The Yeast Centrin, Cdc31p, and the Interacting Protein Kinase, Kic1p, Are Required for Cell Integrity

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Abstract. Cdc31p is the yeast homologue of centrin, a highly conserved calcium-binding protein of the calmodulin superfamily. Previously centrins have been implicated only in microtubule-based processes. To elucidate the functions of yeast centrin, we carried out a two-hybrid screen for Cdc31p-interacting proteins and identified a novel essential protein kinase of 1,080 residues, Kic1p (kinase that interacts with Cdc31p). Kic1p is closely related to *S. cerevisiae* Ste20p and the p-21–activated kinases (PAKs) found in a wide variety of eukaryotic organisms. Cdc31p physically interacts with Kic1p by two criteria; Cdc31p coprecipitated with GST–Kic1p and it bound to GST–Kic1p in gel overlay assays. Furthermore, GST–Kic1p exhibited in vitro kinase activity that was *CDC31*-dependent. Although *kic1* mutants were not defective for spindle pole body duplication, they exhibited a variety of mutant phenotypes demonstrating that Kic1p is required for cell integrity. We also found that *cdc31* mutants, previously identified as defective for spindle pole body duplication, exhibited lysis and morphological defects. The *cdc31 kic1* double mutants exhibited a drastic reduction in the range of permissive temperature, resulting in a severe lysis defect. We conclude that Kic1p function is dependent upon Cdc31p both in vivo and in vitro. We postulate that Cdc31p is required both for SPB duplication and for cell integrity/morphogenesis, and that the integrity/morphogenesis function is mediated through the Kic1p protein kinase.

Key words: microtubule organizing center • spindle pole body • budding • actin • cell wall

THE microtubule-based cytoskeleton in eukaryotic cells is organized and regulated by microtubule organizing centers (MTOC).[1] The MTOC in animal cells is the centrosome, and the equivalent organelle in the yeast *Saccharomyces cerevisiae* is the spindle pole body (SPB) (for reviews see Rose et al., 1993; Winey and Byers, 1993; Kellogg et al., 1994). The centrosome consists of centrioles embedded in an amorphous pericentriolar material. In yeast, the SPB is a trilaminar structure embedded in the nuclear envelope; it contains an inner plaque that nucleates the nuclear microtubules and an outer plaque that organizes the cytoplasmic microtubules (Byers and Goetsch, 1974, 1975). Despite the structural differences between the centrosome and SPB, they have equivalent functional roles and organize the microtubules that carry out microtubule-mediated processes.

The centrosome is a complex organelle consisting of many proteins, most of which are unidentified. One of the few known conserved centrosomal proteins is centrin/caltractin, which was originally identified in the basal body of the unicellular alga *Chlamydomonas reinhardtii* (Huang et al., 1988*a*; Salisbury et al., 1988). In *Chlamydomonas*, centrin is essential for accurate basal body duplication and separation (Taillon et al., 1992) as well as for microtubule severing (Sanders and Salisbury, 1989, 1994). Centrin homologues have been identified in numerous organisms (Levy et al., 1996), including yeast (Baum et al., 1986), mice (Ogawa and Shimizu, 1993), and humans (Lee and Huang, 1993; Errabolu et al., 1994; Middendorp et al., 1997). Centrin is a small calcium-binding protein that is a member of the calmodulin superfamily of proteins (Huang et al., 1988*b*; Salisbury et al., 1988).

Calmodulin is also a conserved centrosomal protein (for review see Means, 1994). In yeast, there is a single essential calmodulin gene, *CMD1* (Davis et al., 1986). Cmd1p

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1. *Abbreviations used in this paper*: 5-FOA, 5-fluoro-orotic acid; DAPI, 4′,6′-diamidino-2-phenylindole; DIC, differential interference contrast; GST, glutathione-S-transferase; MAP kinase, mitogen-activated protein kinase; MTOC, microtubule organizing centers; PAK, p-21–activated kinases; SPB, spindle pole body; YPD, yeast extract with peptone and dextrose.

localizes to the SPB (Geiser et al., 1993; Stirling et al., 1994), and some temperature-sensitive *cmd1* mutants affect SPB function (Geiser et al., 1993; Ohya and Botstein, 1994). In addition to its localization to the SPB, Cmd1p localizes to regions of cell growth (Brockerhoff and Davis, 1992); in unbudded cells, it concentrates in a patch at the presumptive bud site and then, in small buds, it accumulates at cortical sites in the bud tip. As the bud enlarges, Cmd1p disperses before concentrating again at the site of cytokinesis in the neck. This localization pattern is similar to actin cortical patches, and the localization of actin and calmodulin in the bud are interdependent (Brockerhoff and Davis, 1992). At the bud tip, calmodulin binds to the unconventional myosin Myo2p (Brockerhoff et al., 1994), and its localization is largely dependent on Myo2p (Stevens and Davis, 1998). Temperature-sensitive *cmd1* mutations affect actin organization, calmodulin localization, and bud emergence, in addition to SPB function (Davis, 1992; Ohya and Botstein, 1994). Therefore, calmodulin has multiple essential functions that correlate with its multiple sites of intracellular localization.

Yeast centrin, encoded by the *CDC31* gene, is essential for SPB duplication (Schild et al., 1981; Baum et al., 1986). Mutations in *CDC31* block the earliest steps in the duplication of the SPB and lead to enlargement of the remaining unduplicated SPB (Byers, 1981). Mutations in the *KAR1* gene result in SPB phenotypes indistinguishable from those of *cdc31* mutants (Rose and Fink, 1987). Kar1p is also a component of the SPB (Vallen et al., 1992; Spang et al., 1995), and is required to localize Cdc31p to the SPB (Vallen et al., 1994; Biggins and Rose, 1994; Spang et al., 1993, 1995). However, Cdc31p also shows significant localization away from the SPB (Spang et al., 1993; Biggins and Rose, 1994) and recent results show that the majority of centrin in other organisms is also found elsewhere in cells (Paoletti et al., 1996). Therefore, like calmodulin, centrin may have multiple functions.

In this paper, we report the characterization of a protein kinase that interacts with Cdc31p. Identified in a two-hybrid screen for Cdc31p-interacting proteins, Kic1p is an essential protein kinase that phosphorylates substrates in a Cdc31p-dependent manner. Characterization of *kic1* mutants shows that the gene is necessary for cell integrity or morphogenesis but not for SPB duplication. In addition, re-examination of *cdc31* mutants revealed that they also affect cell integrity and morphogenesis. These results indicate that Cdc31p most likely acts through Kic1p to regulate cell integrity or morphogenesis. This is the first example of a centrin acting as a regulator of a protein kinase and the first known function for centrin separate from its roles in microtubule organizing centers.

Materials and Methods

Strain Constructions and Microbiological Techniques

All yeast strains used in this study are listed in Table I. All standard microbiological techniques and media are described in Rose et al. (1990). β-Galactosidase liquid assays were performed on mid-exponential cultures of MY3366, MY3370, MY3470, MY3473, and MY3582 as described in Rose et al. (1990). For plate dilutions, single colonies were picked and resuspended in 200 μl of sterile H_2O in microtiter wells in a 10-fold dilution series. Cells were replica plated to YPD with or without 1 M sorbitol at 23°, 30°, or 37°C and photographed after 4 d.

To construct a *kic1*Δ strain, pMR3062 was digested with XbaI and transformed into MY3492, a wild-type diploid strain. Genomic DNA from Ura^+ transformants was prepared as described in Rose et al. (1990), and the presence of the deletion was confirmed by Southern analysis of the DNA. Tetrad analysis of the diploid (MY3605) determined that *KIC1* is an essential gene. A *LEU2*-marked *kic1*Δ was generated by transforming a PvuII fragment containing *LEU2* obtained from pRS405 (Sikorski and Hieter, 1989) into MY3605 and selecting Leu^+ transformants that were Ura^-. The presence of the deletion in this strain (MY3606) was confirmed by showing that tetrads segregated 2:2 for viability. In other analyses, the *kic1*Δ::*LEU2* mutant was kept alive by a *URA3* plasmid containing wild-type *KIC1* (pMR3026). This strain was sensitive to 5-fluoro-orotic acid (5-FOA).

Plasmid Constructions and DNA Manipulations

All DNA manipulations were carried out as described in Sambrook et al. (1989). All enzymes were obtained from New England Biolabs Inc. (Beverly, MA) and were used according to the supplier's specifications. Plasmids are listed in Table II and/or described where appropriate below.

Primers were from the Princeton University sequencing facility or from GIBCO BRL (Gaithersburg, MD). A Gal4–Cdc31p fusion plasmid, pMR2716, was constructed by isolating the entire *CDC31* gene from pMR2298 (Biggins and Rose, 1994) as a NdeI–SalI fragment, with the NdeI site filled in using the Klenow fragment of DNA polymerase I. This was ligated into plasmid pGBT9 which had been digested with EcoRI, filled in using the Klenow fragment of DNA polymerase I, and then digested with SalI.

The full-length *KIC1* gene was isolated from a YCp50 library (Rose et al., 1987) by plating colonies on LB ampicillin plates and then replica printing onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) as described in Scidmore (1993). Two DNA fragments from the *KIC1* gene, a 2.5-kb BamHI–EagI fragment and a 600-bp BamHI–EagI fragment, were labeled by the random primer method (Feinberg and Vogelstein, 1983) and used to probe the membranes by hybridization. Plasmids from candidate positives were restriction mapped to confirm the presence of the full-length *KIC1* gene. Two clones were obtained containing the entire *KIC1* gene, pMR3015 and pMR3016. Since these plasmids contained >15-kb inserts, smaller subclones were constructed to facilitate further manipulations. The 3.1-kb BamHI fragment from pMR3016 containing the COOH-terminal region of *KIC1* was ligated into pRS416 (Sikorski and Hieter, 1989) at the BamHI site. Plasmids were obtained with the fragment in both orientations, pMR3020 and pMR3021. To regenerate the full-length *KIC1* gene, pMR3020 was digested with XbaI and SphI and ligated with the 2.9-kb XbaI–SphI fragment containing the NH_2-terminal coding region of *KIC1* obtained from pMR3015. The resulting plasmid, pMR3026, contains 5.9 kb of DNA containing the 3.9-kb *KIC1* gene.

The glutathione-S-transferase (GST)-tagged versions of Kic1p were generated as follows. First, the catalytic domain was PCR amplified using primers 5′ (CCTCTAGACATGACGACGAAGCCAC) and 3′ (GGGAAGCTTCTCCTTT*GGATCC*TCATC) and plasmid pMR3015 as template. The 5′ primer had an XbaI site (*underlined*) engineered just upstream of the starting ATG codon and the 3′ primer had a HindIII site engineered (*underlined*) next to a BamHI site (*italics*) contained within *KIC1* to allow subsequent cloning. The 790-bp PCR product was subsequently digested with XbaI and BamHI and ligated into XbaI–BamHI cut pRS416 (Sikorski and Hieter, 1989). The resulting plasmid, pMR3027, was then digested with BamHI to allow the remaining 3.1 kb of the *KIC1* gene obtained from pMR3015 to be ligated in as a BamHI fragment. This yielded the final plasmid, pMR3040, which contains the full-length *KIC1* gene with a XbaI site near the ATG. To generate a fusion of GST to full length Kic1p, an ~4-kb XbaI–HindIII DNA fragment containing the entire *KIC1* gene was isolated from plasmid pMR3040 and ligated into XbaI–HindIII cut pEG(KT) (Mitchell et al., 1993) to generate pMR3041. To construct a fusion between GST and the catalytic domain of Kic1p, the 790-bp catalytic domain PCR product was digested with XbaI and HindIII and ligated into the XbaI–HindIII cut vector pEG(KT) to yield plasmid pMR3030.

