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Characterization of *mec1* Kinase-Deficient Mutants and of New Hypomorphic *mec1* Alleles Impairing Subsets of the DNA Damage Response Pathway

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DNA damage checkpoints lead to the inhibition of cell cycle progression following DNA damage. The *Saccharomyces cerevisiae* Mec1 checkpoint protein, a phosphatidylinositol kinase-related protein, is required for transient cell cycle arrest in response to DNA damage or DNA replication defects. We show that *mec1* kinase-deficient (*mec1kd*) mutants are indistinguishable from *mec1*Δ cells, indicating that the Mec1 conserved kinase domain is required for all known Mec1 functions, including cell viability and proper DNA damage response. Mec1kd variants maintain the ability to physically interact with both Ddc2 and wild-type Mec1 and cause dominant checkpoint defects when overproduced in *MEC1* cells, impairing the ability of cells to slow down S phase entry and progression after DNA damage in G_1 or during S phase. Conversely, an excess of Mec1kd in *MEC1* cells does not abrogate the G_2/M checkpoint, suggesting that Mec1 functions required for response to aberrant DNA structures during specific cell cycle stages can be separable. In agreement with this hypothesis, we describe two new hypomorphic *mec1* mutants that are completely defective in the G_1/S and intra-S DNA damage checkpoints but properly delay nuclear division after UV irradiation in G_2. The finding that these mutants, although indistinguishable from *mec1*Δ cells with respect to the ability to replicate a damaged DNA template, do not lose viability after UV light and methyl methanesulfonate treatment suggests that checkpoint impairments do not necessarily result in hypersensitivity to DNA-damaging agents.

DNA is prone to alterations, and genomic integrity is ensured by DNA repair systems removing DNA damage and by surveillance mechanisms, known as DNA damage checkpoints, delaying cell cycle progression in response to DNA insults. These mechanisms contribute to the maintenance of genome stability, since they ensure that damaged DNA molecules are neither replicated nor segregated to daughter cells until repaired. Failure to respond properly to DNA damage is a hallmark of cancer cells (reviewed in reference 56).

Cell cycle progression can be transiently arrested by checkpoints at different stages, depending on the cell cycle phase at which DNA alterations occur. In fact, delay of G_1/S transition or slowing down of progression through S phase takes place when DNA is damaged in G_1 or during DNA synthesis, respectively, thus preventing replication of damaged templates (38, 47). Furthermore, when DNA is damaged in G_2 or when DNA replication is incomplete, segregation of damaged or incompletely replicated chromosomes is prevented by delaying nuclear division, thus linking entry into mitosis to proper completion of S phase (58, 59, 60).

Studies of the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* play an important role in the identification of DNA damage checkpoint proteins and in unraveling checkpoint mechanisms. The budding yeast *RAD9*, *RAD24*, *RAD17*, *MEC3*, and *DDC1* genes are necessary for DNA damage checkpoint response and are thought to act early in the DNA damage-induced signaling pathways (reviewed in references 27, 30, and 57). Both Ddc1 and Rad17 are structurally related to the sliding-clamp protein PCNA (proliferating cell nuclear antigen) (50), whose homotrimers form a structure that encircles DNA and tethers DNA polymerase δ to DNA during DNA replication (reviewed in reference 55). This homology and the finding that Ddc1 physically interacts with Rad17 and Mec3 (23, 35) raise the possibility that the Ddc1-Rad17-Mec3 complex may also form clamp-like structures that participate in the recognition and/or processing of damaged DNA.

Central to this signal transduction network is the Mec1 protein, a member of the evolutionarily conserved phosphatidylinositol 3-kinase motif family (6, 19, 21, 62), including *S. cerevisiae* Tel1 (17, 34), *S. pombe* Rad3 (4), *Drosophila melanogaster* Mei-41 (20), and human ATM (45), ATR (4), and DNA-PK (12). *MEC1*, as well as human ATM and *S. pombe* Rad3, is required for all known DNA damage checkpoints and for response to incomplete DNA replication. Moreover, the ATM gene is mutated in the familial neural degeneration and cancer-predisposition syndrome ataxia telangiectasia (45). Due to the lack of human ATR mutant cells, the functional role of ATR in the checkpoint pathway is not fully understood. However, overexpression of kinase-defective mutant ATR in wild-type cells abrogates G_2/M arrest after exposure to ionizing radiation and increases the sensitivity of cells to ionizing radiation and UV light (9), suggesting some overlapping functions of ATM and ATR.

In addition to its involvement in the checkpoint responses, budding yeast Mec1 is essential for cell viability. However, its essential function, but not its checkpoint functions, can be

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bypassed by increasing the intracellular concentration of deoxyribonucleotide triphosphates (dNTPs), either by overexpression of *RNR* genes encoding ribonucleotide reductase (13) or by deletion of the *SML1* gene (64), which negatively affects dNTP pools (7).

In *S. cerevisiae*, several key regulators of the Mec1-dependent signaling pathway, like Rad9, Ddc1, and Ddc2, become phosphorylated in a Mec1-dependent manner in response to DNA damage. The study of the interdependency of these phosphorylation events suggests that Mec1 is implicated in the DNA damage-sensing pathways (14, 35, 48, 49, 53). Moreover, since Mec1 physically interacts with Ddc2 (Lcd1), which is also necessary for all of the DNA integrity checkpoints (36, 42) and undergoes Mec1-dependent DNA damage-induced phosphorylation independently of all of the other known checkpoint proteins, the Mec1-Ddc2 complex might respond to DNA insults independently of the other checkpoint factors (36).

Also, Rad53 and Chk1 undergo Mec1-dependent phosphorylation in response to DNA damage (2, 43, 44) and appear to act downstream of Mec1. Whereas Rad53 is required for all of the DNA integrity checkpoints, Chk1 is specifically required to prevent nuclear division in *cdc13* mutants at nonpermissive temperatures, presumably through phosphorylation of the anaphase inhibitor Pds1 (10, 44).

Although phosphorylation of several key regulators in response to DNA damage or a replication block is Mec1 dependent, less is known about the requirement for the Mec1 kinase domain in activation of the DNA damage checkpoints and whether the cell cycle phases at which DNA alterations occur might influence the chance to activate the checkpoint response. To address these points, we generated and characterized two *mec1kd* alleles specifically altered in the Mec1 conserved kinase domain and searched for new *mec1* mutants specifically altered in subsets of DNA damage checkpoint pathways. We show that the Mec1 conserved kinase domain is essential for all of the functions of Mec1. Moreover, overproduction of the Mec1kd mutant forms has a dominant-negative effect specifically on the cell response to DNA damage in G_1 or during S phase. We also describe two new hypomorphic *mec1* mutants that appear to be completely defective in the G_1/S and intra-S checkpoints but proficient in the G_2/M checkpoint, suggesting that the Mec1 functions required for response to DNA alterations in the different cell cycle stages are separable.

MATERIALS AND METHODS

Yeast strains and media. The genotypes of all of the yeast strains used in this study are listed in Table 1. All of the yeast strains were derivatives of W303 (*MAT***a** or *MAT*α *ade2-1 can1-100 trp1-1 leu2-3,112 his3-11,15 ura3*).

To obtain strains YLL516, YLL517, and YLL518, carrying, respectively, the *GAL1-MEC1*, *GAL1-mec1kd1*, and *GAL1-mec1kd2* alleles at the *URA3* chromosomal locus, strain K699 was transformed with *Nco*I-digested plasmids pML236, pML237, and pML238, respectively. Strains DMP3055/8B and DMP3058/13B were derived from crosses of strain DMP683.8/3D with YLL516 and YLL517, respectively. The *MEC1* and *SML1* deletions (28) and the *DDC2-HA3*, *MEC1-MYC18*, and *MEC1-HA9* alleles (36) were constructed as previously described. Strains DMP3295/8B, DMP3296/3C, and DMP3297/6D, carrying the *DDC2-MYC18* allele at the *DDC2* chromosomal locus, and strain YLL839, carrying the *CHK1-HA3* allele at the *CHK1* chromosomal locus, were generated by the PCR one-step tagging method (22) using, respectively, plasmids 3746 and 3748 (K. Nasmyth, Institute of Molecular Pathology, Vienna, Austria) as templates and oligonucleotides PRP179 (5′-CTT GAG TCA AAA TCA TTC GAT CTA ACC ACA CTA GAG GAG GCC GAT TCA TTA TAT ATC TCA ATG GGA CTG TCC GGT TCT GCT GCT AG-3′) and PRP180 (5′-ATA TAG TTA ATA TTA AGC ATT ACA AGG TTT CTA TAA AGC GTT GAC ATT TTC CCC TTT TGA TTG TTG CCC CTC GAG GCC AGA AGA C-3′) (*DDC2-MYC18*) or oligonucleotides PRP217 (5′-CTT TAG AAT GGA GAA GAT TGT TCA AGA AAA TTT CAA CTA TCT GTA GGG ATA TTA TCC TAT TCC CAA CTC CGG TTC TGC TGC TAG-3′) and PRP218 (5′-ATA AGT AGA AAG AAT TTT TTT TTT TTG ATC AGT GCA TCT TAA CCC TTC TTT TGT CTC CAT TTT TTC CTC GAG GCC AGA AGA C-3′) (*CHK1-HA3*) as primers. Strains DMP3412/1A and DMP3412/6C were meiotic segregants from a cross between strains YLL839 and DMP683.8/3D. Strains DMP3455/9A and DMP3459/17C were meiotic segregants from crosses of strains DMP3058/13B and DMP3055/8B with strain DMP3412/6C, respectively. Strain DMP3432/7A was a meiotic segregant from a cross between strains DMP3058/13B and DMP3412/6C, followed by deletion of *MEC1* and *SML1* as described by Longhese et al. (28). The *DDC2-HA3*, *DDC2-MYC18*, *MEC1-MYC18*, and *MEC1-HA9* alleles are fully functional, since strains carrying them at the corresponding chromosomal loci were indistinguishable from the wild type with respect to viability, growth rates at any temperature, and sensitivity to UV radiation, methyl methanesulfonate (MMS), and hydroxyurea (HU). Since *CHK1* alterations do not cause obvious phenotypes but do impair Pds1 phosphorylation (44), we verified that DNA damage-induced Pds1 phosphorylation was unaffected in *CHK1-HA3* cells.

