

Antagonism of Chk1 Signaling in the G₂ DNA Damage Checkpoint by Dominant Alleles of Cdr1

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ABSTRACT

Activation of the Chk1 protein kinase by DNA damage enforces a checkpoint that maintains Cdc2 in its inactive, tyrosine-15 (Y15) phosphorylated state. Chk1 downregulates the Cdc25 phosphatases and concomitantly upregulates the Wee1 kinases that control the phosphorylation of Cdc2. Overproduction of Chk1 causes G₂ arrest/delay independently of DNA damage and upstream checkpoint genes. We utilized this to screen fission yeast for mutations that alter sensitivity to Chk1 signaling. We describe three dominant-negative alleles of *cdr1*, which render cells supersensitive to Chk1 levels, and suppress the checkpoint defects of *chk1Δ* cells. *Cdr1* encodes a protein kinase previously identified as a negative regulator of Wee1 activity in response to limited nutrition, but Cdr1 has not previously been linked to checkpoint signaling. Overproduction of Cdr1 promotes checkpoint defects and exacerbates the defective response to DNA damage of cells lacking Chk1. We conclude that regulation of Wee1 by Cdr1 and possibly by related kinases is an important antagonist of Chk1 signaling and represents a novel negative regulation of cell cycle arrest promoted by this checkpoint.

GENOME integrity is essential for normal tissue homeostasis. DNA damage, either from extrinsic sources such as radiation and genotoxic drugs or from intrinsic sources from metabolism and DNA replication, poses a threat to the stable inheritance of the genome. Not surprisingly, therefore, cells have evolved a plethora of pathways to sense and repair DNA damage and also to alter cell cycle progression to allow time for DNA repair to complete. The latter, known as DNA damage checkpoints, respond to many different lesions throughout the cell cycle and ultimately regulate the cyclin-dependent kinases that drive the cell cycle transitions.

The transition from G₂ into mitosis is controlled by the cyclin B/Cdc2 kinase. Cdc2 is the catalytic subunit of this complex, although its kinase activity requires its binding to the cyclin partner. While Cdc2 levels remain constant throughout the cell cycle, cyclin B accumulates during interphase, forms complexes with Cdc2, and is rapidly destroyed by ubiquitination at the metaphase-to-anaphase transition. As cyclin B/Cdc2 complexes form, they are subjected to an inhibitory tyrosine phosphorylation on residue 15 (Y15) by specific tyrosine kinases, generally known as Wee1. Such inactive complexes accumulate during G₂, and when conditions are appropriate for mitotic entry, Y15 is dephosphorylated by the

Cdc25 phosphatases, and cells rapidly enter mitosis (NURSE 1990; DUNPHY 1994).

DNA damage in S-phase and G₂ activates a checkpoint pathway that is largely conserved from the fission yeast *Schizosaccharomyces pombe* through to humans (O'CONNELL *et al.* 2000). The effector of this signaling cascade is a serine/threonine protein kinase, Chk1. Activation of Chk1 by DNA damage requires several protein complexes, including the PCNA-related 9-1-1 complex, its loader Rad17-Rfc2-5, and the PI-3K-related kinases ATM and ATR, which phosphorylate Chk1 on C-terminal residues, an event required for its activation. Several mediator proteins are also required for Chk1 activation, including the BRCT-domain proteins Crb2 and Cut5 in *S. pombe* (O'CONNELL *et al.* 2000) and BRCA1, Mdc1, and TopBP1 in metazoans (GARCIA *et al.* 2005). When Chk1 is overexpressed, it can signal a G₂ delay/arrest independently of DNA damage and these upstream regulators (WALWORTH *et al.* 1993).

Activated Chk1 ensures a delay in G₂ until completion of DNA repair by enforcing the maintenance of Cdc2 Y15 phosphorylation. This is achieved through regulation of both Wee1 kinases and Cdc25 phosphatases (O'CONNELL *et al.* 2000). In *S. pombe*, phosphorylation of Wee1 by Chk1 leads to a transient stabilization of this short-lived protein and hence to increased cellular levels of Wee1 kinase (O'CONNELL *et al.* 1997; RALEIGH and O'CONNELL 2000). Similarly, Chk1 has been implicated in the regulation of Wee1 homologs in *Xenopus* (LEE *et al.* 2001; STANFORD and RUDERMAN 2005), *Drosophila* (PRICE *et al.* 2000; STUMPF *et al.* 2004),

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and humans (LI *et al.* 2002). For Cdc25, its phosphorylation by Chk1 in *S. pombe* leads to its nuclear exclusion and catalytic inactivation (FURNARI *et al.* 1999; LOPEZ-GIRONA *et al.* 1999, 2001). In mammals, there are three isoforms of Cdc25, Cdc25A, -B, and -C. Each can be phosphorylated by Chk1, although only Cdc25A is essential for cell cycle progression in the mouse (FERGUSON *et al.* 2005). Chk1 phosphorylation of Cdc25A leads to its degradation by ubiquitin-dependent proteolysis (SORENSEN *et al.* 2003; XIAO *et al.* 2003). Either mode of regulating Cdc2 Y15 phosphorylation is sufficient for a checkpoint delay; that is, cells lacking Wee1 or cycling independently of Cdc25 are largely checkpoint proficient. When both are absent, and the cell cycle is driven only by cyclin accumulation, the checkpoint fails, and cells become hypersensitive to DNA damage (RALEIGH and O'CONNELL 2000).

Inactivation of Chk1 is both necessary and sufficient to signal resumption of the cell cycle through activation of Cdc2 (LATIF *et al.* 2004). This is achieved via dephosphorylation of the activating phospho-residues by Dis2, a protein phosphatase 1 (PP1) homolog, in *S. pombe* (DEN ELZEN and O'CONNELL 2004), and by PP1 and the p53-target PPMID (Wip1) in humans (LU *et al.* 2005). How this is spatially and temporally regulated is not clear at this stage, and it remains likely that additional mechanisms to antagonize Chk1 signaling exist. Other mechanisms of regulating Cdc2 activity have been described. These include regulation of Wee1 activity by the Cdr kinases in *S. pombe* under conditions of limited nutrition, especially nitrogen starvation, where downregulation of Wee1 is an important event in advancing mitotic entry to achieve G₁ cell cycle arrest (YOUNG and FANTES 1987; FEILOTTER *et al.* 1991; COLEMAN *et al.* 1993; PARKER *et al.* 1993; WU and RUSSELL 1993). Further, Polo kinases play an important role in upregulating Cdc25 activity at mitotic entry, and regulation of Polo kinases has been implicated in DNA damage checkpoint proficiency (BARR *et al.* 2004). Although cells lacking Chk1 fail to delay in response to DNA damage, it is not known whether other pathways converging on Cdc2 activation contribute to the delay in wild-type cells.

We have used genetic screens in *S. pombe* to attempt to identify additional regulators and effectors of the G₂ DNA damage checkpoint. Here we describe a screen in which we have isolated mutations that alter the cellular sensitivity to ectopic Chk1 expression. We isolated three alleles of *cdr1* that render cells supersensitive to Chk1. These mutations suppress the DNA damage sensitivity of mutants in the checkpoint pathway. We show that the *cdr1* mutations are dominant-negative alleles, linking Cdr1 for the first time to the DNA damage response, in addition to its role in nutritional signaling. Upon limited nutrition, particularly nitrogen deprivation, Cdr1 phosphorylates and negatively regulates the kinase activity of Wee1, thus activating Cdc2, and cells enter

into mitosis at a smaller cell size (FEILOTTER *et al.* 1991; COLEMAN *et al.* 1993; WU and RUSSELL 1993). As *cdr1Δ* does not suppress the checkpoint mutations, this dominant-negative effect may extend to other related protein kinases. Finally, upregulation of Cdr1 leads to a checkpoint defect, DNA damage hypersensitivity, and exacerbation of the defects of *chk1Δ* cells. We conclude that Cdr1 and related kinases antagonize Chk1 in the regulation of Wee1 in the context of the DNA damage checkpoint.