To generate the weak *kic1-1* mutation in the catalytic domain, site-directed mutagenesis was performed on the *KIC1* gene. Glutamate 198, which corresponds to the invariant residue in subdomain VIII of kinases (Hanks et al., 1988; Lindberg et al., 1992), was changed to alanine by PCR mutagenesis. An ~570-bp DNA fragment within the *KIC1* catalytic do-

Table I. Strains Used in This Study

Strain	Genotype
Y526	*MAT*α *leu2 his3 trp1 gal4 gal80 gal1-lacZ::URA3*
MY3278	same as Y526 [pMR2716]
MY3279	same as Y526 [pGBT9]
MY3370	same as Y526 [pGBT9 + pGAD424]
MY3366	same as Y526 [pMR2716 + pGAD424]
MY3473	same as Y526 [pGBT9 + pMR2929]
MY3470	same as Y526 [pMR2716 + pMR2929]
MY3582	same as Y526 [pHS14 + pMR2929]
MY3492	*MAT***a**/*MAT*α *lys2-801/+ trp1*Δ*63/trp1*Δ*63 leu2/leu2 ura3/ura3 ade2-101/ADE2 his3*Δ*200/his3*Δ*200*
MY3605	same as MY3492 but *kic1*Δ::*URA3/KIC1*
MY3606	same as MY3492 but *kic1*Δ::*LEU2/KIC1*
MY3636	*MAT*α *ura3-52 leu2-3,112 ade2-101 his3*Δ*200 trp1*Δ*63 kic1*Δ::*LEU2*; [pMR3026]
MY4124	same as MY3492 [pEG(KT), *URA3* 2μ P_{GAL}*GST*]
MY4125	same as MY3492 [pMR3030, *URA3* 2μ P_{GAL}*GST-KIC1 kinase domain*]
MY4126	same as MY3492 [pMR3041, *URA3* 2μ P_{GAL}*GST-KIC1*]
MY4127	same as MY3492 [pMR3141, *URA3* 2μ P_{GAL}*GST–kic1-1*]
MY3899	*MAT***a** *ura3-52 leu2-3,112 ade2-101 his3*Δ*200 cdc31-1* [pMR3041]
MS4048	*MAT***a** *ura3-52 leu2-3,112 ade2-101CDC31-16* [pMR3041]
MS10	*MAT***a** *ura3-52 leu2-3,112 ade2-101*
MY3779	*MAT***a** *ura3-52 leu2-3,112 ade2-101 lys2-801 his3*Δ*200 kic1*Δ::*LEU2*; [pMR3141]
MY4066	*MAT*α *ura3-52 leu2-3,112 ade2-101his3*Δ*200/trp1*Δ*63 kic1*Δ::*LEU2*; [pMR3308]
MY4065	same as MY4066, but [pMR3310]
MY4063	same as MY4066, but {pMR3311]
MY4064	same as MY4066, but [pMR3309]
MY6306	same as MY4066, but [pMR4136]
MY3873	*MAT***a** *ura3-52 leu2-3,112 ade2-101 his3*Δ*200 cdc31-1*
MY2260	*MAT*α *ura3 ade2 met2 cyh*^{r} *cdc31-2*
MY2261	*MAT*α *ura3 leu2 ade2 trp1 cyh*^{r} *cdc31-5*
MS3510	*MAT*α *ura3-52 leu2-3,112 ade2-101CDC31-16*
MY4977	*MAT*α *ura3-52 leu2-3,112 ade2-101 trp1*Δ*63 kic1*Δ::*LEU2 cdc31-1*; [pMR3141]
MY4123	same as MY3492 [pMR3071]
MY5493	same as MY3492 [pMR3071 + pMR2345]

Y526 was from S. Fields. All other strains were constructed for this study. Strains are listed in the order that they appear in the text.

main was PCR amplified using a 5′ primer, CTTCTAGACATGACGACGAAGCCAC, containing an engineered XbaI site (*underlined*), and a 3′ primer, CCTTCCATGATCAC**TGC**TGGAGCC. The 3′ primer spanned a BclI site within the *KIC1* gene (*underlined*). The 3′ primer was designed with a mutation, which changed TTC (encoding glutamate), to TGC (encoding alanine, *bold*). PCR using these primers was performed on pMR3015, the product was subsequently digested with XbaI and BclI, and then ligated into plasmid pMR3040 that had been digested with XbaI and BclI. This generated plasmid pMR3115, a centromere-based plasmid containing the full-length *KIC1* gene with a mutation at amino acid 198. To construct a GST fusion containing this mutation, the ~4-kb XbaI–HindIII fragment was isolated from pMR3115 and ligated into pEG(KT), which had been digested with XbaI and HindIII to generate pMR3141. The plasmids were transformed into yeast strains and protein expression was confirmed by Western blotting with the appropriate antibodies.

To generate the strong *kic1-101* mutation in the catalytic domain, site-directed mutagenesis was performed on the *KIC1* gene. The ATP binding pocket sequence GRGKFGVV was changed to GRSKFGVG using the oligonucleotide GTTATTGGGCGAAGTAAATTTGGTGTGGGTTATAAGGGCTAT and the *dut-ung* mutagenesis method (Bio-Rad Laboratories, Hercules, CA). Analogous mutations were sufficient to reduce the kinase activity of skeletal muscle phosphorylase kinase to negligible levels (Lee et al., 1992).

To generate temperature-sensitive mutant alleles, *KIC1* was amplified using mutagenic PCR conditions (Leung et al., 1989). For these experiments, *KIC1* was subcloned from pMR3026 into pRS414 to make plasmid pMR3256. PCR reactions contained 10 ng of plasmid pMR3256 linearized with EcoRI, 200 ng of forward and reverse primers, 1 mM each dGTP, dCTP, dTTP, 0.2 mM dATP, 3.0–4.0 mM $MgCl_2$, 0.3–1.0 mM $MnCl_2$, and 5 U TaqI DNA Polymerase. For mutagenesis, the coding sequence of the gene was divided into three regions, A, B, and C. Region A was primed with the forward primer (5′-GAGGCAACTCGTCCTACGTGAA) and the reverse primer (5′-GATCGTCTGCTGACAATCTCTC). Region B was primed with the forward primer (5′-GCGCTGGTGGTTCACTGC) and the reverse primer (5′-GCCGGAAACAGATGAGCC). Region C was primed with the forward primer (5′-GGAAGCAGCGGTAGTAACACTG) and the reverse primer (5′-CTTGCGCAGTCGAGTTC-

Table II. Plasmids Used in This Study

Plasmid	Relevant yeast markers
pEG(KT)	*URA3* 2μ P_{GAL}*GST*
pGAD424	*LEU2* 2μ P_{ADH}*GAL4*(*768–881*)
pGBT9	*TRP1* 2μ P_{ADH}*GAL4*(*1–147*)
pHS14	*TRP1* 2μ *GAL4–CMD1*
pMR2345	*LEU2* 2μ *CDC31*
pMR2716	*TRP1 GAL4–CDC31*
pMR2929	*LEU2 GAL4–KIC1*
pMR3026	*URA3 CEN4 ARS1 KIC1*
pMR3030	*URA3* 2μ P_{GAL} *GST–KIC1* (*kinase domain*)
pMR3041	*URA3* 2μ P_{GAL} *GST–KIC1*
pMR3062	*URA3 kic1*Δ
pMR3071	*URA3 CEN4 ARS1* P_{GAL}*–KIC1*
pMR3141	*URA3* 2μ P_{GAL} *GST–kic1-1*
pMR3256	*TRP1 CEN4 ARS1 KIC1*
pMR3308	*TRP1 CEN4 ARS1 kic1-4*
pMR3309	*TRP1 CEN4 ARS1 kic1-2*
pMR3310	*TRP1 CEN4 ARS1 kic1-3*
pMR3311	*TRP1 CEN4 ARS1 kic1-5*
pMR4136	*TRP1 CEN4 ARS1 kic1-101*

pGBT9 and pGAD424 were from S. Fields; pHS14 was from H. Sundberg and T. Davis; pEG(KT) was from R. Deschenes; all other plasmids are from the Rose Laboratory.

CATGT). Reactions were subjected to 30 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min. Mutant alleles were recovered according to the gap repair method of Muhlrad et al. (1992). Competent MY3636 cells (containing pMR3026; *URA3 CEN KIC1*) were co-transformed with PCR product from region A, B or C and pMR3256 (*TRP1 CEN KIC1*) gapped at the corresponding region. Region A plasmids were gapped with NarI and MscI. Region B plasmids were gapped with BsiWI and MscI. Region C plasmids were gapped with MscI and BstBI. Repair of the gapped plasmids by homologous recombination with the PCR products generated Trp^+ CEN plasmids that were selected on medium lacking tryptophan. Trp^+ transformants were then transferred to 5-FOA medium to select for Ura^- segregants. Strains containing only the pMR3256-PCR mutants were then replica plated to YPD plates at 23°C, at 14°, 23°, 30°, and 37°C to screen for temperature-sensitive mutants.

To generate a *kic1* deletion plasmid, the upstream flanking region of *KIC1* was isolated as a NarI–XbaI 340-bp fragment from pMR3015 and ligated into ClaI–XbaI cut pRS406 (Sikorski and Hieter, 1989). This plasmid, pMR3029, was digested with XbaI–SacII and 1.0 kb downstream flanking region of *KIC1* (containing the last 325 bps of *KIC1*) was isolated from pMR3021 as a XbaI–SacII fragment. These were ligated to generate pMR3062, a *URA3*-marked integrating vector which can be digested with XbaI for integration.

Two-Hybrid Screen

The two-hybrid screen was based on the system of Fields and Song (1989). The relevant plasmids are indicated in Table II. Strains were constructed by transformation of plasmids into reporter strain Y526, which contains a GAL_{UAS}-lacZ reporter integrated at the *URA3* locus. As a preliminary control, MY3278 (Y526 containing the Gal4–Cdc31p fusion) was tested for β-galactosidase activity and none was detected (see Table III), confirming that the Gal4–Cdc31p fusion did not activate on its own. Western blotting of extracts prepared from MY3278 confirmed expression of intact Gal4–Cdc31p fusion protein. To screen for Cdc31p-interacting proteins, MY3278 was transformed with two-hybrid libraries (YL-1, YL-2, and YL-3) obtained from S. Fields (University of Washington, Seattle, WA). Transformants were plated on selective media and grown at 30°C. The colonies were replica printed to X-GAL plates (Rose et al., 1990) at 30°C and scored daily for blue color for 7 d. Colonies that turned blue were colony purified on selective media and then replica printed to X-GAL plates to confirm that they produced β-galactosidase. Colonies that re-tested twice after colony purification were subsequently plated on synthetic complete media lacking leucine, which selected for the library plasmid but not the Gal4–Cdc31p fusion plasmid. The colonies were tested for loss of the Gal4–Cdc31p plasmid, and those that lost the plasmid and maintained the library plasmid were tested on X-GAL plates for β-galactosidase expression. Plasmids that did not turn blue on X-GAL when the Gal4–Cdc31p plasmid was absent were then isolated from yeast by the method of Hoffman and Winston (1987) and amplified in bacteria. The plasmids were retransformed into strain MY3278 (containing Gal4–Cdc31p) and strain MY3279 (containing Gal4) and subsequently tested for β-galactosidase expression. Plasmids that turned strain MY3278 blue and strain MY3279 white on X-GAL plates were considered to be Cdc31p-interacting proteins. Approximately 500,000 colonies were screened and 4 plasmids were isolated. These plasmids were sequenced with a *GAL4* primer encoding amino acids 856–861, 5′-TACCACTACAATGGATG. Sequencing was performed using Sequenase version 2.0 (United States Biochemical, Cleveland, OH) according to manufacturer's specifications.

