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The *cdr2*$^+$ Gene Encodes a Regulator of G_2/M Progression and Cytokinesis in *Schizosaccharomyces pombe*

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Schizosaccharomyces pombe cells respond to nutrient deprivation by altering G_2/M cell size control. The G_2/M transition is controlled by activation of the cyclin-dependent kinase Cdc2p. Cdc2p activation is regulated both positively and negatively. *cdr2*$^+$ was identified in a screen for regulators of mitotic control during nutrient deprivation. We have cloned *cdr2*$^+$ and have found that it encodes a putative serine-threonine protein kinase that is related to *Saccharomyces cerevisiae* Gin4p and *S. pombe* Cdr1p/Nim1p. *cdr2*$^+$ is not essential for viability, but cells lacking *cdr2*$^+$ are elongated relative to wild-type cells, spending a longer period of time in G_2. Because of this property, upon nitrogen deprivation *cdr2*$^+$ mutants do not arrest in G_1, but rather undergo another round of S phase and arrest in G_2 from which they are able to enter a state of quiescence. Genetic evidence suggests that *cdr2*$^+$ acts as a mitotic inducer, functioning through *wee1*$^+$, and is also important for the completion of cytokinesis at 36°C. Defects in cytokinesis are also generated by the overproduction of Cdr2p, but these defects are independent of *wee1*$^+$, suggesting that *cdr2*$^+$ encodes a second activity involved in cytokinesis.

INTRODUCTION

The eukaryotic cell cycle is regulated by a number of evolutionarily conserved gene products. The fission yeast, *Schizosaccharomyces pombe*, has been used extensively as a model organism to isolate and study these genes, especially those involved in the progression from G_2 to M. The best described of these is *cdc2*$^+$, which encodes a cyclin- dependent protein kinase, whose periodic activation controls progression into both M and S phases of the cell cycle (Nurse and Bissett, 1981; Piggot *et al.*, 1982). Mutational analyses of *S. pombe* have identified temperature-sensitive alleles of *cdc2* that arrest in G_2, unable to progress into M phase, as well as alleles that arrest in both G_2 and G_1, unable to progress into M or S phase (Nurse and Bissett, 1981).

Other regulators of G_2/M progression have also been identified, and several of these encode regulators of Cdc2p (reviewed by Berry and Gould, 1996). *cdc13*$^+$ encodes a B-type cyclin that binds to Cdc2p as a positive regulatory subunit (Booher and Beach, 1988; Hagan *et al.*, 1988; Booher *et al.*, 1989). The gene products of *wee1*$^+$ and *cdc25*$^+$ regulate activation of Cdc2p by phosphorylation (inhibitory) and dephosphorylation (activating) of Cdc2p Y15, respectively, which sets the timing of mitosis (reviewed by Morgan, 1995; Berry and Gould, 1996). Y15 phosphorylation is not only a critical control point for G_2/M progression, but it also determines cell size at mitosis (Gould and Nurse, 1989) and thus provides a link between mitotic control and growth.

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S. pombe cells grow by tip extension, such that increasing cell mass is reflected by increasing cell length (Mitchison, 1957). At the end of the cycle, cells "fission" or divide medially, generating two identical daughter cells. *S. pombe* cells are also able to adjust cell length by changing the timing of mitosis (Fantes, 1977). For a population of cells to be able to maintain an average cell size, cells born with a smaller-than-average length must spend more time growing, before initiating mitosis and dividing, than do cells born with an average cell length. The reverse is true for cells born with a larger-than-average cell length.

The sizing mechanism linking growth to division, or the G_2/M cell size checkpoint, has been studied extensively in *S. pombe* through the analysis of mutant alleles of *wee1* (Nurse, 1975; Fantes and Nurse, 1978; Thuriaux *et al.*, 1978; Nurse and Thuriaux, 1980; Fantes, 1981; Russell and Nurse, 1987a). The G_2/M cell size checkpoint is missing in *wee1* mutants, so that cells initiate mitosis at a length much shorter than wild type (Fantes and Nurse, 1978). Because *wee1* cells spend a longer time than wild type in G_1, it was also determined that a G_1/S cell size checkpoint exists, forcing cells to achieve a critical cell length in G_1 before progressing into S phase. Since the G_2/M cell size checkpoint is missing in *wee1* mutants, it is thought that this checkpoint operates through Wee1p-inhibitory phosphorylation of Cdc2p Y15.

For both immediate and long-term survival, *S. pombe* cells must be able to respond to changes in nutrition by changing the rate of growth and division (Fantes and Nurse, 1977). Nutritional changes trigger the G_2/M cell size control mechanism, shifting the size required for mitotic progression so that cells growing in rich medium divide with a longer cell size, whereas cells growing in poor medium divide with a smaller cell size. Also, when faced with severe shortages of nitrogen, *S. pombe* cells are able to adapt by altering G_2/M cell size control so that cells arrest in G_1 with a much reduced cell size. If the cells are sexually competent and the proper mating partner is present, they differentiate sexually and mate (reviewed by Egel, 1989). However, if there is no sexual partner present, the arrested cells enter a long-term state of dormancy, also referred to as G_0 or quiescence. Cells arrested in G_0 are able to survive over extended periods of time and are resistant to environmental stresses such as heat shock.

In a search for potential regulators of the G_2/M cell size control, Young and Fantes (1984, 1987) carried out a genetic screen in which they isolated mutants that were unable to alter G_2/M size control in response to nitrogen deprivation. It was anticipated that such a screen would identify genes involved in G_2/M progression control and also those involved in nutritional sensing and monitoring. To date, only one complementation group from this screen, *cdr1*$^+$ (changed division response), has been further characterized (Russell and Nurse, 1987b; Feilotter *et al.*, 1991; Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993; Wu *et al.*, 1996; Belenguer *et al.*, 1997; Rupes *et al.*, 1997; Wu and Russell, 1997). *cdr1*$^+$ was also isolated as *nim1*$^+$ (new inducer of mitosis), a multicopy suppressor of *cdc25*-22 (Russell and Nurse, 1987b). Like all *cdr* mutants, *cdr1/nim1* mutant cells are unable to respond properly to nitrogen deprivation and arrest as large cells in G_2 rather than as smaller cells in G_1 (Young and Fantes, 1984, 1987; Belenguer *et al.*, 1997; Wu and Russell, 1997). Moreover, cycling *cdr1/nim1* cells are longer than wild type, suggesting that their mitotic control has been altered. Molecularly, it has been shown that *cdr1/nim1* encodes a serine-threonine protein kinase and acts as a mitotic inducer by negatively regulating Wee1p activation; thus, it is a component of mitotic control (Russell and Nurse, 1987b; Feilotter *et al.*, 1991; Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993).

We present the characterization of a second complementation group isolated in the Young and Fantes screen. Similar to *cdr1/nim1*, *cdr2* mutant cells initiate mitosis with a cell length longer than wild type and arrest in G_0 from G_2 with a large cell size in response to nitrogen deprivation (Young and Fantes, 1984, 1987; Rupes *et al.*, 1997). We have found that *cdr2*$^+$ is nonessential and is predicted to encode a serine-threonine protein kinase. We present genetic evidence to demonstrate that *cdr2*$^+$ functions in G_2 as a negative regulator of *wee1*$^+$. We also show that *cdr2*$^+$ has an additional role in cell division. Cells lacking Cdr2p or overexpressing *cdr2*$^+$ fail to undergo cytokinesis and septation normally; these defects are independent of Wee1p function.

MATERIALS AND METHODS

Yeast Methods and Strains

S. pombe strains used in this study are listed in Table 1. Strains were grown in yeast extract medium, minimal medium with appropriate supplements, or minimal medium lacking ammonium chloride as the nitrogen source (Moreno *et al.*, 1991). Crosses were performed on glutamate medium (minimal medium lacking ammonium chloride and containing 0.01 M glutamate, pH 5.6). Tetrad analysis was performed as described (Moreno *et al.*, 1991). Yeast transformations were performed by electroporation (Prentice, 1991). Genomic DNA was isolated as described (Moreno *et al.*, 1991; Hoffman, 1993).

Molecular Biology Techniques

All plasmid manipulations and bacterial transformations were by standard techniques (Sambrook *et al.*, 1989). Essential features of plasmid construction are described below. All sequencing was performed using Sequenase 2.0 (United States Biochemical, Cleveland, OH) according to manufacturer's instructions. All PCR reactions except those for the construction of epitope-tagged strains were performed using *Taq* DNA polymerase and the GeneAmp PCR kit (Perkin Elmer-Cetus, Norwalk, CT) in a PTC-100 programmable thermal controller (PTC-100; MJ Research, Watertown, MA) pro-

Table 1. *S. pombe* strain list

Strain	Genotype		Source
GL122	h$^-$	*cdc25*-22::*p*RIP2*cdc*25$^+$ *leu1*-32 *ura4*-D18*his3*-237	Paul Russell
Q1045	h$^+$	*cdc*25-22r1	This study
Q1046	h$^-$	*cdr*2$^+$::pcdr2.2*leu1*-32	This study
KGY1	h$^-$	*leu* 1-32	Paul Nurse
KGY4	h$^-$	*wee1*::*ura4*-$^+$ *ura4*-D18 *leu1*-32	Paul Nurse
KGY19	h$^-$	*cdc*2-33 *leu1*-32	Paul Nurse
KGY43	h$^+$	*cdc*25-22 *leu1*-32	Paul Nurse
KGY68	h^{90}		Paul Nurse
KGY69	h$^+$	975	Paul Nurse
KGY137	h$^+$/h$^-$	*ura4*-D18/*ura4*-D18 *leu1*-32/*leu1*-32 *ade6*-210/*ade6*-216	Paul Nurse
KGY246	h$^-$	*leu1*-32, *ura4*-D18, *ade6*-210	Paul Nurse
KGY347	h$^-$	*cdc13*-117 *ura4*-D18 *leu1*-32 *ade6*-216	Paul Nurse
KGY460	h$^+$	*wee1-50 ura4*-D18 *leu1*-32	Paul Nurse
KGY519	h$^-$	*cdr2*-96 *leu1*-32	Young and Fantes, 1987
KGY531	h$^-$	*cdc2*-22 *ura4*-D18 *leu1*-32 *ade6*-210	Paul Nurse
KGY1475	h$^+$	*cdr2*::*ura4*$^+$ *ura4*D18	This study
KGY1476	h$^-$	*cdr2*::*ura4*$^+$ *ura4*D18	This study
KGY1480	h^{90}	*cdr2*::*ura4*$^+$ *ura4*D18	This study
KGY1519	h$^-$	*cdr2*::*ura4*$^+$ *ura4*-D18 *leu1*-32	This study
KGY1628	h$^-$	HA*cdr2*$^+$	This study
KGY1630	h$^+$/h$^-$	*cdr2*$^+$/*cdr2*::*ura4*$^+$ *ura4*-D18/*ura4*-D18 *leu1*-32/*leu1*-32 *ade6*-210/*ade6*-216	This study
KGY1631	h$^-$	*cdc*25-22 *cdr2*::*ura4*$^+$	This study
KGY1632	h$^-$	*cdc2*-33 *cdr2*::*ura4*$^+$	This study
KGY1633	h$^-$	*cdc2*-22 *cdr2*::*ura4*$^+$	This study
KGY1634	h$^-$	*cdc13*-117 *cdr2*::*ura4*$^+$	This study
KGY1635	h$^-$	*wee1*::*ura4*$^+$ *cdr2*:;*ura4*$^+$	This study
KGY1636	h$^-$	*wee1*-50 *cdr2*::*ura4*$^+$	This study
KGY1637	h$^-$	*cdc*25-22::*p*RIP2*cdc*25$^+$ *cdr2*::*ura4*$^+$	This study