MATERIALS AND METHODS

Fission yeast methods: All strains used were derivatives of 972 *h⁻* and 975 *h⁺*, and detailed genotypes are listed in the supplemental material at <http://www.genetics.org/supplemental/>. Standard procedures and media were used for growth and genetic manipulation (MORENO *et al.* 1991). Methods for transformation, microscopy, and DAPI staining have been described previously (O'CONNELL *et al.* 1997). For *nmt1* promoter induction experiments, exponentially growing cells were washed three times in medium lacking thiamine and then inoculated into defined medium lacking thiamine and collected after 15–18 hr. For testing of dominance without background sporulation, *h⁻/h⁻* diploids were constructed by protoplast fusion and selected by cross complementation of *leu1-32* and *his3-D1* markers.

Screening to identify the location of the D8 mutation: On the basis of the recessive lethal phenotype of the *D8* strain, suppressed by the tRNA *sup3-5*, we designed a plasmid shuffle screen. For this we cloned an *adh1*-driven herpes simplex virus 1 thymidine kinase (TK) gene (*Padh1::TK*) (KIELY *et al.* 2000) into the *Sad* site of pREP5 (*sup3-5*, *nmt1*). *ade6-704* cells were transformed with this plasmid, conferring adenine prototrophy (*sup3-5* suppression of *ade6-704*) and sensitivity to the thymidine analog (+)-5-fluoro-2'-deoxyuridine (FUdR), which, when phosphorylated by *Padh1*-driven TK, inhibits thymidylate synthetase and blocks dTTP synthesis, killing the cells. This strain was crossed to *nmt1::chk1(int)sup3-5 D8 ade6-704 ura4-D18* to derive *D8* (pREP5-*sup3-5-Padh1::TK ade6-704 ura4-D18* progeny (*i.e.*, no longer containing *sup3-5 nmt1::chk1*). The presence of the *D8* mutation, now suppressed by the *sup3-5* gene within the *Padh1::TK* construct, was confirmed by transformation with pREP41X::*chk1*. This strain was then transformed with a *ura4*-based genomic library (BARBET *et al.* 1992), and transformants were selected on minimal medium with adenine to allow loss of the *sup3-5* marker (within pREP5-*Padh1::TK* plasmid) with *D8*'s recessive lethality, presumably now rescued by a library plasmid. Such events were screened for by replica plating to minimal medium with adenine (to allow *ade6-704* cells to grow in the absence of *sup3-5*) and 50 μM FUdR to select for the absence of *Padh1::TK*. Surviving colonies, now adenine auxotrophs, had library plasmid-borne rescue of the *D8*-associated lethality. From this, a total of five independent clones of *cdc5* were obtained. However, further analysis (see RESULTS) showed that the *cdc5* mutation in *D8* was not the cause of the supersensitivity to Chk1, but rather due to the closely linked *cdr1* gene, and that the *D8* strain contained these two linked mutations.

DNA damage survival assays: For ultraviolet-C (UV-C) assays, 100, 1000, and 10,000 cells were plated in triplicate on yeast extract plus supplements (YES) agar (90-mm plate) or on EMM2 agar without thiamine for the overexpression of Cdr1 protein and irradiated in a Stratalinker. Colonies were counted and the percentage of survival was expressed as a proportion of unirradiated controls after 4 days at 30°. For

methyl methanesulfonate (MMS) sensitivity assays, cultures were diluted to an OD₅₉₅ of 0.1 (2×10^6 cells/ml) and 5 μ l of 10-fold serial dilutions were spotted onto YES, or EMM2 agar for the overexpression of Cdr1 protein, containing the indicated concentrations of MMS. Plates were incubated for 4 days at 30°.

Construction of *cdr1*-Flag3-His6 strains: A Flag₃-His₆ tag was integrated into the 3'-end of the *cdr1* ORF using PCR products derived from pFA6a-Flag₃-His₆-kanMX6 (MORIKAWA *et al.* 2004). G418-resistant colonies were selected and checked for correct integration in the genome by PCR with primers inside and outside the ORF of the *cdr1* gene and by Western blotting. We confirmed that the tagged protein was functional by proficiency for G₁ arrest upon nitrogen depletion. A PCR product from this strain that spanned 300 bp on each side of the integration was derived and used to tag the dominant *cdr1* alleles by homologous recombination.

Protein methods: For detection of Chk1, GST-Cdr1, Cdc2, or Wee1-His₆HA₃, frozen cells were disrupted with glass beads using a bead beater and extracted into lysis buffer 1 (8 M urea, 100 mM Na₂HPO₄, 10 mM Tris-HCl, pH 8.0). The extract was cleared by centrifugation at 13,000 \times *g* for 15 min, and the supernatant was boiled in SDS sample buffer. Protein extracts were run on 7% (Wee1) or 10% (Chk1, GST-Cdr1, Cdc2) SDS-PAGE gels and transferred to nitrocellulose membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11, and 10% methanol for 1 hr. Overexpressed Chk1 was detected by rabbit polyclonal anti-Chk1 (O'CONNELL *et al.* 1997), HA epitope with 12CA5 (Roche), GST with monoclonal anti-GST (Santa Cruz, B14), Y15 phosphorylated Cdc2 (Cell Signaling Technology), total Cdc2 (PSTAIRE, Santa Cruz), and tubulin as a loading control by monoclonal- α -tubulin (Sigma, St. Louis, B-5-1-2). For Cdr1-Flag₃-His₆, the extracts were prepared in lysis buffer 2 (10 mM NaPO₄, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM EDTA, 50 mM NaF, 2 mM DTT) and 5 \times protease inhibitor cocktail (Sigma). Cdr1-Flag₃-His₆ was immunoprecipitated with anti-Flag M2 agarose (Sigma). Extracts were run on a 7% gel and transferred as above, and the Flag-Tag was detected with the anti-Flag M2 (Sigma). Immune complexes were detected with horseradish-peroxidase-linked secondary antibodies (Amersham, Buckinghamshire, UK) followed by chemiluminescence. Dephosphorylation of Wee1 was performed as described (O'CONNELL *et al.* 1997). For Cdr1 autophosphorylation assays, wild-type and mutant GST-Cdr1 expressed from the *nmt1* promoter was recovered on glutathione sepharose from 500 μ g of extract prepared in lysis buffer 2. After extensive washing in lysis buffer 2 and kinase buffer (50 mM Tris, pH 8, 10 mM MgCl₂, 1 mM MnCl₂, 1 mM DTT), cold ATP was added to 100 μ M together with 10 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) and incubated at 30° for 30 min. Reactions were stopped by boiling in sample buffer and separated on SDS-PAGE gels, and the signal quantified with a Bio-Rad (Hercules, CA) FX phosphorimager.