To generate the *CHK1* chromosomal deletion, a *chk1*Δ*::HIS3* cassette was constructed by PCR using the pFA6a-*HIS3* plasmid (54) as a template and oligonucleotides PRP190 (5′-TAT CAT AAG TTG CTG TAT ATG GGC AGC ACG TAT TAC TAT GAG TCT CGT ACG CTG CAG GTC GAC-3′) and PRP191 (5′-TGT CTC CAT TTT TTT CAG TTG GGA ATT AGG ATA ATA TCC CTA CAG ATA GTA TCG ATG AAT TCG AGC TCG-3′) as primers, followed by transformation of strain K700 with the PCR product, giving rise to strain DMP3274/5A, where the 1,540 bp of the *CHK1* coding region were replaced with the *Kluyveromyces lactis HIS3* gene. Strain DMP3287/2C was a meiotic segregant from a cross between strains DMP3274/5A and DMP3055/8B. Strains DMP3288/5A and DMP3288/8C were meiotic segregants from a cross between strains DMP3058/13B and DMP3274/5A. Strain YLL769 was obtained by transforming strain YLL517 with plasmid pML240. Details of strains carrying the *mec1kd*, *mec1-100*, and *mec1-101* alleles are given in the paragraphs describing the generation of the mutant alleles.

The accuracy of all gene replacements and integrations was verified by Southern blot analysis or PCR. The standard yeast genetic techniques and media used were described by Rose et al. (41). Cells were grown in YEP medium (1% yeast extract, 2% Bacto Peptone, 50 mg of adenine per liter) supplemented with 2% glucose (YEPD), 2% raffinose (YEP-raf), or 2% raffinose and 1% galactose (YEP-raf-gal). Transformants carrying the KanMX4 cassette were selected on YEPD plates containing G418 (United States Biological) at 400 μg/ml.

Plasmids. Plasmid pML224, used to generate plasmids pML228.1 and pML229.3 (see next paragraph) and carrying the C-terminal region of *MEC1*, was originated by inserting into the *Kpn*I-*Bam*HI sites of plasmid YIplac211 (16) the 1,243-bp *Kpn*I-*Bam*HI *MEC1* fragment from plasmid pML79 (29). To construct plasmid pML227 (*LEU2 CEN4 MEC1*), the 8,358-bp *Xba*I-*Spe*I fragment containing the whole *MEC1* coding region and the 385 bp upstream of the *MEC1* ATG codon was cloned into the *Spe*I site of plasmid YCplac111 (16), followed by excision of the *Sal*I-*Nar*I fragment. To construct plasmid pML236 (YIp5 *URA3 GAL1-MEC1*), in which a 7,437-bp fragment extending from the *MEC1* ATG codon to the *Sac*I site downstream to the *MEC1* stop codon is fused to the *GAL1* promoter, the *Sph*I-*Spe*I 8,372-bp fragment from plasmid pML225 (*URA3 CEN4 GAL1-MEC1*) (36) was cloned into the *Nhe*I-*Sph*I sites of plasmid YIp5. Plasmids pML230 and pML231, used to generate plasmids pML237 and pML238, were constructed by cloning, respectively, the 588-bp *Kpn*I-*Sac*II fragment from plasmids pML228.1 and pML229.3 into the *Kpn*I-*Sac*II sites of plasmid pML225. To construct plasmids pML237 (YIp5 *URA3 GAL1-mec1kd1*) and pML238 (YIp5 *URA3 GAL1-mec1kd2*), the *Sph*I-*Spe*I 8,372-bp fragments from plasmids pML230 (*URA3 CEN4 GAL1-mec1kd1*) and pML231 (*URA3 CEN4 GAL1-mec1kd2*), respectively, were cloned into the *Nhe*I and *Sph*I sites of YIp5. Plasmid pML240 (*CEN4 LEU2 GAL1-MEC1*) was obtained by cloning a 7,437-bp fragment extending from the *MEC1* ATG codon to the *Sac*I site downstream to the *MEC1* stop codon into the YCplac111 plasmid. To obtain plasmid pML239, the 8,093-bp *Spe*I-*Spe*I fragment containing the whole *MEC1* gene from plasmid pML79 was cloned into the *Xba*I site of plasmid pGEM4.

Generation and transplacement of the *mec1kd* alleles. Plasmids pML228.1 and pML229.3, containing, respectively, the base substitutions resulting in the Mec1kd1 D2243E and Mec1kd2 D2224A amino acid changes, were generated by PCR site-directed mutagenesis using plasmid pML224 as a template and oligonucleotides PRP154 (5′-CGG GTA AAG TTC TTC ATG TAG AAT TCG ACT GTT TAT TTG AGA AAG-3′) and PRP155 (5′-CTT TCT CAA ATA AAC

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
K699	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3*	29
K700	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3*	29
DMP2750.1	*MAT***a**/*MATα ade2-1*/*ade2-1 can1-100*/*can1-100 his3-11,15*/*his3-11,15 leu2-3,112*/*leu2-3,112 trp1-1*/*trp1-1 ura3*/*ura3 MEC1-HA9::LEU2::mec1*/*MEC1-MYC18::LEU2::mec1*	This study
DMP2872/8B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC1-HA2::LEU2::ddc1 mec1kd1 sml1Δ::KanMX4*	This study
DMP2872/4A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC1-HA2::LEU2::ddc1 sml1Δ::KanMX4*	This study
DMP2876/3A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC1-HA2::LEU2::ddc1 meclkd2 sml1Δ::KanMX4*	This study
DMP2882/2C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC1-HA2::LEU2::ddc1 mec1Δ::HIS3 sml1Δ::KanMX4*	This study
DMP2885.4	*MAT***a**/*MATα ade2-1*/*ade2-1 can1-100*/*can1-100 his3-11,15*/*his3-11,15 leu2-3,112*/*leu2-3,112 trp1-1*/*trp1-1 ura3*/*ura3 mec1kd1-HA9::LEU2::mec1*/*MEC1-MYC18::LEU2::mec1*	This study
DMP2893.1	*MAT***a**/*MATα ade2-1*/*ade2-1 can1-100*/*can1-100 his3-11,15*/*his3-11,15 leu2-3,112*/*leu2-3,112 trp1-1*/*trp1-1 ura3*/*ura3 mec1kd2-HA9::LEU2::mec1*/*MEC1-MYC18::LEU2::mec1*	This study
DMP3048/5B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1Δ::HIS3 sml1Δ::KanMX4 DDC2-HA3::URA3*	This study
DMP3055/8B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1- MEC1::URA3 DDC2-HA3::URA3*	This study
DMP3058/13B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-mec1kd1::URA3 DDC2-HA3::URA3*	This study
DMP3274/5A	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 chk1Δ::HIS3*	This study
DMP3287/2C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-MEC1::URA3 DDC2-HA3::URA3 chk1Δ::HIS3*	This study
DMP3288/5A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3 chk1Δ::HIS3*	This study
DMP3288/8C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-mec1kd1::URA3 DDC2-HA3::URA3 chk1Δ::HIS3*	This study
DMP3295/8B	*MAT***a** *ade2-1 can1-100 his3-11,15 trp1-1 leu2-3,112 ura3 MEC1-HA9::LEU2::mec1 DDC2-MYC18::HIS3*	This study
DMP3296/3C	*MAT***a** *ade2-1 can1-100 his3-11,15 trp1-1 leu2-3,112 ura3 mec1kd1-HA9::LEU2::mec1 DDC2-MYC18::HIS3 sml1Δ::KanMX4*	This study
DMP3297/6D	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1kd2-HA9::LEU2::mec1 DDC2-MYC18::HIS3 sml1Δ::KanMX4*	This study
DMP3343/6C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3 mec1-100::LEU2::mec1Δ*	This study
DMP3344/4A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3 mec1-101::LEU2::mec1Δ*	This study
DMP3412/1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3 CHK1-HA3::URA3*	This study
DMP3412/6C	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3 CHK1-HA3::URA3*	This study
DMP3432/7A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-mec1kd1::URA3 mec1Δ::HIS3 sml1Δ::KanMX4 DDC2-HA3::URA3 CHK1-HA3::URA3*	This study
DMP3455/9A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-mec1kd1::URA3 DDC2-HA3::URA3 CHK1-HA3::URA3*	This study
DMP3459/17C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-MEC1::URA3 DDC2-HA3::URA3 CHK1-HA3::URA3*	This study
YLL334	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC1-HA2::LEU2::ddc1*	29
YLL447.32	*MAT***a**/*MATα ade2-1*/*ade2-1 can1-100*/*can1-100 his3-11,15*/*his3-11,15 leu2-3,112*/*leu2-3,112 trp1-1*/*trp1-1 ura3*/*ura3 MEC1*/*MEC1-MYC18::LEU2::mec1*	This study
YLL476.34	*MAT***a**/*MATα ade2-1*/*ade2-1 can1-100*/*can1-100 his3-11,15*/*his3-11,15 leu2-3,112*/*leu2-3,112 trp1-1*/*trp1-1 ura3*/*ura3 MEC1*/*MEC1-HA9::LEU2::mec1*	This study
YLL490	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1Δ::HIS3 sml1Δ::KanMX4*	28
YLL516	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-MEC1::URA3*	This study
YLL517	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-mec1kd1::URA3*	This study
YLL518	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-mec1kd2::URA3*	This study
YLL683.8/3D	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3*	36
YLL683.8/4A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3*	36
YLL750	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1Δ::KanMX4 mec1-100::LEU2::mec1Δ*	This study
YLL753	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1Δ::KanMX4 mec1-101::LEU2::mec1Δ*	This study
YLL769	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1-mec1kd1::URA3* pML240 [CEN4 LEU2 GAL1-MEC1]	This study
YLL839	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 CHK1-HA3::URA3*	This study

AGT CGA ATT CTA CAT GAA GAA CTT TAC CCG-3′) or oligonucleotides PRP152 (5′-GGC CAT ATA TTA GGT CTA GGT GCT AGG CAC TGT GAA AAC ATA TTA-3′) and PRP153 (5′-TAA TAT GTT TTC ACA GTG CCT AGC ACC TAG ACC TAA TAT ATG GCC-3′) as primers to obtain the *mec1kd1* or *mec1kd2* allele, respectively. The presence of the expected mutations in the above plasmids was assessed by DNA sequencing of the entire PCR fragments.