Protein Techniques

Inducible yeast GST–Kic1p fusion proteins were constructed to confirm that Kic1p interacts with Cdc31p. The NH_2-terminal catalytic domain (amino acids 1–263) and the full-length Kic1p protein (amino acids 1–1,080) were both cloned into a yeast GST fusion vector under the control of the galactose-inducible *GAL1* promoter (see above). To determine whether these constructs were functional we tested whether they suppressed a deletion of *KIC1*. Accordingly, the plasmids were transformed into a heterozygous diploid (*KIC1/kic1*Δ*::LEU2*) and the transformants were sporulated. All viable spores containing the *kic1*Δ also contained the full-length GST–Kic1p plasmid, indicating that this fusion can suppress the *kic1*Δ. In addition, the GST–Kic1p fusion could suppress on either glucose or galactose plates at 30°C and 37°C, suggesting that the fusion must be expressed at high enough levels even in the presence of glucose. In contrast, neither the catalytic domain fusion plasmid nor the GST plasmid could suppress the *kic1*Δ on glucose or galactose plates.

GST fusion proteins were purified by the method of Lauzé et al. (1995) with slight modifications. Strains were grown in synthetic complete media lacking uracil with 2% raffinose to early exponential phase. The production of the GST fusions was induced by the addition of 2% galactose to the media for ~4 h. The cells were then harvested and resuspended in breaking buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 1% Triton X-100) and cell extracts were prepared. The extracts were incubated either with glutathione–agarose or with overnight at 4°C. When α-GST antibodies were used, the extracts were centrifuged for 5 min in a microfuge at 4°C to precipitate particulate matter, and the supernatants were then incubated with protein A–Sepharose beads for 2 h at 4°C. The beads were washed 2× in breaking buffer, 2× in breaking buffer with 0.1% Triton X-100, 2× in breaking buffer with 0.02% Triton X-100, and 2× in breaking buffer without detergent. The washed beads were then stored at 4°C and used in subsequent kinase assays. For coprecipitation experiments, the GST–agarose beads were resuspended in sample buffer and the proteins were separated on denaturing polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Inc.) and Western blotted with anti-Cdc31p antibodies to determine whether Cdc31p co-purified with Kic1p. As a control, extracts from cells expressing intact GST were analyzed in parallel. For protein microsequencing, proteins were blotted to PVDF membranes and the sequence determined at the Princeton University Protein Microsequencing Facility. For some Western blot experiments, yeast extracts were prepared by the method of Ohashi et al. (1982). Anti-GST antibodies were obtained from James Broach (Princeton University, Princeton, NJ) or GIBCO BRL and used at a 1:1,000 dilution. Anti-Cdc31p antibodies (Biggins and Rose, 1994) were used at a 1:500 dilution for Western blotting. The gel overlay assays were performed as described in Biggins and Rose (1994).

Kinase Assays

Kinase assays were performed by the method of Lauzé et al. (1995), with slight modifications, using α-GST antibodies to purify the fusion proteins. The assays were performed in a final volume of 25 μl using 5–10 μl of protein A–conjugated beads containing bound GST fusion proteins (~50 ng of fusion protein). The reactions were preincubated in buffer without ATP (50 mM Hepes, pH 7.4, 0.1 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF) sometimes containing either 100 μM $CaCl_2$ or 1 mM EGTA for 30 min at 23° or 37°C. Unlabeled ATP was added to a final concentration of 10 mM and [^{32}P]γ-ATP was added to a final concentration of 0.4 nM. Approximately 0.5 μg of myelin basic protein was added to the reactions. The reactions were incubated at 23° or 37°C for 30 min and terminated by the addition of 10 μl of 2× sample buffer. The samples were separated on denaturing polyacrylamide gels (the unincorporated radioactivity was cut from the bottom of the gel), stained in Coomassie stain (Sambrook et al., 1989), and then dried and autoradiographed at −70°C. Chemicals were obtained from Sigma (St. Louis, MO) and isotopes were obtained from DuPont-NEN (Boston, MA). Kinase assays were quantified using a Molecular Devices PhosphorImager (Sunnyvale, CA).

Microscopic Analysis

For cell cycle analysis, strains were grown to mid-exponential phase in YPD at 23°C and then shifted to YPD with or without 1 M sorbitol. After 4 or 8 h, cells were harvested and fixed with 3:1 methanol/acetic acid. Cells were stained with 10 μg/ml DAPI (4′,6′-diamidino-2-phenylindole) for 30 min at room temperature and analyzed using DIC (differential interference contrast) or fluorescence microscopy. For actin staining, cells were fixed by adding formaldehyde directly to the culture to a final concentration of 4% and incubating at 23°C for 1 h. Fixed cells were permeabilized by incubation with 25 μg/ml Zymolyase 100,000 (ICN Pharmaceuticals Inc., Costa Mesa, CA) in 1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5, and 25 mM β-mercaptoethanol at 37°C for 30 min. Permeabilized cells were stained for immunofluorescence as described by Kilmartin and Adams (1984). Anti-actin antibodies B28 were a gift from T. Wang and A. Bretscher (Cornell University, Ithaca, NY) and used at a dilution of 1:50. FITC-conjugated goat anti–rabbit antibody was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and used at 1:25 dilution. DAPI was obtained from Accurate Chemicals and Scientific Corp. (Westbury, NY).

For electron microscopy cells were grown to mid-exponential phase at 23°C, shifted to 37°C for 8 h, and then fixed and stained as described in Gammie et al. (1998).

Results

A Two-Hybrid Screen to Isolate Cdc31p-interacting Proteins

A two-hybrid screen was performed to isolate potential Cdc31p-interacting proteins. The full-length *CDC31* gene was fused to the DNA binding domain of the *GAL4* gene to generate an in-frame fusion. A library of plasmids containing random genomic fusions to the Gal4p-activating domain was transformed into a strain expressing Gal4–Cdc31p. Four potential Cdc31p interacting clones were isolated. Three of the plasmids did not contain gene fusions but the fourth corresponded to a gene called *NRK1*, previously deposited in Genbank/EMBL/DDBJ by Y. Fukami (Kobe, Hyogo, Japan). No information about the function of the gene had been published; however its sequence indicates that it is an asparagine-rich kinase. We therefore named the gene *KIC1* to indicate its role as a kinase that interacts with Cdc31p. *KIC1* corresponds to the open reading frame YHR102W identified by the yeast Genome Sequencing Project.

Liquid β-galactosidase assays were performed to confirm the interaction between Cdc31p and Kic1p. As reported in Table III, neither the Gal4–Cdc31p fusion nor the Gal4–Kic1p fusion plasmids caused any β-galactosidase expression on their own. Expressed in a strain together, they produced 141 units of β-galactosidase activity, >1,000 times above the background. Because of the the homology between Cdc31p and calmodulin, we also tested whether the Kic1p kinase interacted with calmodulin by the two-hybrid test. A yeast Gal4–calmodulin fusion did not confer any β-galactosidase activity when coexpressed with Gal4–Kic1p (see Table III), indicating that the Kic1p interaction was specific to Cdc31p.

Kic1p Is an Essential PAK Kinase

The full-length *KIC1* gene was isolated from a genomic yeast library by hybridization with a DNA probe from the two-hybrid clone. An *URA3*-marked deletion of the gene was constructed in a diploid by a one-step gene replacement (Rothstein, 1991). Tetrad analysis of the heterozygous diploid (MY3605) demonstrated that *KIC1* is essential for viability. Microscopic examination of the inviable spores determined that they contained four or eight cells, indicating that *KIC1* does not affect germination. In addition, an equivalent *kic1*Δ::*LEU2* heterozygous diploid strain was constructed (MY3606). Leu$^+$ haploid spores from this diploid (e.g., MY3636) were kept alive by a *URA3* plasmid containing wild-type *KIC1*. MY3636 was inviable when the *KIC1* plasmid was lost, over a wide range of temperatures (14°, 23°, and 37°C) on both rich (YPD) and synthetic defined media (see Materials and Methods). We therefore concluded that *KIC1* is an essential gene.

The NH_2-terminal region of Kic1p contains a 280–amino acid domain designated the "catalytic" domain (see Fig. 1) because it contains every residue conserved in protein kinases (Hanks et al., 1988; Lindberg et al., 1992). The putative ATP-binding domain consists of the GXGXXG se-

Table III. Two-Hybrid Interaction between Cdc31p and Kic1p

Strain	Binding domain	Activation domain	Specific activity
MY3370	Gal4	Gal4	0.1
MY3366	Gal4–Cdc31p	Gal4	0.1
MY3473	Gal4	Gal4–Kic1p	0
MY3470	Gal4–Cdc31p	Gal4–Kic1p	141
MY3582	Gal4–calmodulin	Gal4–Kic1p	0.1

Liquid β-galactosidase assays were performed on the indicated strains, which contained various Gal4 DNA–binding domain fusions and activation domain fusions as indicated. Specific activity is expressed as nmoles/min/mg protein (units). The plasmids contained in Y526 are as follows: pGBT9 (Gal4p DNA–binding domain), pGAD424 (Gal4p-activation domain), pMR2716 (Gal4 DNA–binding domain Cdc31p), pMR2929 (Gal4-activation domain Kic1p) and pHS14 (Gal4–calmodulin DNA-binding domain; gift of H. Sundberg and T. Davis).