grammed as follows: 94°C, 1 min; 50°C, 2 min; 72°C, 2 min (40 cycles); 72°C, 10 min. TaqPlus Precision (Stratagene, La Jolla, CA) was used to amplify the *cdr2HA* sequence in a PTC-100 programmable thermal controller (PTC-100; MJ Research) programmed as follows: 95°C, 1 min; 65°C–1°C per cycle, 1 min; 72°C, 5 min (15 cycles); 95°C, 1 min; 50°C, 1 min; 72°C, 5 min (30 cycles); 72°C, 10 min.

Physiological Experiments

For analysis of synchronous cell populations, 4 l of cells were grown to midlog phase (8×10^6 cells/ml) at 30°C in minimal medium. Cells were separated on the basis of size by centrifugal elutriation in an elutriator rotor (JE 5.0; Beckman, Fullerton, CA). Cells synchronized in early G_2 were collected in minimal medium and split into two cultures. Cells from each culture were collected on filters and released into either minimal medium or minimal medium lacking nitrogen at 30°C. Synchrony was monitored at 20- and 30-min intervals by scoring 100 cells for the presence of a septum. Samples were collected periodically for determination of cell length, DNA content, cell number increase, binculeate cells, and protein analysis.

For asynchronous nitrogen deprivation experiments, cells were grown to midlog phase in minimal medium at 30°C. Cells were collected on filters and then inoculated into minimal medium lacking nitrogen. Samples were collected periodically and were processed to determine DNA content.

To determine long-term viability and resistance to heat shock in response to nitrogen deprivation, cells were grown and deprived of nitrogen as described (Su *et al.*, 1996). For viability, samples were collected periodically over a 20-d time course, diluted appropriately, and plated in triplicate on YE plates at 25°C, and colonies were counted 5 d later. For heat shock, cells were incubated at 42°C for 5 min, diluted appropriately, and plated in triplicate on YE plates at 25°C, and colonies were counted 5 d later.

To determine total cell number, cells were collected and fixed in 0.12 M NaCl, 3% formaldehyde, diluted appropriately, sonicated briefly, and then counted in triplicate using the Coulter Multisizer II (Coulter Electronics, Hialeah, FL). Total cell number was taken as the average of each triplicate.

To determine DNA content, cells were fixed in ice-cold 70% ethanol, washed in 50 mM sodium citrate, incubated with 0.1 μg/ml RNase A in 50 mM sodium citrate for 2 h at 37°C, and then stained with 1 μM Sytox green (Molecular Probes, Sunnyvale, CA) for 2 h. Cells were sonicated and analyzed by flow cytometry as described (Sazer and Sherwood, 1990).

To determine cell length, fixed cells were measured microscopically using phase optics and a 100× objective with an eyepiece drum micrometer. To determine average cell length, 100 cells were measured. To determine septation length, 50 cells containing a septum were measured.

Microscopy

All light and fluorescence microscopy was performed on a Zeiss microscope (Axioskop; Carl Zeiss, Thornwood, NY) using appropriate filters. Cells were either fixed in 100% methanol at −20°C for 8 min or in 30% formaldehyde for 10 min at room temperature and then washed with PBS and processed as described by Balasubramanian *et al.* (1997). To visualize DNA and/or cell and septal material, cells were fixed in methanol or formaldehyde and stained with DAPI and/or Calcofluor.

Cloning and DNA Sequence of cdr2+

The *cdr2*-96 *cdc25*-22r1 *leu1*-32 (Q1045) strain was transformed with a *S. pombe* genomic library that was prepared from *Hin*dIII and *Sau*3A partially digested genomic DNA inserted into pWH5 (Wright *et al.*, 1986; Hudson *et al.*, 1990; Young and Beach, unpublished results). Transformants were selected at 25°C for 2 d on minimal medium lacking leucine and then shifted to 35°C. Plasmids were recovered from viable colonies and were transformed into the *cdr2*-96 *leu1*-32 strain (KGY519). Only one plasmid (pcdr2.1) was able to restore the nitrogen deprivation response of *cdr2*-96, and this plasmid was subjected to further subcloning to identify a minimal complementing fragment of 3.0 kilobases (kb).

To characterize the complementing DNA, the 3.0-kb genomic fragment was further subcloned, and appropriate plasmids were sequenced in both directions to generate 2656 base pairs (bp) of sequence. This sequence contained a single open reading frame (ORF) of 2247 bp but terminated without a stop codon. Comparison of this sequence to the *S. pombe* sequence database revealed that this sequence was identical to a sequence contained on cosmid c57A10 from chromosome I. Cosmid c57A10 contained the complete ORF of 2325 bp.

For integration mapping, a *leu1*-32 strain (KGY1) was transformed with linearized pcdr2.2. Transformants were selected on minimal medium lacking leucine. The resulting Leu$^+$ strain (Q1046) was crossed to *cdr2*-96 *leu1*-32 (KGY519), to verify cosegregation of the Cdr2$^+$ phenotype with the Leu$^+$ phenotype. Only Cdr2$^+$ Leu$^+$ and Cdr2$^-$ Leu$^-$ progeny were produced, indicating that pcdr2.2 had integrated within or very close to the *cdr2*$^+$ locus.

Deletion of cdr2+

To generate a full-length *cdr2*$^+$ construct containing 5′- and 3′-flanking genomic sequence, the 3.0-kb fragment containing 2247 bp of *cdr2*$^+$ sequence and 400 bp of 5′-flanking sequence was subcloned into pSK (Stratagene) generating pMB17. Next, a *Nde*I site was introduced at the initiating codon of the *cdr2*$^+$ sequence, and two endogenous *Nde*I sites were removed by site-directed mutagenesis (Chameleon Double Stranded Mutagenesis Kit; Stratagene) in pMB17 generating pKG939. To generate the 3′-coding region missing from pMB17, the oligonucleotides CSB51 (5′-CGTATGAATGAGAATGA-3′) and CSB52 (5′-CCTTGCTCTAGACATGCAAG-3′) were used to PCR amplify from genomic DNA a 1300-bp fragment consisting of 876 bp of the 3′-*cdr2*$^+$ coding region plus 424 bp of 3′-flanking genomic sequence. After digestion with *Xba*I, the 3′-fragment was subcloned into pMB939, exchanging the incomplete 3′-end of the *cdr2*$^+$ coding region with the complete 3′-coding sequence plus flanking sequence, generating pKG943. pKGY943 was sequenced to verify accuracy of the *cdr2*$^+$ coding region.

To generate a deletion construct containing *cdr2*$^+$ flanking sequence but lacking the *cdr2*$^+$ coding region, the *cdr2*$^+$ coding sequence plus flanking sequences were first shuttled into pTZ from pKG943 as a *Sac*I–*Sma*I fragment to generate a construct containing a unique *Eco*RV site (pKG935). pKG935 was digested with *Nde*I and *Eco*RV, removing all but the last 289 bases of the *cdr2*$^+$ coding sequence, and replaced with a 1.8-kb fragment containing the *ura4*$^+$ gene, generating pKG1275 (see Figure 2A). Digestion of pKG1275 with *Sma*I and *Sac*I liberated the deletion fragment, which was transformed into a *ura4-D18/ura4-D18* diploid strain (KGY137). Ura$^+$ diploid transformants were selected on minimal medium lacking uracil and were subjected to tetrad analysis. All spores were viable, and the Ura$^+$ prototrophs were subjected to Southern hybridization analysis to verify deletion of the *cdr2*$^+$ sequence.

Conjugation Efficiency Assay

To determine conjugation efficiency, homothallic *cdr2::ura4*$^+$ (KGY1480) and wild-type homothallic (KGY68) strains were plated onto glutamate agar for 48 h. Samples were analyzed under light microscopy, and conjugation efficiency was determined as described (Wu and Russell, 1997).

Overexpression Analysis

To determine whether the nitrogen deprivation defect of the *cdr2* null could be rescued by overexpression of genes encoding mitotic components, a *cdr2::ura4*$^+$ *ura4*-D18 *leu1*-32 strain (KGY1519) was transformed with plasmids containing cDNAs encoding *cdc2*$^+$, *cdc13*$^+$, and *cdr2*$^+$ under control of the *S. pombe nmt1* (no message in thiamine) thiamine repressible promoter (Maundrell, 1993). To generate pREP1*cdr2*$^+$ (KG947), pKG939 was digested with *Xba*I, treated with Klenow, and then digested with *Nde*I. The resulting fragment was subcloned into *Nde*I/*Sma*I-digested pREP1. The transformed strains were grown to midlog at 30°C in minimal medium containing thiamine, recovered on filters, released into minimal medium containing thiamine but lacking nitrogen, and incubated at 30°C for 48 h. Rescue was determined by phenotype and by DNA content. To determine whether overexpression of *cdc25*$^+$ could rescue the nitrogen deprivation defect of the *cdr2* null, a *cdc25*-22 strain containing *cdc25*$^+$ under control of the *nmt1* promoter integrated at the *cdc25* locus (GL122) was crossed to the *cdr2* null. The resulting double mutant (KGY1637) was grown in minimal medium containing thiamine to midlog at 25°C, washed to remove the thiamine, and then incubated in minimal medium for 18 h to induce expression of *cdc25*$^+$. Cells were then collected on filters, released into minimal medium lacking nitrogen, and incubated for 48 h at 25°C.

