Cds1 Phosphorylation by Rad3-Rad26 Kinase Is Mediated by Forkhead-associated Domain Interaction with Mrc1*

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The protein kinase Cds1 is an effector of the replication checkpoint in the fission yeast Schizosaccharomyces pombe. Cds1 is required to stabilize stalled replication forks, and it helps to prevent the onset of mitosis until the genome is fully replicated. Mrc1 (mediator of the replication checkpoint-1) and Rad3-Rad26 kinase are required for Cds1 activation, but exactly how Mrc1 mediates Cds1 activation is unknown. Here we show that Mrc1 is required for the initial threonine 11 phosphorylation of Cds1 by Rad3-Rad26. Mrc1 specifically interacts with the forkhead-associated (FHA) domain of Cds1 in yeast two-hybrid assays. Mutations in the FHA domain that abolish this interaction also eliminate Thr-11 phosphorylation of Cds1. Weak Thr-11 phosphorylation of a “kinase-dead” mutant of Cds1 is rescued by co-expression of wild type Cds1. The requirement for Mrc1 in the replication checkpoint can be partially eliminated by expression of a Rad26-Cds1 fusion protein. These findings suggest that recognition of Mrc1 by the FHA domain of Cds1 serves to recruit Cds1 to Rad3-Rad26. This interaction mediates the initial Thr-11 phosphorylation of Cds1 by Rad3-Rad26 with subsequent intermolecular phosphorylation events leading to full activation of Cds1.

Accurate DNA replication of a eukaryotic genome, a remarkable accomplishment in the best of circumstances, is often complicated by DNA adducts or protein complexes that obstruct replisomes and thereby cause fork arrest (1, 2). Replication forks may also stall as a result of sNTP starvation. Stalled forks are thought to be inherently unstable structures prone to regression, rearrangement, or collapse (3). These events threaten genome stability. In these situations, a sensor response system known as the DNA replication checkpoint steps into delay mitosis, stabilizes the fork, and facilitates resumption of fork progression in a manner that preserves genetic information (1, 2).

Studies of the budding yeast Saccharomyces cerevisiae and the fission yeast Schizosaccharomyces pombe have uncovered networks of checkpoint sensor, transducer, and effector proteins that are also conserved in mammals. Central to these networks are large phosphoinositide 3'-kinase-like kinases (PIKKs),1 Rad3 and Tel1 in fission yeast, Mec1 and Tel1 in budding yeast, and ATR and ATM in humans (4). The Rad3/Mec1/ATM subfamily of PIKKs act together with regulatory subunits (ATRIP for ATR, Rad26 for Rad3, and Ldc1/Ddc2/Pie1 for Mec1) to phosphorylate downstream targets. These targets include the effector kinases of the checkpoints, Cds1 and Chk1 in fission yeast, Rad53 and Chk1 in budding yeast, and Chk2 (Cds1) and Chk1 in humans (5).

In fission yeast, Cds1 enforces the replication checkpoint (6–8), whereas Chk1 enforces the G2-M DNA damage checkpoint (9). The critical role of Chk1 in the DNA damage checkpoint in fission yeast is straightforward; it delays the onset of mitosis by inhibiting activation of the cyclin-dependent kinase Cdc2 (10). Cds1 has a more complicated set of functions, being required to stabilize stalled forks (11), as well as working with Chk1 to prevent the onset of mitosis (7, 8). Exactly how Cds1 stabilizes arrested forks is unknown, but it appears to regulate several DNA repair proteins that may be involved in maintenance of stalled forks (12, 13).

