MDC1 Regulates DNA-PK Autophosphorylation in Response to DNA Damage*

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DNA damage initiates signaling events through kinase cascades that result in cell cycle checkpoint control and DNA repair. However, it is not yet clear how the signaling pathways relay to DNA damage repair. Using the repeat region of checkpoint protein MDC1 (mediator of DNA damage checkpoint protein 1), we identified DNA-PKcs/Ku as MDC1-associated proteins. Here, we show that MDC1 directly interacts with the Ku/DNA-PKcs complex. Down-regulation of MDC1 resulted in defective phospho-DNA-PKcs foci formation and DNA-PKcs autophosphorylation, suggesting that MDC1 regulates autophosphorylation of DNA-PKcs following DNA damage. Furthermore, DNA-PK-dependent DNA damage repair is defective in cells depleted of MDC1. Taken together, these results suggest that the MDC1 repeat region is involved in protein-protein interaction with DNA-PKcs/Ku, and MDC1 regulates DNA damage repair by influencing DNA-PK autophosphorylation. Therefore, MDC1 acts not only as a mediator of DNA damage checkpoint but also as a mediator of DNA damage repair.

DNA damage response pathways contain many mediator proteins, such as Rad9, Mrcl, Claspin, and 53BP1, which are important for the integration and amplification of DNA damage signals (1). Recent studies suggest that mediator of DNA damage checkpoint protein 1 (MDC1) regulates many aspects of DNA damage response pathways, such as intra-S phase checkpoint, G2/M checkpoint, and radiation-induced apoptosis (2–8). Many proteins involved in DNA damage response pathways, such as ATM, BRCA1, Chk2, NBS1/MRE11/Rad50, interact with MDC1 (2–6). In response to DNA damage, MDC1 forms nuclear foci at the sites of DNA damage. The formation of MDC1 foci requires H2AX (2, 6), with MDC1 in turn regulating foci formation of NBS1/MRE11/Rad50 and BRCA1 (2, 3, 5). In addition, MDC1 regulates the phosphorylation of BRCA1, Chk1, and SMCl in response to ionizing radiation (2, 5).

Similar to other mediator proteins, MDC1 contains several protein-protein interaction domains, such as the FHA domain and the BRCT domain. It is believed that MDC1 functions as an adaptor protein, recruiting downstream proteins to upstream kinases, such as ATM/ATR, and facilitating signal transduction following DNA damage. In addition to the FHA domain and BRCT domain, MDC1 contains 14 repeat sequences (aa 1124–1697), with no apparent homology to other known proteins in the data base. We hypothesized that the repeat region of MDC1 is also involved in protein-protein interaction. Here, we report that this repeat region of MDC1 directly interacts with Ku/DNA-PK, and this interaction is required for efficient DNA-PK autophosphorylation and DNA damage repair.

EXPERIMENTAL PROCEDURES

Cells, Constructs, and Antibodies—HeLa cells were purchased from National Cell Culture Center (Minneapolis, MN). V3.3, V3.155, CHO-K1, and XRS6 cells were kindly provided by Dr. Penny Jeggo. GST-MDC1 repeat region was generated by subcloning a fragment of MDC1 cDNA encoding aa 1148–1610 into pGex5X3 vector. Wild-type and deletion mutation (ΔR) of MDC1 were cloned in pcDNA3.1/V5His vector (Invitrogen). The sequence of MDC1 siRNA was described previously (4). Rabbit anti-MDC1, anti-phospho-Thr2609 DNA-PK and anti-H2AX antibodies were described previously (5, 9). Anti-MDC1 monoclonal antibodies were generated by Mayo Antibody Core Facility using standard procedure. Anti-DNA-PK antibodies were purchased from NeoMarkers (Fremont, CA). Anti-Ku80 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY).

Double Strand Break (DSB) Repair Assay—Cells were twice transfected with control siRNA or MDC1 siRNA as described previously (5). 72 h after initial transfection, cells were irradiated (40 Gy) and left to recover for the indicated time. Cells were then trypsinized and resuspended in agarose plugs. The plugs were loaded onto 0.8% agarose gel, and pulse field gel electrophoresis was performed.