To analyze the phenotype of *cdr2*$^+$ overexpression in wild-type cells and in the absence of *wee1*$^+$ activity, a wild-type strain (KGY246) and the *wee1::ura4 ura4*-D18 *leu1*-32 strain (KGY4) were each transformed with pREP1 vector alone and pREP1*cdr2*$^+$ (KG947). The transformed strains were grown at 25°C in minimal medium containing thiamine to midlog, collected, washed to remove the thiamine, and then released into minimal medium lacking thiamine for 18 h.

Construction of Epitope-tagged Strains

To construct a genomic 3′-3XHA epitope-tagged *cdr2*$^+$ strain (KGY1628), a PCR amplification-based strategy was utilized as described previously (Bähler *et al.*, 1998). Oligonucleotides 5′-cdr2long (5′-CGGCATCCAGACCTGTTTCTCGAATGAGTGTAAGTAGTAGTCCTTTTGCTGTATTTCGTCAACGACAATCCGTCCAAAGTCGGATCCCCGGGTTAATTAA-3′) and 3′-cdr2long (5′-CCAAAGCATCACGAGAAAAATGAAGTTTGCAAAGGTTTTGGAGAATCAAAAAAAAATGATAATAATAATAAAAGAATGAATTCGAGCTCGTTTAAAC-3′) were used to PCR amplify a fragment containing a 3XHA epitope tag kanamycin resistance (*kan*MX6) cassette flanked on either side with *cdr2*$^+$ 3′-genomic sequence. A wild-type strain (KGY246) was transformed with the amplified fragment, plated onto YE medium overnight, and then replica plated onto YE medium containing the drug G418 to select for recombinants. Recombinants were outcrossed to wild type to confirm segregation of the *kan*MX6 marker and were screened by immunoblotting with the 12CA5 monoclonal antibody specific to the hemagglutinin (HA) epitope. Proper integration of the HA epitope in *cdr2*$^+$ was confirmed by Southern hybridization analysis and functionality was assessed by phenotype.

Southern Hybridization Analysis

Approximately 0.5 μg of genomic DNA was digested overnight at 37°C, size fractionated on an 0.8% agarose gel, and transferred to a GeneScreen Plus membrane (New England Nuclear Life Science Products, Boston, MA) overnight. The membrane was treated for 1 h in hybridization buffer (5× Denhardt's solution, 0.5% SDS, 5× SSPE, and 100 μg of hydrolyzed yeast RNA per ml), and then overnight at 65°C in hybridization buffer containing a random-primed α-[^{32}P]dCTP-labeled probe (*redi*Prime; Amersham, Arlington

Heights, IL). The membrane was then washed two times for 30 min at 65°C in 2× SSC–0.2% SDS. Hybridizing bands were detected by autoradiography.

Immunoblotting

S. pombe denatured lysates were prepared as described (Gould *et al.*, 1991). Approximately 2.4×10^8 cells were lysed, and total protein in each lysate was determined by BCA (BCA Protein Assay Reagent Kit; Pierce Chemical, Rockford, IL). For Western blot analysis, lysates were resolved by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Blots were probed with either monoclonal antibodies specific to the HA epitope (12CA5) at 2 μg/ml, PSTAIRE domain of cyclin-dependent kinases (Yamashita *et al.*, 1991; Sigma Chemical, St. Louis, MO) at 2 μg/ml, or a polyclonal antibody specific to *S. pombe* Cdc13p (GJG56, Den Haese and Gould, unpublished results). Antibody GJG56 was affinity purified as described previously (Olmsted, 1981) and used at 1:100 dilution. In *S. pombe*, the PSTAIRE antibody detects two PSTAIRE motif-containing proteins, p34 (Cdc2p) and p31, which encodes a PSTAIRE-related protein (Tournier *et al.*, 1997). Primary antibodies were followed with the appropriate peroxidase-conjugated secondary antibody (Sigma). Reactive proteins were visualized by enhanced chemiluminescence (Amersham Life Sciences). For quantitation of immunoblotting data, ECL Plus reagents (Amersham Life Sciences) were used. Data were collected on a Molecular Dynamics Storm instrument and quantified by ImageQuant version 1.1.

RESULTS

cdr2$^+$ *Encodes a Putative Serine-Threonine Protein Kinase*

Since none of the previously characterized *cdr2* mutant alleles imparted a conditional phenotype, we took advantage of the strong negative interaction between *cdr2* and *cdc25* mutant alleles to clone the *cdr2*$^+$ gene (Young and Fantes, 1987). A double mutant between *cdr2*-96 and a non-temperature–sensitive allele of *cdc25*, *cdc25*-22r1 (Hudson *et al.*, 1990), was constructed. While viable at 25°C, *cdr2*-96 *cdc25*-22r1 is temperature sensitive for growth at 35°C. This strain was transformed with a *S. pombe* genomic library (Wright *et al.*, 1986; Young and Beach, unpublished results), and only one transformant that grew at 35°C contained a plasmid that was also able to restore the ability of *cdr2*-96 to arrest with a small cell size in response to nitrogen deprivation. The complementing plasmid was further characterized to identify a minimal rescuing fragment of 3.0 kb. Integration mapping confirmed that the 3.0-kb fragment contained the *cdr2*$^+$ gene and not a high-copy suppressor.

Sequencing of the 3.0-kb genomic rescuing fragment uncovered a continuous ORF of 2247 bp that did not contain a stop codon. When this ORF was compared with the *S. pombe* sequence database, an identical sequence was found on cosmid c57A10 from *S. pombe* chromosome I. The complete *cdr2*$^+$ ORF encoded a protein of 775 amino acids with a predicted molecular weight of 85,917 (Figure 1A). Further comparison of the Cdr2p amino acid sequence to sequences in the databases revealed the presence of amino- terminal motifs that are signatures of protein kinase catalytic domains (Hanks *et al.*, 1988). The putative catalytic domain of Cdr2p showed highest sequence similarity to the Snf1p subfamily of serine-threonine protein kinases of which the *Saccharomyces cerevisiae* carbon catabolite derepressing kinase Snf1p is the prototypical member. Members of the Snf1p subfamily to which Cdr2p has high similarity in the catalytic domain include the *S. cerevisiae* protein Gin4p, Cdr1p/Nim1p in *S. pombe*, and the Cdr1p/Nim1p *S. cerevisiae* homologue, Hsl1p (Figure 1B) (Russell and Nurse, 1987b; Feilotter *et al.*, 1991; Ma *et al.*, 1996; Atman and Kellogg, 1997). Although limited, Cdr2p also has sequence similarity to Gin4p outside the catalytic domain (Figure 1C).

Analysis of a cdr2 Null Mutant

To determine the phenotype of a strain in which *cdr2*$^+$ has been deleted, the one-step gene disruption method was used to replace one copy of the *cdr2*$^+$ coding sequence with the *S. pombe ura4*$^+$ selectable marker as described in MATERIALS AND METHODS. We found that *cdr2*$^+$ is a nonessential gene; Southern hybridization analysis of genomic DNA isolated from a Ura$^+$ haploid colony confirmed that the *cdr2*$^+$ coding sequence had been replaced by *ura4*$^+$ (Figure 2B). However, cells lacking *cdr2*$^+$ were longer than wild type, septating at an average length of 19.5 μm in minimal medium, the same length at which *cdr2*-96 septates, while wild-type cells septated at an average of 13.0 μm (Figure 2C).

The original *cdr2* mutant alleles were isolated by virtue of their inability to respond normally to nitrogen deprivation (Young and Fantes, 1984, 1987). Subsequent analyses demonstrated that the *cdr2* mutants were longer than wild-type cells under all growth conditions. Moreover, it was determined that during nitrogen deprivation, *cdr2* mutants failed to arrest in G_1 and instead arrested in G_2 (Young and Fantes, 1984, 1987; Rupes *et al.*, 1997). To determine whether the *cdr2* null strain behaved similarly, we performed flow cytometry on *cdr2* null and wild-type strains undergoing nitrogen deprivation. Over the course of the experiment, wild-type cells became short and rounded (Figure 2D). The majority of the cells contained a 1 N content of DNA, demonstrating that these cells had arrested in G_1. In contrast, the majority of *cdr2* null cells remained longer than wild-type cells and contained a 2N content of DNA. Hence, the *cdr2* null cells behaved identically to *cdr2*-96 cells in these assays.

cdr2 Mutants Arrest in G_0 in Response to Nitrogen Deprivation

S. pombe cells responding to nitrogen deprivation have two choices: they either enter G_0, or they initiate sexual differentiation if the proper mating partner is

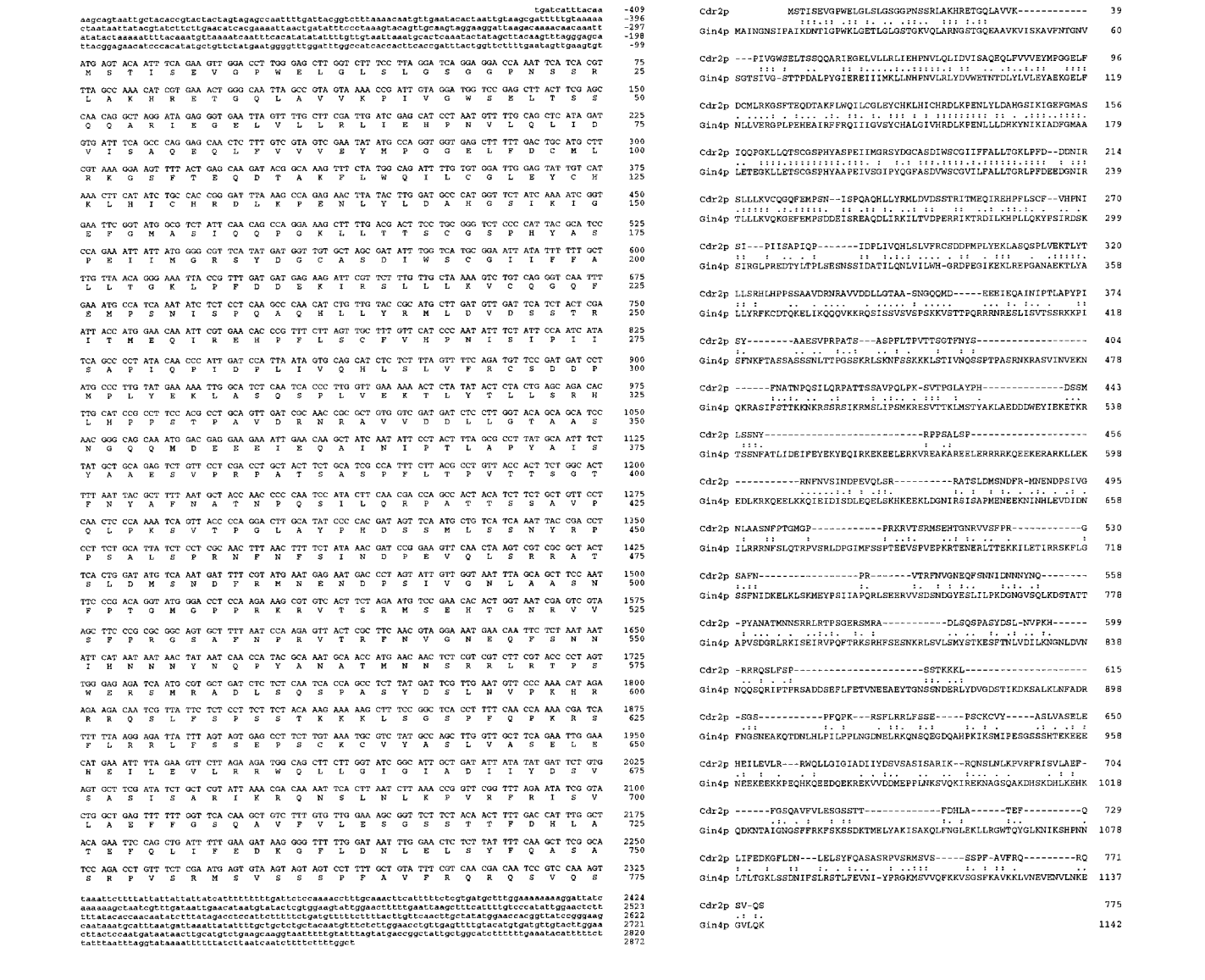

Figure 1.