Rad3 controls both Cds1 and Chk1 (1). Insight into how Rad3 specifically activates Cds1 in S phase was provided by the discovery of Mrc1 (14, 15). Mrc1, so named because it is a mediator of the replication checkpoint, is required for activation of Cds1 but not Chk1. Mrc1 is expressed only during S phase in fission yeast (15), a finding that explains why Cds1 activation is restricted to S phase. Mrc1 was proposed to recruit Cds1 to stalled replication forks to mediate phosphorylation of Cds1 by Rad3-Rad26 kinase. In support of this model, Cds1 was found to interact with Mrc1 in yeast two-hybrid assays (16). Phosphorylation of Mrc1 by Rad3-Rad26 is required for Cds1 activation in fission yeast (17). A similar relationship exists between Rad53, Mrc1, and Mec1-Ddc2 in budding yeast (18). In budding yeast, Mrc1 moves with replication forks during S phase, and it appears to be a component of the replisome complex (18, 19). Mrc1 proteins are highly divergent, and it is not yet clear whether they have true functional homologs in multicellular organisms. Claspin, a protein first identified in Xenopus through its physical association with Chk1 (20), has weak sequence similarity to yeast Mrc1 proteins and may be the functional homolog of Mrc1 (14). Claspin is required for Chk1 activation during replication arrest in Xenopus egg extracts (20–22), and recent studies have provided evidence that Claspin is required for the replication checkpoint in human cells (23).

1 The abbreviations used are: PIKK, phosphoinositide 3'-kinase-like kinase; FHA, forkhead-associated; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia-related; ATRIP, ataxia telangiectasia-related interacting protein; TAP, tandem affinity purification; TEV, tobacco etch virus; HU, hydroxyurea; DAPI, 4', 6-diamidino-2-phenylindole; DTT, dithiothreitol; pThr, phosphothreonine.

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Activation of Cds1 homologs is a multistep process that can involve both trans-phosphorylation by PIKKs as well as intermolecular autophosphorylation between Cds1 molecules. In the case of human Chk2, phosphorylation of Thr-68 by ATM or ATR is crucial for Chk2 activation (24–27). Thr-68 is part of a TQ motif that is a preferred substrate sequence phosphorylated by PIKKs (28, 29). An equivalent position in fission yeast Cds1, Thr-11, is phosphorylated by Rad3 in vivo and is required for Cds1 activation in vivo (16). Similarly, efficient activation of budding yeast Rad53 requires one or more N-terminal TQ motifs that are potential substrate phosphorylation sites of Mecl (30). Studies of Chk2 and Rad53 (and the related kinase Dun1 in budding yeast) have shown that intermolecular autophosphorylation is vital for activation of Cds1 homologs (30–34). Intermolecular autophosphorylation appears to occur on the aforementioned N-terminal TQ motifs as well as threonine residues within the activation loop of the catalytic domain (34). These reactions require a forhead-associated (FHA) domain that is located between the N-terminal TQ motifs and the C-terminal catalytic domain. The FHA domain is a phosphoprotein recognition domain that mediates specific phosphorylation-dependent interactions between proteins (35). A functional FHA domain is essential for activation of Cds1 and its homologs (12, 31, 33, 34). In the case of human Chk2, it appears that a phosphorylated Thr-68 site on one Chk2 molecule interacts with the FHA domain of another Chk2 molecule. This interaction leads to the formation of Chk2 oligomers and efficient intermolecular autophosphorylation (31, 33, 34). Chk2 oligomerization may further regulate hChk2 activation, because Chk2 can also phosphorylate its FHA domain in vitro, and these phosphorylations lead to dissociation of hChk2 dimers (31, 33, 34). Transient transfection assays have indicated that most nonfunctional FHA mutations of Chk2 do not impair Thr-68 phosphorylation, suggesting that the FHA domain is not essential for the initial Thr-68 phosphorylation but may instead be required for Chk2 dimerization (33). In the case of budding yeast Rad53, a C-terminal FHA domain mediates binding to Rad9. This binding is abolished by mutating multiple TQ/SQ motifs in Rad9, suggesting that phosphorylation of Rad9 by Mec1 leads to recruitment of Rad53 and activation of Rad53 by intermolecular autophosphorylation (36, 37). Another possibility that is not mutually exclusive is that phosphorylated Rad9 serves as an adapter that delivers Rad53 to Mec1 (38). Neither human Chk2 or fission yeast Cds1 have a C-terminal FHA domain and the Rad9-related protein Crb2 in fission yeast is not involved in Cds1 activation (11), thus the Rad9-mediated mechanism of Rad53 activation may be unique to budding yeast. Exactly how Mrc1 mediates Cds1 activation in fission yeast or Rad53 activation in budding yeast is unknown. One possibility is that Mrc1 recruits Cds1/Rad53 to upstream PIKK-like kinases. In this model, Mrc1 should be required for the initial Thr-11 phosphorylation of Cds1 by Rad3-Rad26 in fission yeast. In this study we provide evidence that the association of Mrc1 to the Cds1 FHA domain mediates the initial Thr-11 phosphorylation of Cds1. Furthermore, we show that expression of a Cds1-Rad26 fusion protein substantially bypasses the requirement for Mrc1 in the replication checkpoint. These findings support the model that the major function of Mrc1 is to recruit Cds1 and present it as a substrate for Rad3-Rad26. These studies provide new insights into the mechanism of signal transduction in the replication checkpoint in fission yeast and may help to understand how Chk2 is regulated in humans.
was abolished in mrc1Δ cells (Fig. 1B). These findings indicate that Mrc1 is essential for the initial Thr-11 phosphorylation of Cds1 that is catalyzed by Rad3-Rad26.