Nitrogen deprivation: Midlogarithmic asynchronous cultures were washed three times in EMM2 medium (MORENO *et al.* 1991) lacking ammonium chloride and then inoculated into minimal medium with and without ammonium chloride to an OD₅₉₅ of 0.1. The cells were collected at 24 and 48 hr and fixed in 70% ethanol. Cells were resuspended in 50 mM sodium citrate, pH 6.5, 20 μ g/ml propidium iodide, and 40 μ g/ml RNaseA and incubated at 37° overnight. DNA content was determined using a FACSCalibur (Becton Dickinson) and CellQuest software.

RESULTS

Isolation of mutants with altered sensitivity to Chk1:

To screen for novel regulators and effectors of Chk1

signaling, we sought to identify genes that modulate the duration of a Chk1-mediated G₂ delay. To this end, we constructed a strain of *S. pombe* in which Chk1 is expressed from the *nmt1* promoter, integrated in single copy using the *sup3-5* tRNA suppressor (KOHLEI *et al.* 1979). In the presence of thiamine, the *nmt1* promoter is repressed (MAUNDRELL 1993) and this level of Chk1 expression does not alter cell cycle progression. Upon withdrawal of thiamine, the derepression of the *nmt1* promoter after four to five cell cycles leads to a G₂ delay; cells are viable but enter mitosis at an increased cell length as a result of a longer G₂ period. A small percentage of the cells die under these conditions, leading to pink colonies on agar plates containing the vital dye phloxin B, compared to wild-type colonies, which are white under these conditions (Figure 1A). This strain was then mutagenized with nitrosoguanidine for several independent screens (A-E) totaling ~300,000 colonies, and mutants were selected that were either resistant or supersensitive to *nmt1*-driven Chk1 in the absence of thiamine, although they remained identical to parental cells in the presence of thiamine (see MATERIALS AND METHODS). Twelve mutants that were resistant to Chk1 overexpression were isolated. Eleven of these were alleles of *rad24*, which encodes a 14-3-3 isoform that mediates Chk1 signaling via interaction with Chk1 and its substrates (CHEN *et al.* 1999; O'CONNELL *et al.* 2000), and will be described in another article.

Of the supersensitive class, three strong mutants (B3, C47, and D8) were isolated from three independent screens (B, C, and D) that were inviable on plates lacking thiamine, although they phenocopied the parental strain in the presence of thiamine (Figure 1A). Genetic analysis showed that these mutations were in the same gene, shown below to be *cdr1*. These cells were examined by microscopy and shown to arrest as highly elongated cells in the absence of thiamine (Figure 1B), which was confirmed to be a G₂ cell cycle arrest by flow cytometry of DNA (not shown). By Western blotting of extracts with affinity purified anti-Chk1 antibodies we confirmed that the Chk1 supersensitive mutants did not arise via upregulation of Chk1 expression, which was unchanged compared to controls (Figure 1C).

We next crossed these strains to wild-type cells to isolate the single mutations away from the integrated *sup3-5 nmt1::chk1*, which was easily achieved for B3 and C47. To confirm that the single mutants retained the hypersensitivity to Chk1, they were transformed with a plasmid in which Chk1 is expressed from an attenuated *nmt1* promoter (pREP41X::*chk1*) that, as an episome, directs ~10-fold lower levels of Chk1 expression compared to the single integrated wild-type *nmt1* promoter (FORSBURG 1993). In wild-type cells, this level of Chk1 expression results in a modest G₂ delay, although the level was lethal to the B3 and C47 strains (Figure 2A). For D8, tetrad analysis showed that this mutation was lethal in the absence of the *sup3-5 nmt1::chk1* construct;

germinated spores arrested as highly elongated, uninucleated cells (not shown). As this strain was isolated on the basis of hypersensitivity to Chk1, we hypothesized that this lethality may be due to the absence of the *sup3-5* marker used to select the *nmt1::chk1* integration. This was indeed the case, and *D8* could be propagated in cells containing *sup3-5* but not *nmt1::chk1*. We transformed this strain with pREP41X::*chk1*, and this too was lethal in the absence of thiamine (not shown).

An important component of our screen was that, for mutants to be retained for further analysis, they needed to be normal in the absence of Chk1 overexpression. To confirm that this was the case, we assayed cell cycle pro-

gression in the single mutants. Although each mutant divided at a cell length of $\sim 18 \mu\text{m}$ compared to wild-type cells, which divide at $14 \mu\text{m}$ (Figure 2B), by monitoring cell numbers in exponentially growing cultures we found that each mutant had a normal cell doubling time (Figure 2C). Therefore, the slightly elongated nature of these cells is a result of a larger birth size rather than an extended growth period without division. We therefore conclude that these strains are specifically supersensitive to Chk1 signaling.

We next assessed whether these mutations were dominant or recessive. We were surprised to observe that each heterozygous diploid retained the supersensitivity to Chk1 expression, albeit to a slightly lesser extent than the haploids on plates (supplemental Figure 1 at <http://www.genetics.org/supplemental/>). Microscopy and FACS confirmed that these heterozygotes retained G₂ arrest upon Chk1 overexpression, whereas wild-type diploid cells overexpressing Chk1 underwent a modest delay (supplemental Figure 1B at <http://www.genetics.org/supplemental/> and data not shown). Thus, each allele is dominant for supersensitivity to Chk1, which could result either from a gain-of-function or from dominant interference with a wild-type allele.

The supersensitive mutants are dominant-negative alleles of *cdr1*: As these alleles are dominant for supersensitivity to Chk1, we could not identify the gene by conventional complementation screens. However, the lethal cell cycle arrest of *D8* that was suppressed by *sup3-5* was recessive in diploids (data not shown). *sup3-5* is a tRNA suppressor that inserts serine residues opposite UGA termination codons (KOHLEI *et al.* 1979), and thus we knew the nature of the lethal mutation in *D8*. We devised a plasmid shuffle screen to replace an episomal *sup3-5* allele with genomic clones (see MATERIALS AND METHODS). From this screen we obtained several clones of *cdc5*, an essential gene required for pre-mRNA splicing (OHI *et al.* 1994). Sequencing of the *cdc5* gene