present. Cells arrested in G_0 are able to maintain viability over long periods of time and are able to withstand extracellular stresses such as heat shock (reviewed by Egel, 1989). To examine the ability of *cdr2* cells to enter G_0 from G_2, long-term viability and heat-shock tolerance were measured in *cdr2* null and wild-type strains undergoing nitrogen deprivation. There was no significant difference in the long-term viability of the *cdr2* null strain compared with wild type (Figure 3A), nor was there a difference in their ability to withstand heat shock over time (Figure 3B). This demonstrates that the *cdr2* null is able to enter G_0 from G_2, which is similar to the phenotype exhibited by *cdr1/nim1* and also a subset of sterile mutants such as *nuc2* (Kumada *et al.*, 1995; Belenguer *et al.*, 1997; Wu and Russell, 1997).

cdr2$^+$ *Is Not Required for Sexual Differentiation*

In wild-type *S. pombe*, nutrient deprivation is a necessary prerequisite for sexual differentiation. Lack of nitrogen lowers intracellular cAMP levels by 50% (Maeda *et al.*, 1990; Kawamukai *et al.*, 1991; Mochizuki and Yamamoto, 1992). This, in turn, triggers the induction of *ste11*$^+$, whose protein product induces transcription of genes required for sexual differentiation (Sugimoto *et al.*, 1991). To determine whether *cdr2*$^+$ played a role in this response, we examined the induction of *ste11*$^+$ mRNA. We found that the transcription of *ste11*$^+$ mRNA was up-regulated in both wild-type and *cdr2* null strains upon nitrogen deprivation (our unpublished results).

In order for *S. pombe* cells to mate, they must first arrest in G_1 in response to nitrogen deprivation. This is a critical step because each cell must have a 1C DNA content before undergoing conjugation and nuclear fusion. Although the induction of *ste11*$^+$ appeared normal, we still might expect *cdr2* null cells to have a mating defect because of their inability to arrest in G_1. To examine this question, we generated a homothallic, or self-mating, *cdr2* null strain and compared its mating efficiency to a wild-type homothallic strain. As illustrated in Figure 3C, there was no significant difference between the mating efficiencies of wild type and *cdr2* null strains. The ability of the *cdr2* null cells to mate at levels similar to wild type suggests that the *cdr2* null cells must be able to arrest in G_1 under conditions suitable for mating, i.e., low nitrogen levels and the presence of pheromones expressed from cells of the opposite mating type (reviewed by Neilsen and Davey, 1995).

The G_2 Delay Exhibited by the cdr2 Null Is Exacerbated during Nitrogen Deprivation

Since the *cdr2* null was not defective in undergoing sexual differentiation in limiting nitrogen, or arresting in G_0 in response to nitrogen deprivation, it seemed unlikely that *cdr2*$^+$ functioned as a nutritional sensor or monitor. Another possibility for the inability of the *cdr2* null cells to arrest in G_1 is a defect in G_2/M progression. The longer cell length of the *cdr2* null suggests that it takes a longer period of time for cells lacking *cdr2*$^+$ to initiate mitosis. Such a delay might inhibit the ability of the *cdr2* null cells to alter mitotic control in response to nitrogen deprivation.

To carefully measure the ability of cells to undergo mitosis in response to nitrogen deprivation, we utilized cultures synchronized in the cell cycle by centrifugal elutriation. Either wild-type or *cdr2* null strains were grown to midlog phase in minimal medium at 30°C. Newborn cells, which we found using the new DNA dye, Sytox green, were actually in S phase (see Figure 4F), were selected by centrifugal elutriation and collected on filters, and then released immediately into either minimal medium or minimal medium lacking a nitrogen source. Samples were collected periodically during a 48-h time course to follow cell length, DNA content, cell number increase, and time of septation, and for protein analysis.

Wild-type cells inoculated into minimal medium began to septate at 100 min, with a septation peak at 160 min for the first cell cycle and at 340 min for the second cell cycle (Figure 4A). The peaks of binucleate cells were at 140 and 340 min (Figure 4B). Similarly, the *cdr2* null cells inoculated into minimal medium exhibited a peak of septation at 180 min in the first cell cycle and at 360 min in the second (Figure 4A). The binucleate peaks were at 160 and 340 min (Figure 4B).

Cells deprived of nitrogen behaved differently. Nitrogen-deprived wild-type cells exhibited a significant delay in septation: they did not begin to septate until 180 min, exhibiting a peak of septation at 210 min, after which the synchrony of the culture was lost (Figure 4C). The peak of binucleates was similarly delayed, exhibiting a first peak at 180 min (Figure 4D). The *cdr2* null cells inoculated into minimal medium without nitrogen did not begin to septate until 210

Figure 1 (facing page). Sequence analysis of *cdr2*$^+$. (A) Nucleotide sequence and predicted amino acid sequence of *cdr2*$^+$. Sequencing of the *cdr2*$^+$ complementing DNA generated bases −409 to +2247 that did not include a stop codon. Comparison of this sequence to the *S. pombe* sequence database revealed that it was identical to a ORF on cosmid c57A10 from chromosome I. Bases +2248 to +2872 were obtained solely from the sequence of cosmid c57A10. The GenBank accession number for the *cdr2*$^+$ sequence is AF092508. (B) Alignment of the Cdr2p putative catalytic domain with the Snf1p subfamily members *S. cerevisiae* proteins Hsl1p and Gin4p and *S. pombe* Cdr1p/Nim1p. Subdomains conserved among protein kinases I–XI are indicated by black lines (Hanks *et al.*, 1988). Amino acid residues conserved in three of four sequences are highlighted in black. (C) Amino acid sequence alignment of Cdr2p and Gin4p. Identical residues are indicated by colons (:). Similar residues are indicated by a period (.).

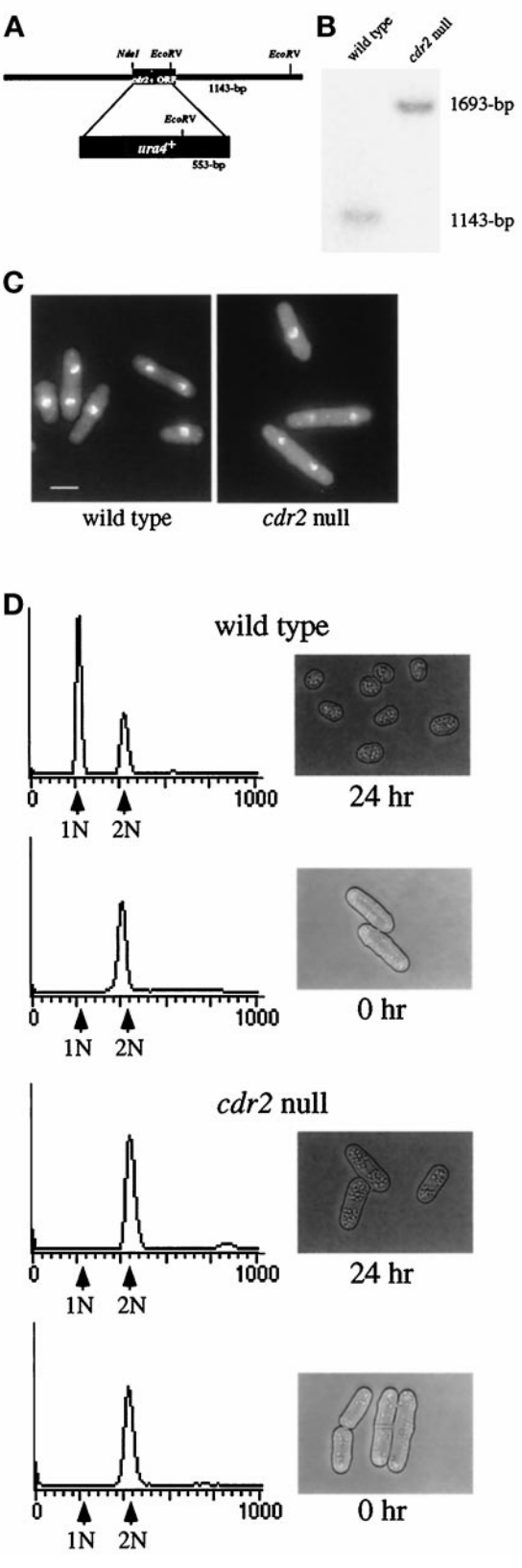

Figure 2.

min, and then only a few cells were able to septate at any given time, suggesting that the synchrony of the culture was lost by the time the first cells initiated septation (Figure 4C). The binucleate peak also appeared later (Figure 4D). That the cell cycle delays occurred in G_2 rather than in mitosis was confirmed by determining that the appearance of mitotic spindles was also delayed (our unpublished results).

By determining cell number increases and DNA content of the cells throughout this experiment, we found that wild-type cells deprived of nitrogen underwent two rounds of cell division; cell number approximately quadrupled (Figure 4E). Interestingly, using the improved DNA stain, Sytox green, rather than propidium iodide, we were able to discern in this and other experiments (our unpublished results) that the smallest *S. pombe* cells isolated by centrifugal elutriation were in S phase rather than G_2. After completion of DNA replication, they divided synchronously and then underwent a final cell cycle and arrested in G_1 (Figure 4F). In contrast to wild-type cells, the *cdr2* null cells underwent only one round of cell division, doubling their cell number over the course of the experiment (Figure 4E). Because they did not go through another round of mitosis, they were arrested in G_2 (Figure 4F).