**FHA Domain Is Required for Thr-11 Phosphorylation of Cds1**—FHA domains are protein-protein interaction modules that bind phosphotheorine (pThr) residues in target ligands (44). They are domains of ~100–180 amino acids that form an 11-stranded β-sandwich. An intact FHA domain is required for activation and function of Cds1 (12). These findings imply that Cds1 activation requires an FHA domain interaction with a phosphorylated protein. However, transient transfection assays have shown that many mutations in the FHA domain of hChk2 that are predicted to impair binding to phosphopeptide ligands do not abolish Thr-68 phosphorylation (33). These findings indicated that a functional FHA domain is not necessary for the initial Thr-68 phosphorylation of Chk2 that is catalyzed by PIKKs (33). However, one shared limitation of this and similar investigations was that they overexpressed mutant forms of Chk2. Protein overexpression can sometimes obscure regulatory relationships. In the case of the Chk2/Cds1, this concern is reasonable because Cds1 overexpression suppresses the HU-sensitive phenotype of mrc1Δ cells in fission yeast (15).

We therefore decided to investigate whether a functional FHA domain is required for Thr-11 phosphorylation of Cds1 in mutant strains that expressed all proteins at endogenous levels. We first examined a previously described FHA mutation of Cds1 (Cds1-S78AH81A; noted as cds1-FHA*) (12), in which the conserved amino acids Ser-78 and His-81 in the FHA domain were both mutated to alanine. The cds1-FHA* strain is very sensitive to HU, and the mutant Cds1 protein is not activated in response to HU treatment (12). Immunoblotting with Cds1 antisera showed that Cds1-FHA* protein was expressed at the same level as wild type Cds1, although Cds1-FHA* had an altered electrophoretic mobility (Fig. 1B). Immunoblotting with phospho-Thr-11 antibodies showed that Thr-11 was not phosphorylated in cds1-FHA* cells (Fig. 1B). These results indicated that the initial Thr-11 phosphorylation of Cds1 by Rad3 requires an intact FHA domain.

These results prompted a more detailed analysis of the FHA domain. Structural information based on NMR spectroscopic and x-ray crystallographic analyses was used to produce a modified sequence alignment of FHA domains from fission yeast Cds1, budding yeast Rad53, and Chk2 proteins from human, mouse, and Xenopus (Fig. 2A) (33, 35, 44, 45). Based on this information, we created mutations at conserved residues that are located within the β strands (H81A and I84A) or within loops connecting β strands that are expected to make contact with a phosphopeptide ligand (R64A, S78A, and N107A) (44) (Fig. 2A). Crystallographic analyses indicated that a conserved Arg residue (Arg-70 in Rad53 and Arg-64 in Cds1) is pivotal for direct FHA domain interactions with the phosphate group of pThr-containing peptide ligands (35, 46, 47). These mutations were introduced into the genomic locus of cds1*. All mutant forms of Cds1 except for Cds1-I84A were expressed at wild-type levels (Fig. 2C). The Cds1-I84A mutation probably affected protein stability, because mutations of residues that form the β-sandwich structure of the FHA domain also destabilized hChk2 protein (33).