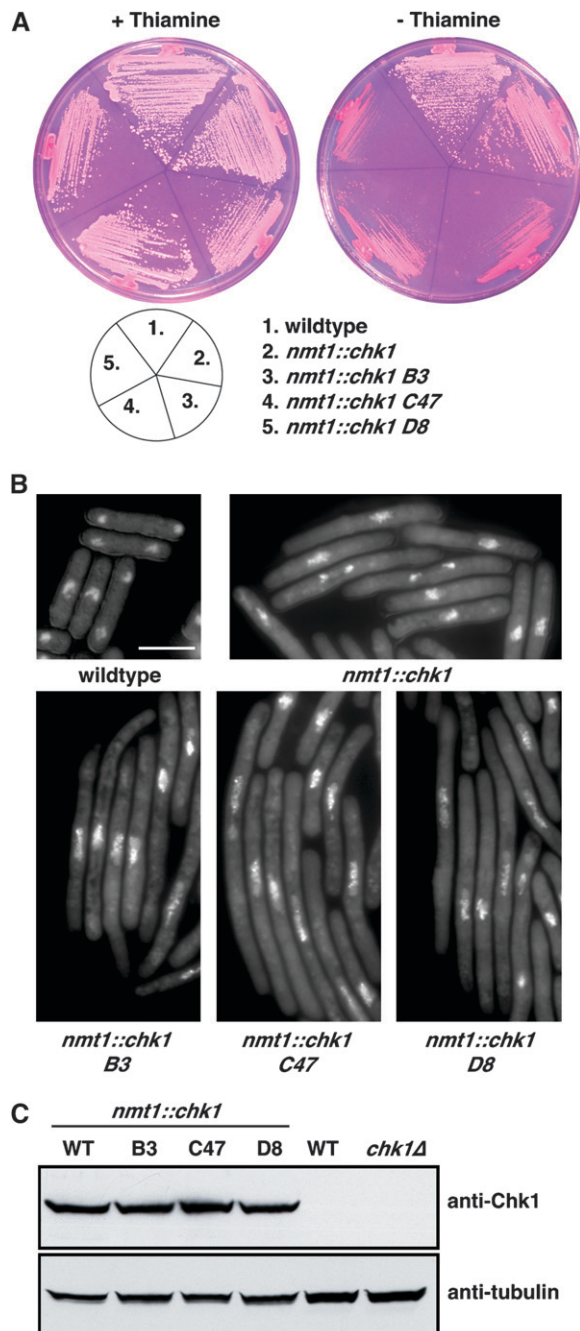


FIGURE 1.—Isolation of mutants supersensitive to Chk1 overexpression. (A) The indicated strains were streaked onto minimal media containing phloxin B together with thiamine (*nmt1* promoter repressed) or without thiamine (*nmt1* promoter derepressed), and the plates were incubated at 30° for 4 days. Note that the residual growth of mutants on plates lacking thiamine is from a heavy inoculum from a plate containing thiamine and also that the *nmt1* promoter takes about five cell cycles to fully derepress. These inviable cells are stained with phloxin B and cannot be propagated on a fresh plate. (B) Cells were grown to midlogarithmic phase in the presence of thiamine, washed three times in thiamine-free medium, and then grown in the absence of thiamine for 18 hr at 30°. Cells were fixed with formaldehyde and stained with DAPI. Cytoplasmic staining is from mitochondrial genomes. Bar, 10 μm . (C) The same cultures as in B were harvested and snap frozen. Extracts were prepared and analyzed by Western blotting using anti-Chk1 polyclonal antibodies, which can detect only overexpressed Chk1. The filter was stripped and probed with antitubulin antibodies.

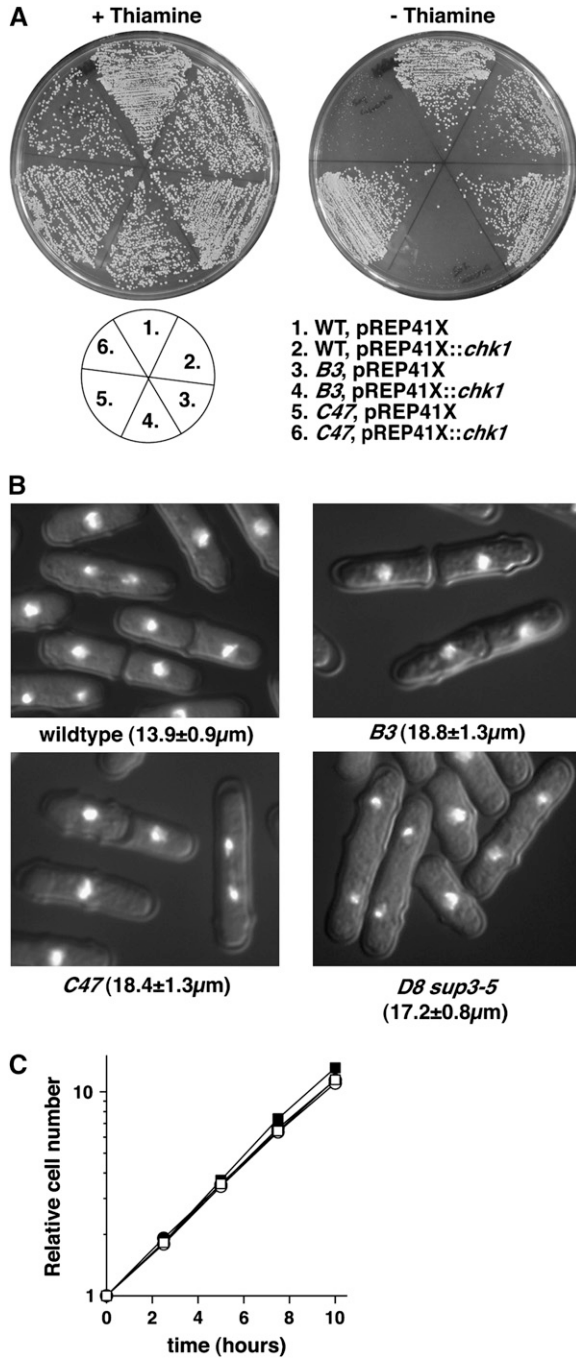


FIGURE 2.—Normal cell cycle progression of the supersensitive mutants in the absence of Chk1 overexpression. (A) *B3* and *C47* were separated from *sup3-5 nmt1::chk1* by tetrad dissection and transformed with pREP41X::*chk1* (medium strength *nmt1*) together with vector and wild-type controls. Sensitivity to Chk1 overexpression in the presence and absence of thiamine was assayed as described in Figure 1. (B) Midlogarithmic cultures of the indicated strains were fixed with formaldehyde and stained with DAPI, and the mean length at division (±standard deviation, $n = 100$) was measured with an eyepiece micrometer. (C) Wild type (□), *B3* (●), *C47* (■), and *D8 sup3-5* (○) were grown to midlogarithmic phase, and cell cycle progression was assayed by triplicate cell number determination using a Coulter counter for 10 hr.

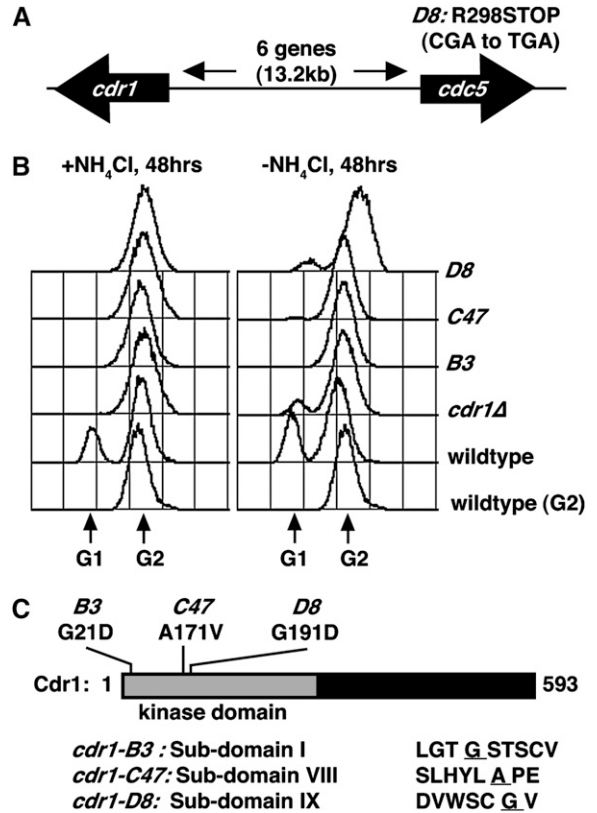


FIGURE 3.—The Chk1 supersensitive mutants are alleles of *cdr1*. (A) A plasmid shuffling screen (see MATERIALS AND METHODS) was used to identify a termination codon in *cdc5* (CGA to TGA) in *D8* cells, but *cdc5* mutations were not present in *B3* or *C47*. The *cdr1* locus is located 13.2 kb to the left of *cdc5* on chromosome I. (B) The mutants exhibit a changed division response (Cdr) phenotype. Cultures were started at 2×10^6 cells/ml and grown for 48 hr to saturation in the presence or absence of NH₄Cl, fixed with 70% ethanol, and processed for DNA content determination by FACS. Note the absence of a G₁ peak in *cdr1* mutants and the *cdr1Δ* control. (C) Mutations in the Cdr1 catalytic domain (subdomains I, VIII, and IX; substituted amino acid is underlined) were present in our *cdr1* mutants.