Transformation of diploid strain W303 with *Xho*I-digested plasmids pML228.1 and pML229.3 generated *MEC1*/*mec1kd1::URA3* and *MEC1*/*mec1kd2::URA3* heterozygous strains, respectively. Meiotic tetrads from these strains contained only two viable spores carrying the *MEC1* allele, while no viable *mec1kd1* or *mec1kd2 URA3* spores were found. Transformation with the above plasmids of a diploid *sml1Δ::KanMX4*/*SML1* heterozygous strain generated an *SML1*/*sml1Δ::KanMX4 MEC1*/*mec1kd::URA3* strain. Several meiotic tetrads from this strain contained more than two viable spores, and viable *mec1kd::URA3 sml1Δ::KanMX4* segregants were present with the frequency expected for cosegregation of the two unlinked *mec1kd* and *sml1Δ* alleles. Strains DMP2872/8B and DMP2876/3A, in which the *MEC1* chromosomal copy was replaced with the *mec1kd1* and *mec1kd2* alleles, respectively, were obtained by two-step replacement, by transforming a *MEC1 DDC1-HA2 sml1Δ* strain with *Xho*I-digested plasmids pML228.1 and pML229.3, followed by excision of the *URA3* marker. Similarly, to obtain strains DMP3296/3C and DMP3297/6D, in which the *MEC1* chromosomal copy was replaced with the *mec1kd1-HA9* and *mec1kd2-HA9* alleles, respectively, a *MEC1-HA9::LEU2::mec1 DDC2-MYC18::HIS3 sml1Δ* strain was transformed with *Xho*I-digested plasmids pML228.1 and pML229.3, followed by excision of the *URA3* marker.

Search for new *mec1* mutants. Mutagenesis of the *MEC1* gene was performed by PCR using standard PCR conditions as described by Umezu et al. (52). Primers PRP161 (5′-ATG GAA TCA CAC GTC AAA TAT C-3′) and PRP162 (5′-GAG AAG TGT CTA ATA AAG CAC C-3′) were used to amplify the region between positions +1 and +3307 (gap A), primers PRP163 (5′-CGG AGA AAG CAG ACA GAA AG-3′) and PRP164 (5′-GGG CCA CGT TCA TGT CAA AT-3′) were used to amplify the region between positions +3207 and +6034 (gap B), and primers PRP165 (5′-CAA ACG AGG ATC CAT TAA GGA-3′) and PRP166 (5′-CCA AAA TGG AAG CCA ACC AAT-3′) were used to amplify the region between positions +4747 and +7104 (gap C). PCR mixtures for each set of primers (25 μl) contained 1.25 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 10 ng of template DNA (pML239), 1 μM each primer, 200 μM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. Twenty independent reaction mixtures were prepared for each set of

primers. Strain YLL490 (*mec1*Δ *sml1*Δ) was cotransformed with gap A PCR products and the *Stu*I-*Stu*I fragment of pML227 (YCplac111 *CEN4 LEU2 MEC1*), lacking 2,358 bp between positions +476 and +2834 of the *MEC1* coding region, or with gap B or gap C PCR products and the *Nru*I-*Not*I fragment of pML227, lacking 304 bp between positions +5271 and +5575 of the *MEC1* coding region, in order to obtain reconstruction of the whole *MEC1* coding region by gap repair. Leu^+ transformants were tested for the ability to grow on YEPD plates after UV irradiation (50 J/m^2) or in the presence of MMS (0.01%) or HU (100 mM) at 25°C. None of the several *mec1* mutants identified were confirmed to be specifically hypersensitive to MMS or UV light, while the *mec1-100* and *mec1-101* mutants were weakly hypersensitive to HU, but not to MMS and UV light, and were further analyzed. To obtain stable *mec1-100* and *mec1-101* mutants, the 7,876-bp *Nco*I-*Eco*RI fragments from plasmids pML254.1 and pML253.1, containing, respectively, the whole *mec1-100* and *mec1-101* alleles, were cloned into the *Sph*I-*Eco*RI sites of the YIplac128 (*LEU2*) integrative plasmid to generate plasmids pML258.51 and pML266.46, respectively. *Spe*I digestion was then used to direct the integration of these plasmids into the *MEC1* promoter region of *mec1*Δ *sml1*Δ strain YLL490, giving rise to strains YLL750 and YLL753, carrying, respectively, the *mec1-100* and *mec1-101* alleles as the sole complete *mec1* alleles at the *MEC1* chromosomal locus. *SML1* strains DMP3343/6C and DMP3344/4A were meiotic segregants from crosses of strain YLL683.8/3D with strains YLL750 and YLL753, respectively, and their phenotypes were indistinguishable from those of strains YLL750 and YLL753, indicating that the effects of the *mec1-100* and *mec1-101* alleles are not influenced by *SML1*.

Other techniques. Synchronization experiments, immunoprecipitations, Western blot analysis, and kinase assays were performed as previously described (36).

RESULTS

Alteration of the Mec1 conserved kinase domain impairs all Mec1 functions. In order to investigate whether Mec1 functions as a protein kinase in establishing the DNA integrity checkpoints, we generated the mutations *mec1kd1* and *mec1kd2*, which cause the amino acid changes D2243E and D2224A, respectively, in the Mec1 putative kinase domain (see Materials and Methods). The same amino acid changes in the *S. pombe* Rad3 lipid kinase domain affected Rad3 function (4). When we analyzed the in vivo consequences of these mutations, we found that both *mec1kd* alleles resulted in cell lethality that was suppressed by deletion of the *SML1* gene (see Materials and Methods), similarly to the *MEC1* deletion (64) and to another recently described *mec1kd* allele (D2224A N2229K) (31). Furthermore, viable *mec1kd1 sml1*Δ and *mec1kd2 sml1*Δ strains were as hypersensitive as a *mec1*Δ *sml1*Δ strain to UV light, MMS, and HU (Fig. 1A).

As shown in Fig. 1, *mec1kd1 sml1*Δ cells were as defective as *mec1*Δ *sml1*Δ cells at all known DNA damage checkpoints. In fact, when *mec1kd1 sml1*Δ or *mec1*Δ *sml1*Δ G_1-arrested cells were UV irradiated and then released from the block, both entry into S phase (Fig. 1B, middle) and budding kinetics (Fig. 1C) were much faster and cell survival was much lower (3.4 and 2.5%, respectively) than in wild-type and *sml1*Δ mutant cell cultures under the same conditions (87 and 89% cell survival, respectively). Furthermore, when α-factor-synchronized *mec1kd1 sml1*Δ or *mec1*Δ *sml1*Δ cells were released from G_1 arrest in the presence of MMS, they doubled their DNA content within 30 min and progressively lost viability (both already down to 10% cell survival at 30 min), whereas MMS-treated wild-type and *sml1*Δ cells progressed through S phase very slowly, completing DNA replication only after 150 min (Fig. 1B, bottom) without losing viability. Finally, *mec1kd1 sml1*Δ, as well as *mec1*Δ *sml1*Δ, cells released from G_2 arrest after UV irradiation lost viability and divided nuclei much faster than similarly treated wild-type and *sml1*Δ cells, which maintained high cell survival and delayed nuclear division compared to unirradiated cells (Fig. 1D).

Since activation of DNA damage checkpoint pathways leads to Mec1-dependent phosphorylation of Rad53 (reviewed in references 27, 30, and 57), we analyzed the Rad53 phosphorylation pattern as a means by which to uncover alterations of Mec1 functions. As shown in Fig. 1E, the inability of *mec1kd1* cells to arrest cell cycle progression after DNA damage correlated with impaired Rad53 phosphorylation, since no phosphorylated Rad53 was detectable in *mec1kd1 sml1*Δ or *mec1*Δ *sml1*Δ cells after DNA damage in G_1 or G_2 or during S phase.

A *mec1kd2 sml1*Δ strain was also subjected to all of the above-described analyses, and its behavior was always indistinguishable from that of the *mec1kd1 sml1*Δ strain (data not shown). Thus, the Mec1 kinase domain is required both to sustain cell viability and for proper DNA damage response.

The kinase-deficient Mec1kd1 and Mec1kd2 variants still physically interact with both Ddc2 and wild-type Mec1. We have previously shown that Mec1 physically interacts with Ddc2 and that its associated kinase activity is capable of phosphorylating Ddc2 in vitro and is impaired by the *mec1kd* mutations (36). As shown in Fig. 2A, we further confirmed this point, since phosphorylated, Myc-tagged Ddc2 was detected when in vitro kinase assays were performed on immunoprecipitates containing hemagglutinin (HA)-tagged Mec1 but not when the same assays were performed on HA-tagged Mec1kd immunoprecipitates, although similar amounts of Myc-tagged Ddc2 coprecipitated with either the Mec1 or the Mec1kd protein. Thus, both mutations completely abolish the Mec1-associated kinase activity, further strengthening the hypothesis that the kinase is Mec1 itself. Accordingly, Mallory and Petes (31) recently showed that a Mec1 variant with two amino acid substitutions (D2224A and N2229K) in the kinase domain lost the ability to phosphorylate the mammalian protein PHAS-I (phosphorylated heat- and acid-stable protein I) in vitro.

Since it was shown that multiple *S. pombe* Rad3 molecules may be present in complexes (4), we asked whether Mec1 could also form homomeric complexes and whether the Mec1kd variants might still be present in these complexes. To this end, we performed immunoprecipitation assays on protein extracts from untreated and MMS-treated diploid cells carrying fully functional *MEC1-HA9* and *MEC1-MYC18* alleles at the two *MEC1* chromosomal loci. As shown in Fig. 2B, Mec1 molecules can self-associate, since Mec1-MYC18 was specifically recognized by the anti-MYC antibodies in Mec1-HA9 immunoprecipitates, and anti-HA antibodies detected Mec1-HA9 in Mec1-MYC18 immunoprecipitates, independently of DNA damage. Furthermore, when the heterozygous diploid *MEC1-MYC18*/*mec1kd1-HA9* and *MEC1-MYC18*/*mec1kd2-HA9* strains were used in analogous immunoprecipitation assays, Mec1-MYC18 was specifically recognized by the anti-MYC antibodies in both Mec1kd1-HA9 and Mec1kd2-HA9 immunoprecipitates, and anti-HA antibodies detected Mec1kd1-HA9 and Mec1kd2-HA9 in Mec1-MYC18 immunoprecipitates (Fig. 2B), indicating that both Mec1kd inactive forms are still able to interact with wild-type Mec1.