Serial dilutions of wild type and cds1 mutant cells were spotted on media containing increasing concentrations of HU (Fig. 2B). The cds1-R64A, cds1-S78A, cds1-H81A, and cds1-N107A cells were extremely sensitive to HU, displaying a phenotype that was comparable to cds1Δ cells. The cds1-I84A cells were moderately sensitive to HU, being able to grow on 5 mM HU but not 10 mM HU plates.

Immunoblotting with the phospho-Thr-11-specific antibodies A-Sepharose-bound Cds1 antibodies at 4 °C for 2 h. Protein A-Sepharose was washed three times with lysis buffer followed by three times with kinase buffer (10 mM HEPES-KOH, pH 7.5, 75 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, 1 mM DTT). 20 μl (50% slurry) of protein A-Sepharose pellet was incubated with 10 μl of 2× kinase buffer containing 5 μCi of [γ-32P]ATP, 1 μl of 2× ATP, 5 μl of myelin basic protein (1 mg/ml stock) at 30 °C for 15 min. The reaction was stopped by the addition of 20 μl of 2× SDS sample buffer. Samples were boiled and subjected to SDS-PAGE in 15% gel.

**RESULTS**

**Mrc1 Is Essential for Thr-11 Phosphorylation of Cds1**—Activation of Cds1 and its homologs is thought to be a multistep process that involves PIKK-dependent phosphorylation of one or more N-terminal TQ motifs followed by intermolecular autophosphorylation. In fission yeast, Rad3 phosphorylates Thr-11 in vitro, and this site is required for Cds1 activation in vivo (16). In principle, Mrc1 might act by facilitating initial Thr-11 phosphorylation by Rad3-Rad26, or it could act after this event to promote intermolecular autophosphorylation, or it could act in an unknown way to promote Cds1 activation. To determine how Mrc1 functions in the Cds1 activation mechanism, we investigated if Mrc1 is required for the initial Thr-11 phosphorylation catalyzed by Rad3.

Analysis of Thr-11 phosphorylation required the production of antibodies that specifically recognized the Thr-11 phosphorylated form of Cds1. These phosphospecific antibodies were affinity purified with the Thr-11 phosphopeptide and depleted with unphosphorylated Thr-11 peptide (see “Materials and Methods”). The specificity of these antibodies was demonstrated by their ability to recognize Cds1, but not Cds1-T11A (Thr-11 mutated to Ala), after HU treatment (Fig. 1A). These antibodies were used to examine Thr-11 phosphorylation in several mutant strains. Analysis of rad3Δ cells confirmed that HU-induced Thr-11 phosphorylation required Rad3 (Fig. 1B). These results strengthened evidence that Rad3 phosphorylates Thr-11 in response to HU (16). A similar analysis was carried out with mrc1Δ cells. We observed that Thr-11 phosphorylation...
was carried out with the mutant strains. All of the FHA mutants were severely defective in Thr-11 phosphorylation (Fig. 2D). The data with the cds1-I84A mutant were uncertain, however, because the low abundance of Cds1-I84A protein may have precluded detection of Thr-11 phosphorylation. In addition to Cds1-I84A, Cds1-H81A may also be below the detection limit (Fig. 2D). We also observed that only wild type Cds1 protein displayed reduced electrophoretic mobility in response to HU treatment (Fig. 2C). Reduced electrophoretic mobility is a consequence of activating phosphorylation (7, 16); thus, it appears that all the mutants, including cds1-I84A, were unable to activate Cds1 in response to HU treatment.

