild type, indicating a low cell lysis defect (Figure 3F). In contrast, several alleles in the middle of the protein caused a high number of cells to exhibit the intense yellow fluorescence indicative of a loss of cell integrity (*cdc31-5*, *-134*, and *-145*; Figures 2B and 3G). These results indicate that the central region of the protein is particularly important for association with proteins required for cell integrity.

Previous analysis showed that the G2/M-arrested cells of *cdc31-1* have a disrupted actin cytoskeleton (SULLIVAN *et al.* 1998). Instead of actin rings around the bud neck, characteristic of large-budded cells, these cells had actin patches scattered throughout the mother and daughter cells. We examined actin localization in the new *cdc31* alleles and found that whereas unbudded and small-budded cells had a wild-type actin cytoskeleton, the large-budded cells in all alleles had the same pattern of scattered actin patches as *cdc31-1* (data not shown). Therefore, dis-

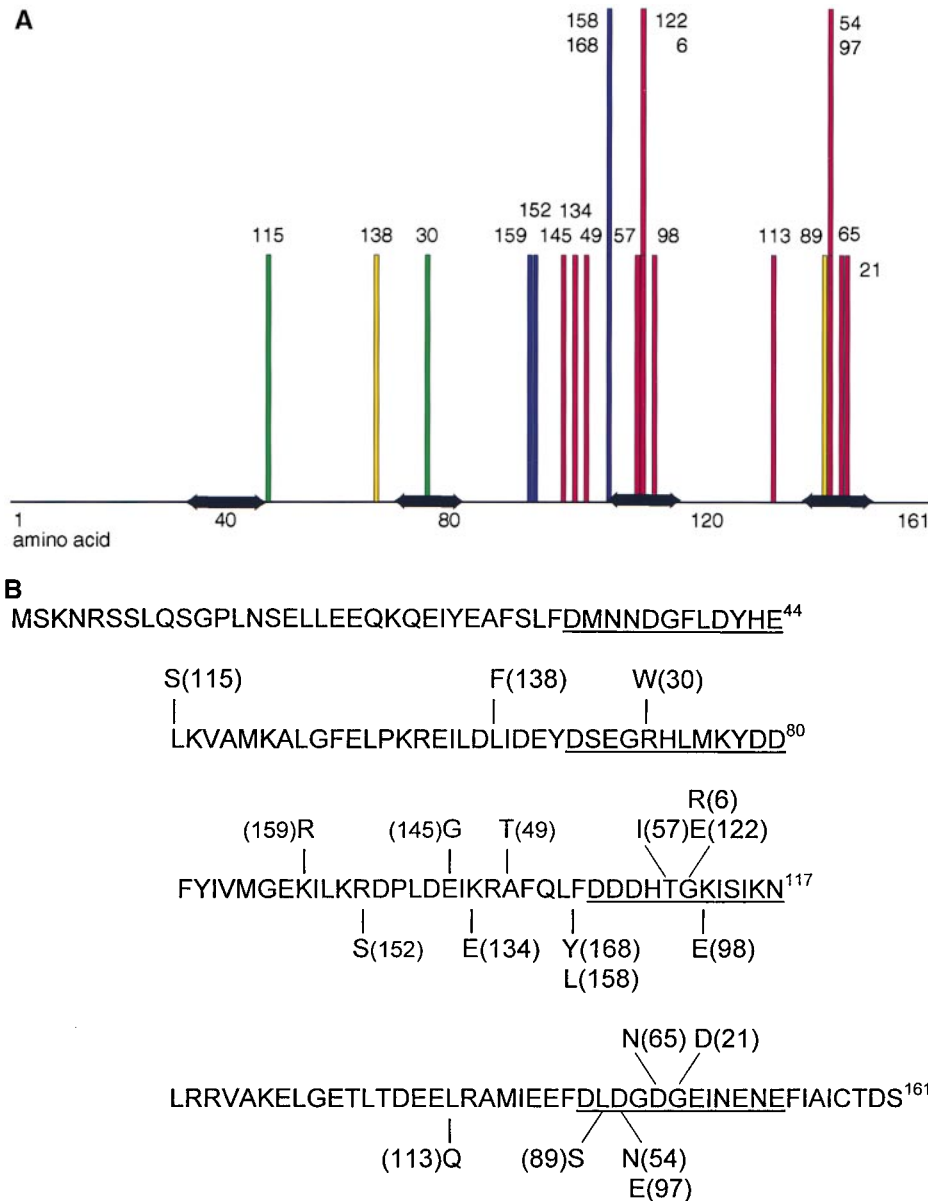


FIGURE 1.—(A) Linear representation of Cdc31p. The entire protein (amino acids 1–161) is shown. The numbers below the horizontal line depict the approximate amino acid positions. The vertical bars represent mutation of an amino acid that resulted in a temperature-sensitive phenotype and the height of each bar represents the number of different alleles recovered at the same position. The number by each bar represents the allele number. The black arrows represent the positions of the four EF hands. (B) The position of the mutations on the amino acid sequence of Cdc31p. The letters above the sequence represent the amino acid substitutions at each position. The numbers in parentheses represent the allele numbers. (C) Distribution of residues on the predicted three-dimensional structure of Cdc31p mutations, which resulted in temperature-sensitive phenotype. Each part represents a 90° rotation to its neighbor. The color coding corresponds to the predicted functions that are disrupted by each allele. Green depicts alleles that may be disrupted for novel functions, yellow depicts alleles that may be disrupted for functions downstream in the SPB duplication pathway, blue depicts alleles that may be disrupted for Kic1p-related functions, and red depicts alleles that may be disrupted for multiple functions (see DISCUSSION). (D) Distribution of residues in the inner cavity of Cdc31p. The two images are rotated by 180° and the front half of the protein has been excluded from each image. (E) Distribution of Phe residues on the predicted three-dimensional structure of Cdc31p.

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 *CDC31* mutations. 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 *kar1-Δ17* 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 *kar1-Δ17*. The *cdc31* alleles fell into four groups with respect to genetic interactions with *kar1-Δ17* (Figure 4). In the first group of alleles, *cdc31-6*,