High levels of kinase-deficient Mec1kd1 protein in *MEC1* cells cause damage-resistant DNA replication. Although the *mec1kd* alleles behave recessively when present in single copy in a *mec1kd*/*MEC1* heterozygous strain (data not shown), the

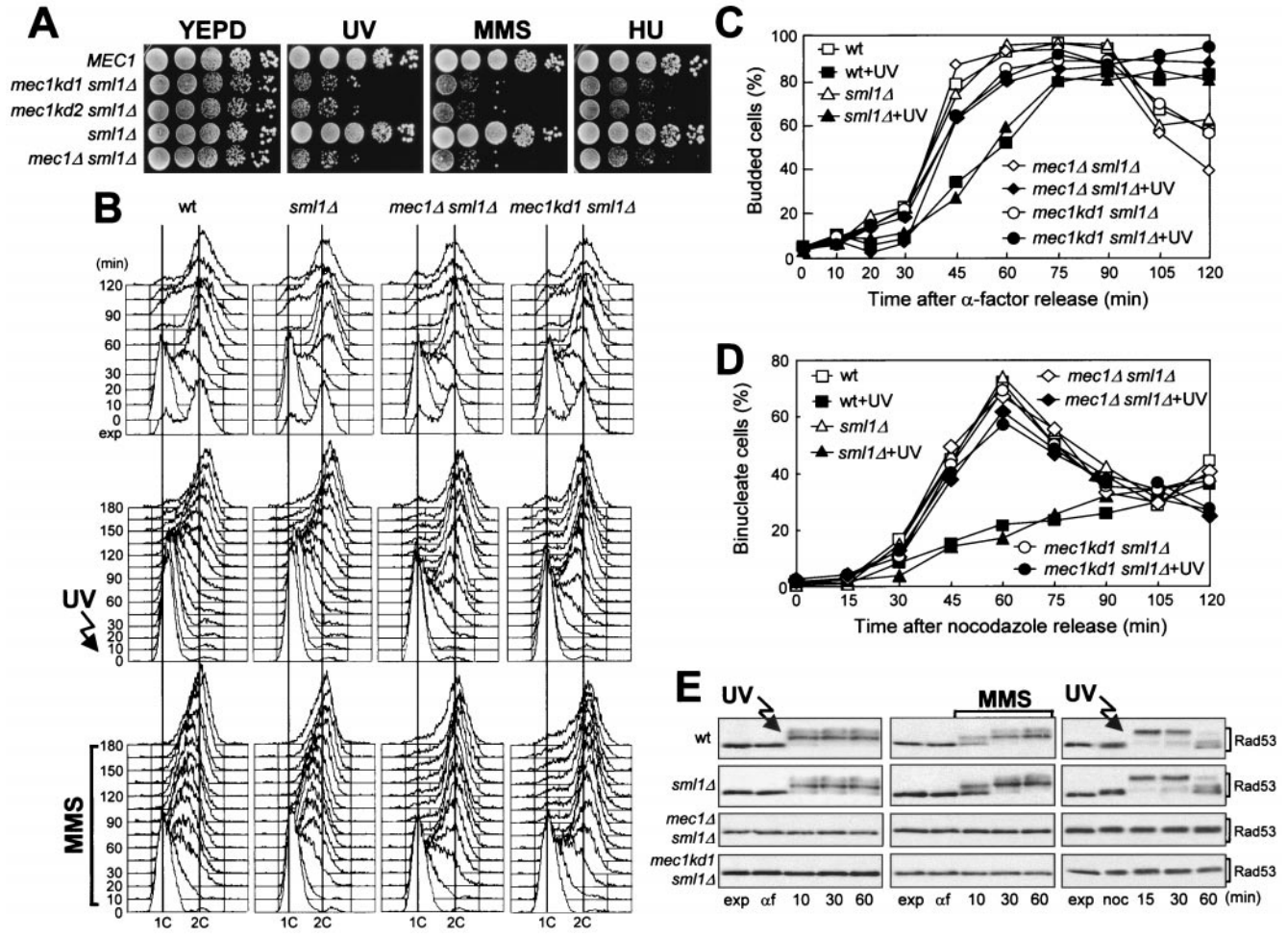

FIG. 1. *mec1* kinase deficiency mutations impair all known DNA damage checkpoints. (A) Serial dilution of cultures of wild-type (wt) YLL334, *mec1kd1 sml1*Δ DMP2872/8B, *mec1kd2 sml1*Δ DMP2876/3A, *sml1*Δ DMP2872/4A, and *mec1*Δ *sml1*Δ DMP2882/2C cells growing exponentially in YEPD were spotted on YEPD plates with or without MMS (0.005%) or HU (5 mM). One YEPD plate was UV irradiated (30 J/m^2) (UV). (B to E) The strains used were wild-type YLL334, *sml1*Δ DMP2872/4A, *mec1*Δ *sml1*Δ DMP2882/2C, and *mec1kd1 sml1*Δ DMP2872/8B. (B and C) α-Factor-synchronized cell cultures were UV irradiated (40 J/m^2) prior to the release from α-factor in YEPD or were released in YEPD containing 0.02% MMS. (B) Samples of untreated (top), UV-irradiated (middle), or MMS-treated (bottom) cells were taken at the indicated times after release into the cell cycle and analyzed by fluorescence-activated cell sorter. (C) Untreated or UV-irradiated (+UV) cell cultures were scored for the percentage of budded cells at the indicated times. (D) Cell cultures were arrested with nocodazole (noc) and UV irradiated (50 J/m^2) prior to the release in YEPD at time zero. Propidium iodide staining was used to directly visualize nuclear division at the indicated times after release from nocodazole in unirradiated and UV-irradiated (+UV) cultures. The survival levels of UV light-treated wild-type, *sml1*Δ, *mec1kd1 sml1*Δ, and *mec1*Δ *sml1*Δ cells were 78, 90, 8.3, and 7.3%, respectively. (E) Extracts from the above-described G_1 UV light-treated (left) or MMS-treated (middle) or G_2 UV light-treated (right) cell cultures were analyzed by Western blot assay with anti-Rad53 antibodies. exp, exponentially growing cells.

finding that their kinase-deficient gene products are still able to interact in vivo with both wild-type Mec1 and Ddc2 (Fig. 2) led us to ask whether their overexpression might affect the response to DNA damage in the presence of physiological amounts of wild-type Mec1. To address this point, *MEC1* strains carrying *GAL1-MEC1*, *GAL1-mec1kd1* and *GAL1-mec1kd2* gene fusions at the *URA3* locus were first assayed for sensitivity to genotoxic agents under galactose-induced conditions. As shown in Fig. 3A, high levels of inactive Mec1kd proteins in a *MEC1* background have a dominant-negative effect, since wild-type cells overproducing Mec1kd1 or Mec1kd2 were more sensitive to HU, MMS, and UV light than otherwise isogenic wild-type or *MEC1*-overexpressing strains. This hypersensitivity can be suppressed by increasing the level of wild-type Mec1, since *MEC1* cells concomitantly expressing the *GAL1-mec1kd1* and *GAL1-MEC1* fusions were as sensitive as the wild type to HU, MMS, and UV light (Fig. 3A). Therefore, high levels of the kinase-defective variants might determine a dominant defect in the response to DNA damage by competing with wild-type Mec1 molecules.

We then analyzed the checkpoint-mediated cell cycle arrest in *MEC1* cells overproducing the Mec1kd variants. When G_1-arrested, galactose-induced *MEC1 GAL1-mec1kd1* cell cultures were UV irradiated prior to release from the block, they not only lost viability (24% survival) but progressed into the cell cycle, reaching a 2C DNA content after 75 min, faster than

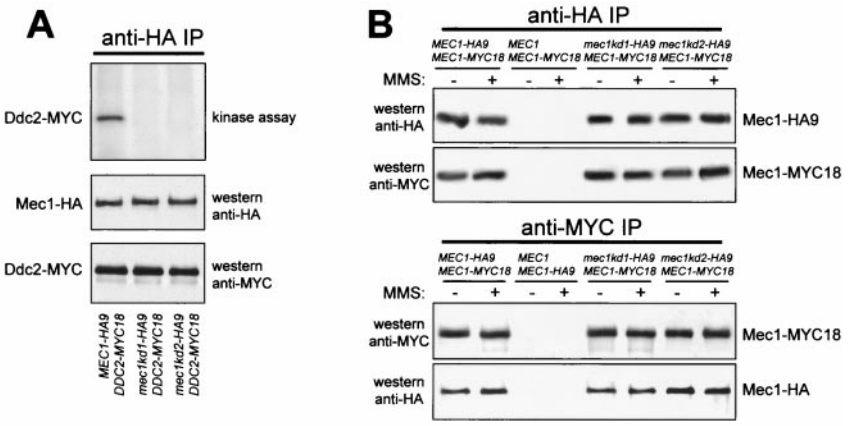

FIG. 2. Kinase activity and interactions of Mec1kd variants. (A) HA-tagged Mec1 or Mec1kd proteins (Mec1-HA) were immunoprecipitated with anti-HA antibodies (anti-HA IP) from protein extracts prepared from exponentially growing cells concomitantly expressing Mec1-HA9 and Ddc2-MYC18 (DMP3295/8B), Mec1kd1-HA9 and Ddc2-MYC18 (DMP3296/3C), or Mec1kd2-HA9 and Ddc2-MYC18 (DMP3297/6D) from the *MEC1* and *DDC2* promoters, respectively, as indicated at the bottom. Kinase assays were performed on anti-HA immunoprecipitates, and the results are shown at the top. The same immunoprecipitates were also analyzed by Western blot assay using the antibodies indicated on the right side of the middle and bottom parts of the panel. (B) Immunoprecipitations with anti-HA (anti-HA IP) or anti-MYC (anti-MYC IP) antibodies were performed on extracts from exponentially growing untreated (−) or MMS-treated (+; 0.02% MMS for 1 h) diploid cells with the genotypes indicated in the top part of the panels. Mec1-HA9 and Mec1-MYC18 were then detected by Western blot analysis of the immunoprecipitates by using anti-HA and anti-MYC antibodies. The genotypes of the strains used were *MEC1-HA9*/*MEC1-MYC18* (DMP2750.1), *MEC1*/*MEC1-MYC18* (YLL447.32), *MEC1*/*MEC1-HA9* (YLL476.34), *mec1kd1-HA9*/*MEC1-MYC18* (DMP2885.4), and *mec1kd2-HA9*/*MEC1-MYC18* (DMP2893.1).

similarly treated wild-type and *MEC1 GAL1-MEC1* cells, which completed DNA replication only after 120 min (Fig. 3B, middle) and maintained high cell survival (79 and 85%, respectively). Furthermore, when G_1-arrested *MEC1 GAL1-mec1kd1* cells were released from the block in the presence of MMS under galactose-induced conditions, they progressed through S phase much faster than similarly treated wild-type and *MEC1 GAL1-MEC1* cells (Fig. 3B, bottom) and progressively lost viability (already down to 30.5% cell survival at 30 min), while the viability of the MMS-treated wild-type and *MEC1 GAL1-MEC1* cells was substantially unaffected throughout the experiment. Furthermore, Ddc2 phosphorylation was abolished and Rad53 phosphorylation was severely affected in both of the above-described UV light- and MMS-treated *MEC1 GAL1-mec1kd1* cell cultures, compared to similarly treated wild-type and *MEC1 GAL1-MEC1* cells (data not shown). Therefore, high levels of the kinase defective Mec1kd1 protein in *MEC1* cells have dominant-negative effects on checkpoint response, impairing the ability of cells to regulate DNA replication, as well as to promote Rad53 and Ddc2 phosphorylation when DNA is damaged in G_1 or during S phase.