As mentioned above, wild-type cells deprived of nitrogen alter cell size control and divide at a reduced cell size (Fantes and Nurse, 1977). To determine whether *cdr2* null cells were similarly able to alter cell size control upon nitrogen deprivation, mean cell lengths were determined throughout this synchronous cell experiment (Figure 4G). The newborn wild-type cells isolated by centrifugal elutriation were, on average, 6.7 μm in length. At the first round of division, the average length of cells containing a septum was 7.9 μm. This represents a reduction from ~13.0 μm at septation of wild-type cells grown in minimal medium. After 24 h and 48 h of nitrogen starvation, the mean cell lengths of wild-type cells had diminished to 4.4 μm and 3.9 μm, respectively. Newborn *cdr2* null cells isolated by centrifugal elutriation were

Figure 2. Deletion of *cdr2*$^+$. (A) Diagram of the *cdr2* deletion construct. (B) Southern blot of a wild-type and a *cdr2::ura4*$^+$ strain (KGY246 and KGY1519). The 1143-bp band represents the wild-type *cdr2*$^+$ locus while the 1693-bp band represents the disrupted *cdr2::ura4*$^+$ locus. (C) Phenotype of the *cdr2* null cells. Wild-type and *cdr2::ura4*$^+$ strains (KGY69 and KGY1475) were grown at 30°C to midlog in YE, fixed with formaldehyde, and stained with DAPI to visualize DNA. Scale bar, 5 μm. (D) Response of *cdr2* null and wild-type cells to nitrogen deprivation. Wild-type (upper panels) and *cdr2* null (lower panels) strains (KGY69 and KGY1475) were grown at 30°C to midlog, harvested by filtration, washed, and released into minimal medium lacking nitrogen. Samples were collected at 0 and 24 h for DNA content (left-hand panels) and to visualize the phenotype of the cells by light microscopy (right-hand panels).

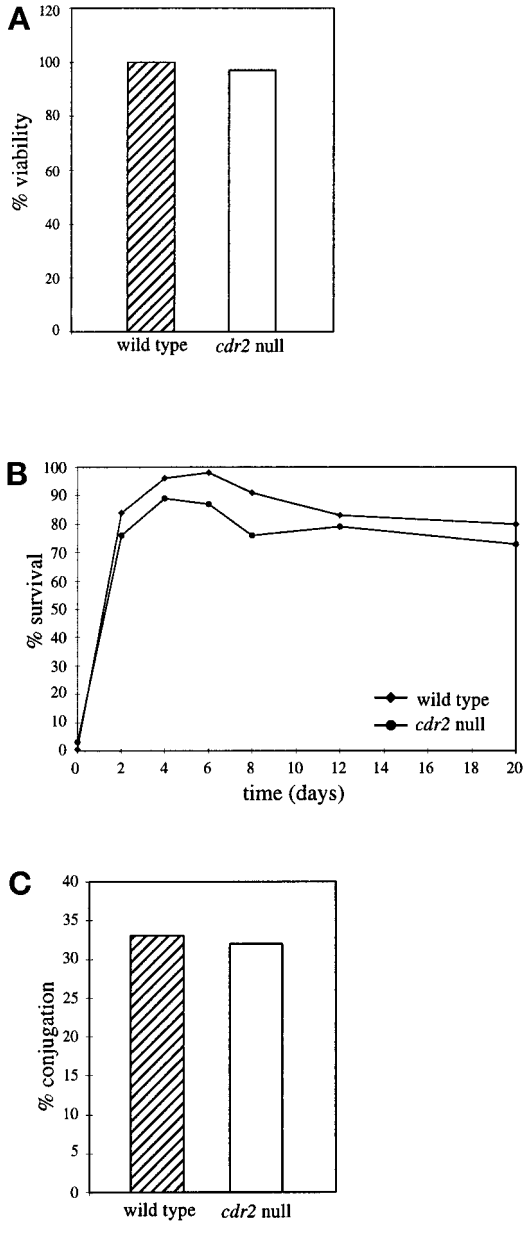

Figure 3. Cells lacking *cdr2*$^+$ are able to enter quiescence upon nitrogen deprivation. Wild-type and *cdr2* null strains (KGY69 and KGY1475) were grown at 30°C to midlog, filtered, washed, and released into minimal medium lacking nitrogen. Cells were incubated for a total of 20 d with samples collected periodically to determine total viability (A) and heat shock resistance (B). (C) Conjugation assay. Homothallic wild- type and *cdr2* null strains were plated onto glutamate agar and incubated at 25°C for 48 h.

11.0 μm in length. At the first round of division, the average length of cells containing a septum was 16.0 μm. This is somewhat reduced from the average septation length of 19.5 μm in nitrogen-containing minimal medium. After 24 and 48 h of nitrogen starvation, the mean cell lengths of the *cdr2* null cells were 9.4 and 9.6, respectively. Thus, *cdr2* null cells do adjust cell size at division in response to nitrogen deprivation but not to the same proportion as wild-type cells.

Genetic Interactions with Mitotic Control Genes

To better understand the role of *cdr2*$^+$ in mitotic control, we examined genetic interactions between the *cdr2* null and a variety of mitotic control mutants. *cdr2* mutants display strong negative interactions with mutations in *cdc25* and lower the restrictive temperature of alleles of *cdc2* and *cdc13* (Young and Fantes, 1987). As mentioned previously, the *cdr2* null appeared phenotypically similar to the initially characterized *cdr2* mutant alleles. We found that the *cdr2* null displayed genetic interactions with alleles of *cdc25*, *cdc2*, and *cdc13* similar to those found with the *cdr2* mutant alleles (Table 2). Interestingly, mutations in *wee1* or dominant alleles of *cdc2* are epistatic to mutations in *cdr2*, including the *cdr2* null; cells lacking both *wee1* and *cdr2* were small in length (Young and Fantes, 1987; Figure 6B). This result would be consistent with the possibility that Cdr2p acts as a negative regulator of Wee1p.

To determine whether overexpression of several known mitotic control genes could compensate for the lack of *cdr2*$^+$, the cDNAs encoding *cdr2*$^+$, *cdc2*$^+$, *cdc13*$^+$, and *cdc25*$^+$, under control of the *S. pombe nmt1* (no message in thiamine) thiamine-repressible promoter (Maundrell, 1993), were overexpressed in the *cdr2* null strain. Only overexpression of *cdr2*$^+$ or *cdc13*$^+$ could restore the wild-type response to nitrogen deprivation; these cells arrested in G_0 with a small cell size that was indistinguishable from wild type (Figure 5A).

The ability of additional Cdc13p to restore the wild-type response to nitrogen deprivation suggested that Cdc13p levels may be altered in the *cdr2* null. The level of Cdc13p is highly regulated. During mitosis, Cdc13p levels decline (Booher *et al.*, 1989; Hayles and Nurse, 1995; Creanor and Mitchison, 1996). Also, Cdc13p is degraded in response to nitrogen deprivation (Broek *et al.*, 1991). To determine whether Cdc13p levels are perturbed in the *cdr2* null strain, Cdc13p levels were assayed from samples collected from the same experiment described in Figure 4. We found that Cdc13p was degraded with similar kinetics in both the wild-type and the *cdr2* null strains (Figure 5B). Quantification of the Cdc13p signal standardized with the Cdc2p loading control indicated that in both wild-type and *cdr2* strains, the percent of Cdc13p signal dropped to 8 and 10% of starting levels, respectively, by 180 min. Since *cdr2* null cells must grow substantially before initiating septation even during nitrogen deprivation, as shown in Figure 4, this timely degradation of Cdc13p partially explains why *cdr2* null cells struggle to initiate mitosis after nitrogen deprivation and also might explain why increased levels of Cdc13p are sufficient to rescue this defect of the *cdr2* null.

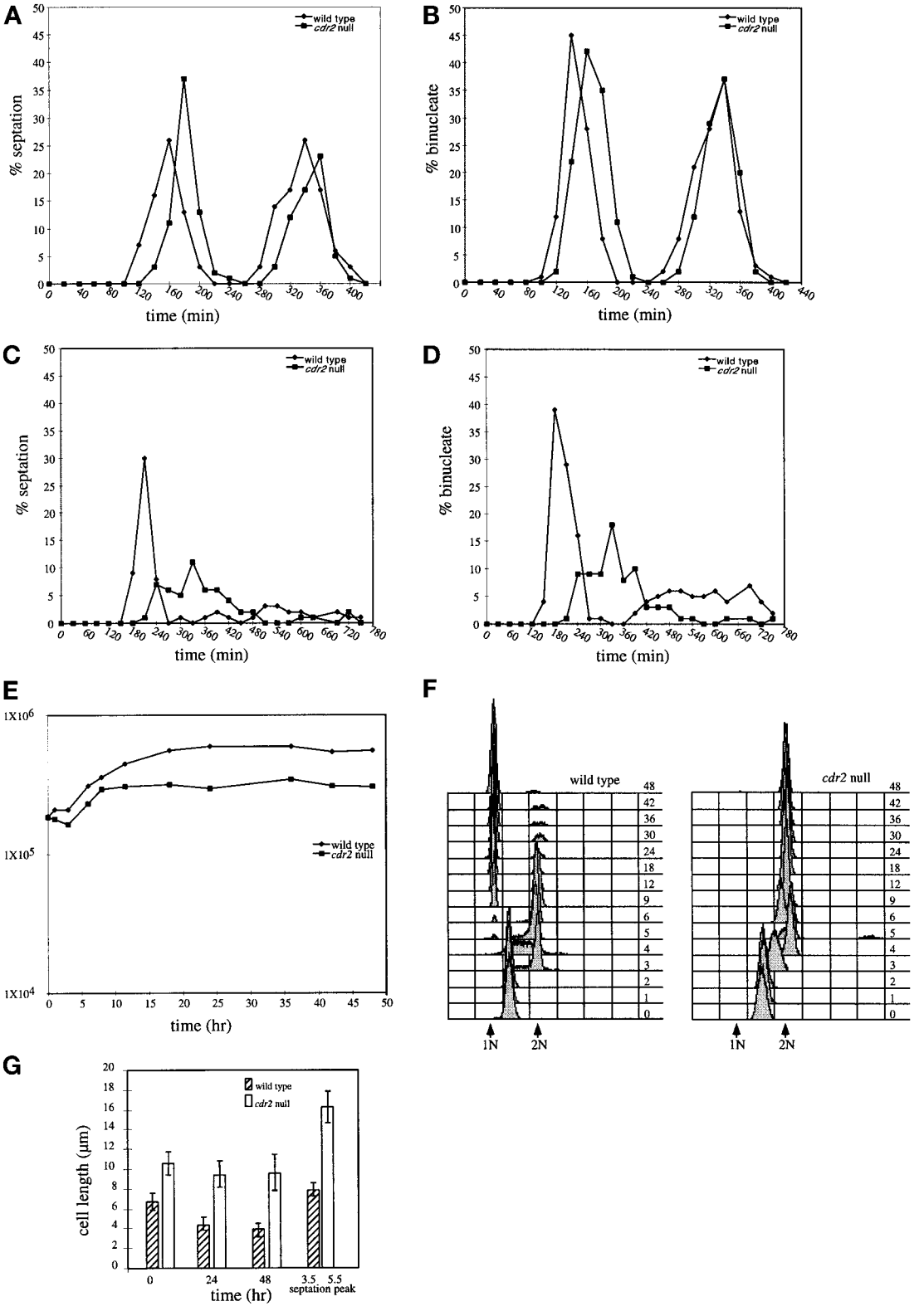
A
% septation
time (min)
wild type
cdr2 null
B
% binucleate
time (min)
C
% septation
time (min)
D
% binucleate
time (min)
E
1X10^6
1X10^5
1X10^4
time (hr)
F
wild type
cdr2 null
1N
2N
G
cell length (μm)
time (hr)
0
24
48
3.5 5.5
septation peak