in the *D8* strain indeed showed a CGA-to-TGA mutation (Figure 3A), truncating Cdc5 after amino acid 298 of 757. We were somewhat surprised by this finding, however, as no link between Cdc5 and Chk1 signaling is apparent. Moreover, we failed to find mutations in *cdc5* in the *B3* or *C47* strains, even though no wild-type recombinants were recovered from crosses between different Chk1 supersensitive alleles (>20,000 spores tested). Linkage analysis could not separate the recessive lethality of *D8* from its dominant supersensitivity to Chk1. We concluded, therefore, that the supersensitivity to Chk1 must arise from a mutation very tightly linked to *cdc5*, and not from the *cdc5* mutation. That is, the *D8* strain fortuitously contained two linked mutations: one in *cdc5* and one in another gene that was the cause of the supersensitivity to Chk1.

While sequencing nearby candidate genes, we noted that saturated cultures of the supersensitive mutants were not the characteristically small cells seen in saturated wild-type cultures. Under conditions of limited nutrition, which results from either culture saturation or can be induced by nitrogen withdrawal, *S. pombe* cells advance the timing of mitotic entry, and as G₂ is the major growth period, these cells become smaller and eventually undergo G₁ cell cycle arrest (YOUNG and FANTES 1987). FACS analysis confirmed an absence of G₁ cell cycle arrest in the mutant strains (Figure 3B), which is reminiscent of cells lacking either of two related protein kinases, Cdr1 and Cdr2, which derive their name from this changed *d*ivision *r*esponse (YOUNG and FANTES 1987). Inspection of the genes within the vicinity of *cdc5* showed that *cdr1* was separated from *cdc5* by six genes >13.2 kb (Figure 3A). Normally this distance would be separable by recombination and *cdr1* has not been implicated in checkpoint signaling, but we nevertheless sequenced *cdr1* in each of the mutants. Indeed, each allele contained single amino acid substitutions in largely invariant residues of the first, eighth, and ninth kinase subdomains (Figure 3C). Thus, the mutations are alleles of *cdr1* and were therefore named *cdr1-B3*, *-C47*, and *-D8*. Given the highly conserved nature of the catalytic subdomains within serine/threonine protein kinases (HANKS *et al.* 1988) and the dominance of the supersensitive to the Chk1 phenotype and the Cdr phenotype in the saturated cultures, these are likely to be dominant-negative *cdr1* alleles.

The *cdr1* mutants were then crossed to a number of checkpoint and cell cycle mutants to establish whether a normal pattern of signaling was present in these cells and to attempt to decipher where in the checkpoint pathway Chk1 signaling is upregulated. Although Cdr1 is known to regulate Wee1 (COLEMAN *et al.* 1993; PARKER *et al.* 1993; WU and RUSSELL 1993), we needed to address whether this was the sole cause of the supersensitive phenotype. Cell cycle delay/arrest resulting from Chk1 overexpression is independent of upstream checkpoint *rad* genes (WALWORTH *et al.* 1993). Similarly, the *cdr1* mutants did not require upstream components such as Rad3, the 9-1-1 complex, or the BRCT-domain protein Crb2 to exhibit supersensitivity to Chk1 overexpression (not shown). However, cells lacking the Cdc2 Y15 kinase Wee1 or the interacting 14-3-3 protein Rad24 are largely insensitive to Chk1 overexpression (O'CONNELL *et al.* 2000). Double mutants were therefore constructed between each *cdr1* allele and *wee1*Δ or *rad24*Δ, and sensitivity to Chk1 overexpression from the integrated *nmt1::chk1* allele was assayed. Both mutations suppressed the supersensitivity of the dominant *cdr1* mutants, which were capable of forming colonies on medium lacking thiamine (supplemental Figure 2 at <http://www.genetics.org/supplemental/>) and were no longer elongated (not shown). These data confirm that the *cdr1* mutants exert their phenotype at the level of Wee1.

The dominant alleles of *cdr1* bypass G₂ DNA damage checkpoint defects: We next constructed double mutants between each *cdr1* allele and *rad3*Δ, *rad17*Δ, *crb2*Δ, and *chk1*Δ (without *nmt1::chk1*). Figure 4 shows that the *cdr1* mutations indeed partially suppressed the hypersensitivity of *chk1*Δ cells to the alkylating agent MMS and to UV-C irradiation. Similar partial suppression was also observed with *rad3*Δ, *rad17*Δ, and *crb2*Δ double mutants (not shown). The *cdr1* mutants themselves showed wild-type sensitivities to a range of DNA-damaging agents. This was expected, given their sensitized response to Chk1 overexpression, which, if anything, should further enforce the checkpoint response to DNA damage.

While Wee1 is implicated in checkpoint signaling in several systems, Cdr1 has been implicated in cell cycle control only in response to limited nutrition, which does not occur in any of the experiments using Chk1 overexpression or checkpoint activation in midlogarithmic cultures. We therefore wanted to assess whether the dominant-negative *cdr1* alleles exerted their effects only on Cdr1, or whether other related kinases may also be dominantly interfered with. We favored the latter hypothesis, as we did not obtain recessive alleles of *cdr1* (or *cdr2*) in our screen. Although *cdr1*Δ cells are somewhat more sensitive to Chk1 overexpression than wild-type cells, they do form colonies on media lacking thiamine, although they are stained with phloxin B, indicating the presence of some dead cells (Figure 4C). Further, *cdr1*Δ was not capable of suppressing the MMS or UV-C hypersensitivity of *chk1*Δ cells (Figure 4, D and E). Therefore, these alleles dominantly interfere with molecules other than Cdr1 alone.

Biochemical analysis of Cdr1 mutants: Cdr1 exists in cells as multiple phospho-isoforms generated, at least in part, by autophosphorylation (WU *et al.* 1996). We did not observe changes in the multiple Cdr1 species upon Chk1 activation by irradiation or overexpression (data not shown), showing that at least autophosphorylation was unaffected. There have been several reports regarding phosphorylation of Wee1 by Cdr1 using recombinant proteins produced in insect cells (COLEMAN *et al.* 1993; PARKER *et al.* 1993; WU and RUSSELL 1993), but not using Cdr1 isolated from *S. pombe* extracts. We attempted to reproduce these experiments using immunoprecipitated endogenous or overexpressed Cdr1 and bacterially expressed Wee1 as substrate, but were unable to demonstrate significant kinase activity under any condition. Thus, while it is possible that Cdr1 activity is modulated by checkpoint signaling, this does not involve changes in Cdr1 protein levels or autophosphorylation. Although autophosphorylation assays are less quantitative than when excess substrate is provided, these observations lend further support to the notion that these mutations dominantly interfere with Cdr1-mediated Wee1 regulation.