The DNA damage checkpoint defects of *MEC1 GAL1-mec1kd1* cells appeared to be less severe than those of *mec1*Δ cells, suggesting that the presence of physiological amounts of wild-type Mec1 may contribute to partial activation of the DNA damage response in galactose-induced *MEC1 GAL1-mec1kd1* cells. Indeed, cells overproducing Mec1kd1 in a *mec1*Δ *sml1*Δ background were more sensitive to HU, MMS, and UV light than otherwise isogenic cells overproducing Mec1kd variants in a *MEC1* background and were indistinguishable from *mec1*Δ *sml1*Δ cells (Fig. 3A). Moreover, when *GAL1-mec1kd1 mec1*Δ *sml1*Δ cells were released from G_1 arrest after UV irradiation or in the presence of MMS under galactose-induced conditions, they progressed through S phase faster than similarly treated *MEC1 GAL1-mec1kd1* cells (Fig. 3B), and DNA damage-induced Rad53 phosphorylation was completely abolished (data not shown). Therefore, the residual activation of the DNA damage response in *MEC1 GAL1-mec1kd1* cells was dependent on the presence of wild-type Mec1.

High levels of Mec1kd1 in *MEC1* cells do not affect the delay of nuclear division caused by UV irradiation in G_2. The above-described dominant effects of Mec1kd overproduction were limited to the checkpoints controlling S phase entry and progression. In fact, *MEC1 GAL1-mec1kd1* galactose-induced cell cultures released from a nocodazole-induced G_2 arrest after UV irradiation underwent a delay in nuclear division comparable to that observed in wild-type and *MEC1 GAL1-MEC1* cells under the same conditions (Fig. 3C, top), although they showed a premature disappearance of DNA damage-induced Rad53 phosphorylated forms (Fig. 3C, bottom). The activation of the G_2/M checkpoint in *MEC1* cells overproducing Mec1kd1 was likely due to the presence of wild-type Mec1, since similarly treated *GAL1-mec1kd1 mec1*Δ *sml1*Δ cells divided nuclei much faster than did *MEC1 GAL1-mec1kd1* cells (Fig. 3C, top) and phosphorylation of Rad53 and Ddc2 was completely abolished (Fig. 3C, bottom), as can be observed in *mec1*Δ cells under the same conditions (Fig. 1). Therefore, physiological levels of Mec1 in cells overproducing Mec1kd1 might be sufficient to activate Rad53 and/or other proteins specifically required to prevent nuclear division when DNA is damaged in G_2. Since Rad53 phosphorylation after UV irradiation in G_2 was reduced prematurely in *MEC1* cells with high levels of Mec1kd1 (Fig. 3C, bottom), the G_2 DNA damage-induced cell cycle arrest of these cells might at least partially depend on proteins acting independently of Rad53. One possible candidate was the Chk1 kinase, which is phosphorylated in a Mec1-dependent manner and is specifically required to prevent an-

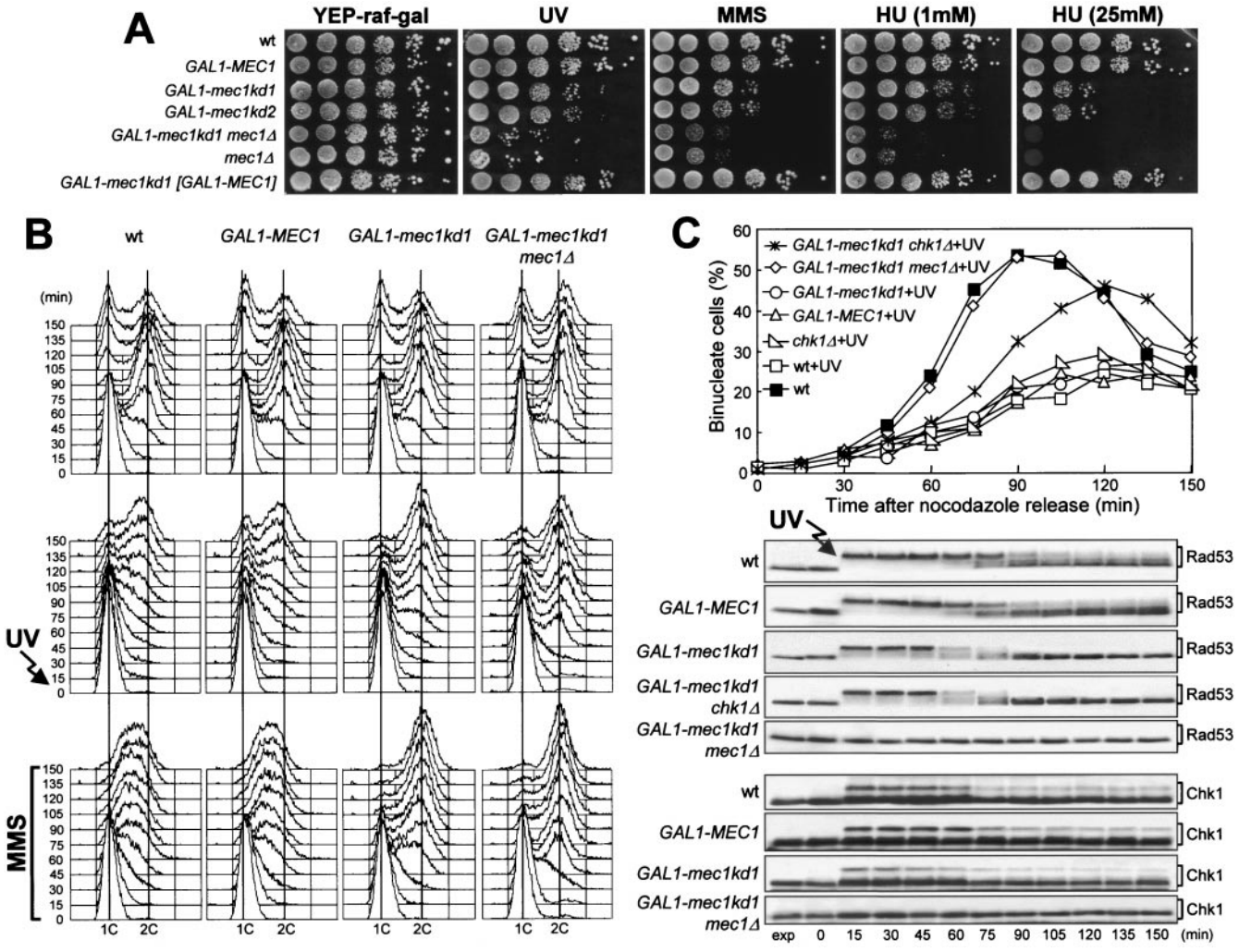

FIG. 3. Dominant-negative effect of *mec1kd1* overexpression. (A) Serial dilutions of exponentially growing (in YEPD) cultures of wild-type (wt) K699, *GAL1-MEC1* YLL516, *GAL1-mec1kd1* YLL517, *GAL1-mec1kd2* YLL518, and *GAL1-mec1kd1* [*GAL-MEC1*] YLL769 cells, all carrying the *MEC1* allele at the *MEC1* chromosomal locus, and *GAL1-mec1kd1 mec1*Δ DMP3432/7A and *mec1*Δ YLL490 cells, both also carrying the *sml1*Δ allele, were spotted on YEP-raf-gal plates with or without MMS (0.005%) or HU. One YEP-raf-gal plate was UV irradiated (40 J/m^2) (UV). (B) Cultures of wild-type DMP3412/1A, *GAL1-MEC1* DMP3459/17C, and *GAL1-mec1kd1* DMP3455/9A cells, all carrying the *MEC1* allele at the *MEC1* chromosomal locus, and *GAL1-mec1kd1 mec1*Δ DMP3432/7A cells, also carrying the *sml1*Δ allele, logarithmically growing in YEP-raf, were synchronized with α-factor 2.5 h after addition of galactose to 1%. Cell cultures were released from α-factor at time zero into YEP-raf-gal medium with or without 0.02% MMS. One-third of each synchronized culture was UV irradiated (40 J/m^2) prior to release. Samples of untreated (top), UV-irradiated (middle), or MMS-treated (bottom) cultures were taken at the indicated times after the release from α-factor and analyzed by fluorescence-activated cell sorter. (C) Cultures of wild-type DMP3412/1A, *GAL1-MEC1* DMP3459/17C, *GAL1-mec1kd1* DMP3455/9A, *GAL1-MEC1 chk1*Δ DMP3287/2C, *chk1*Δ DMP3288/5A, and *GAL1-mec1kd1 chk1*Δ DMP3288/8C cells, all carrying the *MEC1* allele at the *MEC1* chromosomal locus, and *GAL1-mec1kd1 mec1*Δ DMP3432/7A cells, also carrying the *sml1*Δ allele, logarithmically growing in YEP-raffinose, were synchronized with nocodazole 2 h after addition of 1% galactose and UV irradiated (50 J/m^2) prior to release in YEP-raf-gal medium. Nuclear division (top) was directly visualized at the indicated times in untreated and UV light-treated (+UV) cultures by propidium iodide staining. Protein extracts (bottom) from the UV light-treated cell cultures were analyzed by Western blot assay using anti-Rad53 and anti-HA (Chk1) antibodies. exp, exponentially growing cells.

aphase entry in *cdc13* mutants at restrictive temperatures, independently of Rad53 (44). Indeed, when galactose-induced cell cultures were released from a nocodazole-induced G_2 arrest after UV irradiation, *MEC1 GAL1-mec1kd1 chk1*Δ cells divided nuclei faster than *MEC1 GAL1-mec1kd1* cells, although Rad53 phosphorylation was not further affected (Fig. 3C) and deletion of *CHK1* per se was not sufficient to impair either the DNA damage-induced Rad53 phosphorylation or the checkpoint-mediated delay in nuclear division after DNA damage in G_2 (Fig. 3C). While the overall amount of Chk1 phosphorylation after UV irradiation was reduced in *MEC1 GAL1-mec1kd* cells compared to wild-type and *GAL1-MEC1* cells during the above-described synchronization experiments (Fig. 3C, bottom), the Chk1 phosphorylated forms persisted until the end of the experiment and were dependent on wild-type Mec1, since they were completely absent in *GAL1-mec1kd1 mec1*Δ *sml1*Δ cells under the same conditions (Fig. 3C, bottom).