Figure 4.

Table 2. Genetic interactions between *cdr2* null and mitotic control genes

Strain	25°C	29°C	32°C	36°C
Wild type	+	+	+	+
cdr2 null	+	+	+	+
cdc13-117	+	+	+	−
cdc2-22	+	+	+	−
cdr2 null *cdc13*-117	+	+	−	−
cdr2 null *cdc2*-22	+	+	−	−

Strains were streaked out onto YE media and incubated for 2 d at 25°C, replica plated to YE, and shifted to the indicated temperature for 24 h. + Indicates that the colonies were viable at the indicated temperature; − indicates that the colonies were inviable at the indicated temperature.

cdr2 Mutants Have an Additional Defect in Cell Division

Although no temperature-sensitive phenotype was initially ascribed to the *cdr2* mutants (Young and Fantes, 1987), we observed that the *cdr2* null strain exhibited temperature-associated growth abnormalities. When incubated at 36°C, *cdr2* null cells were more elongated and showed defects in cytokinesis; cells contained multiple septa and misplaced septa and septum material (Figure 6A). However, these defects did not result in lethality and were only observed when cells were incubated in YE. Moreover, the defects at 36°C were suppressed by the addition of sorbitol to the medium (our unpublished results).

Since $cdr2^+$ activity is not required in the absence of $wee1^+$ with respect to cell length, we wanted to know whether loss of $wee1^+$ activity also suppressed the cytokinesis defect of the *cdr2* null. To determine this, the *cdr2* null *wee1–50* double mutant was incubated at 36°C in YE. As can be seen in Figure 6B, loss of $wee1^+$ did not suppress the cytokinesis defect associated with loss of $cdr2^+$. The double mutant not only had defects in cytokinesis but also accumulated multiple nuclei. The same was found to be true in a *cdr2* null *wee1* null double mutant (our unpublished results).

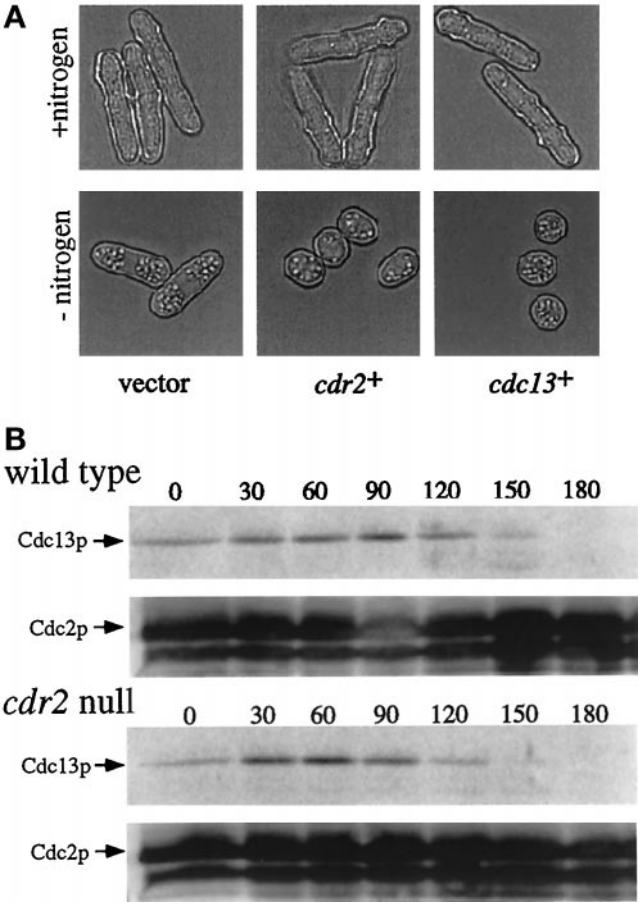

Figure 5. Cdc13p levels in the *cdr2* null. (A) Overexpression of $cdc13^+$ in the *cdr2* null. A *cdr2* null *leu1*-32 strain was transformed with either empty pREP1 vector (left panel), pREP1$cdr2^+$ (middle panel), or pREP1$cdc13^+$ (right panel). Transformants were selected and grown to midlog in selective medium. Transformants were then collected, filtered, washed, and released into selective medium lacking nitrogen for 48 h. Micrographs were taken just before nitrogen deprivation (top panels; +nitrogen) and after 48 h (bottom panels; −nitrogen). (B) Detection of endogenous Cdc13p during nitrogen deprivation. Cell samples acquired from the experiment described in Figure 4 were analyzed for Cdc13p levels at 30-min intervals for 3 h. Cdc2p served as a loading control.

Figure 4 (facing page). Mitotic response to nitrogen deprivation. Wild-type and *cdr2* null strains (KGY69 and KGY1475) were grown to midlog phase in minimal medium, and then separated on the basis of size by centrifugal elutriation. The smallest newborn cells were collected and split between two cultures, and each half was filtered, washed, and released into minimal medium (A and B) or minimal medium lacking nitrogen (C, D, E, F, and G). Samples were collected initially every 20 min for 6 h (A and B) or every 30 min for 12 h (C and D), and the percentage of binucleate (B and D) and cells containing a septum (A and C) were determined microscopically. From 12–48 h, samples were collected less frequently, and total cell number (E) and DNA content (F) were determined by Coulter counting and flow cytometry, respectively. Samples of cells grown in medium lacking nitrogen were also collected to measure average cell length at 0, 24, and 48 h and cell length at the first peak of septation (3.3 h for wild type and 5.5 h for *cdr2* null) (G).

The inability of the loss of *wee1* function to suppress the cytokinesis defect of the *cdr2* null suggested the possibility that $cdr2^+$ has two separate roles: one in the decision to enter mitosis, dependent on $wee1^+$, the other in cytokinesis, independent of $wee1^+$.

Overexpression of $cdr2^+$ Has a Dominant Negative Effect

To further characterize the function of $cdr2^+$, we overexpressed the $cdr2^+$ gene in a wild-type strain. If $cdr2^+$ functions as a negative regulator of $wee1^+$, similar to $cdr1^+/nim1^+$, then we might expect overexpression of $cdr2^+$ to cause cells to become wee in size, as in the case of $cdr1^+$ overexpression (Russell and Nurse,