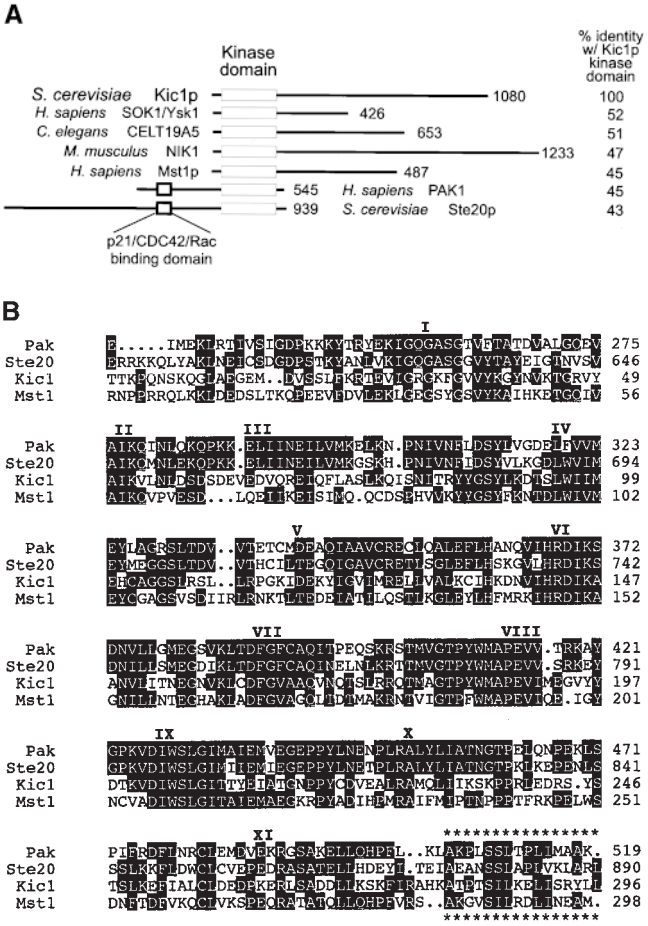

Figure 1. Kic1p kinase catalytic domain. (*A*) The Kic1p-kinase domain is aligned with its closest homologues: SOK1/Ysk1 (Pombo et al., 1996), CELT19A5 (Wilson et al., 1994), NIK1 (Su et al., 1997), MST1 (Creasy and Chernoff, 1995), PAK1 (Brown et al., 1996), and Ste20p (Leberer et al., 1992). The length in amino acids is indicated at the end of the bars. The percentage of identical residues with the Kic1p kinase domain is shown in the far right. (*B*) The NH_2-terminal amino acids of Kic1p are aligned with the catalytic domains of MST1 from *H. sapiens*, PAK1 from *H. sapiens*, and Ste20p from *S. cerevisiae*. Identical residues are indicated by the black boxes and amino acid number is indicated on the right. The 11 subdomains conserved in all kinases are indicated (Hanks et al., 1988; Lindberg et al., 1992). A short domain of additional homology is marked by asterisks above and below the text.

quence motif at position 30 and invariant lysine and aspartate residues at positions 52 and 144, respectively (Hanks et al., 1988). Kic1p is predicted to be a serine/threonine kinase based on the presence of the sequences DIKAAN (amino acids 144–149) and GTPYWMAPE (amino acids 181–189). In addition to the NH_2-terminal catalytic domain, Kic1p contains an 800–amino acid COOH-terminal tail that does not display homology to any other proteins in the database.

Homology searches indicate that the kinase domain of Kic1p shares between 43% and 52% homology with members of the extended PAK/Ste20 kinase family (Fig. 1 *A*). Kic1p, SOK1, CELT19A5, MST1, MST2, and Ste20p, are examples of PAKs that share a common 11–amino acid motif (AKPXSILXD/ELI) just COOH-terminal to the kinase domain (Fig. 1 *B*). This domain is unique to PAK kinases and is similar to a recently identified Ste20/PAK kinase sequence shown to interact with G protein β-subunits (Leeuw et al., 1998). Beyond these regions, Kic1p shows no significant homology to any other known proteins.

Despite the abundance of PAK kinases, relatively little is known about their specific functions. SOK1/YSK1 is a human kinase that is activated three- to sevenfold by oxidant stress (Pombo et al., 1996; Osada et al., 1997). CELT19A5 is a putative open reading frame (ORF) identified in the *C. elegans* sequencing project (Wilson et al., 1994). NIK1 is a mouse kinase that interacts with the SH3 domains of Nck, an adapter protein that is the common target for a number of cell surface receptors (Su et al., 1997). MST1 and MST2 are human kinases that are not involved in the regulation of any known mammalian MAPK pathway, but MST1 activity is apparently stimulated by protein phosphatase 2A (Creasy and Chernoff, 1995). Perhaps more relevant to Kic1p function, PAK1, MUK2, mPAK-3, and β-PAK (Manser et al., 1994, 1995; Bagrodia et al., 1995; Brown et al., 1996; Osada et al., 1997) are all thought to be activated by p21/CDC42/Rac and are implicated in rearrangements of the actin cytoskeleton. Interestingly, hPAK1 and mPAK-3 can both substitute for Ste20p in yeast (Bagrodia et al., 1995; Brown et al., 1996). Ste20p is involved in signal transduction during the yeast pheromone activated mating response (Ramer and Davis, 1993; Herskowitz, 1995). Although Ste20p and the PAKs contain p21/CDC42/Rac-binding sites, there are no predicted binding sites in Kic1p and the other Ste20-like kinases. In *S. cerevisiae* there are at three additional Ste20p-related protein kinases (for review see Hunter and Plowman, 1997): Sps1p, which is induced during meiosis and required for spore wall formation; Cla4p, which is required for cytokinesis; and Skm1p, whose function is unclear but which may be involved in morphogenesis. Kic1p and Sps1p are more closely related to each other than to Ste20p (45% identity over the catalytic domain) and share a similar NH_2-terminal location for the kinase domain. The two other protein kinases are more distantly related to Kic1p and, like Ste20p, have Cdc42-binding sites and COOH-terminal catalytic domains.

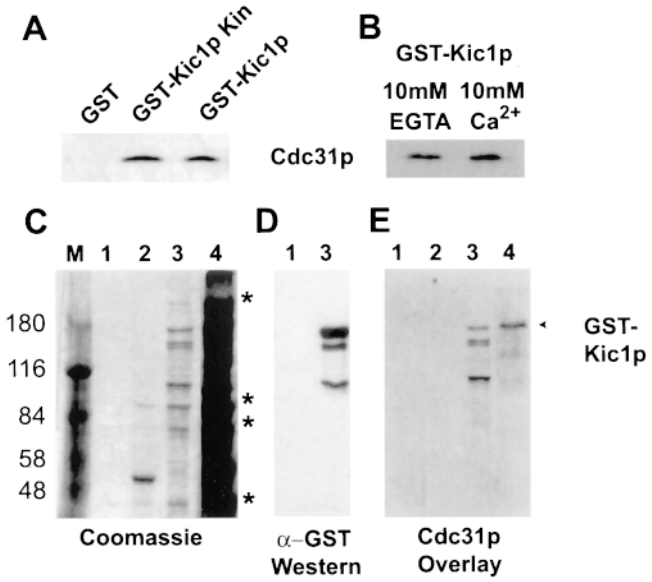

Figure 2. Cdc31p binds Kic1p directly and is part of a larger complex. (*A*) Cdc31p coprecipitates with Kic1p. GST, GST catalytic domain, and GST full-length Kic1p fusion proteins were purified from yeast (strains MY4124, MY4125, and MY4126, respectively) using glutathione–agarose under native conditions, separated on denaturing gels, and then Western blotted with anti-Cdc31p antibodies. Cdc31p band is indicated for part *A* and *B*. (*B*) Cdc31p interaction with Kic1p is calcium independent. The full-length Kic1p fusion protein was purified from MY4126 in the presence of 10 mM EGTA or 10 mM calcium. Equal amounts of purified Kic1p were run on a gel and Western blotted with anti-Cdc31p antibodies. (*C*) Coomassie staining of the pure GST–Kic1p fusion proteins as well as copurifying proteins. Purified GST (lane *1*, strain MY4124), GST–catalytic domain (lane *2*, strain MY4125), and GST full-length fusion proteins (lane *3*, MY4126) were separated on an 8% denaturing gel. Lane *4* contains the total yeast extract from MY4126 expressing GST fused to full-length Kic1p. Copurifying peptides of 200, 80, 70, and 40 kD are indicated with asterisks. The GST protein is not observed because it is <30 kD and therefore ran off the gel. Higher percentage gels were run to monitor GST purification (not shown). *M*, protein molecular weight markers. (*D*) Anti-GST Western blotting showing full-length GST–Kic1p and several degradation products. Lanes marked *1* and *3* are as in *C*. (*E*) Cdc31p binds to full-length GST–Kic1p and its degradation products. Fusion proteins were transferred to membrane and a gel overlay was performed with radiolabeled Cdc31p (Biggins and Rose, 1994). Lanes are marked as in *C*.

Kic1p Interacts Directly with Cdc31p

The two-hybrid analysis suggested that Cdc31p and Kic1p formed a complex in vivo. We wanted to confirm that these proteins interacted by other methods. Inducible GST–Kic1p fusion plasmids were transformed into wild-type strains and protein extracts were prepared using glutathione–agarose. We found that Cdc31p copurified with both the Kic1p catalytic domain fusion (amino acids 1–263) and the full-length Kic1p fusion (Fig. 2 *A*). The interaction was specific because Cdc31p did not coprecipitate with GST alone (Fig. 2 *A*). In an attempt to determine the relative amounts of each protein in the complex, extracts from wild-type cells (not expressing GST–Kic1p) were immunoprecipitated with α-Cdc31p antibodies. The majority of the endogenous Kic1p protein coprecipitated

with Cdc31p, as determined by Western blotting with α-Kic1p antibodies (data not shown). These results indicate that a significant fraction of Kic1p is associated with Cdc31p in vivo and therefore the interaction is likely to be functionally relevant.

Since Cdc31p is a calcium-binding protein (Spang et al., 1993; Biggins and Rose, 1994), we tested whether the interaction with Kic1p depended on the presence of calcium. Full-length GST–Kic1p fusion was precipitated using glutathione–agarose in the presence of either 10 mM calcium or 10 mM EGTA. We found that equivalent amounts of Cdc31p copurified with Kic1p in the presence of EGTA or calcium (Fig. 2 *B*), indicating that the Kic1p–Cdc31p interaction is calcium independent.

To determine whether Kic1p and Cdc31p were part of a larger complex in vivo, we examined the precipitates by Coommassie blue staining (Fig. 2 *C*). Several additional proteins coprecipitated with the GST–Kic1p fusions but not with GST alone (Fig. 2 *C*). Three major bands coprecipitated with both the full-length and the catalytic domain fusion, migrating at 40, 70, and 80 kD (Fig. 2 *C*). In addition, a minor protein of 200 kD also coprecipitated with the full-length fusion protein. We performed peptide microsequencing on the coprecipitating bands. The 70- and 200-kD proteins were NH_2-terminally blocked. The 80-kD protein was determined to be the heat shock protein Hsc82p (Borkovich et al., 1989), a member of the Hsp90 class. It is of interest that an Hsp90p also coprecipitates with centrin purified from *Xenopus* extracts (Uzawa et al., 1995), suggesting that centrins may interact with Hsp90s in all eukaryotes. By Western blotting we determined that the additional bands present in the full-length GST–Kic1p fusion lanes were proteolytic fragments (see Fig. 2 *D*, lane *3*).