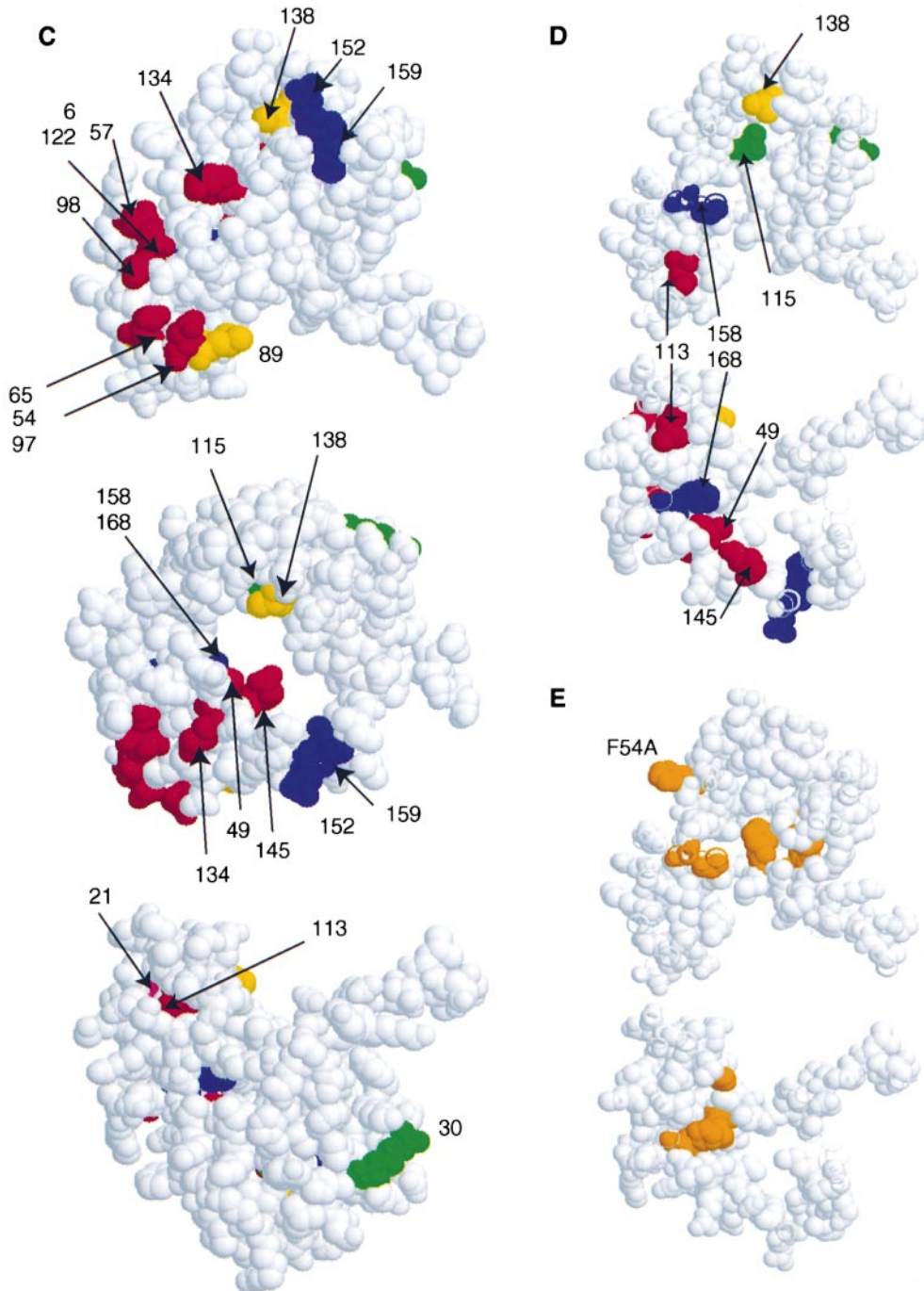


FIGURE 1.—Continued.

-115, and -152, the temperature spectrum was not altered by the presence of *kar1-Δ17* (Figure 4A); these alleles did not show genetic interactions with *kar1-Δ17*. The second group of alleles, *cdc31-21*, -49, -57, -65, -89, -125, -145, -158, -159, and -168 were synthetically lethal with *kar1-Δ17*; 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 *kar1-Δ17* 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 *kar1-Δ17* 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 *kar1-Δ17* 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 *kar1-Δ17* and *CDC31-16* (VALLEN *et al.* 1994). In contrast, cosuppression between *cdc31-113* and *kar1-Δ17* was dominant to *KARI*, but recessive to *CDC31* (Figure 4C and data not shown). The cosuppression in the *cdc31-*

TABLE 1
Amino acid substitutions in the temperature-sensitive
***CDC31* alleles**

Allele no.	Amino acid no.	Amino acid change
-115	45	L → S
-138	64	L → F
-30	73	R → W
-159	91	K → R
-152	92	R → S
-145	97	E → G
-134	99	K → E
-49	101	A → T
-158	105	F → L
-168	105	F → Y
-57	110	T → I
-122	111	G → E
-6	111	G → R
-98	112	E → K
-113	134	I → Q
-89	143	L → S
-54	144	D → N
-97	144	D → E
-65	146	D → N
-21	147	G → D

134 kar1-Δ17 double mutant was recessive to both *KARI* and *CDC31*. Finally, *kar1-Δ17* 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-Δ17* double mutants:

To further analyze the basis of the genetic interactions between *kar1-Δ17* 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 (23°) and the semipermissive (35°) temperatures. The *cdc31-138 kar1-Δ17* mutant had a higher level of large-budded cells (60%) compared to either single mutant [*kar1-Δ17* (22%) and *cdc31-138* (18%)] at 35°.

We also analyzed the alleles that did not show any growth phenotypes in combination with *kar1-Δ17*. Double mutants between alleles *cdc31-6*, *-115*, and *-152* and *kar1-Δ17* 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-Δ17* (Table 2C), suggesting that they suppress the growth defect of *kar1-Δ17* 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 Cdc31 protein. 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,