As another independent assay of Cds1 function, we carried out immunoblot analysis of the S-phase-specific cyclin Cig2. The cig2 gene is periodically expressed in S phase (43); hence Cig2 protein should accumulate in cells that are arrested at the S phase checkpoint. As expected, we observed a large accumulation of Cig2 in wild type cells treated with HU (Fig. 2C). In contrast, Cig2 abundance was unchanged in cds1Δ cells treated with HU (Fig. 2C). Likewise, no accumulation of Cig2 was detected in strains that had mutations in the FHA domain of Cds1 (Fig. 2C).

These studies provided strong evidence that the integrity of the FHA domain is essential for the initial Thr-11 phosphorylation of Cds1. These findings are consistent with the idea that the FHA domain must interact with a pThr-containing protein as a prerequisite for Thr-11 phosphorylation by Rad3. Mrc1 is the best candidate for a Cds1 FHA ligand, because Mrc1 is required for Thr-11 phosphorylation (Fig. 1B), and it is phosphorylated by Rad3 (17).

**Mrc1 Specifically Interacts with the Cds1 FHA Domain**—We have shown by yeast two-hybrid assay that Mrc1 interacts with...
the N-terminal region of Cds1 that contains the FHA domain (15). Analysis of the *pha* allele indicated that the interaction required an intact FHA domain (17). To extend and correlate these studies with defects in Thr-11 phosphorylation, we examined whether the new FHA domain mutants impaired the two-hybrid interaction with Mrc1. As shown in Fig. 3, Mrc1 and the N-terminal region of wild type Cds1 (Cds1N) displayed a positive two-hybrid interaction that was absent in the negative controls, as described previously (15). Four mutated Cds1N derivatives (Cds1N-R64A, Cds1N-S78A, Cds1N-H81A, and Cds1N-I84A) were tested for their interaction with Mrc1. Interestingly, two-hybrid interactions between Mrc1 and Cds1N were lost in three FHA mutants (R64A, S78A, and H81A). Only the I84A mutation did not affect this interaction (Fig. 3). This pattern was consistent with the observations that all FHA mutants except *cds1-I84A* were highly sensitive to HU (Fig. 2B). These results indicated that Mrc1 specifically interacts with the Cds1 FHA domain and that Arg-64, Ser-78, and His-81 residues in the Cds1 FHA domain are important for interaction with Mrc1. Taken together, the results described in Figs. 1–3 supported a model in which the FHA domain of Cds1 binds to Mrc1, thereby recruiting Cds1 to Rad26. The interaction of Mrc1 with either Rad3 or Rad26 may be very weak or transient, or occur indirectly through an intermediate. As an alternative method to test our hypothesis, we asked whether the requirement for Mrc1 might be bypassed by expressing chimeric Cds1-Rad26 protein in which genomic *cds1* was modified to encode Cds1 fused at its C terminus to Rad26 and allowing Rad3-Rad26 to phosphorylate Thr-11 of Cds1.

**Cds1 Kinase Activity Is Required for Amplification of Thr-11 Phosphorylation**—In human cells, ATM was shown to phosphorylate hChk2 at Thr-68, a site equivalent to Thr-11 in fission yeast Cds1 (31, 33). Phosphorylation of Thr-68 of hChk2 was found to be important for hChk2 oligomerization and autophosphorylation via the FHA domain for full activation. Interestingly, we found that Thr-11 phosphorylation was strikingly reduced but not abolished in cells that expressed the Cds1 “kinase-dead” allele *cds1-D312E* (*cds1-KD*) (12) (Fig. 1B). This result suggested that Cds1 kinase activity was required to reach full Thr-11 phosphorylation in vivo. We therefore tested whether the Thr-11 phosphorylation of Cds1-D312E required Rad3 and Mrc1. As shown in Fig. 4A, HU-dependent Thr-11 phosphorylation of Cds1-D312E was eliminated in *rad3Δ* and *mrc1Δ* cells. These results supported the model in which initial Thr-11 phosphorylation of Cds1 is amplified by intermolecular autophosphorylation in a Cds1 activation loop.

