DNA Binding Domain in the Replication Checkpoint Protein Mrc1 of Schizosaccharomyces pombe*

The replication checkpoint is activated when replication forks are obstructed by DNA lesions or protein complexes bound to DNA or when DNA synthesis is repressed by the limited availability of deoxyribonucleotides. This checkpoint preserves genome integrity by stabilizing stalled forks and delaying the onset of mitosis. In the fission yeast Schizosaccharomyces pombe, Mrc1 is a replication checkpoint adaptor protein that allows the sensor kinase Rad3-Rad26 to activate the effector kinase Cds1. In Saccharomyces cerevisiae, Mrc1 associates with replication forks and co-precipitates with the DNA replication protein Cde45. Whether or not Mrc1 interacts directly with DNA is unknown. Here we define a ~150 amino acid DNA binding domain (DBD) in the N-terminal region of S. pombe Mrc1. The DBD interacts preferentially with branched DNA structures in vitro. Deletion of the DBD or point mutations that diminish its DNA binding activity render cells sensitive to the replication inhibitor hydroxyurea. These mutations also impair the replication checkpoint arrest. The DBD has a helix-loop-helix motif that is predicted to bind DNA. This motif is conserved in the recently identified N-terminal DBD of human Claspin, a presumptive homolog of yeast Mrc1 proteins.

The fission yeast Schizosaccharomyces pombe has been a valuable model system for studies of genome surveillance and checkpoint mechanisms. These systems control cell cycle progression while regulating DNA repair and DNA replication. Two distinct DNA structure checkpoints, the DNA replication checkpoint and the DNA damage checkpoint, have been defined in fission yeast (1–3). The DNA damage checkpoint, which responds to double strand breaks and other abnormal DNA structures, functions primarily in G2 phase. The DNA replication checkpoint, which is activated when replication forks stall, functions specifically in the DNA synthesis (S) phase of the cell cycle. Central elements of these two checkpoints include two phosphatidylinositol 3-kinase-like kinase family members, Rad3 (an ataxia telangectasia-related homolog) and Tel1 (an ataxia telangectasia homolog), as well as the Rad9-Rad1-Hus1 checkpoint clamp and a checkpoint clamp loader that contains Rad17 and four replication factor-C subunits. Rad3 acts in a complex with Rad26 to phosphorylate the effector protein kinases Chk1 and Cds1 (4–7). Chk1 is an effector of the DNA damage checkpoint, whereas Cds1 is specific for the replication checkpoint. Chk1 and Cds1 enforce checkpoint arrest of the cell cycle by regulating Cdc25 and Mdk1 (8–14). Cds1 has an additional role in promoting recovery from replication fork arrest (8, 10).

Checkpoint adaptor or mediator proteins are required to transmit the checkpoint signal from the sensor phosphatidylinositol 3-kinase-like kinases to the effector kinases Cds1 and Chk1. In fission yeast, Crb2 is the adaptor protein that connects the Rad3-Rad26 complex to Chk1 (15, 16), whereas Mrc1 (mediator of replication checkpoint) mediates signal transduction from Rad3-Rad26 to Cds1 (17, 18). In both fission yeast and budding yeast, Mrc1 was identified as a vital component of the system that activates Cds1 (or its budding yeast homolog Rad53) in response to replication arrest (17, 18). Cds1 phosphorylation by Rad3-Rad26 is mediated by an interaction involving the forkhead-associated domain of Cds1 with Mrc1 (7). Genetic and physiological studies indicate that Mrc1 also has a role in preserving genome integrity that is independent of the effector kinases of the replication checkpoint (19–22). Studies in fission yeast and budding yeast have shown that Mrc1 is a physiologically important substrate of checkpoint sensor kinases (20, 23). In fission yeast, Mrc1 is phosphorylated in response to replication arrest in a Rad3/Tel1-dependent manner (23). Two (S/T)Q clusters are required for the electrophoretic mobility shift induced by this phosphorylation. Threonine 645, a site in the first (S/T)Q cluster, is required to mediate interaction with the forkhead-associated domain of Cds1. Serine 604, located in the second (S/T)Q cluster, appears to be involved in the stable association of Mrc1 with chromatin. These findings identify two mechanisms of controlling the function of Mrc1 in the replication checkpoint response.