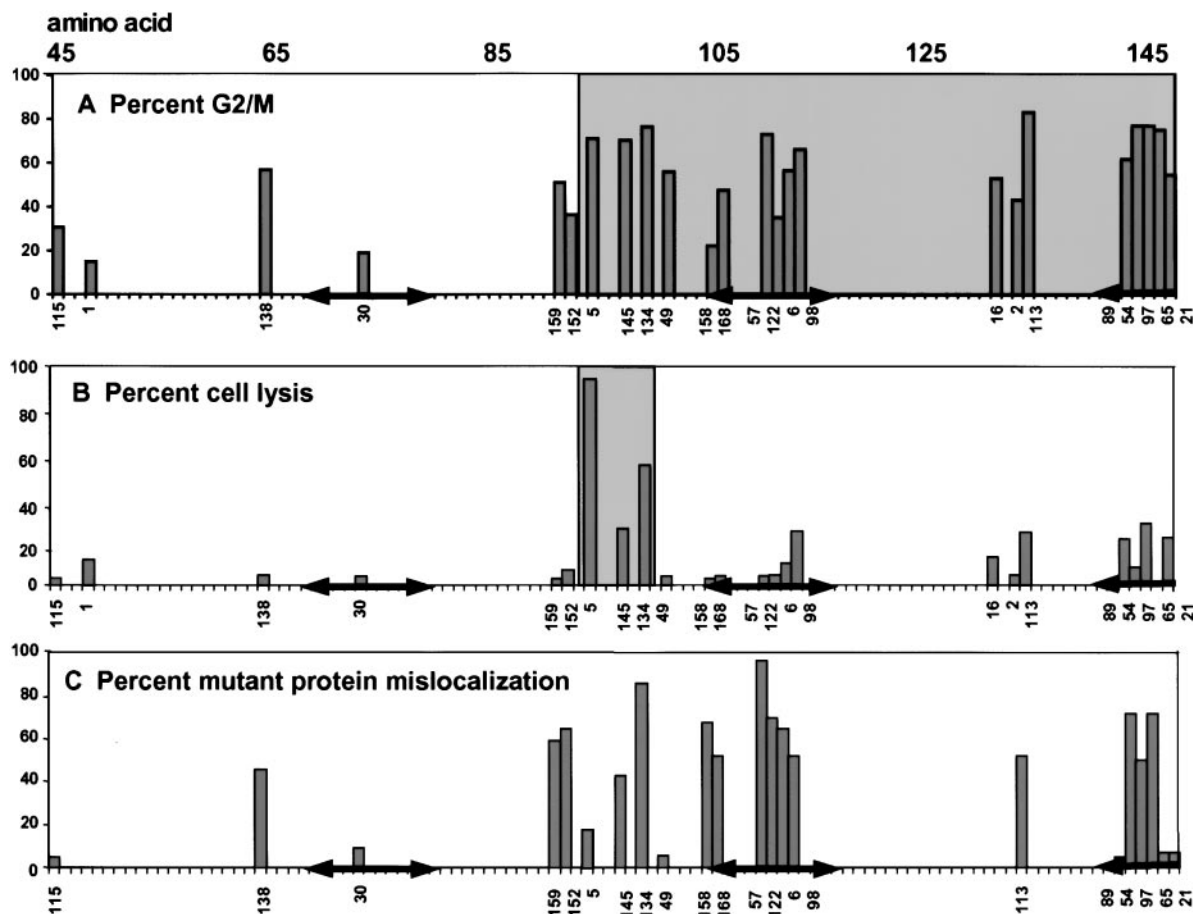


FIGURE 2.—G2/M arrest and cell lysis phenotypes are not correlated. Each part is a separate linear representation of Cdc31p. Amino acids 45–147 are depicted, because mutations in the first 44 and the last 14 amino acids were not recovered. The vertical bars depict the position of each mutation and the 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°. 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°. Alleles in the center of the protein (gray shading) had higher cell lysis than 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°. Results obtained from cultures grown at 30° 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 ($\sim 25\%$ 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