Although Kic1p and Cdc31p interact by the two-hybrid test and coimmunoprecipitation, these assays do not distinguish whether the interaction is via a direct or indirect interaction. Therefore, a gel blot overlay assay was performed. This technique was successful in detecting Cdc31p binding to the Kar1 fusion proteins expressed in yeast and in bacteria (Biggins and Rose, 1994; Spang et al., 1995). Both the catalytic domain and full-length GST–Kic1p fusions were purified from yeast under native conditions (Fig. 2 *E*) and binding to radiolabeled Cdc31p purified from bacteria was assayed. We found that Cdc31p bound directly to the full-length GST–Kic1 protein (and proteolytic fragments), but not to the Kic1p catalytic domain (Fig. 2 *E*). Cdc31p binding to GST–Kic1p was also detected in a total yeast protein extract expressing the GST–Kic1p fusion (Fig. 2 *E*, lane *4*). In addition, Cdc31p bound to a 116-kD protein band, which might correspond to wild-type Kic1p (Fig. 2 *E*, lane *4*). In the control experiment, Cdc31p did not bind to purified GST alone (data not shown). Because Cdc31p did not bind directly to the catalytic domain fusion in the gel blot experiment, the Cdc31p-binding site is most likely COOH-terminal to residue 263. This is consistent with the Gal4p fusion protein, which starts at amino acid 260 of the kinase. Interestingly, adjacent to the kinase domain is a stretch of 14 amino acids, LKELISRYLLFRDK, that is 64% similar (50% identical) to the Cdc31p-binding domain described for Kar1p, LIESKWHRLLFHDK (Vallen et al., 1994; Spang et al., 1995). This sequence is just COOH-terminal to, and partially overlaps, the potential G protein β-subunit consensus (Leeuw et al., 1998) described above (Fig. 1 *B*).

Kic1p Has Kinase Activity In Vitro, Which Depends upon Cdc31p

To confirm that Kic1p was a kinase, we tested whether Kic1p had kinase activity in vitro. The GST–Kic1p fusion proteins were purified from yeast using α-GST antibodies and tested for kinase activity using myelin basic protein (MBP) as substrate. We found that GST–Kic1p was associated with a high level of protein kinase activity (Fig. 3 *A*). The level of activity was the same in either 1 mM EGTA or 100 μM calcium, indicating that the Kic1p kinase activity is not regulated by calcium levels. The purified GST protein control exhibited only a slight background kinase activity (Fig. 3 *A*).

A GST–Kic1 mutant protein was constructed to determine whether the kinase activity was due to Kic1p or another copurifying kinase. Site-directed mutagenesis was performed on the *KIC1* gene to change the conserved glutamate in subdomain VIII of the catalytic region (amino acid 189) to an alanine. The corresponding mutation in cAMP-dependent protein kinase reduced but did not eliminate kinase activity (Gibbs and Zoller, 1991). This G189A mutation was made in the GST–Kic1p plasmid and the mutant protein was designated GST–Kic1-1p. Equal amounts of wild-type GST–Kic1p and GST–Kic1-1p were purified from strains grown at 23°C and tested for autophosphorylation and MBP phosphorylation at 23° and 37°C (Fig. 3 *B*). The mutant GST–Kic1-1p fusion protein showed reduced kinase and autophosphorylation activity at 23°C and kinase activity that was further reduced at 37°C (Fig. 3 *B*). Therefore, the kinase activity could be attributed to GST–Kic1p and not to a coprecipitating kinase.

Since Kic1p interacts with Cdc31p we wished to determine if the kinase activity required functional Cdc31p. To test this we purified wild-type GST–Kic1p from *CDC31*, *cdc31-1*, and *CDC31-16* strains. Both *cdc31-1* and *CDC31-16* strains are temperature sensitive for growth at 37°C. GST–Kic1p fusions were purified from each of these strains at the permissive temperature and then GST–Kic1p kinase activity was assayed as above (Fig. 3 *C*). GST–Kic1p purified from both *cdc31-1* and *CDC31-16* strains showed reduced kinase activity at 23°C (~20% and 40%, respectively compared with the wild type) and activity was further reduced to background levels at 37°C (Fig. 3 *C*). Therefore we concluded that Kic1p kinase activity is dependent upon Cdc31p.

KIC1 Is Required for Cell Integrity and Morphogenesis

To understand the functional role of *KIC1* and its interactions with *CDC31*, we generated additional temperature-sensitive alleles. Random PCR mutagenesis of *KIC1* produced 58 different temperature-sensitive alleles. The mutants were divided into three classes. Class A mutants grew poorly at 37°C. Class B mutants showed no growth at 37°C, but the growth defect was suppressed by 1 M sorbitol (an osmotic stabilizer). Class C mutants failed to grow at 37°C but were not suppressed by 1 M sorbitol. Two examples of each class are shown in Fig. 4 in order of their

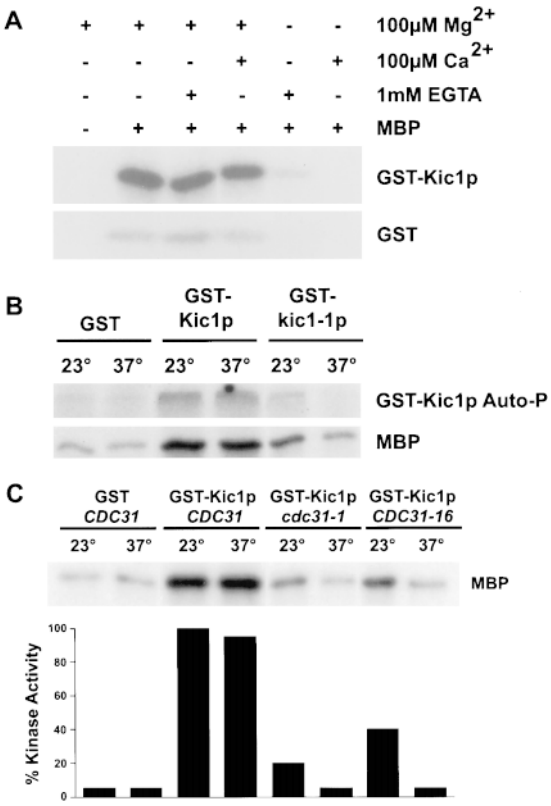

Figure 3. GST–Kic1p kinase activity is Cdc31p-dependent, but calcium-independent. (*A*) GST–Kic1p and GST alone were expressed in yeast (strains MY4126 and MY4124, respectively) and purified under native conditions by immunoprecipitation with anti-GST antibodies. The kinase activity of GST–Kic1p and GST preparations was assayed at 23°C using γ-labeled ATP. The conditions used are shown above the gel with the addition (+) or omission (−) of the following: the test substrate MBP (myelin basic protein), Mg^{2+}, Ca^{2+} or EGTA. (*B*) The kinase activity in the reactions is Kic1p specific. GST, GST-Kic1p, and GST-Kic1-1p were expressed in yeast (strains MY4124, MY4126, and MY4127, respectively) and purified under native conditions by immunoprecipitation with anti-GST antibodies. Kinase assays were performed in standard kinase buffer (see Materials and Methods) using equal amounts of GST–Kic1p or GST–Kic1-1p (proteins amounts were normalized by Coomassie staining and anti-GST Westerns). Protein preparations were assayed for kinase activity at 23°C and 37°C. Both autophosphorylation (auto-P) of GST-Kic1p and phosphorylation of MBP are shown. (*C*) Cdc31p function is required for GST–Kic1p kinase activity. GST was purified from a *CDC31* strain (MY4124) and GST-Kic1p was purified from *CDC31*, *cdc31-1*, and *CDC31-16* strains (MY4126, MY3899, and MS4048, respectively) under native conditions by immunoprecipitation with α-GST antibodies. Strains containing either *cdc31-1* or *CDC31-16* are temperature sensitive for growth at 37°C. Equivalent amounts of GST–Kic1p kinase were used in each assay. Phosphorylation of MBP is shown. GST–Kic1p kinase activity is reduced in the presence of mutant Cdc31-1p or Cdc31-16p at 23°C and is at background levels at 37°C. Kinase activities were quantified by PhosphorImager analysis (see Materials and Methods).

severity, class A being the least severe and class C the worst.

Surprisingly, the kinase-down mutant *kic1-1* (Fig. 3 *B*) was found to be a member of the least severe mutant class (class A), growing poorly at 23° and 37°C, but like wild type at 30°C (data not shown). To determine the phenotype of a mutant with a more severe defect in kinase activity, we constructed a mutation in the ATP-binding pocket that is expected to completely block kinase activity (based on Lee et al., 1992). This mutation, designated *kic1-101*, was expressed from a CEN plasmid and found to partially complement a complete deletion of *kic1*. Although viable, the *kic1-101* mutant exhibits the most severe defect among our collection of mutants, apart from the deletion (Fig. 4). The Gal4–Kic1p fusion plasmid isolated from the two-hybrid screen also complemented a complete deletion of *kic1* and resulted in a mutant phenotype indistinguishable from *kic1-101*. This construct contained only the last 20 amino acids of the kinase domain and the entire COOH-terminal portion of the Kic1p protein. These results demonstrate that the kinase activity of Kic1p is critically important, although not essential, for normal cell function. In addition, they suggest that the non-kinase domain may have a separate important role in cell growth.

Cell cycle analysis demonstrated that the *kic1* mutations resulted in multiple severe morphological defects. Mutants from each class were found to have similar phenotypes. For simplicity, only one mutant from each class is described. The *kic1-1* (class A), *kic1-3* (class B), and *kic1-2* (class C) strains were shifted to 37°C for 8 h, and then analyzed microscopically (Table IV). Wild-type strains were found to have the same cell cycle distributions at 23° and 37°C in both the presence and absence of 1 M sorbitol. Accordingly, only the data for wild type at 23°C in the absence of 1 M sorbitol is shown (Table IV, line *1*). In contrast to the wild type, the class A *kic1-1* mutant grew in chains and clusters of cells, each of which contained a nucleus (Table IV, lines *2–4*; Fig. 5). Many of the cells had abnormally wide bud necks (Fig. 5, *arrows*), and three percent of the cells had lysed (Table IV, line *3*). These results suggested that the *kic1* class A mutations resulted in defects in cell wall assembly and cell separation. The more severe class B *kic1-3* mutant had a strong lysis phenotype at 37°C as evidenced by the frequent presence of cell "ghosts" and cell debris (Fig. 5, *asterisks*; Table IV, lines *5–7*). The inviability and lysis defect of the *kic1-3* mutant was suppressed by the presence of 1 M sorbitol. Similar to the class A mutants, unlysed *kic1-3* cells exhibited wide bud necks at 37°C. When the lysis defect was osmotically suppressed with sorbitol, the *kic1-3* mutant took on the appearance of a class A mutant, growing in clusters and chains (Fig. 5). The *kic1-3* mutant cultures also contained cells with small buds that had completed anaphase (divided nuclei, Table IV, line *6*). The observation that there was no accumulation of binucleate cells with large buds suggested that the mutants might be defective for bud growth. The class C *kic1-2* mutant behaved very much like *kic1-3* at 37°C except that its growth defect was not suppressed by the addition of 1 M sorbitol (Fig. 5; Table IV, lines *8–10*). In the presence of sorbitol, the *kic1-2* mutants were larger and round in appearance but most cells were lysed (Fig. 5, *asterisks*).