Each Cdr1 mutant protein showed altered migration on SDS-PAGE, the most extreme being *cdr1-D8*

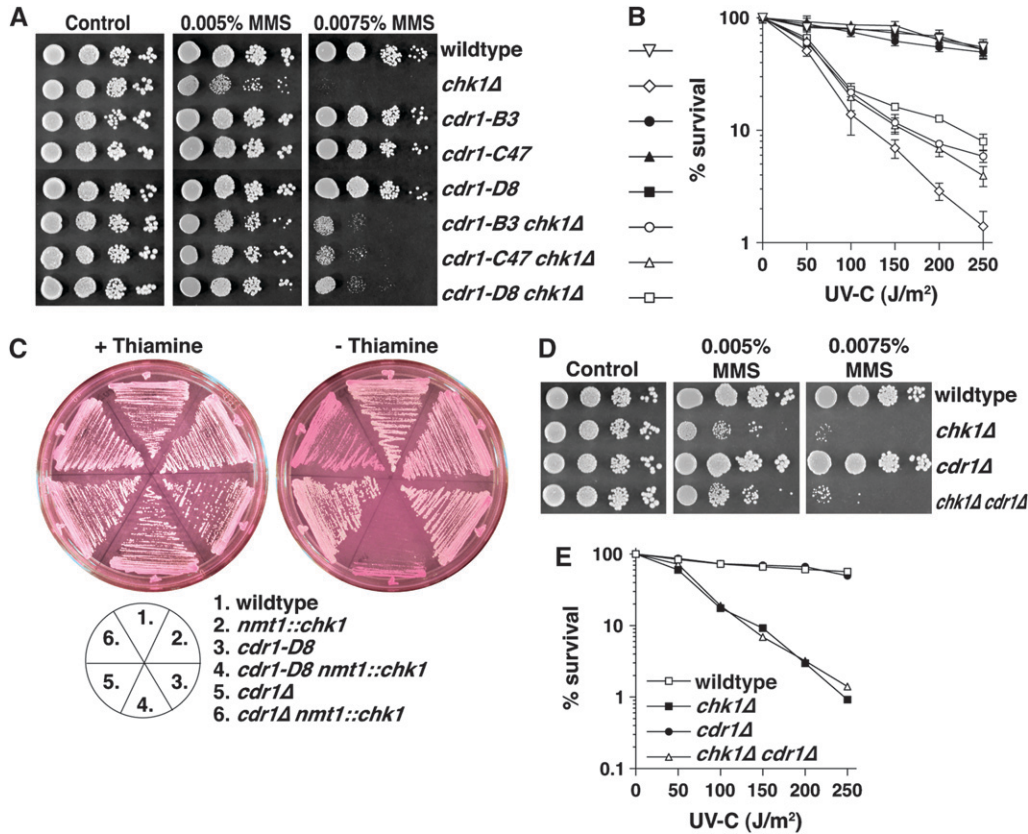


FIGURE 4.—Dominant-negative *cdr1* mutations partially suppress the MMS and UV-C hypersensitivity of *chk1Δ*. (A) Ten-fold serial dilutions of the indicated strains were spotted onto YES agar with a range of concentrations of MMS, and colonies were allowed to form over 4 days at 30°. (B) UV-C survival assays of the same series of strains shown in A. (C) Plate assays of sensitivity to Chk1 overexpression were carried out as described in Figure 1. Note that *cdr1Δ* shows an intermediate sensitivity to Chk1 compared to wild-type and *cdr1-D8* controls. (D) MMS sensitivity and (E) UV-C survival was assayed as in A and B. Note that *cdr1Δ* does not partially suppress *chk1Δ*.

(Figure 5A), which is similar to that described for the kinase-dead *cdr1-K41A* mutation (WU *et al.* 1996). It is notable that *cdr1-D8* gave the strongest suppression of *chk1Δ* and the other checkpoint mutants (Figure 4) and consistently had the strongest sensitivity to Chk1 upon replica plating. *In vitro* autophosphorylation assays using GST-Cdr1 fusion proteins purified from *S. pombe* extracts showed a significant reduction of activity in *cdr1-B3* and *-C47* and almost complete loss of activity in *cdr1-D8* (Figure 5B).

Cdr1 is known to negatively regulate Wee1 via phosphorylation of residues in the C-terminal catalytic domain of Wee1 (COLEMAN *et al.* 1993; PARKER *et al.* 1993). We next monitored phosphorylation of endogenous Wee1 in cells overproducing wild-type and mutant Cdr1 proteins from the *nmt1* promoter. Wee1 migrates as a prominent band with a slower migrating smear that is due to phosphorylation (O'CONNELL *et al.* 1997). Wild-type Cdr1 overexpression resulted in a significant reduction in Wee1 mobility that was confirmed to be hyperphosphorylation using calf-intestinal phosphatase (Figure 5, C and D). Overexpression of Cdr1-B3 or *-C47* did not affect Wee1 phosphorylation, whereas overexpression of Cdr1-D8 consistently reduced basal phosphorylation of Wee1 (Figure 5C). Moreover, wild-type Cdr1 overexpression results in a “wee” phenotype (WU *et al.* 1996) and a reduction in Cdc2Y15 phosphorylation (Figure 5C). Conversely, the

overexpression of the dominant alleles resulted in a Wee1-dependent cell cycle delay (Figure 5E and data not shown). Together these data show that the *cdr1* alleles isolated in our screen show poor kinase activity toward Wee1 *in vivo* and exert dominant-negative effects acting through Wee1 regulation.

Cdr1 overexpression results in DNA damage checkpoint defects: As the above data show that Cdr1 functions downstream of Chk1, most likely through regulation of Wee1, we asked whether upregulation of Cdr1 could overcome a G₂ DNA damage checkpoint in otherwise wild-type cells. Expression of Cdr1 from the *nmt1* promoter causes a “wee” phenotype due to shortening of G₂ by Wee1 downregulation (WU *et al.* 1996). We found that this level of overexpression also caused a hypersensitivity to both MMS and UV-C (Figure 6, A and B). The degree of sensitivity was similar to that of *wee1Δ* cells. Like *wee1Δ* (WALWORTH *et al.* 1993), Cdr1 overexpression also exacerbated the sensitivity of *chk1Δ* cells. While *wee1Δ* cells do show some cell cycle delay in response to DNA damage (BARBET and CARR 1993), the kinetics of cell cycle arrest are somewhat delayed (RALEIGH and O'CONNELL 2000), especially when considering the shortened G₂ period of these cells. Both *wee1Δ* and *nmt1::cdr1* cells showed a significant percentage of “cut” cells following irradiation (Figure 6C), which, in *S. pombe*, is indicative of progression through mitosis prior to completion of DNA repair.