The apparent vertebrate homolog of Mrc1 is Claspin. Claspin was first identified in Xenopus egg extracts through its association with Chk1 (24). It was initially surprising that Mrc1 and Claspin interact with different effector kinases, but this pattern is consistent with a large number of studies that have shown Chk1 having a role in the replication checkpoint in Xenopus and mammals (25). Claspin is phosphorylated in response to replication fork arrest in Xenopus egg extracts. This phosphorylation is required for Claspin’s association with Chk1 and for Chk1 activation (24, 26).

Claspin associates with chromatin during S phase in a manner that depends upon the pre-replication complex and the DNA replication protein Cdc45, but not on the ataxia telangiectasia-related protein or replication protein A (26). Claspin’s chromatin association increases in response to replication arrest. These findings are consistent with the evidence that Mrc1 co-precipitates with Cdc45 in budding yeast cell lysates (21) and with chromatin immunoprecipitation studies that showed that Mrc1 moves along chromosomes in conjunction with DNA replication proteins during S phase (20, 21). These findings

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have suggested that Mrc1 is a non-essential replisome subunit that is ideally placed to mediate the interaction between sensor and effector kinases at stalled replication forks.

Although there is evidence that Mrc1 associates with replication forks and interacts with Cdc45, it is not known if the interaction with Cdc45 is required to target Mrc1 to replication forks, nor is it known if Mrc1 associates directly with DNA. In this study we have investigated whether Mrc1 directly associates with DNA. We report that the N-terminal half of Mrc1 binds to DNA directly in vitro and displays a higher affinity for branched DNA structures. The DNA binding domain (DBD)\(^1\) of Mrc1 was mapped to the 160–317 region of Mrc1, and we have identified two amino acids that are critical for the DNA binding activity of Mrc1. These findings correlate remarkably well with a very recent report that the 1–340 region of human Mrc1 binds to branched DNA structures in vitro (27). Importantly, we have found that deletion of the DBD or the mutation of specific residues in the Mrc1 DBD renders cells sensitive to replication fork arrest and unable to properly arrest division in response to fork arrest. These findings suggest that the direct association of Mrc1 with branched DNA at stalled forks is needed for efficient function of the replication checkpoint.

MATERIALS AND METHODS

Fission Yeast Strains, Growth Medium, and Genomic and Molecular Methods—Standard growth media and general biochemical and genetic methods were used. Yeast cultures were grown at 32 °C in YES medium (0.5% yeast extract, 3% glucose, and supplements) or Edinburgh minimal medium unless indicated otherwise. Hydroxyurea (Sigma) was used at the indicated concentration. To mutate Lys-235 and Lys-236 to glutamine, a site-directed mutagenesis kit (Stratagene) was used to mutate the indicated site(s) in the pUC28-mrc1 plasmid. PCR was then used to amplify the 270–1150-nucleotide fragment that had ura4 inserted at nucleotide 660. To delete the region encoding the DNA binding domain (160–284 amino acid) of Mrc1, products from overlapping PCRs were purified and transformed into the same strain. 5-fluoroorotic acid-resistant transformants were selected and mutations were confirmed by sequencing.

RESULTS

DNA Binding Domain in Checkpoint Protein Mrc1—In view of evidence that Mrc1 and Claspin are nuclear proteins that associate with replication forks (17, 20, 21, 23, 28), we decided to investigate whether Mrc1 binds directly to DNA. Full-length Mrc1 expressed in bacteria was insoluble; therefore, we expressed GST fusion proteins that contained the N-terminal 1–610 amino acids of Mrc1 or its C-terminal 560–1019 amino acids. The GST fusion proteins were expressed and purified from bacteria as GST fusion proteins expressing Mrc1 1–610 amino acids (N) or 560–1019 amino acids (C) were purified from bacteria. Equal amount of proteins were detected by Coomassie Blue staining. B, electrophoretic mobility shift assay. Equal amount of proteins (0.5 μg) from the 1–610 fragment (N), the 560–1019 fragment (C), or buffer (−) was mixed with \(^{32}\)P-labeled, 60-bp double-stranded DNA. The protein–DNA complex was separated from free probes by electrophoresis in a 4.5% gel in 0.5x Tris borate-EDTA at 4 °C.