Taken together, the cell cycle analysis suggested that *KIC1* is required for some aspect of morphogenesis and to maintain cell integrity. To characterize this further we examined the mutants by electron microscopy. The cell walls

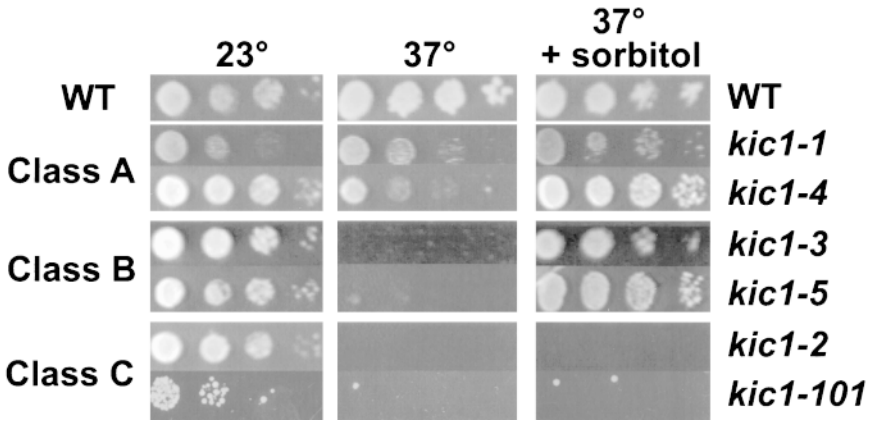

Figure 4. kic1 mutants fall into three classes. Wild-type (MS10), *kic1-1* (MY3779), *kic1-4* (MY4066), *kic1-3* (MY4065), *kic1-5* (MY4063), *kic1-2* (MY4064), and *kic1-101* (MY6306) strains were serially diluted and aliquots were spotted onto YPD or YPD containing 1 M sorbitol media (+ sorbitol) and incubated at the indicated temperatures. At 37°C, class A mutants grew poorly compared with the wild type. Class B mutants fail to grow at 37°C but the growth defect was suppressed by the addition of 1 M sorbitol to the media. Class C mutants failed to grow at 37°C in both the presence and absence of 1 M sorbitol.

in wild type are trilaminate in appearance (for review see Cid et al., 1995). The plasma membrane–cell wall interface stains darkly, whereas the β1-3 glucan–rich middle layer is lighter in appearance. The outer protein-rich mannan layer also stains darkly (Fig. 6). In cross section, the primary septum (PS), composed of chitin, can be seen at the bud neck. Under these conditions, chitin is very electron translucent and typically appears as a thin white line. Surrounding the primary septum at the outer periphery of the bud neck is a ring of chitin, which appears as two white crescents in cross section (Fig. 6, *B* and *D*).

Electron microscopic analysis of the *kic1* mutants revealed a series of distinct cell wall assembly defects. The class A *kic1-1* mutant had a noticeable increase in electron translucent material, presumably chitin, at the bud neck (Fig. 6 *C*, *asterisks*). The extra material was often associ-

Table IV. Cell Cycle Analysis of WT, cdc31, kic1, and cdc31-1 kic2-2 Strains

	Strain	temp	±sorb	⊙	⊙	⊙+⊙	⊙	⊙	lysed
		°C							
(1)	WT	23	−	33	35	0	7	25	0
(2)	*kic1-1*	23	−	40	29	0	9	22	0
(3)		37	−			Clusters & chains			**3**
(4)		37	+			Clusters & chains			0
(5)	*kic1-3*	23	−	21	39	**1**	4	27	0
(6)		37	−	13	8	**12**	2	3	**62**
(7)		37	+			Clusters & chains			0
(8)	*kic1-2*	23	−	28	36	0	8	28	0
(9)		37	−	29	4	**8**	1	2	**57**
(10)		37	+	22	2	**2**	8	2	**54**
(11)	*cdc31-1*	23	−	43	31	0	9	17	0
(12)		37	−	9	2	0	**89**	0	0
(13)		37	+	22	9	0	**67**	2	0
(14)	*cdc31-2*	23	−	33	41	0	6	20	0
(15)		37	−	15	4	0	**72**	0	**9**
(16)		37	+	22	21	0	**54**	3	0
(17)	*cdc31-5*	23	−	40	35	0	5	20	0
(18)		37	−	4	1	0	**39**	1	**55**
(19)		37	+	30	6	0	**30**	2	**32**
(20)	*CDC31-16*	23	−	32	34	0	9	25	0
(21)		37	−	30	7	0	**61**	2	3
(22)		37	+	29	16	0	**53**	2	0
(23)	*cdc31-1 kic1-1*	23	−	25	27	0	20	28	0
(24)		37	−	25	14	0	3	5	**53**
(25)		37	+	21	20	0	12	7	**40**

Wild-type (MS10), *kic1-1* (MY3779), *kic1-3* (MY4065), *kic1-2* (MY4064), *cdc31-1* (MY3873), *cdc31-2* (MS2260), *cdc31-5* (MS2261), *CDC31-16* (MS3510), and *cdc31-1*, *kic1-1* (MY4977) strains were grown to early exponential phase and shifted to the indicated media and temperature for the times shown. Cells were then fixed in 3:1 methanol/acetic acid and stained with the DNA-specific dye DAPI. In each case 200 cells were examined. Where "Clusters & chains" is indicated, cell cycle analysis could not be performed.

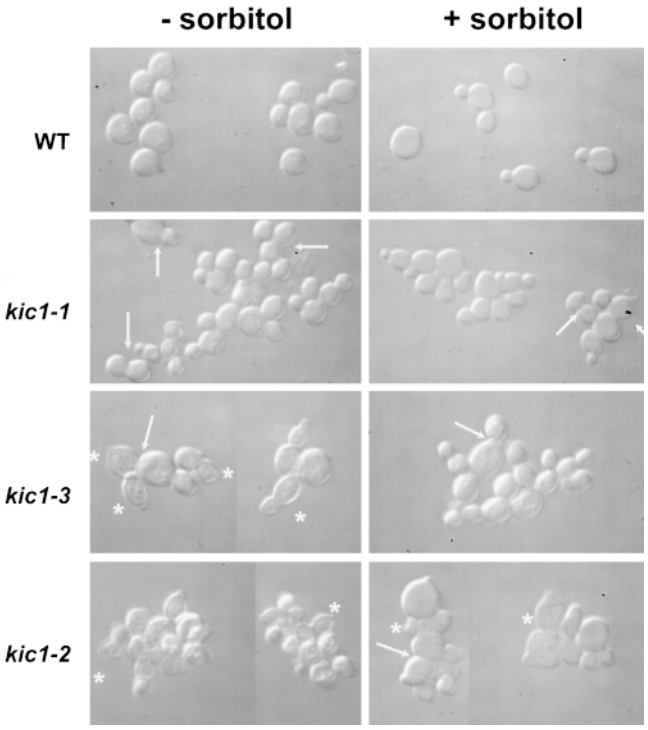

Figure 5. Images of wild-type and *kic1* strains. Wild-type (MS10), *kic1-1* (MY3779), *kic1-2* (MY4064), and *kic1-3* (MY4065) strains were incubated in YPD at 37°C for 8 h, with (+) or without (−) 1 M sorbitol and analyzed microscopically. The asterisks indicate lysed cells or lysed cell contents. Single arrows indicate wide bud necks.

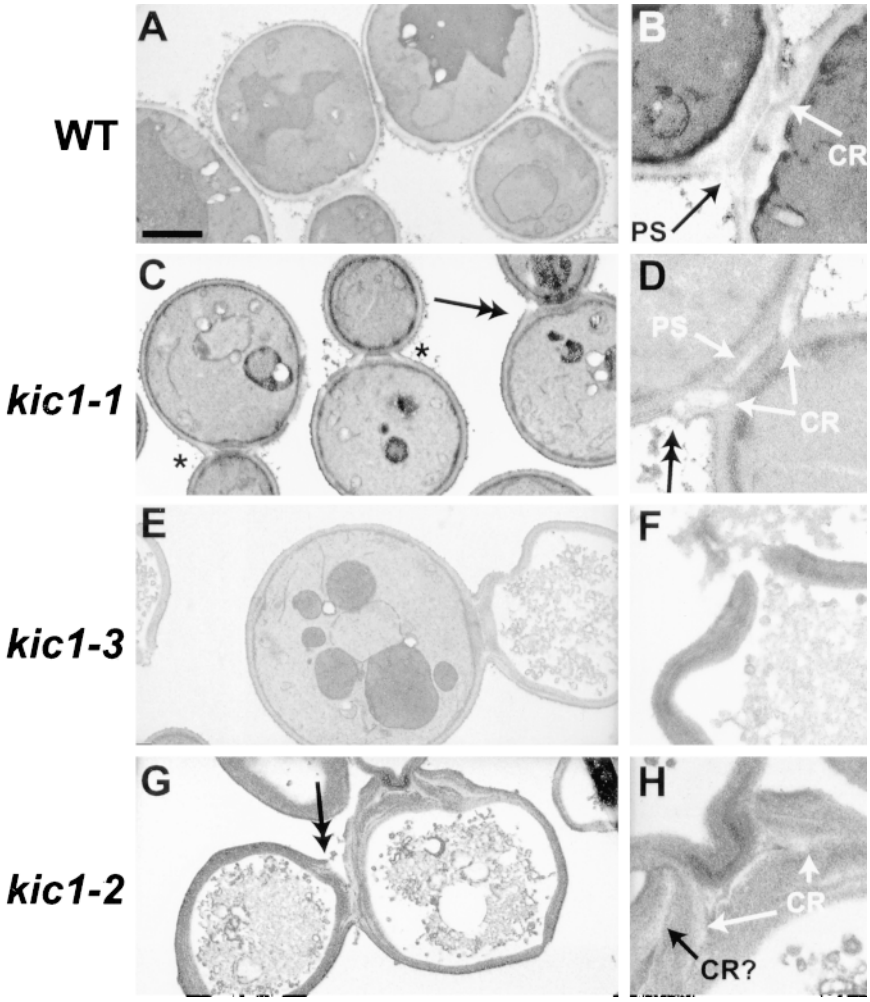

Figure 6. Electron microscopic images of wild-type and *kic1* strains. Wild-type (MS10), *kic1-1* (MY3779), *kic1-2* (MY4064), and *kic1-3* (MY4065) strains were grown to exponential phase at 23°C and then shifted to 37°C for 8 h before being fixed and stained for electron microscopy as described in Gammie et al. (1998). Cells in the *center* and *right columns* are magnified 3.2× compared with the *left column*. Chitin rings (*CR*) and primary septa are indicated (*PS*). "Frayed" or discontinuous cell walls are indicated with double arrows. Bar in left column, 2 μm.

ated with a "frayed" appearance of the cell wall at or near the bud neck (Fig. 6, *C* and *D*, *double arrows*). The primary septa in these cells were thicker than wild type (Fig. 6 *D*, *PS*). In some cases the primary septum appeared as two white lines instead of one.