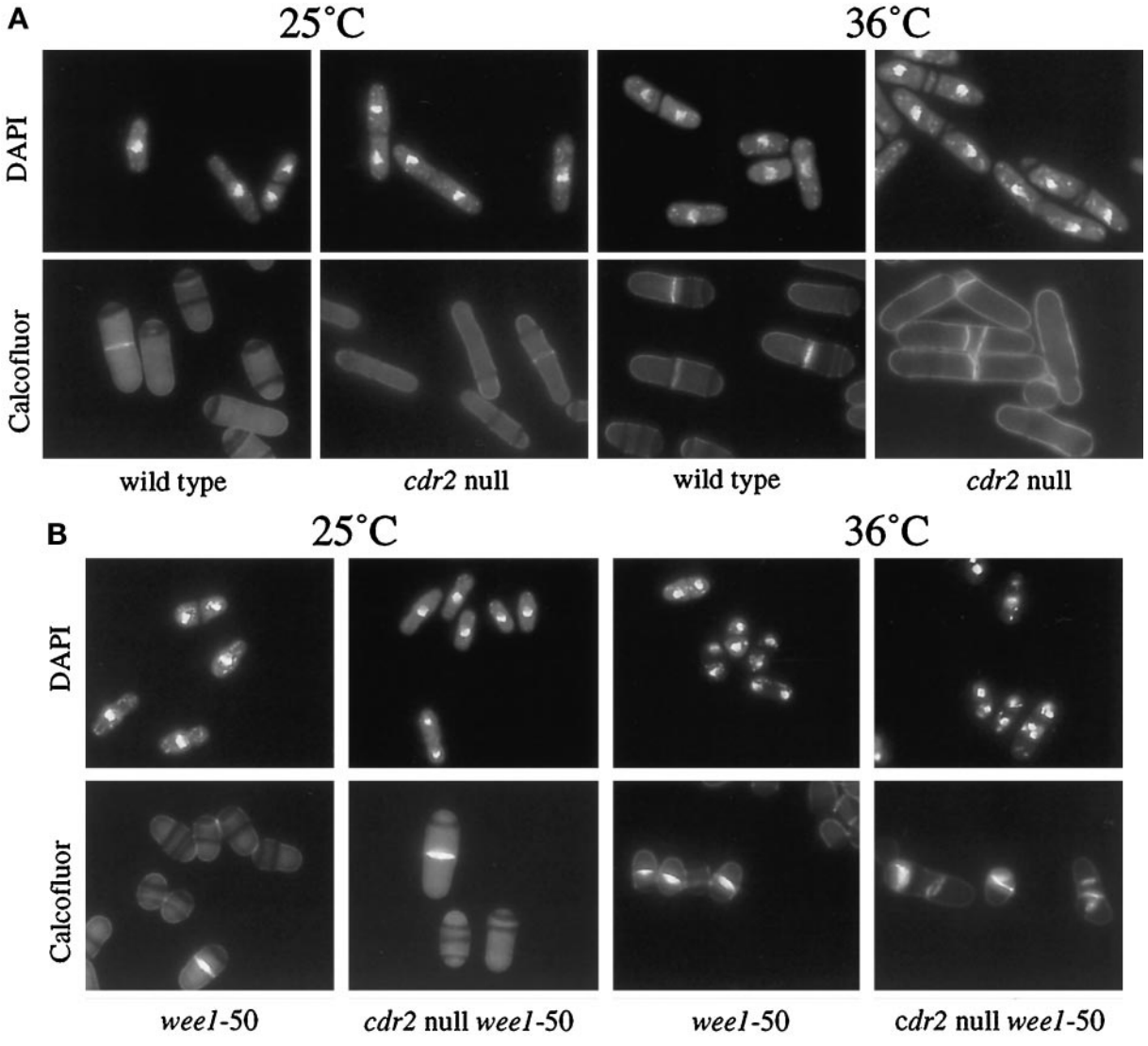

Figure 6. *cdr2* null and *cdr2* null *wee1*-50 strains at 36°C. (A) *cdr2* null at 36°C. Wild-type and *cdr2* null strains (KGY69 and KGY1475) were grown to midlog at 25°C (left panels) and to midlog at 36°C (right panels). Cells were fixed in methanol and then stained with DAPI to visualize the DNA (top panels) or with Calcofluor to visualize cell walls and septa (bottom panels). (B) *wee1*-50 and *cdr2* null *wee1*-50 strains at 36°C. *wee1*-50 and *cdr2* null *wee1*-50 strains (KGY460 and KGY1636) were grown to midlog at 25°C (left panels) and then shifted to 36°C for 6 h (right panels). Cells were fixed in methanol and then stained with DAPI to visualize the DNA (top panels) or with Calcofluor to visualize cell walls and septa (bottom panels).

1987b). Contrary to this expectation, overexpression of *cdr2*$^+$ in the wild-type strain was lethal and generated elongated, highly branched cells that contained two or more septa (Figure 7A). Thus, the overexpression of *cdr2*$^+$ has an apparent dominant negative effect.

To determine whether the *cdr2*$^+$ overexpression phenotype was dependent on *wee1*$^+$, we overexpressed *cdr2*$^+$ in the *wee1* null strain. Overexpression of *cdr2*$^+$ in the *wee1* null was lethal, and these cells displayed septation defects that were similar to the defects that resulted from overexpression of *cdr2*$^+$ in wild-type cells (Figure 7B). However, the *wee1* null cells overexpressing *cdr2*$^+$ did not become elongated, supporting the idea that *cdr2*$^+$ function is dependent on *wee1*$^+$ with respect to cell length, but is independent of *wee1*$^+$ with respect to its role in cytokinesis.

Cdr2p Abundance Is Regulated during Nitrogen Deprivation

The results from the experiments above suggested that Cdr2p is required for cells to initiate mitosis at the ap-

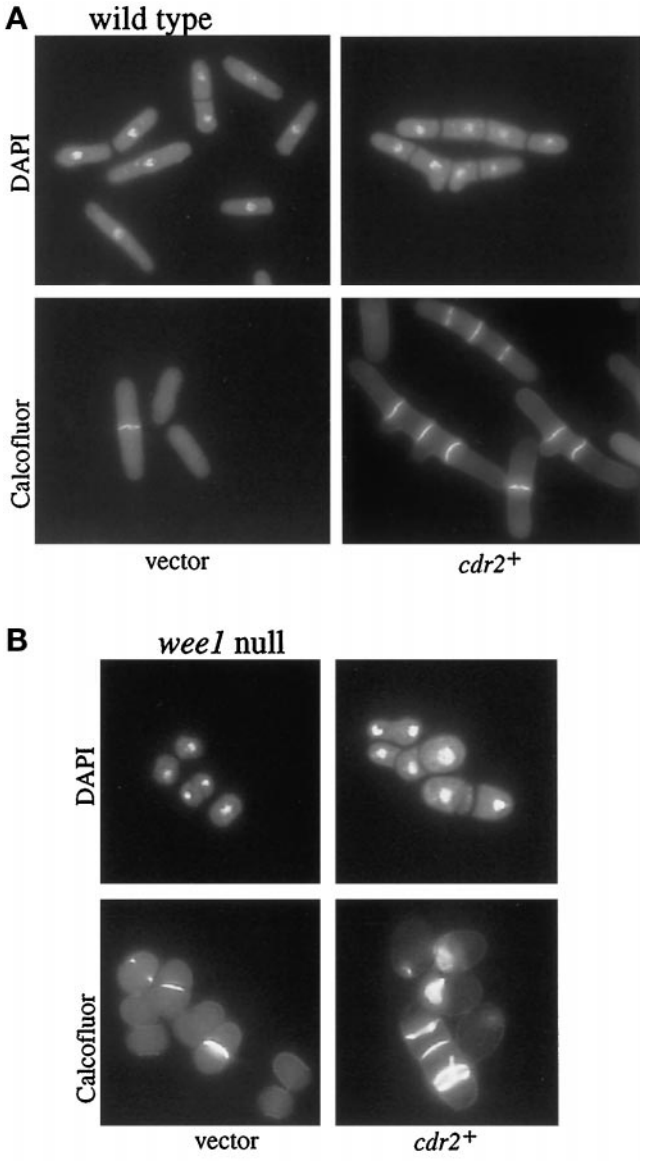

Figure 7. Overexpression of *cdr2*$^+$ in wild-type and *wee1* null cells. A *leu1*-32 strain (KGY246; panel A) and a *wee1* null *leu 1*-32 strain (KGY4; panel B) were transformed with either empty pREP1 vector (left panels) or pREP1*cdr2*$^+$ (right panels). Transformants were selected and grown to midlog in selective medium containing thiamine. Cells were collected, washed, and released into selective medium lacking thiamine for 18 h. Samples were fixed with methanol and stained with DAPI (top panels) to visualize the DNA or Calcofluor (bottom panels) to visualize cell walls and septa.

propriate time. Thus, we might expect Cdr2p abundance to be regulated during the cell cycle and/or during nitrogen deprivation. We first determined that the level of *cdr2*$^+$ mRNA did not vary during the cell cycle or during nitrogen deprivation (our unpublished results).

To measure Cdr2p protein abundance, we generated a C-terminal *HA* epitope-tagged *cdr2*$^+$. The resulting Cdr2p-HA protein was determined to be functional as the tagged strain was indistinguishable from wild type in terms of cell length and response to nitrogen deprivation (our unpublished results). The Cdr2p-HA protein was detected as a doublet migrating at a molecular mass of ~90 kDa using monoclonal antibodies specific to the HA epitope (12CA5). This doublet was detected only in lysates from the tagged strain (Figure 8A).

A synchronous cell population was used to determine the abundance of Cdr2p-HA throughout the cell cycle and during nitrogen deprivation. While Cdr2p-HA levels remained constant throughout the cell cycle (our unpublished results), the level of Cdr2p-HA decreased during nitrogen deprivation but was detectable up to 100 min (Figure 8B). This is contrast to Cdr1p/Nim1p, which is degraded immediately upon release into medium lacking nitrogen (Wu and Russell, 1997). These data also support the hypothesis that Cdr2p acts as a mitotic inducer; Cdr2p is present at the time in which cells are initiating mitosis.

DISCUSSION

cdr2$^+$ was isolated in a screen for genes required for the proper mitotic response to nitrogen deprivation in *S. pombe*. Because the *cdr* mutants were not able to respond properly to nitrogen deprivation, it was predicted that the *cdr*$^+$ genes would be involved in either nutritional sensing and/or mitotic control (Young and Fantes, 1984, 1987). Early evidence supported both predictions; the two complementation groups characterized, *cdr1* and *cdr2*, initiated mitosis with a longer cell length than wild type, indicating a delay in G_2, and they were unable to respond properly to nitrogen deprivation. *cdr1*$^+$, also known as *nim1*$^+$, has been shown to encode a conserved serine-threonine protein kinase that promotes G_2/M progression through the inhibitory phosphorylation of the *wee1*$^+$ gene product, thus fulfilling one of the predictions of the *cdr* screen (Russell and Nurse, 1987b; Feilotter *et al.*, 1991; Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). However, *cdr1*$^+$/*nim1*$^+$ does not appear to have a role in nutritional sensing, and the defect in responding to nitrogen deprivation by *cdr1*$^+$/*nim1*$^+$ mutants is a result of a delay in G_2 (Belenguer *et al.*, 1997; Wu and Russell, 1997). We have continued the characterization of *cdr2*$^+$ and have found that similar to *cdr1*$^+$/*nim1*$^+$, *cdr2*$^+$ acts as an inducer of mitosis. However, unlike *cdr1*$^+$/*nim1*$^+$, *cdr2*$^+$ has an additional role in cytokinesis.

We have found that *cdr2*$^+$ is not an essential gene. The *cdr2* null is phenotypically similar to *cdr2* mutants; *cdr2* null cells septate at the same length as the *cdr2*-96 mutant. Utilizing the *cdr2* null strain, we dissected the involvement of *cdr2*$^+$ in the response of *S. pombe* cells

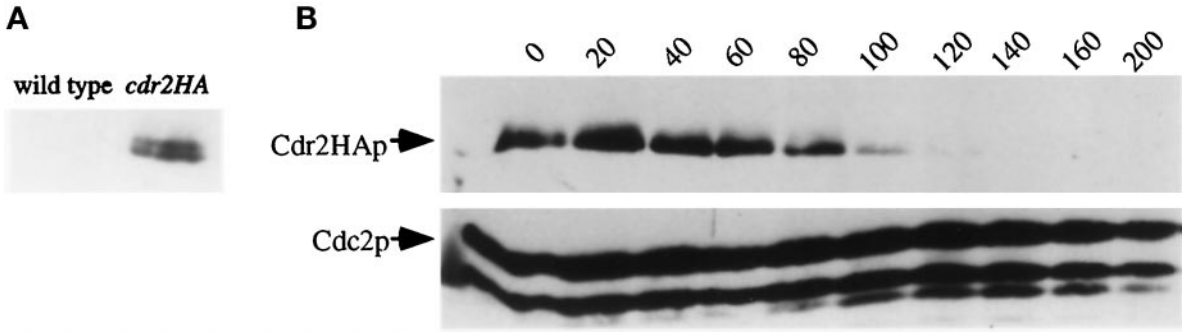

Figure 8. Regulation of Cdr2p levels. (A) Detection of Cdr2p-HA. Protein generated from a HA epitope-tagged *cdr2*$^+$ strain (*cdr2HA*$^+$; KGY1628) and a wild-type strain (KGY68) was probed with the HA antibody (12CA5) to detect Cdr2p-HA. (B) Detection of Cdr2p-HA levels during nitrogen deprivation. The *cdr2HA*$^+$ strain (KGY1628) was grown to midlog in minimal medium, and then separated on the basis of size by centrifugal elutriation. Early G_2 cells were collected, filtered, washed, and released into minimal medium lacking nitrogen. Time points were taken every 20 min for 3 h to analyze Cdr2p-HA levels. Cdc2p served as a loading control.