To further explore the possibility that Cds1 kinase activity is required for full phosphorylation of Thr-11, we monitored Thr-11 phosphorylation of Cds1-D312E in a *cds1-d312E* strain transformed with a plasmid containing *cds1*. Thr-11 phosphorylation of Cds1-D312E was strikingly enhanced in response to HU treatment when Cds1 kinase was supplied on an episomal plasmid (Fig. 4B). This phosphorylation was not seen in cells transformed with an empty vector. These results supported the model that Cds1 kinase activity is required for amplification of Thr-11 phosphorylation.

**Expression of Cds1-Rad26 Fusion Protein Partially Bypasses the Requirement for Mrc1**—We have hypothesized that Mrc1 is an S-phase-specific adaptor protein that transiently interacts with Cds1 and Rad3-Rad26 complex, thereby allowing Rad3-Rad26 to phosphorylate Cds1 (15). We have been unable, however, to detect any association between Mrc1 and either Rad3 or Rad26 by immunoprecipitation and two-hybrid assay. The interaction of Mrc1 with either Rad3 or Rad26 may be very weak or transient, or occur indirectly through an intermediate. As an alternative method to test our hypothesis, we asked whether the requirement for Mrc1 might be bypassed by expressing chimeric Cds1-Rad26 protein in which genomic *cds1* was modified to encode Cds1 fused at its C terminus to Rad26 (Fig. 5A, and “Materials and Methods”). We reasoned that if Mrc1 functions to recruit Cds1 to Rad3-Rad26 complex, then joining Cds1 and Rad26 proteins might bypass the requirement for Mrc1. This *cds1-rad26* fusion strain, which retained intact copies of *rad26* and *mrc1*, appeared fully resistant to 5 mM HU, whereas *rad26Δ*, *cds1Δ*, or *mrc1Δ* cells were unable to form colonies on the same medium (Fig. 5B). Relative to wild type, the *cds1-rad26* strain showed a slight sensitivity to 10 mM HU (data not shown). These observations indicated that the functions of Cds1 that are essential for HU survival were largely intact when it was expressed as a Cds1-Rad26 fusion protein. The *cds1-rad26* construct also suppressed the UV and HU sensitivities of *rad26Δ* cells (data not shown), indicating that the functions of Rad26 that are necessary for the replication and DNA damage checkpoints were also maintained in Cds1-Rad26 fusion protein.

The *cds1-rad26* fusion allele was mated into an *mrc1Δ* background. Remarkably, the *cds1-rad26* construct substantially suppressed the *mrc1Δ* HU-sensitive defect (Fig. 5B). On a 5 mM HU plate, the *cds1-rad26* *mrc1Δ* cells did not grow as well as *cds1-rad26* cells, but they grew much better than *mrc1Δ* cells. Therefore, the function of Mrc1 in the HU response was bypassed, albeit somewhat inefficiently, by fusion of Cds1 to Rad26. The *cds1-rad26* construct did not, however, suppress the HU sensitivity of *rad3Δ* cells (Fig. 5B), indicating...
that rescue of mrc1Δ by the cds1-rad26Δ construct depends on phosphorylation of substrates by Rad3.

Rescue of mrc1Δ by cds1-rad26Δ was also analyzed in checkpoint arrest studies. These checkpoint assays were performed in a chk1Δ background, because functional Chk1 is sufficient to impose a HU-induced cell cycle arrest in mrc1Δ or cds1Δ.
strains (15). As shown in Fig. 5 (C and D), cds1-rad26Δ expressed in an mrc1Δ chklΔ background strongly decreased the frequency of abnormal mitotic “cut” cells in cultures treated with HU.