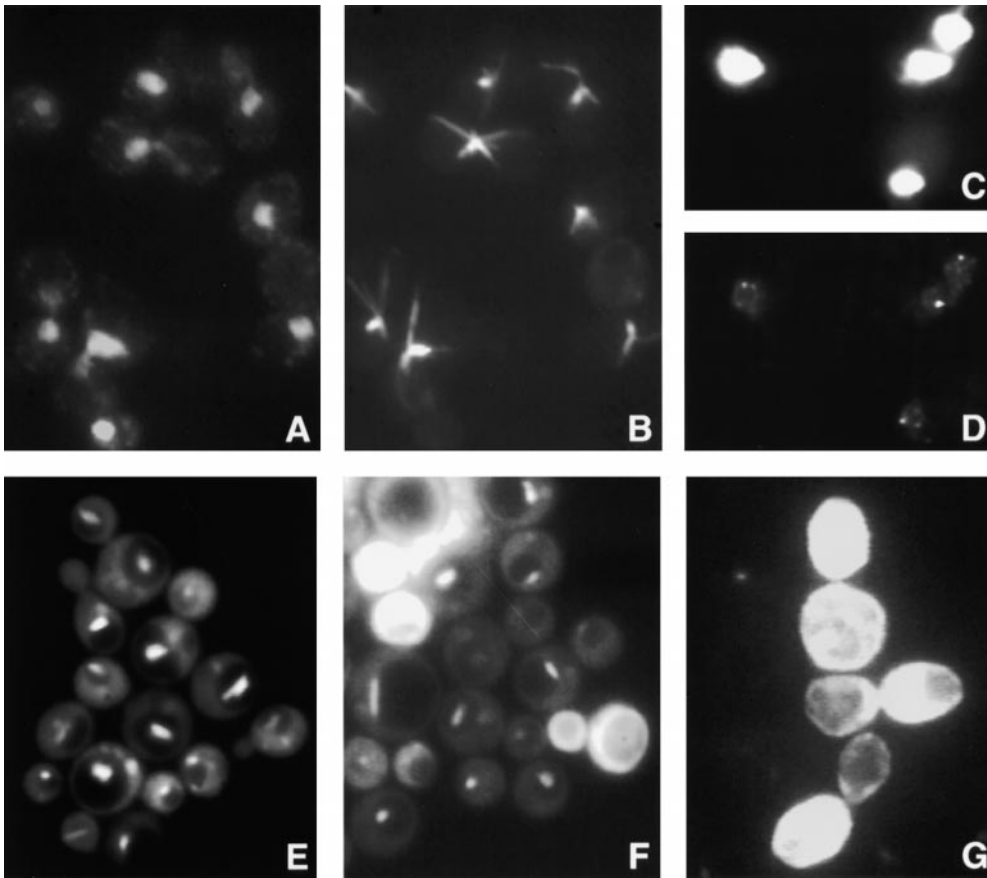


FIGURE 3.—Characteristic *cdc31* phenotypes. (A and B) Mutations in the carboxy-terminal region of Cdc31p caused cells to arrest in G2/M as large-budded cells with one nucleus and a monopolar spindle. Representative *cdc31-6* cells are shown (A) DAPI; (B) tubulin. (C and D) Localization of Cdc31p to 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.

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 30° and 37° (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*, *-168*, 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