to nitrogen deprivation. We found that, like *cdr1/nim1* mutants, the *cdr2* null mutant arrested in G_2 instead of G_1 when starved for nitrogen and entered a state of quiescence or G_0. Since *S. pombe* cells can enter G_0 in response to nitrogen deprivation from either G_1 or G_2 (Castello *et al.*, 1986), the latter usually occurring from carbon deprivation, it is not surprising that *cdr2* mutants are able to enter G_0 from G_2. However, we also found that the *cdr2* null was able to undergo sexual differentiation and produce viable haploid progeny, which is surprising if the *cdr2* null cells had truly arrested in G_2 under the conditions employed in mating assays. However, mating assays differ from nitrogen deprivation experiments in that low amounts of nitrogen are present. Also, pheromones, produced under limiting nutrient conditions (reviewed by Neilsen and Davey, 1995), may help to promote a G_1 arrest in the mating assays. Thus, under these conditions, it is likely that the *cdr2* null cells are able to transiently arrest in G_1 and undergo sexual differentiation. These data indicate that *cdr2*$^+$ is not required for nutritional sensing, and therefore it is not likely that *cdr2*$^+$ encodes a nutritional sensor or monitor.

To show that *cdr2*$^+$ functions in G_2/M progression, we analyzed the mitotic response of wild-type and *cdr2* null cells to nitrogen deprivation. It has been demonstrated previously that asynchronous populations of wild-type cells respond to nitrogen deprivation by immediately altering G_2/M size control and entering mitosis with a reduced cell size (Fantes and Nurse, 1977). In our nitrogen deprivation experiments, wild-type cells entered mitosis with a reduced cell size as seen previously. However, there was an unexpected delay in G_2 before these cells entered mitosis. Since the cells utilized in our experiments were synchronized early in the cell cycle, they had not yet reached the critical size for mitosis. Hence, these uniformly small cells were forced to grow until the proper size was reached, even though the G_2/M size had indeed been reset in response to nitrogen deprivation. When we analyzed asynchronous populations of wild-type cells, we observed the acceleration into mitosis previously described (our unpublished results) (Fantes and Nurse, 1977).

The *cdr2* null strain also exhibited a delay in G_2 in response to nitrogen deprivation, but the delay in the *cdr2* null cells was substantially longer than in wild-type cells. Furthermore, although cell size at mitosis was altered by nitrogen deprivation in the absence of Cdr2p function, the response was not proportionately as large as in wild-type cells. The *cdr2* null cells did eventually undergo mitosis and divide, as reflected by the doubling of cell number, and arrested in G_2 of the following cell cycle. Under the same regimen, wild-type cells nearly quadrupled in number, undergoing an additional round of mitosis and arresting in G_1. These data demonstrate that Cdr2p acts as a mitotic inducer, which is especially important in conditions of limiting nitrogen.

To begin dissecting the molecular nature of Cdr2p's role in promoting mitosis, we examined the genetic relationship between *cdr2* and known mitotic control genes. From the initial characterization of *cdr2*$^+$, it is known that the restrictive temperatures of alleles of *cdc2*, *cdc25*, and *cdc13* mutants are lowered in combination with *cdr2* mutants, demonstrating that they interact genetically (Young and Fantes, 1987). Moreover, we found that ectopic expression of *cdc13*$^+$ was sufficient to rescue the nitrogen deprivation response of the *cdr2* null. This suggested that Cdc13p might be limiting in the *cdr2* null since Cdc13p association with Cdc2p is required for activation of Cdc2p and entry into mitosis (Booher *et al.*, 1989). When the level of Cdc13p was examined in the *cdr2* null, we found that it was the same as in wild type (our unpublished results). Because Cdc13p protein is turned over in response to nitrogen deprivation (Broek *et al.*, 1991), we then tested the possibility that the kinetics of

Cdc13p degradation were altered in the *cdr2* null. The kinetics of Cdc13p degradation in response to nitrogen deprivation were similar in the *cdr2* null and wild-type cells. Since the *cdr2* null cells must grow proportionately longer than wild-type cells before initiating mitosis, the decrease in Cdc13p levels might contribute to their difficulty initiating mitosis. Because of the limitations of detection on immunoblots, we would not argue that these cells are initiating mitosis in the absence of Cdc13p but rather that Cdc13p becomes limiting under these conditions. Hence, addition of excess Cdc13p is sufficient to restore the wild-type response to nitrogen deprivation in *cdr2* null cells.

Genetic interactions between *cdr2* and *wee1* mutants also provide some insight into the molecular role of *cdr2*$^+$ in G_2/M progression. It was demonstrated by Young and Fantes (1987) that *wee1*$^+$ was epistatic to both *cdr1*$^+$/*nim1*$^+$ and *cdr2*$^+$, and they suggested that the *cdr* genes may regulate *wee1*$^+$ activity. Indeed, Cdr1p/Nim1p has been shown to inhibit Wee1p directly by phosphorylation (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). *cdr2*$^+$ also encodes a putative protein kinase with homology to the N-terminal catalytic domain of the Snf1p subfamily of serine-threonine protein kinases. Does *cdr2*$^+$ function as a negative regulator of *wee1*$^+$ as originally suggested? *cdr2*$^+$ overexpression data suggests that it does. Overexpression of *cdr2*$^+$ in wild-type cells led to a delay in G_2, followed by defects in cytokinesis, but no G_2 delay was detected when *cdr2*$^+$ is overexpressed in cells lacking *wee1*$^+$ activity. These data, combined with the epistatic relationship between *wee1* and *cdr2* mutants, demonstrate that *cdr2*$^+$ activity is not required in the absence of *wee1*$^+$ to promote mitosis, and possibly Cdr2p acts as an inhibitor of Wee1p. If *cdr2*$^+$ is acting as an inducer of mitosis acting through *wee1*$^+$, does it do so in a manner identical to *cdr1*$^+$/*nim1*$^+$? Our data suggest not. As mentioned above, overexpression of *cdr2*$^+$ led to a delay in G_2 followed by defects in cytokinesis. This is in sharp contrast to *cdr1*$^+$/*nim1*$^+$ overexpression, which promotes entry into mitosis at a reduced cell size (Russell and Nurse, 1987).

Genetic analysis of *cdr1* and *cdr2* mutants further suggests that *cdr2*$^+$ functions in a pathway separate from *cdr1*$^+$/*nim1*$^+$. Even though a *cdr2* null *cdr1*/*nim1* null double mutant is phenotypically indistinguishable from the *cdr2* null, both *cdr2* and *cdr1/nim1* mutants were sensitive to overexpression of the other (our unpublished results), which demonstrates a lack of dependence between *cdr2*$^+$ and *cdr1*$^+$/*nim1*$^+$. These data suggest that *cdr2*$^+$ and *cdr1*$^+$/*nim1*$^+$ do not function in a linear pathway and act independently to promote mitosis. Since Wee1p is the key inhibitor of Cdc2p activity before mitosis (Russell and Nurse, 1987a), there are probably numerous factors and signal transduction pathways, some yet uncharacterized, that regulate *wee1*$^+$ activity.

The additional role of *cdr2*$^+$ in cytokinesis also supports the idea that *cdr2*$^+$ functions in a pathway separate from *cdr1*$^+$/*nim1*$^+$. Cells lacking *cdr2*$^+$ and cells overproducing *cdr2*$^+$ exhibited defects in forming septa and undergoing cell separation. It appears then that *cdr2*$^+$ is required for proper septum formation and cell separation. At this time, we do not understand the molecular role of *cdr2*$^+$ in cytokinesis, except that it is independent of *wee1*$^+$ activity. Thus, if *cdr2*$^+$ functions to negatively regulate *wee1*$^+$, it is doing so in a manner different from that of *cdr1*$^+$/*nim1*$^+$. It is possible that *cdr2*$^+$ acts indirectly to inhibit *wee1*$^+$. Excess Cdr2p might sequester a Wee1p inhibitory factor that is normally activated by Cdr2p; thus, a G_2 delay is produced when *cdr2*$^+$ is overexpressed. Another possibility is that Cdr2p influences the level of Wee1p. In *S. pombe*, the level of Wee1p protein level is cell cycle regulated; Wee1p levels decrease at mitosis, and this reduced level is maintained into G_1 (Aligue *et al.*, 1997). In this scenario, Cdr2p may facilitate the degradation of Wee1p to promote Cdc2p activation and entry into mitosis.

Interestingly, Cdr2p is most similar to the *S. cerevisiae* protein kinase Gin4p. *GIN4* encodes a nonessential protein kinase that is required for the ability of *NAP1* and *CLB2* to promote normal mitotic progression (Altman and Kellogg, 1997). *S. cerevisiae CLB2* encodes a B-type cyclin analogous to *S. pombe* Cdc13p (Fitch *et al.*, 1992; Richardson *et al.*, 1992), whereas the *NAP1* gene product was identified as a Clb2p-binding protein (Kellogg and Murray, 1995; Kellogg *et al.*, 1995). Gin4p binds Nap1p, and Gin4p phosphorylation and activation in mitosis are dependent on both Nap1p and Clb2p (Altman and Kellogg, 1997). Since we have shown that *cdr2*$^+$ also promotes mitosis, it will be interesting to see whether Cdr2p interacts with a protein similar to Nap1p in *S. pombe* and whether such an interaction influences its function. Our genetic data, however, suggest that at least one function of *cdr2*$^+$ lies upstream of *cdc2*$^+$/*cdc13*$^+$ activation, rather than being dependent upon it.

In conclusion, we have begun characterizing the role of *cdr2*$^+$ in *S. pombe* and have found that, like *cdr1*$^+$/*nim1*$^+$, *cdr2*$^+$ functions as a mitotic inducer that is especially important under nutrient limiting conditions and not as a sensor of nutrient limitation. Unlike *cdr1*$^+$/*nim1*$^+$, which acts directly on *wee1*$^+$ to regulate mitosis, the role of *cdr2*$^+$ in cell cycle regulation is more complex; *cdr2*$^+$ has an additional role in cytokinesis and is required for proper septum formation and cell separation.

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