These findings supported the model that Mrc1 functions to recruit Cds1 toRad3-Rad26 as a substrate. We examined this possibility in more detail by carrying out Cds1 kinase assays. As noted previously, HU activates Cds1 in a Mrc1-dependent manner (14, 15). Significant activation of Cds1-Rad26 was detected in a mrc1Δ background (Fig. 5E). In an mrc1Δ background, specific HU-induced activation of Cds1-Rad26 was also detected, although it was reduced compared with Cds1-Rad26 activation in the mrc1Δ background. Furthermore, expression of Cds1-Rad26 in mrc1Δ cells restored the accumulation of the S-phase-specific cyclin Cig2, an indicator of the cell cycle progression arrest by the replication checkpoint (Fig. 5E). These results showed that the requirement of Mrc1 for Cds1 activation and function was substantially bypassed by physically linking Cds1 to Rad26, suggesting that the function of Mrc1 as an S-phase-specific adaptor is to recruit Cds1 to Rad3-Rad26.

**DISCUSSION**

**Cds1 Activation Requires Phosphorylation of Thr-11 by Rad3-Rad26**—In our previous work, we showed that both Rad3 and ATM phosphorylate a N-terminal fragment of Cds1 that contains TQ motifs at Thr-8 and Thr-11 (16). The T11A mutation abolished in vitro phosphorylation of Cds1 and eliminated Cds1 activation in vivo. The cds1-T11A mutant was profoundly sensitive to HU and defective for the S-M checkpoint. These data indicated that Cds1 activation requires direct phosphorylation of Thr-11 by Rad3. Here we have used phospho-Thr-11 specific antibodies to show that Thr-11 of Cds1 is phosphorylated in vivo in response to HU in a Rad3-dependent manner (Fig. 1). In addition, mass spectrometry analysis of Cds1 has confirmed that HU induces Thr-11 phosphorylation. Collectively, these data provide strong evidence that direct phosphorylation of Thr-11 by Rad3-Rad26 is critical for Cds1 activation.

**Mrc1 Is Required for Thr-11 Phosphorylation of Cds1**—As noted above, activation of Cds1 homologs appears to be a multistep process that involves initial phosphorylation by PIKKs that is followed by intermolecular autophosphorylation. A checkpoint adaptor or mediator protein such as Mrc1 might act at either step. Our studies have shown that Thr-11 phosphorylation is abolished in an mrc1Δ mutant (Fig. 1B). These findings provide strong evidence that Mrc1 is required for the initial Thr-11 phosphorylation catalyzed by Rad3-Rad26. It is a formal possibility that the initial Thr-11 phosphorylation is independent of Mrc1, but the sensitivity of the phospho-Thr-11-specific antibodies is insufficient to detect a small amount of “triggering” Thr-11 phosphorylation. This possibility appears unlikely, because the phospho-Thr-11 antibodies were able to detect Thr-11 phosphorylation of a kinase-dead mutant of Cds1 (Fig. 1B). This mutant is presumably unable to carry out intermolecular autophosphorylation; in fact, its Thr-11 phosphorylation is substantially reduced compared with wild type Cds1 (Fig. 1B). This defect can be substantially corrected by co-expressing wild type Cds1 (Fig. 4B). Thus, the absence of detectable Thr-11 phosphorylation in mrc1Δ cells very probably reflects the fact that Mrc1 is required to mediate the initial Thr-11 phosphorylation of Cds1 by Rad3-Rad26. Of course, these findings do not preclude the possibility that Mrc1 might also have a role in promoting subsequent intermolecular autophosphorylation of Cds1.