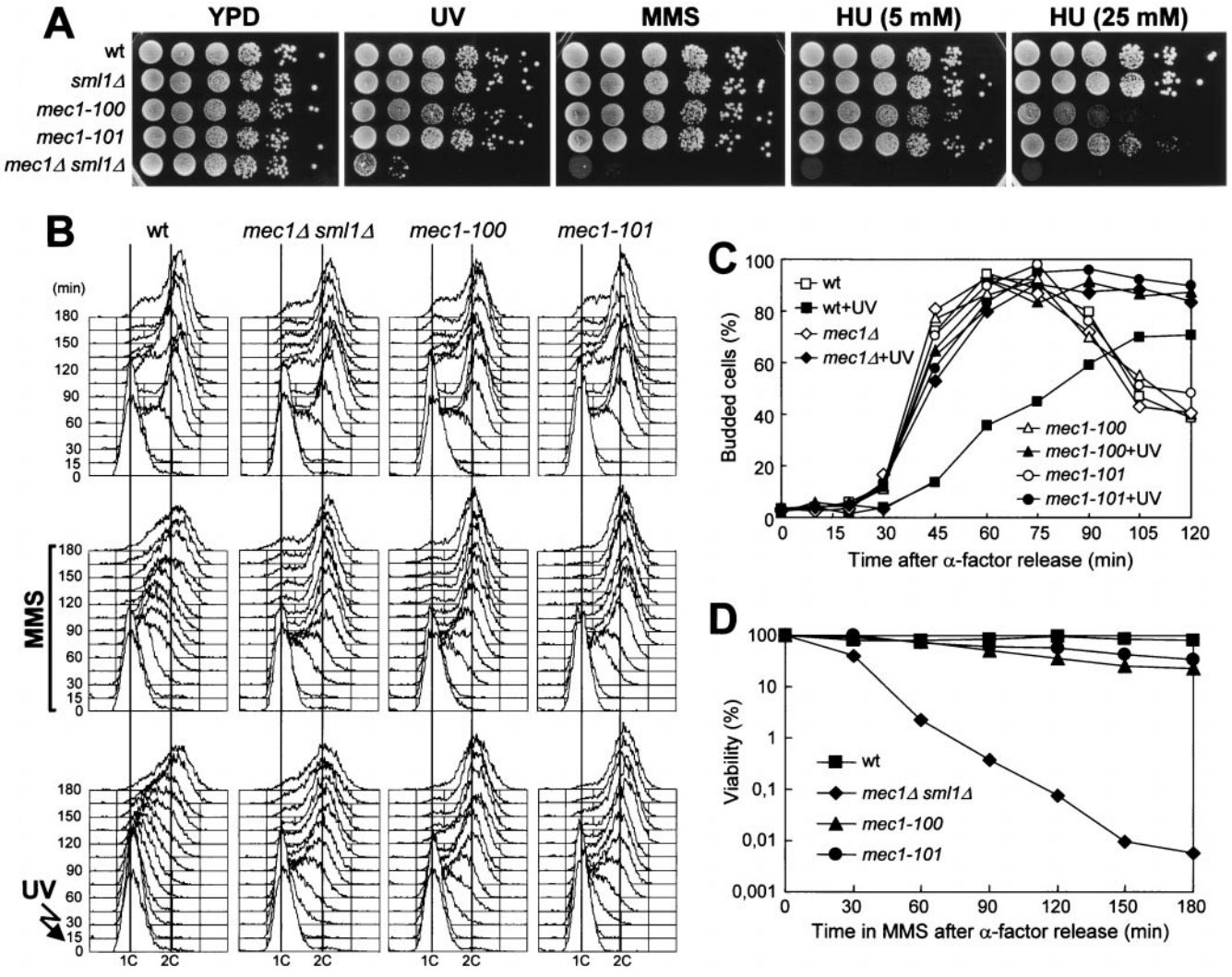

FIG. 4. G_1/S and intra-S DNA damage checkpoints in *mec1-100* and *mec1-101* mutants. The strains used were wild-type (wt) YLL683.8/4A, *mec1*Δ *sml1*Δ DMP3048/5B, *mec1-100* DMP3343/6C, and *mec1-101* DMP3344/4A. (A) Serial dilution of exponentially growing (in YEPD) cell cultures were spotted on YEPD plates with or without MMS (0.005%) or HU. One YEPD plate was UV irradiated (40 J/m^2) (UV). The data presented in panels B, C, and D all come from the same experiment. (B to D) α-Factor-synchronized cells were released from α-factor at time zero in YEPD (top) or in YEPD containing 0.02% MMS (middle) or were UV irradiated (40 J/m^2) prior to the release in YEPD (bottom). (B) Samples of untreated and UV light- and MMS-treated cell cultures were collected at the indicated times after release from α-factor and analyzed by fluorescence-activated cell sorter. (C) Untreated or UV-irradiated (+UV) cell cultures were scored at the indicated times for the percentage of budded cells. (D) Aliquots were removed from the MMS-treated cultures at timed intervals to score for CFU on YEPD plates at 25°C.

New *mec1* mutants impaired in subsets of DNA integrity checkpoint pathways. We have shown that an excess of inactive Mec1kd molecules causes dominant DNA damage-resistant DNA replication, but it is not sufficient to abolish the G_2/M DNA damage checkpoint, suggesting that specific impairment of Mec1 functions may affect the checkpoint response differently, depending on the cell cycle stages at which DNA alterations occur. If this were the case, it should be possible to isolate *mec1* mutants that are defective in slowing down of DNA synthesis but are still able to delay nuclear division in response to DNA damage. Random mutagenesis of the *MEC1* gene and screening for mutants that displayed different patterns of sensitivity to genotoxic agents (see Materials and Methods), allowed us to isolate the *mec1-100* and *mec1-101* mutant alleles. As shown in Fig. 4A, the *mec1-100* and *mec1-101* mutants did not show hypersensitivity to MMS and UV radiation, while they exhibited a limited sensitivity to HU that was much lower than that of *mec1*Δ cells. Both *mec1-100* and *mec1-101* mutants turned out to be completely defective in both the G_1/S and intra-S DNA damage checkpoints. In fact, when *mec1-100* and *mec1-101* G_1-arrested cells were UV irradiated and then released into the cell cycle, they entered S phase (Fig. 4B, bottom) and budded (Fig. 4C) much faster than the wild type, similarly to *mec1*Δ cells, although their cell survival was very similar to that of wild-type cells under the same conditions (75% for *mec1-100*, 80% for *mec1-101*, 87% for wild-type, and 3% for *mec1*Δ *sml1*Δ cells). Furthermore, when α-factor-synchronized *mec1-100* and *mec1-101* cells were released from the G_1 arrest in the presence of MMS, they doubled their DNA content within 45 min, like *mec1*Δ cells,

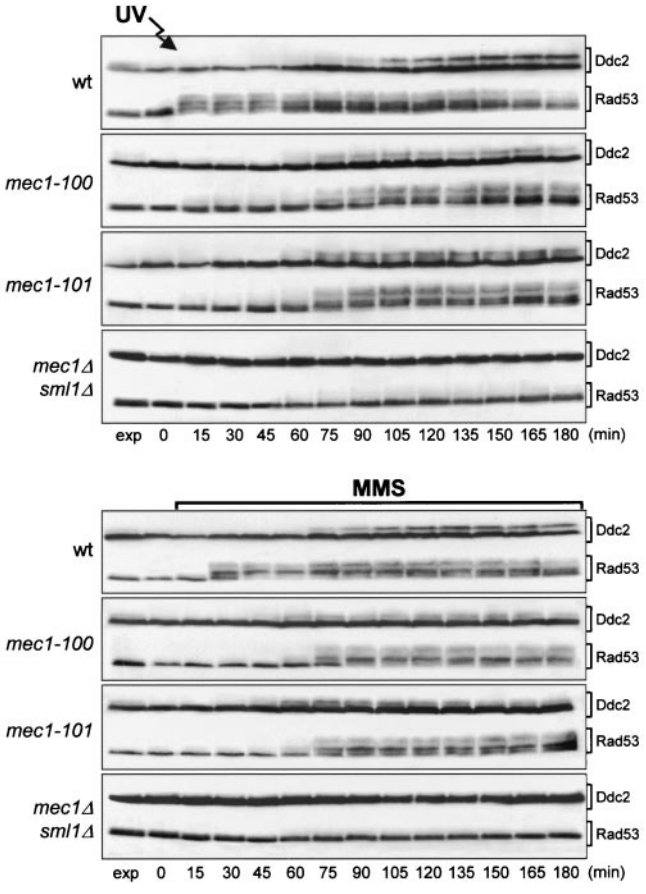

FIG. 5. Rad53 and Ddc2 phosphorylation in *mec1-100* and *mec1-101* mutants after DNA damage in G_1 or during S phase. The strains used were wild-type (wt) YLL683.8/4A, *mec1*Δ *sml1*Δ DMP3048/5B, *mec1-100* DMP3343/6C, and *mec1-101* DMP3344/4A. The data all come from the experiment described in the legend to Fig. 4B, C, and D. Protein extracts from the UV light-treated (top panel) and the MMS-treated (bottom panel) cell cultures were analyzed by Western blot assay using anti-Rad53 and anti-HA (Ddc2) antibodies. exp, exponentially growing cells.