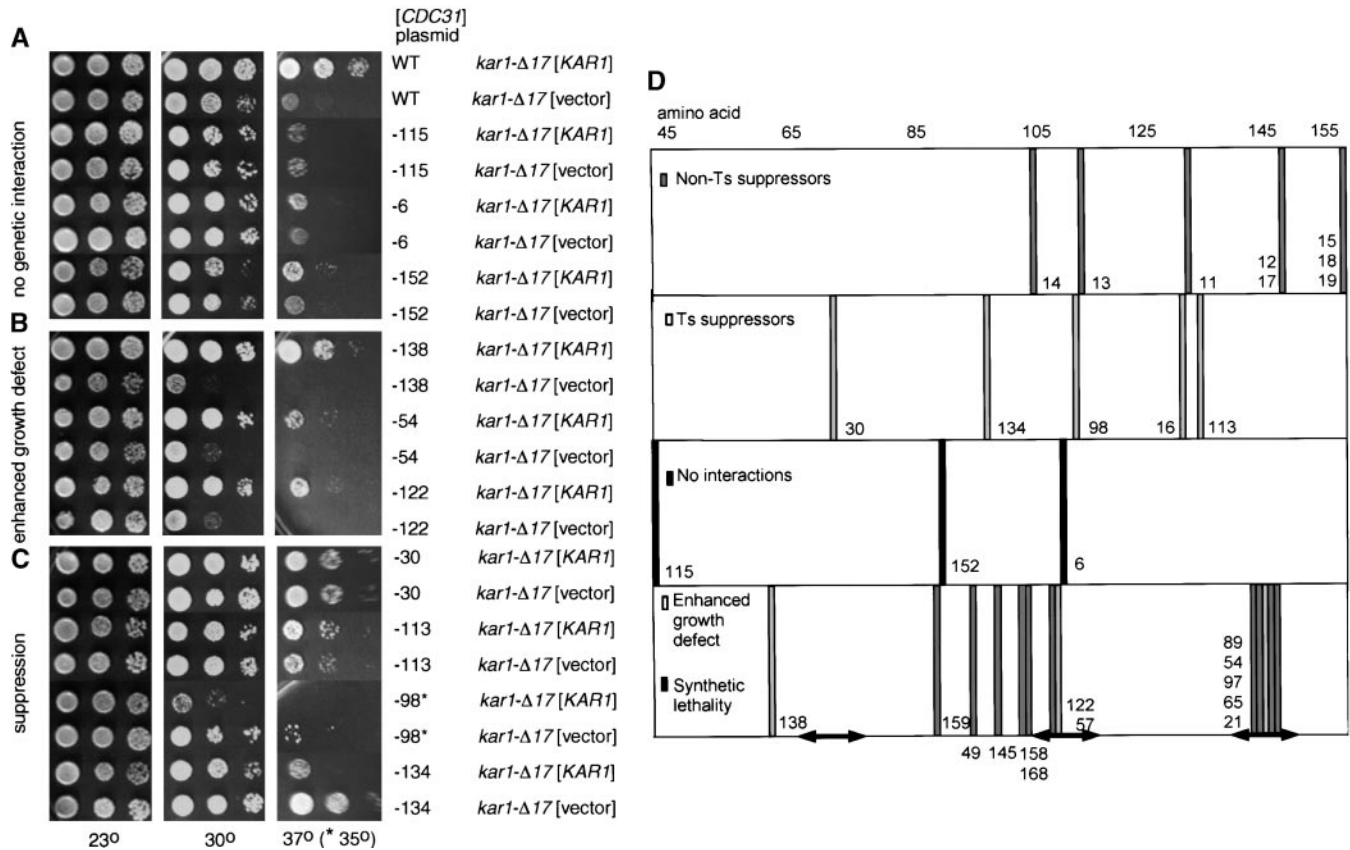


FIGURE 4.—Genetic interactions between *kar1-Δ17* and *cdc31* alleles. The genotypes of each strain are *cdc31Δ::LEU2 kar1-Δ17* [*cdc31** *HIS3 CEN/ARS*], where * represents the different *CDC31* alleles. The column on the right shows the effective genotypes, where strains that are *kar1-Δ17* contain 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 Cmd1p 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

(A) Genotype	8 hr	
	23°	35°
Wild type	18	9
<i>kar1-Δ17</i>	28	22
<i>cdc31-54</i>	21	24
<i>cdc31-54 kar1-Δ17</i>	46	44
<i>cdc31-122</i>	18	20
<i>cdc31-122 kar1-Δ17</i>	49	36
<i>cdc31-138</i>	12	18
<i>cdc31-138 kar1-Δ17</i>	27	60
(B) Genotype	6 hr	
	23°	37°
<i>kar1-Δ17</i>	27	47
<i>cdc31-6 kar1-Δ17</i>	27	50
<i>cdc31-113 kar1-Δ17</i>	32	56
<i>cdc31-115 kar1-Δ17</i>	12	59
<i>cdc31-152 kar1-Δ17</i>	34	54
(C) Genotype	8 hr	
	23°	37°
<i>kar1-Δ17</i>	30	39
<i>cdc31-30 kar1-Δ17</i>	23	20
<i>cdc31-134 kar1-Δ17</i>	10	0
<i>cdc31-98 kar1-Δ17</i>	23	21

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* HIS3 CEN/ARS]*, 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* 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* 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*, *-134*, *-145*, *-57*, *-122*, *-6*, *-98*, *-54*, *-97*, *-65*, and *-113*) showed both Cdc31p mislocalization and high G2/M arrest defects (lines 3, 7, and 8 in Table 3, shown in red in Figure 1). Strikingly, when