The cell wall defects of the class B and C mutants, *kic1-3* and *kic1-2*, were more severe than that of *kic1-1*. Many cells were lysed and the cell walls were grossly abnormal (Fig. 6, *E–H*). Instead of the normal trilaminar appearance, the mutant cell walls were thicker and uniformly electron dense (most apparent in Fig. 6, *F* and *H*). They lacked the distinct electron translucent β-glucan layer seen in wild-type cells. The bud necks of class B and C mutants were also wider and thicker than in wild type. Many had multiple layers of electron-translucent material, presumably chitin, at their necks (Fig. 6, *G* and *H*). The cell walls also had a frayed appearance near the bud necks (Fig. 6 *G*, *double arrows*). These observations are in contrast with the phenotype of *PKC1* mutants. *PKC1* mutants lyse at a point of visible thinning of the cell wall, usually at the tips of small buds. In contrast, the *kic1* mutants showed no visible thinning of the cell wall at the lysis point (Fig. 6 *F*) and lysis typically occurred near the bud necks (data not shown).

CDC31 Is Also Required for Morphogenesis and Cell Integrity

We were surprised to find that the *kic1* mutants exhibited phenotypes that were not like the published phenotypes of *cdc31* mutants (Schild et al., 1981; Baum et al., 1986; Vallen et al., 1994). The *cdc31* mutants arrest as large-budded cells with unduplicated spindle poles. In contrast, after shift to 37°C, the *kic1* mutants grew as chains of cells, each containing a nucleus, indicating they had neither a SPB duplication defect nor underwent cell cycle arrest. Since *CDC31* is required for Kic1p kinase activity (Fig. 3) and the kinase mutations exhibit morphological defects, we reexamined the morphology of four *cdc31* mutants at the non-permissive temperature. The *cdc31-1*, *cdc31-2*, *cdc31-5*, and *CDC31-16* strains were shifted to 37°C for 8 h and analyzed microscopically. As previously published, the *cdc31* mutants were found to be defective in SPB duplication, as evidenced by the accumulation of mononucleate large-budded cells (Table IV, lines *11–22*). However, the *cdc31* mutants also exhibited distinct allele-specific morphological defects at the nonpermissive temperature (Fig. 7).

Most strikingly, the *cdc31-5* mutant showed a severe lysis defect similar to that of the class B and C *kic1* mutants

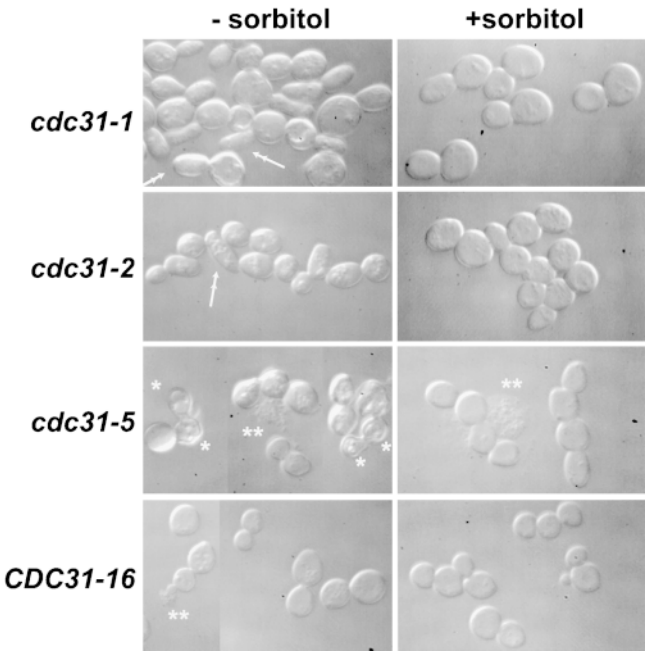

Figure 7. Morphological defects of *cdc31* strains. The *cdc31-1* (MY3873), *cdc31-2* (MS2260), *cdc31-5* (MS2261), and *CDC31-16* (MS3510) strains were incubated in YPD 37°C for 8 h and analyzed microscopically. *Single asterisks*, lysed cells; *double asterisks* indicate cell debris; *double arrows*, long narrow bud.

(Table IV, lines *17–19*; Fig. 7). Lysis was weakly suppressed by the addition of 1 M sorbitol (Fig. 7; Table IV, line *19*). The *cdc31-2* and *CDC31-16* mutants also exhibited weak lysis phenotypes, which were suppressed by 1 M sorbitol (Table IV, lines *14–16*, *20–22*). In Fig. 7, asterisks mark examples of lysed cells, and double asterisks mark cell debris. In addition to the lysis defect, both the *cdc31-1* and *cdc31-2* mutants arrested with an unusual bud morphology; ≤50% of the arrested cells had long narrow buds (Fig. 7, *double arrows*; and Table IV, lines *11–16*). This phenotype was also partially suppressed by the presence of 1 M sorbitol.

Neither the long bud morphology nor lysis has been reported for mutants with defects in spindle assembly that cause a G2/M arrest. We therefore re-examined the large bud arrest phenotype of several yeast β-tubulin mutants (Huffaker et al., 1988) and found no evidence of either elongated buds or cell lysis (data not shown). Furthermore, the long bud phenotype did not lead to lysis in *cdc31-1* (Table IV, lines *11–13*; Fig. 7), nor did lysis of *cdc31-5* and *CDC31-16* require the presence of long buds (Table IV, lines *17-22*; Fig. 7). From these data, we concluded that *CDC31* is also required for cell morphology and integrity.

CDC31 and KIC1 Interact In Vivo

We generated a *kic1-1 cdc31-1* double mutant to look for genetic interactions between *KIC1* and *CDC31*. Neither *kic1-1* nor *cdc31-1* exhibited lysis at 37°C. We reasoned that if the two proteins interact in vivo, then by combining two mild mutations we should be able to generate the lysis defect of the most severe single mutants. When the *cdc31-1 kic1-1* strain was shifted to 37°C it showed a strong lysis defect that was only weakly suppressed by the addition of 1 M sorbitol (Table IV, lines *23–25*; Fig. 8). Thus the double mutant phenotype was equivalent to the severe class C *kic1* mutant. These results strongly suggest that Kic1p and Cdc31p interact in vivo, and that their combined functions are required for normal cell integrity.

The interaction between Kic1p and Cdc31p was further examined by co-overexpression in wild-type cells. When Kic1p was overexpressed, the cultures were viable but grew with elongated cell morphology (Fig. 8) very reminiscent of *cdc31-1* and *cdc31-2* mutants at 37°C (Fig. 7). When Cdc31p was co-overexpressed with Kic1p the cells regained the normal rounded cell morphology (Fig. 8). These results support the view that Kic1p and Cdc31p interact to affect cell morphology.

kic1 Mutants Are Defective for Actin Localization

The cell lysis and abnormal bud phenotypes of *kic1* mutants prompted us to look at their actin distribution. During vegetative growth, actin function and localization is required for normal cell polarity and bud growth (for review see Ayscough and Drubin, 1996). Wild-type cells show a distinct actin localization pattern during the cell cycle (Fig. 9). Actin patches are directed to the bud site and become highly concentrated in the emerging small bud. In large budded cells before cytokinesis, actin redistributes to the bud neck.

All the *kic1* mutants exhibited actin defects (Fig. 9). Small-budded *kic1* cells often had actin at their bud necks (Fig. 9, *asterisks*) instead of being restricted to the bud.

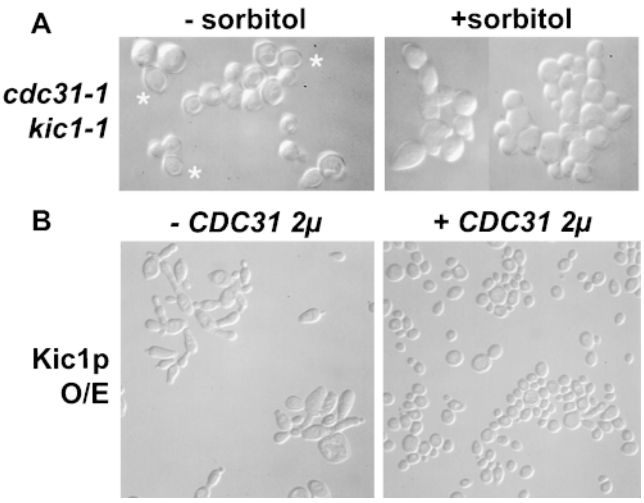

Figure 8. CDC31 and *KIC1* interact in vivo. (*A*) *cdc31-1 kic1-1* double mutants have a synthetic lysis defect. The *cdc31-1 kic1-1* strain (MY4977) was incubated in YPD at 37°C for 8 h in the presence (+) or absence (−) of 1 M sorbitol and analyzed microscopically. *Asterisks*, lysed cells. (*B*) The elongated bud defect of Kic2p overexpression is suppressed by increasing the expression of Cdc31p. Overexpression of the Kic1p (*Kic1p O/E*) protein induces elongated bud growth (−*CDC31 2μ*, strain MY4123) that is suppressed when *CDC31* is expressed off a high copy vector (+*CDC31 2μ*, strain MY5493).

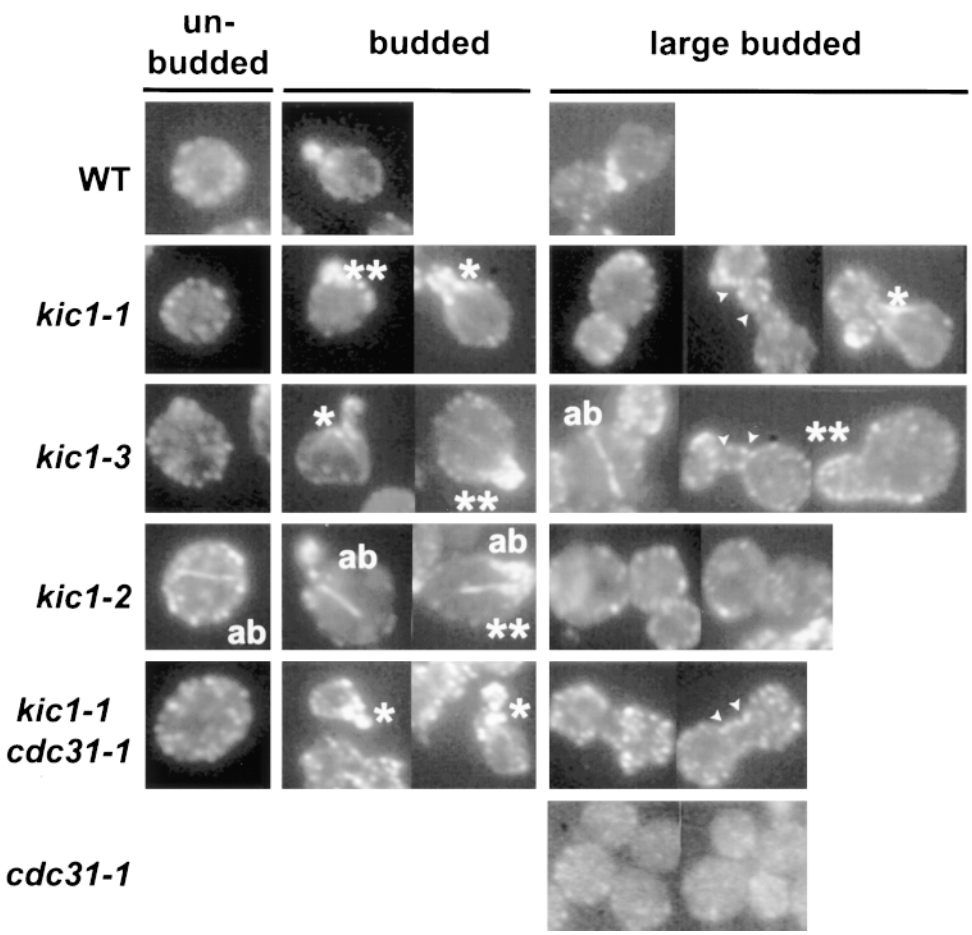

Figure 9. *kic1* and *cdc31* strains have actin localization defects. Wild-type (MS10), *kic1-1* (MY-3779), *kic1-3* (MY4065), *kic1-2* (MY4064), *cdc31-1*(MY3873), and *cdc31-1 kic1-1* (MY4977) strains were grown to exponential phase at 23°C, shifted to 37°C for 4 h, and then prepared for immunofluorescence as described in Material and Methods. A representative gallery of cells (unbudded, budded, and large budded) is shown for each strain. Wild-type (*WT*) cells show a characteristic actin localization pattern during the cell cycle. Unbudded WT cells show no actin concentration. Actin becomes highly concentrated in the emerging small bud of WT cells. Medium-budded WT cells show staining primarily in the bud tip. In WT large-budded cells before cytokinesis, actin redistributes to the septum between the two daughter cells. In the *kic1* mutants actin was often prematurely localized to the necks of emerging buds (*) and buds appeared to be wider than normal (**). In addition, *kic1* mutants also accumulated actin bars (*ab*), which are thought to be agglomerations of improperly folded actin. The *kic1* mutants also accumulated cells with elongated bud necks containing two constrictions (*arrows*).