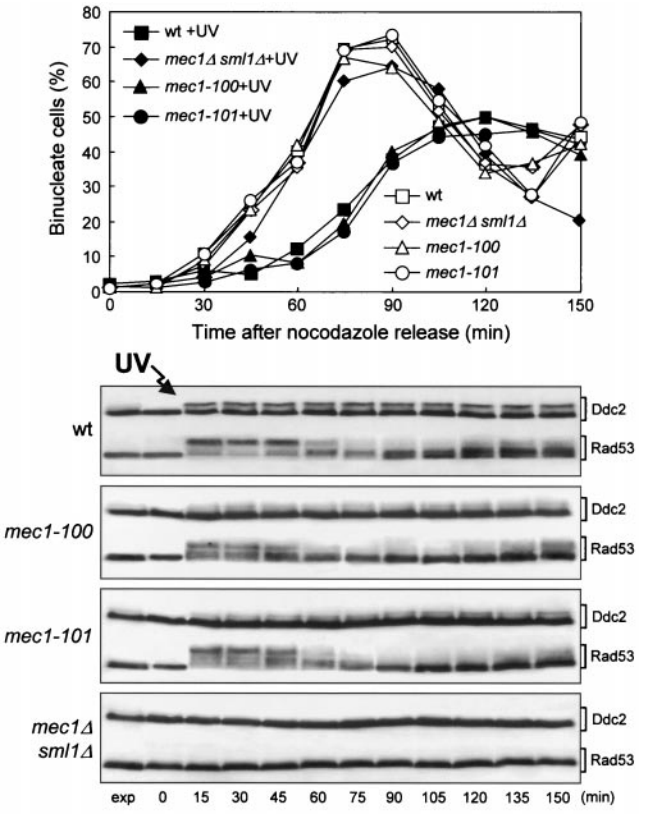

FIG. 6. G_2/M DNA damage checkpoint in *mec1-100* and *mec1-101* mutants. Cultures of wild-type (wt) YLL683.8/4A, *mec1*Δ *sml1*Δ DMP3048/5B, *mec1-100* DMP3343/6C, and *mec1-101* DMP3344/4A cells were arrested with nocodazole and UV irradiated (50 J/m^2) prior to release in YEPD. Kinetics of nuclear division were determined as described in the legend to Fig. 1D in untreated and UV light-treated (+UV) cells and are shown at the top. At the bottom is a Western blot analysis of protein extracts from samples of the UV light-treated cell cultures withdrawn at the indicated times. Rad53 and Ddc2 were detected using, respectively, anti-Rad53 and anti-HA (Ddc2) antibodies. exp, exponentially growing cells.

whereas MMS-treated wild-type cell cultures progressed through S phase very slowly, completing DNA replication only after 150 min (Fig. 4B, middle). On the contrary, the viability of MMS-treated *mec1-100* and *mec1-101* mutant cells was much more similar to that of wild-type cells than to that of *mec1*Δ cells under the same conditions (Fig. 4D). Thus, the new *mec1* mutants were completely unable to delay bud emergence and S phase entry and progression when DNA was damaged in G_1 or during S phase, although their checkpoint defects did not result in loss of viability. These defective checkpoint responses correlated with defects in the extent and/or timing of Ddc2 and Rad53 phosphorylation. In fact, Rad53 phosphorylation was detectable immediately after UV light and MMS treatment of wild-type cells, while it became detectable in *mec1-100* and *mec1-101* mutants only at 75 min (Fig. 5), when cells reached late S or G_2 phase (Fig. 4B). Furthermore, UV light- and MMS-treated *mec1-100* and *mec1-101* cells showed reduced amounts of Ddc2 phosphorylated forms that appeared earlier than in wild-type cells (Fig. 5), reflecting the findings that Ddc2 phosphorylation after DNA damage in G_1 or during S phase becomes detectable in the wild type only when cells reach the G_2 phase (Fig. 4 and 5) (36) and that both mutants reached the G_2 phase earlier than the wild type (Fig. 4B).

As shown in Fig. 6, the *mec1-100* and *mec1-101* mutants were not defective in the G_2/M DNA damage checkpoint. In fact, when *mec1-100* and *mec1-101* cell cultures were released from G_2 arrest after UV irradiation, they showed a delay in nuclear division comparable to that of wild-type cells under the same conditions, as well as immediate induction of Ddc2 and Rad53 phosphorylation (Fig. 6). Thus, the *mec1-100* and *mec1-101* mutants are specifically altered only in subsets of the DNA damage checkpoint pathways responding to DNA damage in G_1 or during S phase.

We also asked whether the *mec1-100* and *mec1-101* mutants were impaired in slowing down of the elongation of mitotic spindles in response to incomplete DNA replication. When cells were released from G_1 arrest in the presence of 200 mM HU, all cell cultures arrested DNA synthesis (Fig. 7A) while spindle elongation took place in the *mec1-100* mutant, along

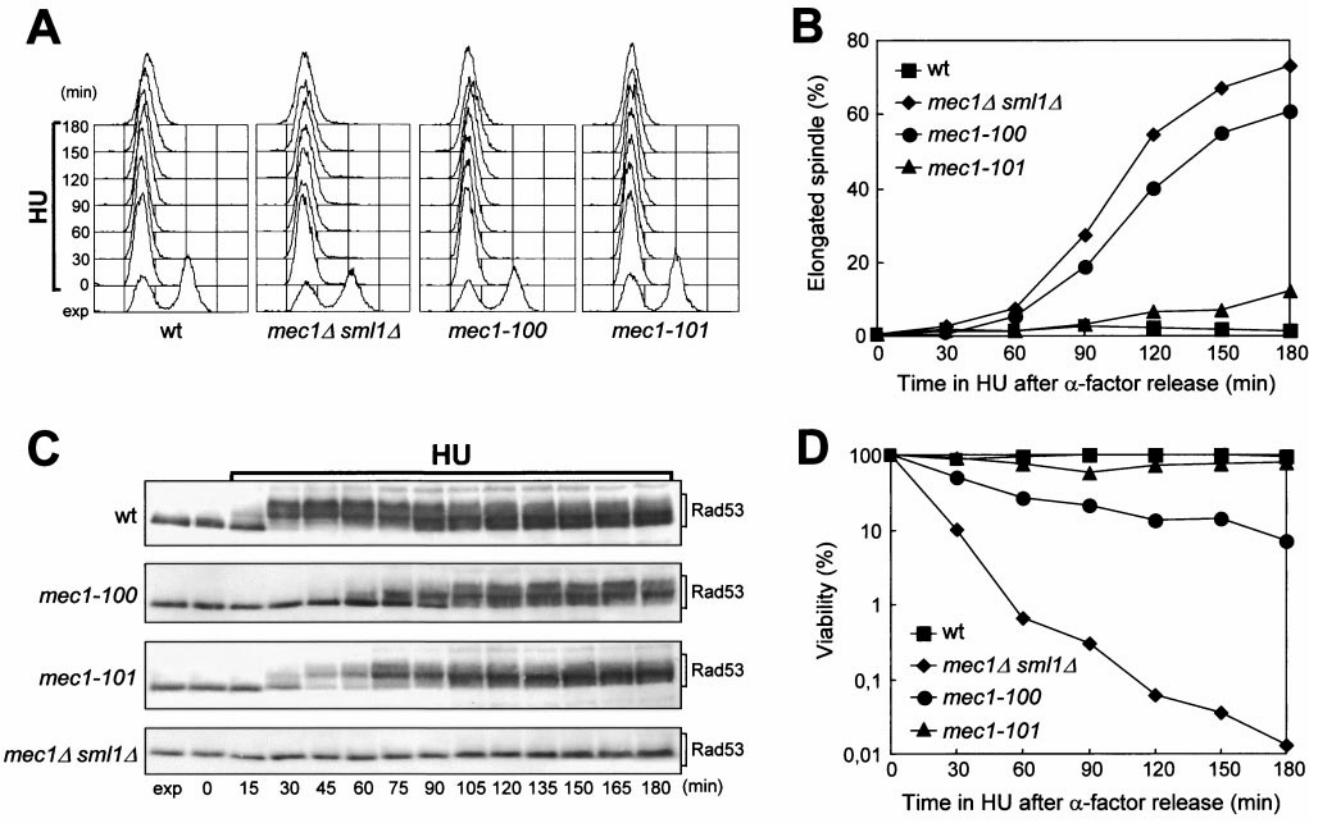

FIG. 7. Response to HU treatment of *mec1-100* and *mec1-101* mutants. Cultures of wild-type (wt) YLL683.8/4A, *mec1*Δ *sml1*Δ DMP3048/5B, *mec1-100* DMP3343/6C, and *mec1-101* DMP3344/4A cells were arrested in G_1 with α-factor and then released at time zero in YEPD containing 200 mM HU. Cell samples were collected at the indicated times after the release from α-factor. The data presented in panels A to D all come from the same experiment. (A) DNA content was analyzed by fluorescence-activated cell sorter. (B) Cells were stained with antitubulin antibodies to score for the percentage of cells with elongated spindles by indirect immunofluorescence. (C) Protein extracts were analyzed by Western blot assay using anti-Rad53 antibodies. exp, exponentially growing cells. (D) Appropriate dilutions were plated on YEPD at 25°C to score for CFU

with aberrant chromosome segregation, with a kinetics only slightly slower than that observed in *mec1*Δ *sml1*Δ cells (Fig. 7B). Conversely, the HU-treated *mec1-101* cells behaved similarly to HU-treated wild-type cells that, as expected, did not elongate the spindles throughout the experiment (Fig. 7B). Moreover, the viability of wild-type and *mec1-101* cells was substantially unaffected by HU, while the *mec1-100* mutant lost viability during HU treatment, although to an extent much less than that of *mec1*Δ *sml1*Δ cells (Fig. 7D). The differences in the abilities of the two mutants to delay S/M transition in response to incomplete DNA replication correlated with differences in HU-induced Rad53 phosphorylation that was consistently delayed in the *mec1-100* mutant compared to wild-type cells, while it was only weakly defective in the *mec1-101* mutant (Fig. 7C).

Determination and comparison of the whole wild-type and mutant *MEC1* coding sequences revealed that the *mec1-100* allele carried two base pair substitutions, resulting in the amino acid changes F1179S and N1700S, while the *mec1-101* allele carried three base pair substitutions, leading to the amino acid changes V225G, S552P, and L781S. The contribution of the single amino acid changes to the mutant phenotypes remains to be established. Alignment of the amino acid sequence of Mec1 with those of *S. pombe* Rad3 and human ATM and ATR indicated that none of the three residues changed by the *mec1-101* mutations is conserved among these proteins. On the contrary, both amino acid changes in the *mec1-100* gene product involve residues that are identical in Mec1 and Rad3 and belong to regions that also appear to be quite well conserved in human ATM and ATR (Fig. 8).