To map the DNA binding domain in Mrc1 more precisely, different regions of Mrc1 were expressed as GST fusion proteins from bacteria and tested for their ability to bind dsDNA. The 1–610 region of Mrc1 bound to dsDNA, whereas the 560–1019 fragment did not interact with dsDNA (Fig. 1B). The 1–610 fragment had little affinity for single-stranded DNA and did not show sequence specificity (data not shown). These results indicated that the 1–610 region of Mrc1 has a DNA binding domain.

The Mrc1 DNA Binding Domain Is Localized in the 160–317 Region—To map the DNA binding domain in Mrc1 more precisely, different regions of Mrc1 were expressed as GST fusion proteins from bacteria and tested for their ability to bind the dsDNA substrate (Fig. 2B). The 1–317 region of Mrc1 bound to dsDNA approximately as well as the 1–610 fragment. The 1–447 and 1–499 region of Mrc1 showed similar binding affinity (data not shown), whereas the 1–250 and 284–499 regions only weakly interacted with the dsDNA substrate (Fig. 2A). Further refinement of these truncations showed that the 160–317 region of Mrc1 interacted with the dsDNA almost as well as the 1–610 and 1–317 fragments (Fig. 2A).

\(^1\) The abbreviations used are: DBD, DNA binding domain; dsDNA, double-stranded DNA; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HU, hydroxyurea.

FIG. 1. Direct DNA binding activity of Mrc1 N terminus. A, GST fusion proteins expressing Mrc1 1–610 amino acids (N) or 560–1019 amino acids (C) were purified from bacteria. Equal amount of proteins were detected by Coomassie Blue staining. B, electrophoretic mobility shift assay. Equal amount of proteins (0.5 μg) from the 1–610 fragment (N), the 560–1019 fragment (C), or buffer (−) was mixed with \(^{32}\)P-labeled, 60-bp double-stranded DNA. The protein–DNA complex was separated from free probes by electrophoresis in a 4.5% gel in 0.5x Tris borate-EDTA at 4 °C.

Immunoblotting—Cells were harvested, washed, and then resuspended in loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.1% bromphenol blue). Cells were boiled at 100 °C for 5 min, subjected to a vortex with glass beads for 5 min, and boiled again for 5 min. Cell lysates were centrifuged for 10 min at 20000 × g. Supernatants were subjected to SDS-PAGE. Immunoblots were blotted with an anti-Myc antibody (9E10, Covance).

Hydroxyurea (HU) Sensitivity and Checkpoint Studies—Cells were grown in a YES medium and then transferred to YES plates with HU at the indicated concentrations. Cells were grown for 2 days before being photographed. To study the significance of the DNA binding activity of Mrc1 in a DNA replication checkpoint, a PCR-based gene targeting method was used to delete chk1 in mrc1 DNA-binding mutants. To study the checkpoint function of Mrc1, log phase cells grown in a YES medium were treated with 12 mM HU for 6 h. Cells were fixed with glutaraldehyde and then stained with 4′,6-diamidino-2-phenylindole before microscopic analysis.

RESULTS

DNA Binding Domain in Mrc1—In view of evidence that Mrc1 and Claspin are nuclear proteins that associate with replication forks (17, 20, 21, 23, 28), we decided to investigate whether Mrc1 binds directly to DNA. Full-length Mrc1 expressed in bacteria was insoluble; therefore, we expressed GST fusion proteins that contained the N-terminal 1–610 amino acids of Mrc1 or its C-terminal 560–1019 amino acids. The GST fusion proteins were expressed and purified from bacteria as shown by Coomassie Blue staining (Fig. 1A). Equal amounts of the purified proteins were incubated with radiolabeled double-stranded DNA (dsDNA) made by annealing two 60-mer oligonucleotides. EMSAs showed that the 1–610 region of Mrc1 bound to dsDNA, whereas the 560–1019 fragment did not interact with dsDNA (Fig. 1B). The 1–610 fragment had little affinity for single-stranded DNA and did not show sequence specificity (data not shown). These results indicated that the 1–610 region of Mrc1 has a DNA binding domain.