This aberrant actin continued to localize to the bud neck during bud growth (see budded panel). Often *kic1* mutant cells had abnormally wide bud-necks (Fig. 9, *double asterisks*). The *kic1* mutant cells also often contained actin bars (Fig. 9, *ab*), that are thought to be agglomerations of improperly folded actin. Actin bars were observed in 7% of *kic1-3* cells ($n = 82$), and 17% of *kic1-2* cells ($n = 62$). Furthermore, many *kic1* cells showed an elongated bud neck when the cell wall was removed (Fig. 9, *arrows*). Finally, in contrast to wild type, actin was absent from the bud necks of large-budded *kic1* mutant cells.

Given the actin phenotype in the large-budded *kic1* mutants, it was of interest to examine actin in a *cdc31* mutant. In the *cdc31* arrested large-budded cells actin was not localized to the mother-bud junction (Fig. 9). This may reflect the failure in cell cycle progression in the *cdc31* mutant. In the unlysed *kic1-1 cdc31-1* double-mutant cells, actin showed an aberrant localization quite similar to the *kic1-1* mutant. Taken together, these data suggest that Cdc31p and Kic1p may function in cell integrity by effecting the localization of the underlying actin cytoskeleton.

Discussion

The identification of a Cdc31p-interacting kinase, Kic1p, marks the first report of a centrin associated with a protein kinase. In addition, this is the first report of a non-SPB (centrosome) function for a centrin protein. We demonstrate that Kic1p's kinase activity is dependent upon Cdc31p and that the kinase domain is required for proper cell wall assembly and cell integrity. Examination of four temperature-sensitive *cdc31* alleles, previously characterized as defective for SPB duplication, revealed that these mutants exhibited allele-specific cell integrity and morphology defects. We conclude that that Cdc31p is critically involved in Kic1p's function in cell integrity and morphogenesis, but that Kic1p is not required for Cdc31p's SPB duplication function. Consistent with this, the combination of *cdc31-1* and *kic1-1*, two alleles that do not generate lysis on their own, resulted in a severe synthetic lysis defect, similar to the most severe *kic1* mutation. The simplest interpretation of these results is that Kic1p and Cdc31p interact, in vivo, to regulate some aspect of cell wall assembly.

The identification of allele-specific cell integrity and morphology defects for different *cdc31* alleles was surprising. The *cdc31-2*, *cdc31-5*, and *CDC31-16* mutants had lysis defects of varying severity, and the *cdc31-1* and *cdc31-2* mutants had elongated bud growth. The variation in *cdc31* phenotypes suggests that *CDC31* might be required for more than one morphological process. One possibility is that, like calmodulin, Cdc31p may interact with several different proteins with distinct functions. Different mutations may have more or less severe defects depending upon the specific interactions that are most affected. It is intriguing that *cdc31-1* maps between the two NH_2-terminal EF-hands (A48T), *cdc31-5* maps to the central α-helical domain (P94S), and both *cdc31-2* and *cdc31-16* map between the two COOH-terminal EF-hands (D131N and E133K, respectively; Vallen et al., 1994). Given the location of the mutations, it is tempting to speculate that the central domain is important for cell integrity, whereas the NH_2-terminal half of the protein preferentially effects bud morphology. Whereas *cdc31-1* does not exhibit a lysis defect on its own, the synthetic lysis defect observed for the *cdc31-1 kic1-1* double mutant suggests that this mutation may partially compromise the activity of Kic1p or the interaction between Kic1p and Cdc31p.

In contrast to the effects of loss of function mutations, when wild-type Kic1p was overexpressed it caused an elongated cell phenotype (Fig. 8 *B*), similar to that observed for *cdc31-1* and *cdc31-2*. Interestingly, co-overexpression of Cdc31p caused cells to regain their normal rounded morphology. If the elongated cell phenotype were simply due to overexpression of Kic1p kinase activity, which is Cdc31p dependent, we would expect that co-overexpression of Cdc31p would increase kinase activity and further exacerbate the elongated cell phenotype. However, we found the opposite to be true. We observed suppression of the elongated cell phenotype, and therefore the phenotype appears to be independent of Kic1p kinase activity. Indeed the elongated cell phenotype was also observed when *kic1-1* (a kinase down mutant) was overexpressed (data not shown). We propose that overexpression of Kic1p titrates Cdc31p away from another protein(s) whose interaction with Cdc31p is required for normal cell morphology. However, we cannot rule out the possibility that overexpression of the non-kinase domain of Kic1p generates the elongated cell phenotype.

Our results complement recent unpublished data from the laboratory of F. Klis (Vossen, J., and F. Klis, personal communication). These researchers independently identified *KIC1* on the basis of the sensitivity of the mutant to calcofluor white. They have also found that *kic1* mutants are highly fragile and defective in cell separation. The mutants were resistant to K1 killer toxin, because of reduced β1-6 glucan in their cell walls. They also found that the cell walls were resistant to Zymolyase, a β1-3 glucanase, indicting that the outer protein or mannan layer was less permeable than in wild-type cells. These results are consistent with our electron microscopy observations that show that *kic1* cell walls are disorganized, lack a wild-type glucan layer, and have increased levels of electron-dense mannans. In addition, these researchers have shown that loss of Kic1p function upregulates the HOG pathway (high osmolarity/glycerol). When the HOG pathway is upregulated, cells produce more of the *EXG1* exoglucanase with reduces β1-6 glucan levels. In addition, upregulation of the HOG pathway triggers accumulation of glycerol in cell increasing their osmotic potentials. This could explain part of the lysis defect of *kic1* mutants. Increased osmotic potential would draw more water into the cells creating additional pressure on the cell walls leading to lysis.

The *kic1* mutants lyse near the bud neck suggesting that they are lysing either at the neck, or at the bud or birth scars. Mutations in chitin deposition in the cell wall often lead to lysis at bud or birth scars which are largely composed of chitin (for review see Cid et al., 1995). Our electron microscopic analysis of the *kic1* mutants identified aberrant electron transparent structures, presumably comprised of chitin, at the bud neck. Mutants sometimes had what appeared to be either multiple primary septa or chitin rings. These structures often appeared frayed and looked like weak spots in the wall. It is important to note that the *kic1* defect is not the same as that caused by mutations in *PKC1*, the gene for protein kinase C (Levin and Bartlett-Heubusch, 1992; for review see Cid et al., 1995). Like *kic1* mutants, *pkc1* mutants exhibit lysis that is suppressed by the addition of 1 M sorbitol to the media. However, unlike *kic1-1* the *pkc1* mutants lyse at the tips of small buds, where there is a noticeable thinning of the cell wall. Furthermore, *pkc1* mutants arrest at the G_2–M transition with duplicated DNA content and small buds, whereas *kic1* mutants do not arrest at the G_2–M transition and complete multiple rounds of the cell cycle. Accordingly, the *kic1* mutants accumulate fewer small-budded cells and the lysis defect is not always suppressed by sorbitol. The *kic1* mutants also exhibit an abnormal bud neck phenotype that is not seen in *pkc1* mutants. Therefore we propose that Kic1p and Pkc1p act in separate morphogenic pathways.

Kic1p is a member of a sub-group of PAK1/Ste20p-like kinases that have NH_2-terminal kinase domains and COOH-terminal regulatory regions. Unlike other members of the PAK1/Ste20p family, this subfamily lacks Cdc42- and Rac-binding domains. The demonstration that the kinase activity of Kic1p is Cdc31p dependent raises the possibility that centrin may regulate the activity of NH_2-terminal Ste20p-like kinases in other organisms just as Cdc42p/Rac regulates the function of the COOH-terminal kinases. Interestingly, Kic1p and a subset of PAK kinases contain an eleven amino acid motif (AKPXSILXD/ELI) that is found just COOH-terminal to the kinase domain (Fig. 1 *B*). It has recently been reported that this sequence regulates the binding of G protein β-subunits to PAK/Ste20 kinases (Leeuw et al., 1998). This domain partially overlaps the putative Cdc31p-binding domain of Kic1p (Vallen et al., 1994; Spang et al., 1995). The G protein β-subunit that binds to Ste20p was Ste4p, a protein of 46 kD. It is tempting to speculate that the 40-kD protein we see associated with Kic1p might be a β-subunit that co-operatively or competitively regulates Kic1p function with Cdc31p.

The class B and C mutants (Table IV) also exhibited mild spindle migration defects, which resulted in small-budded binucleate cells. These may be due to the mislocalization of actin during the stages of bud morphogenesis. Previous work has documented that spindle migration and orientation in yeast requires both functional astral micro-

tubules (Palmer et al., 1992; Sullivan and Huffaker, 1992) and a polarized actin cytoskeleton (Palmer et al., 1992; Wang and Bretscher, 1995). In the absence of any obvious defects in microtubule structure (data not shown), it is reasonable to suggest that the *kic1* binucleate phenotype is due primarily to actin defects. The wide bud neck phenotype could also be caused by the observed actin defects. In many cases actin was found at the bud site and neck after bud emergence had progressed significantly. This is not seen in wild-type cells. Since secretion is actin directed this might lead to increased deposition of cell wall materials. In this respect it is important to note that some PAK kinases have been implicated in regulating actin organization.

Cdc31p is involved in both SPB duplication and cell integrity/morphogenesis. Is this a coincidence or are they coordinated in some way? The earliest step in SPB duplication, the appearance of the SPB satellite body, occurs after telophase but before START. Bud growth is initiated at START and must be completed for proper completion of both the nuclear and cell division pathways. The involvement of Cdc31p in two disparate processes raises the possibility that Cdc31p plays an explicit role in the coordination of these two major cell cycle processes.

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