projected onto the predicted three-dimensional structure of Cdc31p, 8 of these 11 alleles mapped in close proximity, on the same surface on the carboxy-terminal lobe. In addition, *cdc31-113* and *cdc31-21* mapped to the core of the carboxy-terminal lobe, suggesting that they may lead to destabilization of the domain. The carboxy-terminal region of Cdc31p had been previously predicted to play a role in SPB duplication and Kar1p function (BIGGINS and ROSE 1994; VALLEN *et al.* 1994). The surface on the carboxy-terminal lobe may represent the Kar1p-binding site on Cdc31p. Alternatively, this surface may be part of an oligomerization region, because centrins is the major component of contractile filamentous structures associated with MTOCs in Chlamydomonas and mammalian cells (WRIGHT *et al.* 1985; BARON and SALISBURY 1988). The remaining 2 alleles in this class, *cdc31-138* and *cdc31-145*, line the surface of an internal cavity of Cdc31p, analogous to the calmodulin ligand-binding site. Interestingly, these 2 alleles have only a moderate degree of mislocalization as compared to the alleles on the external surface ($\bar{x} = 47\%$ vs. $\bar{x} = 71\%$). One possibility is that they affect binding to additional SPB component(s) that provide a secondary means of Cdc31p localization to the SPB. Alternatively, these alleles may also affect Kar1p binding, if Kar1p and Cdc31p have extensive interactions on different surfaces of Cdc31p.