The Mrc1 DNA Binding Domain Is Localized in the 160–317 Region—To map the DNA binding domain in Mrc1 more precisely, different regions of Mrc1 were expressed as GST fusion proteins from bacteria and tested for their ability to bind the dsDNA substrate (Fig. 2B). The 1–317 region of Mrc1 bound to dsDNA approximately as well as the 1–610 fragment. The 1–447 and 1–499 region of Mrc1 showed similar binding affinity (data not shown), whereas the 1–250 and 284–499 regions only weakly interacted with the dsDNA substrate (Fig. 2A). Further refinement of these truncations showed that the 160–317 region of Mrc1 interacted with the dsDNA almost as well as the 1–610 and 1–317 fragments (Fig. 2A). These findings indi-
cated that amino acids 160–317 are sufficient for Mrc1 DNA binding activity in vitro. To test if the GST moiety is involved in DNA binding, it was removed from the 1–317 fusion fragment. EMSA results indicated that 1–317 alone had the same DNA binding activity as its GST fusion counterpart (Fig. 2A).

Substrate Specificity of Mrc1 DNA Binding Activity—We next investigated whether Mrc1 displays a preference for specific DNA structures. dsDNA, 3'flap, or fork structures were prepared, and equal molar amounts of the substrates were incubated with increasing amounts of the 1–610 fragment and then analyzed by EMSA (Fig. 3). The branched DNA substrates displayed a stronger interaction with the 1–610 fragment, with an apparent preference for the fork substrate over the 3'flap (Fig. 3A). To explore further whether Mrc1 preferentially associates with fork structures, competition experiments were performed with the DNA substrates. The 3'flap structure was radiolabeled with [32P]ATP and co-incubated with increasing amounts of the three unlabelled DNA substrates in the presence of a fixed amount of the Mrc1 1–610 fragment. EMSA showed that the fork structure was the best competitor in this assay (Fig. 3B). The dsDNA structure was the poorest competitor. These experiments demonstrated that the DNA binding domain in the N-terminal region of Mrc1 prefers to interact with branched DNA structures.

Functional Analysis of Mrc1 DNA Binding Domain—To test if the DNA binding domain of Mrc1 is important for Mrc1 function, the DNA sequences that encode the 160–284 region of Mrc1 were deleted from the mrc1+ genomic locus. Cells harboring either the wild type allele (mrc1+), the deletion allele (mrc1Δ), or DBD deletion mutants were compared for their resistance to HU (Fig. 4A). These experiments were done by plating serial dilutions of cells on YES media containing 2 or 5 mM HU. Compared with wild type, mrc1Δ cells were very sensitive to HU, as observed previously (17, 18). Deletion of the Mrc1 DNA binding domain (160–284 amino acids) also made cells sensitive to HU, although to a lesser extent than mrc1Δ cells (Fig. 4A). The sensitivity of mrc1Δ (160–284) cells was not due to mislocalization or destabilization of the mutant Mrc1 proteins, as this protein was nuclear localized (data not shown) and no less abundant than wild type Mrc1 (Fig. 4B).

To further refine the analysis of this region of Mrc1, a smaller deletion allele that removed the sequences that encode amino acids 221–284 was created. This strain showed HU sensitivity that was similar to that of the mrc1Δ (160–284) strain (Fig. 4A). We therefore decided to focus our attention on two lysine residues at positions 235 and 236 in the 221–284 region. Mutations of these lysine residues to glutamate caused an HU-sensitive phenotype that was essentially identical to the 160–284 and 221–284 deletions (Fig. 4A). Thus, these amino acids in the DBD of Mrc1 are important for its function in promoting the survival of fork arrest caused by HU.

FIG. 2. Mapping the Mrc1 DNA binding domain. A, fragments of Mrc1 were expressed and purified from bacteria as GST fusion proteins. The 1–317 GST fusion protein was also incubated with thrombin after purification to remove GST (rightmost lane). The relative amounts of proteins were compared by Coomassie Blue staining. B, EMSA was performed as described in Fig. 1.