Random Integration Assay—Cells were transfected twice with control siRNA or MDC1 siRNA as described previously (5). At the time of second siRNA transfection, cells were cotransfected with linearized pIREs-Puro, kindly provided by Dr. Christopher Ward (Mayo Clinic). 24 h later, cells were plated at different densities in media containing puromycin (2 μg/ml), and colony formation was determined 7–10 days later.

Microhomology Repair Assay—Cells were transfected twice with control siRNA, DNA-PK siRNA, or MDC1 siRNA as described previously (5). Microhomology assay was performed as described previously (10).

RESULTS AND DISCUSSION

To identify proteins that interact with the repeat region of MDC1, we used a GST-MDC1 fragment containing most of the repeat sequence (aa 1148–1610) as an affinity matrix to purify proteins that may associate with MDC1. We identified several checkpoint protein 1; α-amino acids; siRNA, small interfering RNA; Gy, gray; DSB, double strand break; IR, irradiation; GST, glutathione S-transferase; FHA, forkhead-associated; BRCT, BRCA1 carboxyl-terminal; NHEJ, non-homologous end joining.
proteins, especially a protein larger than 250 kDa that specifically interacts with the GST-MDC1 repeat region (GST-MDCR) (Fig. 1A). Mass spectrometry and microsequencing revealed this protein as DNA-PKcs, which is the catalytic subunit of DNA-PK. DNA-PKcs and Ku, the regulatory factors that are composed of heterodimer of 70 and 80 kDa, respectively, form catalytically active DNA-PK (11). Western blot confirmed that GST-MDC1 repeats efficiently pulled down DNA-PKcs and Ku from cell lysates (Fig. 1B). To prove that DNA-PK and MDC1 interact in vivo, we performed communoprecipitation experiment. As shown in Fig. 1C, MDC1 communoprecipitated with DNA-PKcs and Ku. No Ku or DNA-PKcs communoprecipitated with normal rabbit serum (NRS) (Fig. 1C) or with anti-53BP1, anti-BRCA1, and anti-Claspin antibodies (data not shown), suggesting the specificity of MDC1-DNA-PKcs/Ku interaction. The interaction of MDC1 and DNA-PKcs/Ku complex is not dependent upon DNA, since ethidium bromide (100 µg/ml) does not affect the communoprecipitation of MDC1 and DNA-PKcs/Ku (data not shown). Furthermore, the interaction of MDC1 and DNA-PKcs/Ku appears to be constitutive, since we did not observe any changes in this interaction before or after DNA damage (data not shown). We next asked whether the repeat region of MDC1 is required for MDC1-Ku interaction in vivo. Constructs encoding V5 tagged wild-type MDC1 or MDC1 containing a deletion of the repeat region (MDC1delR) were expressed in 293T cells. As shown in Fig. 1D, the binding of Ku to MDC1delR is significantly decreased when compared with its binding to wild-type MDC1, suggesting that the repeat region of MDC1 is important for its interaction with Ku/DNA-PK in vivo.

Because Ku specifically interacts with DNA ends, it is suggested that DNA-PKcs is activated and recruited to DSB through its interaction with the Ku heterodimer (12, 13). However, the mechanism of DNA-PK activation is not clear. Recent studies suggest that DNA-PKcs undergoes autophosphorylation at multiple sites (9, 14–16), and the autophosphorylation of DNA-PKcs is important to DSB repair and cell survival following IR (9, 15, 16). Furthermore, phospho-DNA-PKcs foci colocalize with γH2AX and 53BP1 foci after DNA damage (9), suggesting that phosphorylated DNA-PK localizes at the sites of DNA breaks. As expected, MDC1 foci colocalize with phospho-Thr2609-DNA-PKcs (pT2609DNA-PK) foci following IR (Fig. 2A). No pT2609DNA-PK-foci were detected in DNA-PK-deficient cell line (Ref. 9 and supplementary Fig. S1), suggesting the specificity of the anti-pT2609DNA-PK antibodies. Several lines of evidence suggest that MDC1 foci formation is independent of DNA-PKcs/Ku. A previous study has shown that DNA-PKcs is not required for MDC1 foci formation following IR (3). We also observed normal MDC1 and γH2AX foci formation in DNA-PK-deficient cells (data not shown). In addition, we observed normal MDC1 foci formation in Ku-deficient Xrs6 cells (data not shown). Interestingly, down-regulation of MDC1 expression using siRNA significantly decreased pT2609DNA-PK foci formation following IR (Fig. 2B). The decreased phospho-DNA-PK foci in MDC1-depleted cells could be