The amino-terminal region of Cdc31p mediates novel functions: In two mutants, *cdc31-115* and *-30* (green in Figure 1, line 1 in Table 3), Cdc31p localized to the SPB, and the cells exhibited a low G2/M cell cycle arrest (31 and 35%, respectively), suggesting that these alleles most likely do not affect SPB duplication-related functions. Consistent with this, these alleles map far from the putative Kic1p/Kar1p-binding surface and the alleles with high G2/M arrest. In addition, *cdc31-115* and *-30* did not show defects in cell integrity, Kic1p binding, or Kic1p kinase activity, suggesting that these alleles may affect a novel *CDC31* function. An intriguing possibility is that the function of this region of the protein is related

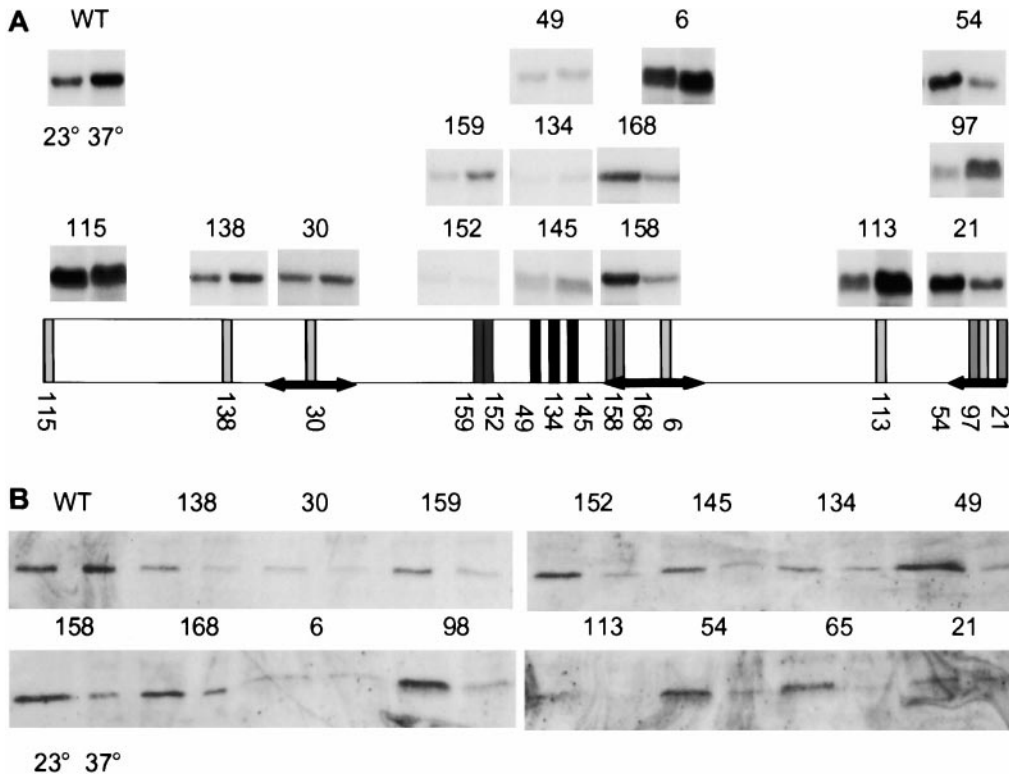


FIGURE 5.—(A) The central part of Cdc31p activates Kic1p kinase activity. The top represents kinase assays performed for each allele at 23° and 37°. The placement of each part corresponds to the position of the allele on the linear representation of the protein. The bottom is a summary of the kinase assay results depicted on the linear sequence of Cdc31p. Each bar corresponds to a mutation. (□) Wild-type Kic1p kinase activity; (▤) temperature-sensitive defect; (■) temperature-independent defect. (B) Steady-state Cdc31p mutant protein levels after growth at 23° or 37° for 4 hr.

to the genetic interactions with the Pkc1p pathway (KHALFAN *et al.* 2000).