FIG. 3. Mrc1 prefers to bind branched DNA structures. A, the same amounts of dsDNA, 3'flap, or fork DNA structures were incubated with increasing amounts of purified GST-Mrc1-(1–610) fusion protein. B, 0.3 μM (final concentration) of 32P-labeled 3'flap DNA was mixed with either a buffer or the indicated amount of unlabeled dsDNA, 3'flap, or fork DNA structures. An equal amount of GST-Mrc1 1–610 fusion protein was added to each mixture. The protein-DNA complexes were separated from free probes by native gel electrophoresis. The signals were quantified with a PhosphorImager. The 32P signals from protein-DNA were divided by the total 32P signals and the percentage of protein-bound, 32P-labeled 3'flap was plotted.
Western blotting analysis using an anti-Myc antibody (grown in rich medium (YES) to log phase and then harvested for effect in the checkpoint arrest to HU. At the 6-h time point, medium supplemented with 12 mM HU for 6 h, and their HZ3484 (by EMSA (its DNA binding activity of Mrc1. The GST-Mrc1 1–610 fragment (affect Mrc1 protein expression. Wild type strain KT2791 (were grown on plates for 2 days at 30 °C. However, in the cells showed the elongated, mononuclear phenotype that is staining and immunofluorescence (Fig. 5). Wild type and septation index was scored by 4 dilutions of wild type strain PR109 (mrc1*), the mrc1 deletion mutants HZ2484 (mrc1:ura4*) and KT2786 (mrc1:kanMX6), the mrc1 DBD deletion mutants HZ2486 (mrc1Δ160–284) and HZ2488 (mrc1Δ221–284), or the point mutant HZ2489 (mrc1-K235E,K236E) were spotted on rich medium plates (YES) supplemented with 0, 2, or 5 mM HU. Cells were grown on plates for 2 days at 30 °C. B, DBD deletion does not affect Mrc1 protein expression. Wild type strain KT2791 (mrc1-13myc) or the mrc1 DBD deletion mutant HZ2494 (mrc1Δ160–284-13myc) was grown in rich medium (YES) to log phase and then harvested for Western blotting analysis using an anti-Myc antibody (top) or anti-tubulin (bottom). C, point mutations within the DBD abolished the DNA binding activity of Mrc1. The GST-Mrc1 1–610 fragment (WT) or its K235E,K236E mutant were purified from bacteria and equal amount of proteins (bottom) were tested for their dsDNA DNA binding activity by EMSA (top).

To test if the K235E and K236E mutations impaired the DNA binding activity of Mrc1, the same mutations were introduced into the construct used to express the 1–610 region of Mrc1 as a GST fusion protein in bacteria. The mutant protein was expressed, purified, and tested for its ability to bind the dsDNA substrate. Consistent with their effect on HU sensitivity, the same mutations also severely decreased the DNA binding activity of Mrc1 (Fig. 4C). Taken together, these results strongly suggested that the DNA binding activity of Mrc1 is important for cell survival in the presence of hydroxyurea.

**Mrc1 DNA Binding Domain Is Important for Its DNA Replication Checkpoint Activity**—The function of Mrc1 in the DNA replication checkpoint can be tested by measuring the septation index in a genetic background that is deficient for the Chk1 DNA damage checkpoint effector kinase (17). To specifically test the functional importance of the DNA binding domain of Mrc1 in its role as a checkpoint adaptor protein, we introduced the chk1Δ allele into the mrc1-K235EK236E strain and a mrc1Δ(160–284) strain. The double mutants were grown in medium supplemented with 12 mM HU for 6 h, and their septation index was scored by 4,6-diamidino-3-phenylindole staining and immunofluorescence (Fig. 5). Wild type and chk1Δ cells showed the elongated, mononuclear phenotype that is expected of cells that have an intact DNA replication checkpoint. However, in the chk1Δ background both the mrc1-K235EK236E and the mrc1Δ(160–284) mutation caused a defect in the checkpoint arrest to HU. At the 6-h time point, ~25% of the chk1Δ mrc1-K235EK236E and chk1Δ mrc1Δ(160–284) cells displayed a “cut” phenotype in which DNA masses were unequally segregated or the nucleus was bisected by the septum.