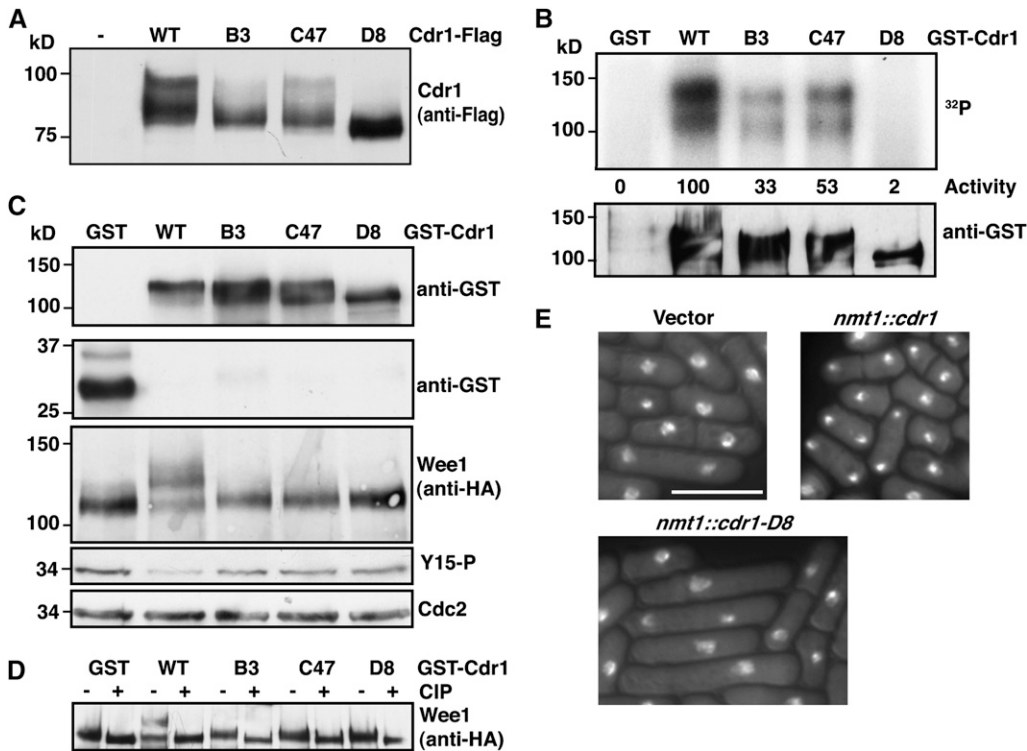


FIGURE 5.—Regulation of Wee1 by Cdr1. (A) Each *cdr1* allele was tagged with His₆Flag₃ at the 3'-end of its endogenous locus. Proteins were immunoprecipitated with anti-Flag M2 beads and detected with M2 antibodies. (B) GST-tagged wild-type and mutant Cdr1 were expressed from the *nmt1* promoter and purified on glutathione sepharose, and auto-phosphorylation was assayed *in vitro* from 50% of the recovered material. "Activity" is the arbitrary units determined by a Bio-Rad FX phosphorimager of the ³²P signal, and levels of GST-Cdr1 in the assays were determined by Western blotting. (C) GST and GST-Cdr1 fusion proteins were expressed from the *nmt1* promoter for 15 hr at 30° in cells expressing Wee1-His₆HA₃ from the endogenous locus. Anti-GST Western blots show GST and GST-Cdr1 levels, and Wee1 is detected with anti-HA antibodies. Identical effects on Wee1 were obtained with untagged Cdr1 constructs. Total Cdc2 and Y15 Cdc2 were detected from the same extracts. (D) Cell extracts were prepared as in C, and Wee1-His₆HA₃ was recovered on Ni-NTA agarose. Samples were split and treated with calf intestinal phosphatase (CIP) or mock treated. (E) Cdr1 and Cdr1-D8 were expressed from the *nmt1* promoter in wild-type cells for 18 hr at 30°, fixed, and stained with DAPI. Bar, 10 μm. Overexpressed Cdr1-B3 and -C47 behave identically to Cdr1-D8 (data not shown).

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Overexpression of wild-type or mutant Cdr1 in *wee1Δ* cells did not affect the response to DNA damage compared to vector controls (data not shown), confirming that the effects of Cdr1 overexpression are via negative regulation of Wee1. Moreover, Cdr1 overexpression suppressed the G₂ delay caused by Chk1 overexpression (not shown). Together, these data show that upregulation of Cdr1 by overexpression can cause checkpoint defects through downregulation of Wee1.

DISCUSSION

Recent years have seen rapid advances in our understanding of cell cycle checkpoints. Although a detailed description of the highly conserved events leading to Chk1 activation has been elucidated, a description of mechanisms by which the checkpoint arrest is terminated remains somewhat rudimentary. Here we have identified a hitherto unknown function for Cdr1, and most likely other Cdr1-related kinases, in the negative regulation of the G₂ DNA damage checkpoint.

Cdr1 was first identified as *nim1*, a truncated clone of *cdr1* that was a high-copy suppressor of the temperature-sensitive G₂ arrest of the *cdc25-22* mutant (RUSSELL and NURSE 1987; FEILOTTER *et al.* 1991). The original mutants in *cdr1*, and a related kinase encoded by *cdr2*,

were identified in a screen for mutants that failed to arrest as small G₁ cells in response to nitrogen deprivation (YOUNG and FANTES 1987). Subsequent experiments using recombinant proteins showed that Cdr1 phosphorylates the catalytic domain of Wee1 and suppresses its kinase activity against Y15 of Cdc2 (COLEMAN *et al.* 1993; PARKER *et al.* 1993; WU and RUSSELL 1993). No direct physical interaction has been reported for the endogenous Wee1 and Cdr1 proteins, which may be technically challenging, as these proteins are labile and of low abundance. Despite this, the above *in vitro* biochemical data support the genetic experiments in *S. pombe* that indicated that *cdr1* acts in opposition to *wee1* to advance mitotic entry under conditions of nitrogen starvation, and overexpression studies showed that no other cellular targets exist (LUNDGREN *et al.* 1991).

In addition to this well-defined role in nutritional signaling, the evidence that Cdr1 negatively regulates checkpoint signaling is as follows: First, the dominant-negative *cdr1* alleles sensitize cells to Chk1 overexpression. Second, these mutations suppress the DNA damage hypersensitivity of *chk1Δ* and mutations in upstream components required for Chk1 activation. We failed to obtain evidence for checkpoint regulation of Cdr1, which also is not regulated through the cell cycle (RUSSELL and NURSE 1987; WU *et al.* 1996). However,