Insights from the phenotypes of *cdc31 kar1-Δ17* double mutants: The new *cdc31* alleles showed a variety of genetic interactions when combined with *kar1-Δ17*. In principle, we imagine three nonexclusive ways to ex-

plain the synthetic lethality and growth defects between *kar1-Δ17* 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 the *kar1-Δ17* mutation may further compromise the Kar1p-Cdc31p interaction, resulting in enhanced growth

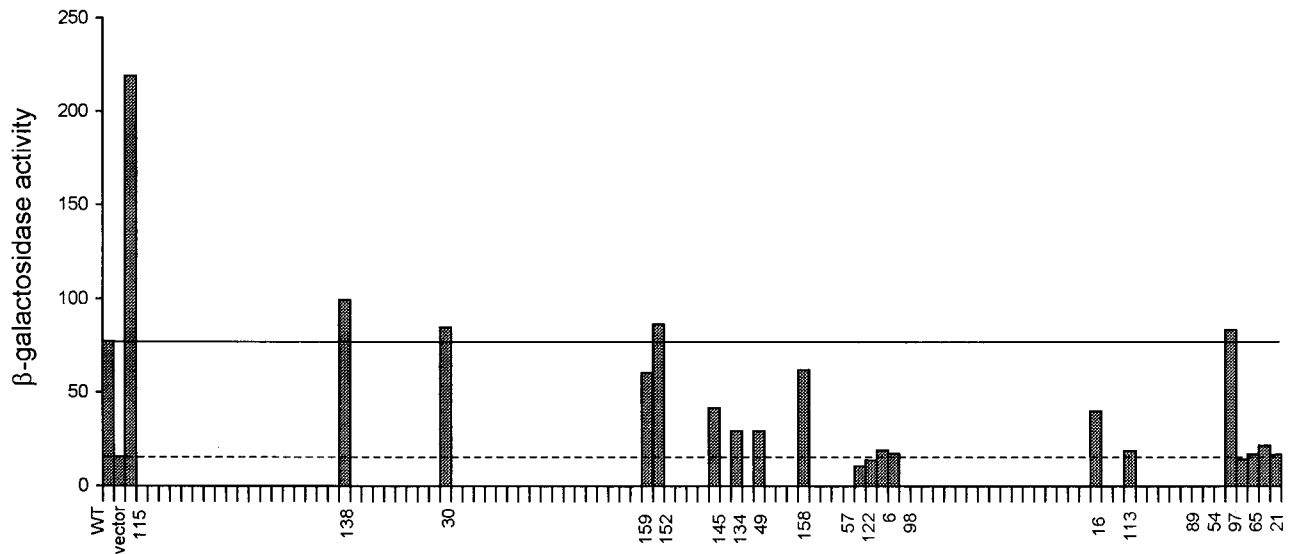


FIGURE 6.—Mutations in the carboxy-terminus of Cdc31p lead to defects in two-hybrid interactions with Kic1p. The x-axis represents the Cdc31 protein. The y-axis represents β-galactosidase activity for each combination of Kic1p and Cdc31 mutant protein. The specific activity is expressed in nanomoles per minute per milligram protein. The wild-type *CDC31* and the vector controls are represented first and the value for each allele is in the position of the mutation. The solid horizontal line represents the level of wild-type β-galactosidase activity whereas the dashed horizontal line represents the level of background β-galactosidase activity.

TABLE 3

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

	Allele	G2/M	Cdc31p localization	Kic1p kinase activity	Kic1p binding	Putative defective function
1	115, 30	+	+	+	+	Novel
2	89	-	+	ND	+	SPB duplication
3	138	-	-	+	+	SPB duplication
4	152, 158, 159, 168	+	-	-	+	Kic1p activation
5	21	-	+	Ts	-	Multiple
6	49	-	+	-	Ts	Multiple
7	134, 145	-	-	-	-/+	Multiple
8	57, 122, 6, 98, 54, 97, 65, 113	-	-	+	-	Kic1p and Kar1p binding

+ 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.

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 *kar1-Δ17* 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 *kar1-Δ17* 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 *kar1-Δ17* 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

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 *cdc31* 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 (*cdc31-158*) or Phe105 → Tyr (*cdc31-168*) 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 Ca²⁺/calmodulin-dependent protein kinase, but not calcineurin (OKANO *et al.* 1998). Strikingly, the analogous mutations in *CDC31*, *cdc31-158* and *cdc31-168*, 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 suppres-

sion (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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