**DISCUSSION**

Recent studies of Mrc1 in budding yeast and Claspin in *Xenopus* egg extracts have provided evidence that these proteins are components of the replisome or are closely associated with the replisome (20, 21, 28). Coupled with earlier studies that showed that Mrc1 is required for the replication checkpoint response (17, 18), these observation raised the central question of whether Mrc1 or Claspin interact with DNA structures at the fork. In this study we have found that an N-terminal 1–610 fragment of fission yeast Mrc1 can directly bind to double strand DNA structures in vitro. This fragment of Mrc1 displayed a preference for branched DNA molecules, a finding consistent with the possibility that Mrc1 is a fork-binding protein. The DBD was further localized to a 150-amino acid fragment in the N terminus of Mrc1.

These observations are remarkably concordant with a recent study of human Claspin (27). This analysis showed that human Claspin is a ring-shaped protein that binds to branched DNA structures with high affinity *in vitro*. These studies implicated the 149–340 region in the N terminus of Claspin as containing sequences essential for DNA binding. As noted previously, Mrc1 and Claspin have very weak sequence similarity (17, 18). Nevertheless, when the DBD regions of *S. pombe* Mrc1 and human Claspin were compared, a region of apparently significant sequence similarity was detected (Fig. 6). Further comparison to other fungal Mrc1 and metazoan Claspin proteins revealed invariant or nearly invariant sequences in this region. Analysis of predicted secondary structures with the Jpred program revealed the presence of a helix-loop-helix motif in the region of highest sequence conservation (Fig. 6). A closely related structure, the helix-hairpin-helix, has been found in many proteins involved in DNA metabolism (29). It is thought to be a structural motif involved in the non-sequence-specific recognition of both single strand and double strand DNA (30). By analogy with the crystal structure of rat DNA polymerase β in a complex with a DNA template primer (31), it has been suggested that the helix-hairpin-helix motif binds to DNA via hydrogen bond-mediated interactions with the DNA-phosphate backbone (30). One protein that contains this motif is fission yeast Mus81, a component of the Mus81-Eme1 endonuclease that cleaves branched DNA structures related to Holliday junctions and replication forks *in vitro* (32–35).
An important question raised by the identification of a DBD in human Claspin and fission yeast Mrc1 is whether this domain is required for the function of these proteins in vivo. We addressed this question by deleting the sequences that encode the DBD in the genomic copy of mrc1 and by making point mutations in this domain. Strains harboring these mutations displayed decreased cell survival in the presence of hydroxyurea. These cells also displayed a defective DNA replication checkpoint arrest in the presence of hydroxyurea. These phenotypes point to the functional importance of the DBD. However, it should be noted that the phenotypes of the DBD mutants are not as severe as those observed in mrc1Δ mutants (17, 18). Presumably, the DBD mutants have retained a partial ability to interact with forks and activate Cds1, perhaps through an association with DNA replication proteins or additional contacts with DNA. One possibility is that a “DBD-less” Mrc1 can interact with forks through an association with Cdc45, although we have been unable to replicate in fission yeast an interaction between Cdc45 and Mrc1 that was found in budding yeast (21). In any case, our studies clearly demonstrate that an intact DBD domain is important for the function of Mrc1 as a protein that protects genome integrity when replication forks are arrested, and, by inference, it is likely that the conserved DBD domain in Claspin plays an important role in human cells.

It is interesting that the properties of Mrc1 DNA binding activity are similar to those of tumor suppressor protein Brca1 (36). Both prefer branched DNA structures to linear DNA, and neither has specificity for nucleotide sequences. The functional significance of the DNA binding activity has yet to be demonstrated, but it is thought that it might play an important role in DNA repair. It will be interesting to refine the analysis of the DNA binding activity of Brca1 to determine whether it shares helix-loop-helix motifs with Mrc1 and Claspin.

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