The FHA Domain of Cds1 Is Essential for Thr-11 Phosphorylation—Previous studies of Chk2 have led to contradictory conclusions regarding the role of the FHA domain in mediating the initial Thr-68 phosphorylation by PIKKs. Several studies reported that the R145W mutant protein was poorly Thr-68-phosphorylated, and its activation was impaired, consistent with this mutation abrogating Chk2 function (31, 33, 34, 48). This FHA domain mutation is particularly interesting, because it is associated with a cancer predisposition phenotype in a subset of Li-Fraumeni patients and a colon cancer cell line (49, 50). However, Chk2-R145W mutant protein is unstable in vivo (33), thus it has been unclear if this mutation only impairs FHA domain function or whether it induces a more drastic conformational change that alters the entire structure of Chk2. In fact, a set of other FHA domain mutations that were predicted to abrogate FHA function, as well as another mutation found in Li-Fraumeni patients (1157T), did not impair Thr-68 phosphorylation in transfected cells (33, 48). These findings suggested that recognition of a mediator or adaptor protein by the FHA domain interaction was not required for phosphorylation of Chk2 by PIKKs.

As noted above, the FHA mutation studies of Chk2 used transient transfection assays that overexpressed Chk2. We have previously reported that Cds1 overexpression bypasses the requirement for Mrc1 (15). This fact indicated that it was necessary to examine FHA mutations in cells that expressed mutant forms of Chk2 at endogenous levels, as we have done here. Strikingly, all the FHA mutations that we examined fully abrogated Thr-11 phosphorylation of Cds1. Some of these mutations align with residues that were mutated in human Chk2 (24). For example, Cds1-R64A is equivalent to Chk2-R117A, and Cds1-R78A is equivalent to Chk2-S140A. These mutations eliminated Thr-11 phosphorylation in Cds1, whereas the equivalent mutations in Chk2 did not impair Thr-68 phosphorylation. Fission yeast and mammalian cells must have a fundamental difference in the requirement for a functional FHA domain to mediate PIKK phosphorylation of Cds1/Chk2, or the observed differences must arise from the variances in the experimental systems.

**Cds1 FHA Domain Mediates Interaction with Mrc1**—We have previously used yeast two-hybrid assays to show that Mrc1 interacts with the N-terminal region of Cds1 that includes the FHA domain (15). Here we showed that Mrc1 does not interact with three of the mutated FHA domains (R64A, S78A, and H81A) of Cds1, indicating that Mrc1 specifically interacts with the Cds1 FHA domain (Fig. 3). These results strongly suggest that a Cds1-Mrc1 interaction, mediated through the FHA domain of Cds1 in a mechanism similar to that proposed for regulation of the Rad9-Rad53 interaction by Mec1 (37), is a prerequisite for the initial Thr-11 phosphorylation of Cds1 by Rad3. We have recently shown that phosphorylation of Mrc1 in response to HU treatment is abolished in a rad3Δ tel1Δ strain (17). These phosphorylations appeared to occur at multiple STQ motifs. The TQ motif at Thr-645 was shown to be very important for Mrc1 function and for a two-hybrid interaction between Mrc1 and the FHA domain of Cds1. These findings suggest that Rad3-dependent phosphorylation of Mrc1 at Thr-645 allows the FHA domain of Cds1 to recognize Mrc1, and through this association Rad3 is able to phosphorylate Cds1.

This model leads to the simple prediction that it might be possible to bypass the requirement for Mrc1 in the replication checkpoint system by physically linking Cds1 to Rad3-Rad26 complex. We carried out this test and found that indeed there was partial rescue of mrc1Δ by expressing Cds1-Rad26 fusion protein (Fig. 5). Importantly, Cds1-Rad26 was expressed from...
the endogenous cds1 promoter at the cds1 genomic locus, so Cds1 was not overexpressed in these studies. The inability of Cds1-Rad26 fusion protein to fully rescue mrc1Δ phenotypes may be simply explained if direct fusion of Cds1 and Rad26 substitutes incompletely for Mrc1 mediated linking of Cds1 and Rad3-Rad26 complex, or it could suggest that Mrc1 has Cds1 independent functions, as indicated by studies of budding yeast Mrc1 (18, 19). Whatever the explanation, the fact that Cds1-Rad26 fusion protein substantially rescues mrc1Δ phenotypes supports the model in which Mrc1 functions as an adaptor or mediator protein to facilitate phosphorylation of Cds1 by Rad3-Rad26.

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