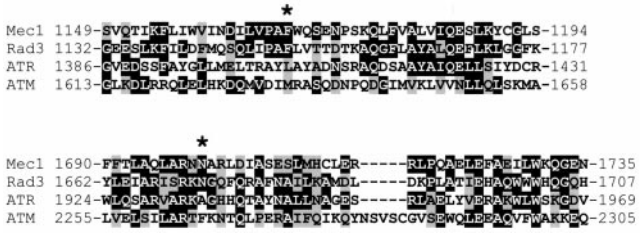

FIG. 8. Amino acid residues changed by the *mec1-100* mutations. The two Mec1 regions containing the *mec1-100*-encoded amino acid changes are shown after alignment of the whole Mec1 amino acid sequence with the *S. pombe* Rad3 and human ATM and ATR amino acid sequences using the ClustalW program. Identical amino acid residues are shaded in black, and similar residues are highlighted in gray. Residues that are changed in the *mec1-100* gene product are marked by asterisks.

DISCUSSION

Although Mec1 is necessary to promote all the known phosphorylation events in the DNA damage checkpoint cascade, little is known about the functions and regulation of Mec1 kinase activity in the activation of the DNA damage response in the different cell cycle phases. We previously showed that a kinase activity dependent on an intact Mec1 kinase domain coimmunoprecipitates with Mec1 (36), and recent work by Mallory and Petes (31) further supports this observation. We now demonstrate that the Mec1 conserved kinase domain is essential for all of the functions of Mec1. In fact, two different Mec1kd variants, in which single amino acid residues in the conserved lipid kinase domain are changed to give kinase-deficient proteins, cause the same effects as the lack of Mec1, resulting not only in hypersensitivity to genotoxic agents and *SML1*-dependent cell lethality but also in a defective DNA damage checkpoint response in all cell cycle phases. Altogether, these data indicate that Mec1 might exert all of its known functions through phosphorylation events. Indeed, we have demonstrated that the kinase activity coprecipitating with Mec1 is able to phosphorylate Ddc2 in vitro (36; this work), thus indicating that Ddc2 may be a target of Mec1 activity in vivo. Both Mec1kd variants completely lose the ability to induce Ddc2 phosphorylation in vitro, although they both still physically interact with Ddc2, indicating that this kinase activity is dependent on the integrity of the Mec1 conserved kinase domain.

Dominant defects caused by *mec1kd* overexpression. Similarly to what was observed when kinase-defective Rad3 and ATR mutant proteins were overproduced (4, 9), high levels of Mec1 kinase-deficient variants in wild-type cells cause dominant-negative effects. In fact, *MEC1* cells overproducing Mec1kd are hypersensitive to DNA-damaging agents and are defective in the slowing down of S phase entry and progression, as well as in Rad53 and Ddc2 phosphorylation, after DNA damage in G_1 or during S phase. Therefore, an excess of Mec1kd proteins in the presence of physiological amounts of wild-type Mec1 may be able to compete for the signals generated by DNA damage, leading to a reduction in the amount of active downstream proteins capable of productively transducing the signal to cell cycle effectors (40). Indeed, we have shown that Mec1 molecules can self-associate and that Mec1-Mec1kd complexes can be formed independently of DNA damage. If Mec1 in vivo functions were dependent on Mec1-Mec1 interaction, Mec1kd overproduction might lead to competition in complex formation, thus reducing the amount of functional Mec1 complexes able to activate downstream effectors like, for example, Rad53. The Mec1kd variants might also titrate Mec1-interacting factors, like Ddc2, into nonfunctional complexes. We found that neither Ddc2 nor Rad53 overproduction can, by itself, suppress the hypersensitivity to DNA-damaging agents or the intra-S checkpoint defect of *MEC1* cells overproducing Mec1kd (V. Paciotti et al., unpublished data). Thus, the dominant effect of Mec1kd overproduction likely involves multiple competition events, or if there is a primary target, it does not seem to be either Ddc2 or Rad53. A search for high-copy-number suppressors of the *MEC1 GAL1-mec1kd* checkpoint defects may help to elucidate this point.

The dominant effects of *mec1kd* overexpression are limited to the DNA damage checkpoints controlling S phase entry and progression. In fact, *MEC1* cells overproducing Mec1kd are still able to activate the G_2/M checkpoint, and this depends on the wild-type Mec1 protein, since *mec1*Δ *sml1*Δ cells overproducing Mec1kd are completely defective in this response. It is interesting that UV light damage in G_2 of *MEC1* cells overproducing Mec1kd allows immediate Rad53 phosphorylation, but the Rad53 phosphorylated forms decrease faster in these cells than in wild-type and *MEC1 GAL1-MEC1* cells under the same conditions. If this implies that Mec1 activity is continuously needed to both activate and maintain the Rad53-dependent checkpoint response, other factors might be required to maintain the G_2 arrest in UV light-treated *MEC1 GAL1-mec1kd* cells. Our data show that the Chk1 protein kinase is necessary for this checkpoint response. In fact, while inactivation of *CHK1* per se is not sufficient to abrogate the UV light-induced G_2/M checkpoint in *MEC1* cells, it leads to premature nuclear division after UV irradiation of *MEC1* cells overexpressing Mec1kd1. Therefore a reduction of Mec1 activity in these cells might uncover the role of Chk1 in this subset of the UV light-induced checkpoint pathways. Conversely, *CHK1* deletion is able to promote nuclear division in *cdc13* mutants also when Mec1 and Rad53 are fully functional (44), suggesting that the amount and quality of DNA damage might determine the ability of cells to activate the *CHK1*-dependent checkpoint response.

Mec1 functions required for checkpoint response to DNA damage in different cell cycle phases. Similar to *MEC1* strains overexpressing the *mec1kd* alleles, both the new *mec1-100* and *mec1-101* mutants are still able to activate the UV light-induced G_2/M checkpoint, while they are completely defective in delaying S phase entry and progression when DNA is damaged in G_1 or during S phase. All of the amino acid substitutions in the Mec1-100 and Mec1-101 proteins are located well outside the conserved Mec1 lipid kinase domain. We cannot exclude the possibility that these amino acid changes can directly affect Mec1 kinase activity, and further work is required to address this point. However, we favor the hypothesis that *mec1-100* and *mec1-101* mutants are defective in interactions with proteins or structures specifically involved in the G_1 and intra-S DNA damage responses and that high levels of Mec1kd variants may titrate molecules important for the above responses into nonfunctional complexes. Moreover, the cell cycle phases at which DNA alterations occur and/or are processed might influence the chance to activate the checkpoint pathways. According to this hypothesis, UV irradiation of G_2-arrested cells results in immediate Mec1-dependent Ddc2 phosphorylation independently of cell cycle progression, while UV irradiation in G_1 is able to induce Ddc2 phosphorylation only when cells are completing S phase or are in G_2 (36), although Ddc2 is strictly required to arrest cell cycle progression in response to DNA damage in all of the cell cycle phases (36). It is therefore tempting to speculate that either DNA damage in G_2 does not require processing in order to be recognized by Mec1 (36) or specific factors are involved in the processing of DNA damage in G_2, allowing easier recognition. If this were the case, it might explain the reduced sensitivity to Mec1 alterations of checkpoint response in G_2 compared to G_1 or S phase.

It is also worth noting that the *mec1-101* mutant that is completely defective in slowing down of S phase progression in

response to DNA damage during DNA synthesis is proficient in arresting spindle elongation in the presence of HU, further supporting the hypothesis that the cellular response to DNA replication blocks or to DNA damage during DNA replication involves different Mec1 functions.

DNA damage checkpoint defects and sensitivity to genotoxic agents. The characterization of the *mec1-100* and *mec1-101* mutants also provides some new data addressing the important question of whether checkpoint impairment renders cells hypersensitive to genotoxic agents. In fact, while these mutants are indistinguishable from *mec1*Δ cells with respect to the ability to replicate a damaged DNA template, they do not show hypersensitivity to UV light and MMS, suggesting that a DNA damage checkpoint defect per se does not necessarily cause hypersensitivity to DNA-damaging agents. It is possible that a functional G_2 checkpoint can compensate for defects in slowing down of DNA replication in the presence of DNA insults (39). In fact, if a failure to control the replication of damaged template DNA results in genetic instability, activation of the G_2/M checkpoint would offer the opportunity to repair strand breaks before sister chromatids are no longer available for repair. If so, the high survival of the *mec1-100* and *mec1-101* mutants after UV light and MMS treatment may correlate with an increased dependence on DNA damage-induced G_2 arrest. However, although *MEC1* cells overexpressing *mec1kd* are still able to activate the G_2/M checkpoint, they show hypersensitivity to DNA-damaging agents, indicating that delay of nuclear division is not always sufficient to compensate for the effects of Mec1 impairment on cell survival after DNA damage. Taken together, our results indicate that when DNA replication occurs in the presence of DNA insults, cell lethality of checkpoint mutants might not be purely a cell cycle transition phenomenon, but other processes might be involved. For example, the inability of these mutants to properly carry out chromosomal replication might result in cell lethality, as also suggested by Desany et al. (13). In this view, the high sensitivity to HU treatment of *mec1*Δ *sml1*Δ cells might be due to failure of replication structures to recover from the effects of nucleotide depletion, instead of depending on the faster cell cycle progression. In fact, the viability of *mec1-100* cells during HU treatment is much higher than that of *mec1*Δ *sml1*Δ cells, although the kinetics of spindle elongation in the presence of HU is almost as fast in *mec1-100* cells as in *mec1*Δ *sml1*Δ cells. Moreover, the sensitivity to genotoxic agents of cells impaired in Mec1 activity may result from the inability to mediate the efficient repair of DNA lesions, leading to a model in which checkpoints are integrated into a larger DNA damage response pathway. Consistent with this hypothesis, recent data have implicated Mec1 in recombination mechanisms. In fact, *MEC1* is absolutely required to induce sister chromatid exchange in nucleotide excision repair-deficient cells (H. Neecke et al., unpublished data) and to promote normal meiotic recombination (18, 51). Moreover, phosphorylation of the Rad55, RPA, and Srs2 proteins, all of which are involved in DNA repair and recombination (1, 37), was found to be Mec1 dependent (3, 5, 25). Finally, the implication of the Mec1 human homologue ATM in the control of recombinational repair has been hinted at by various links recently found (8, 11, 46), by the recombinational abnormalities observed in ataxia telangiectasia patients (32), and by the fact that ATM is required for phosphorylation of NBS1 (15, 26, 61, 63) and BRCA1 (11, 24), both of which regulate the repair of double-strand breaks (DSBs) and the proper cellular response to DNA damage. The findings that ATM is required for homologous recombination-mediated repair of DSBs and is a member of the recombinational repair epistasis group (33) clearly indicate that ATM has a role not only in preventing cells from propagating damaged DNA but also in the processing and repair of DSBs. Our data suggest that this is also the case for Mec1, further strengthening the notion of functional conservation between the human and yeast proteins.

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V.P. and M.C. contributed equally to this work.

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