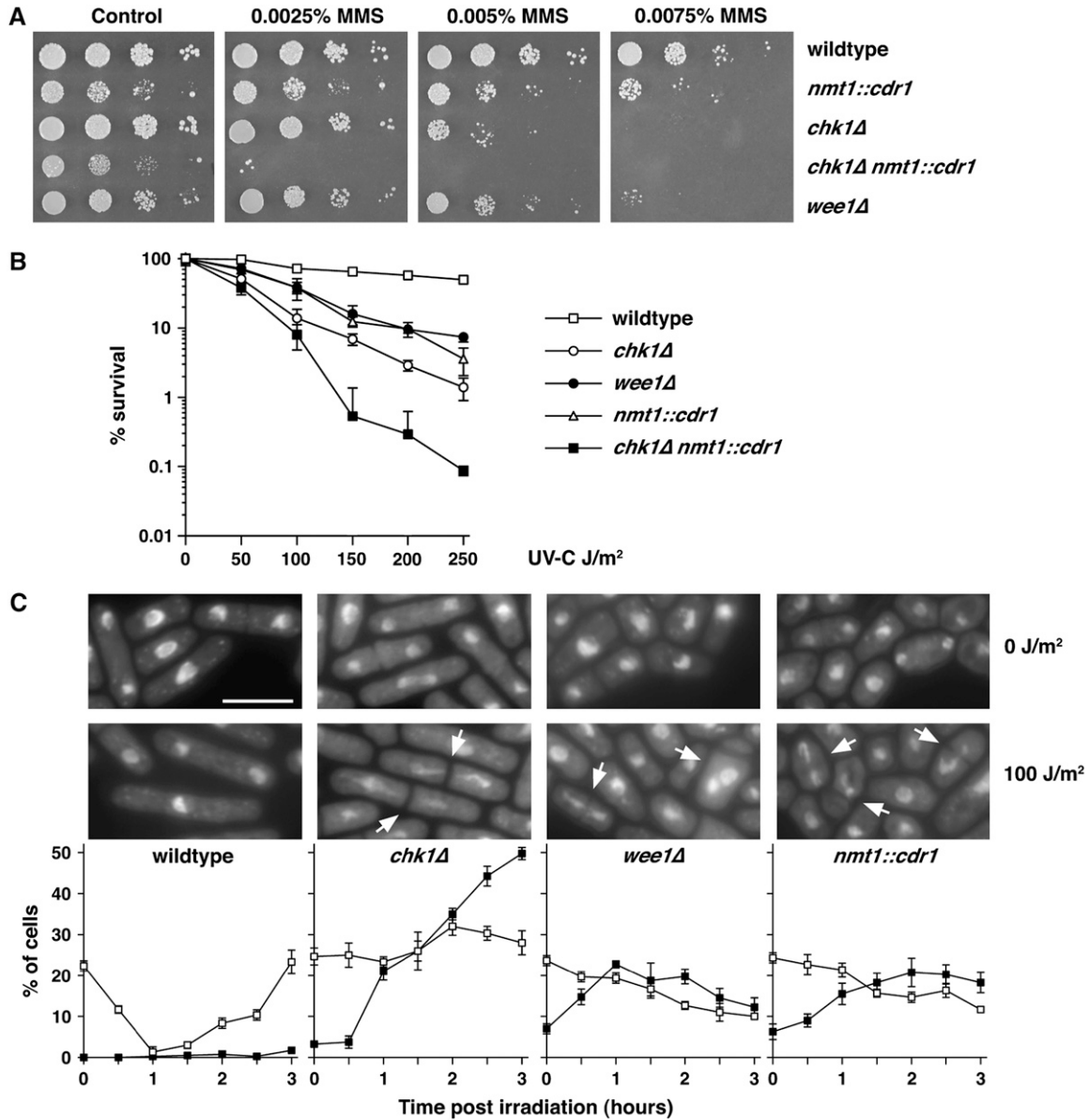


FIGURE 6.—Cdr1 overexpression results in a partially defective checkpoint response. (A) MMS sensitivity and (B) UV-C survival assays were performed as in Figure 4. Note that *nmt1::cdr1* cells exhibit a sensitivity similar to that of *wee1Δ* cells and that Cdr1 overexpression, while somewhat toxic, substantially enhances the MMS and UV-C hypersensitivity of *chk1Δ* cells. (C) The indicated strains were mock irradiated (0 J/m²) or UV-C irradiated (100 J/m²) and grown at 30°. At 30-min intervals for 3 hr, samples were taken and fixed with formaldehyde. Fixed samples were stained with DAPI, and the percentage of cells that were binucleate (□) or exhibited an aberrant mitosis (cut phenotype, ■) were counted in triplicate (± standard deviation, n = 100). A drop in binucleates indicates a cell cycle delay, and aberrant mitoses (arrowed) are indicative of checkpoint failure. Bar, 10 μm.

as Chk1 activity is induced by checkpoint regulation (DEN ELZEN and O'CONNELL 2004), the ratio of Cdr1:Chk1 activity toward Wee1 changes under these conditions. As with *cdr1Δ* (RUSSELL and NURSE 1987; FEILOTTER *et al.* 1991), each dominant allele was synthetically lethal with *cdc25-22* (data not shown), which is also consistent with a lack of restraint on Wee1 activity. Finally, overexpression of Cdr1 renders cells hypersensitive to DNA damage and exacerbates the sensitivity of *chk1Δ* cells. In this regard, Cdr1 overexpression mimics the effects of *wee1Δ* in the context

of checkpoint signaling. Although *wee1Δ* cells retain the ability to delay G₂ progression in response to DNA damage, they are considerably DNA damage hypersensitive and show an induction of mitotic defects following DNA damage. Thus, as *wee1Δ* cells are defective in successfully completing mitosis following DNA damage, we consider this to be a physiologically defective response despite the residual G₂ delay, which is in keeping with data showing that Wee1 homologs are involved in checkpoint signaling in *Drosophila*, *Xenopus*, and humans (PRICE *et al.* 2000; LEE *et al.* 2001; LI *et al.*

2002; STUMPF *et al.* 2004; STANFORD and RUDERMAN 2005). Although Cdr1 overexpression has been shown to cause a "wee" phenotype (RUSSELL and NURSE 1987; WU *et al.* 1996), the only function for Cdr1 determined previously from the *cdr1Δ* cells had been an inability to arrest in G₁ upon nitrogen starvation (YOUNG and FANTES 1987; FEILOTTER *et al.* 1991). All the experiments presented here, except those presented in Figure 3B, have been performed with midlogarithmic cultures with adequate nutrients. Here we show that *cdr1Δ* does not suppress the sensitivity of *chk1Δ* to DNA damage and is not as sensitive to Chk1 overexpression as the dominant-negative *cdr1* alleles. These data explain why our screen did not identify recessive *cdr1* alleles and imply that the phenotypes of these alleles involve interference with additional molecules. Cdr2 is a Cdr1-related kinase that also regulates Wee1 under conditions of nitrogen starvation, although it has a Wee1-independent role in the regulation of cytokinesis (BREEDING *et al.* 1998; KANO and RUSSELL 1998). Budding yeast homologs of Cdr1/2 are similarly involved in cytokinesis (MCMILLAN *et al.* 1999; SHULEWITZ *et al.* 1999), but have not been shown to regulate DNA damage responses, although the DNA damage checkpoint in this organism does not signal via the Wee1 homolog Swe1. We assayed the suppression of MMS and UV-C sensitivity of *cdr2Δ chk1Δ* and *cdr1Δ cdr2Δ chk1Δ*, and in no case did we observe the suppression obtained with the dominant-negative alleles (data not shown). There are several other *S. pombe* protein kinases with significant similarity to Cdr1 ($P = 10^{-47}$ – 10^{-63} by BLAST): Kin1, a protein kinase involved in cell polarity (LEVIN and BISHOP 1990), and Ppk25, Ssp2, and Ppk9, protein kinases of unknown function (BIMBO *et al.* 2005). As the dominant-negative proteins do not accumulate, it is not clear how they exert their dominant effect. This conceivably could be through sequestration of Wee1, although we were not able to show a stable interaction between Wee1 and either wild-type or mutant Cdr1 (data not shown). Alternatively, the mutants could interfere with Cdr1/2- and/or Chk1-mediated Wee1 phosphorylation through transient interaction with Wee1 or upstream regulators, such as Rad24. However, as the dominant alleles suppress *chk1Δ*, a direct effect on Chk1 is not likely. While we did see changes in the mobility of Wee1 in these mutants with and without overexpression (Figure 5C and data not shown), with the multiple kinases impinging on Wee1, a detailed map of phosphorylation will be required to confirm this hypothesis. Nevertheless, our observations confirm the importance of Wee1 regulation in the conveyance of Chk1-dependent checkpoint signals through to Cdc2 to allow time for DNA repair prior to mitotic commitment.

At this stage, the identity of the human homolog of Cdr1 is not clear, although there are several related proteins. However, the high level of conservation of the regulation of the G₂/M transition and the G₂ DNA

damage checkpoint between *S. pombe* and humans suggests that a functional homolog should exist, and it will be important to establish whether it, too, antagonizes Chk1 signaling. Several studies of human cells have shown that inhibition of Chk1 can greatly sensitize tumor cells to genotoxic stresses, particularly in cells lacking p53 function. Thus, there has been great interest in identifying Chk1 inhibitors to be used as a targeted therapy, particularly in chemoresistant tumors. Should Cdr kinases antagonize Chk1 in human cells, then agonists of Cdr kinases may represent an alternative mechanism to achieve this end.

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