Figure 1. MDC1 associates with DNA-PKcs/Ku complex through the repeat region of MDC1. A, Coomassie staining of proteins associated with GST-MDC1 repeat region (GST-MDCR) or GST control affinity column. B, GST-MDCR was incubated with HeLa cell lysate, and proteins bound to GST-MDCR were separated by SDS-PAGE and immunoblotted with anti-DNA-PKcs or anti-Ku80 antibodies. C, HeLa cells were lysed, and cell lysates were immunoprecipitated (IP) with normal rabbit serum (NRS) or anti-MDC1 antibodies and blotted with indicated antibodies. Inputs represent 7% of total proteins used in immunoprecipitation. D, HeLa cells were transfected with plasmids encoding V5-tagged wild-type MDC1 or MDC1 with a deletion of the repeat region (dR). 48 h later, cells were collected and cell lysates were immunoprecipitated (IP) with anti-V5 antibodies, followed by immunoblotting with anti-Ku80 or anti-MDC1 antibodies.

Figure 2. MDC1 regulates DNA-PKcs phosphorylation following DNA damage. A, MDC1 co-localizes with phospho-DNA-PKcs following IR. Irradiated HeLa cells (3 Gy) were fixed 1 h later and immunostained with anti-MDC1 or anti-pT2609DNA-PK antibodies. B, HeLa cells were transfected twice with control siRNA (Cont) or MDC1 siRNA. 72 h after initial transfection, cells were irradiated (3 Gy) and collected 1 h later. Cells were fixed and immunostained with anti-MDC1 or anti-p2609DNA-PK antibodies. C, HeLa cells were transfected twice with control (Cont) or MDC1 siRNA. 72 h later, cells were irradiated (5 Gy) or left untreated. 30 min later, cells were collected, and DNA-PK was immunoprecipitated and blotted with anti-p2609DNA-PK or anti-DNA-PK antibodies (upper two panels). Alternatively, cell lysates were blotted with anti-MDC1 to demonstrate the down-regulation of MDC1 (lower panel).
MDC1 Regulates DNA-PK

caused by defective recruitment of phospho-DNA-PK at the sites of DNA damage or decreased DNA-PK phosphorylation. Therefore, we next examined whether DNA-PKcs phosphorylation requires MDC1. As shown in Fig. 2C, DNA-PKcs phosphorylation at Thr2609, but not DNA-PKcs expression, is decreased in cells transfected with MDC1 siRNA. We also detected decreased DNA-PK phosphorylation at Ser2056 site, another damage-induced phosphorylation site on DNA-PK (data not shown). However, DNA-PK kinase activity was not affected by the depletion of MDC1 (supplementary Fig. S2). Taken together, these results suggest that MDC1 regulates DNA-PKs autophosphorylation following IR.

Many genetic and biochemical studies have established an essential role of DNA-PK in DSB repair (17). The autophosphorylation of DNA-PKcs is important for DSB repair and cell survival in response to ionizing radiation (9, 15, 16). Since MDC1 regulates DNA-PKcs autophosphorylation, it is possible that MDC1 regulates DNA-PK-dependent DSB repair. We performed pulse field gel electrophoresis to assay the kinetics of DSB repair in cells transfected with control siRNA or MDC1 siRNA. We observed a mild defect in DSB repair in MDC1 siRNA-transfected cells (supplementary Fig. S3), suggesting that MDC1 is not essential for overall DSB joining but is instead involved in certain aspects of DNA end-joining. To investigate whether MDC1 is involved in error-free end-joining mediated by DNA-PK, we used a previously established plasmid-based end-joining assay for non-homologous end joining (NHEJ) (10). In this assay, linearized pDVG94 plasmid was used to transfect cells. If cells are defective in direct error-free end-joining, an alternative microhomology-based joining will be used, therefore creating a BstXI site. In cells that contain mutations of DNA-PK, Ku, XRCC4, or DNA ligase IV, a dramatic increase in the alternative microhomology-based joining has been observed (10), suggesting the specific requirement of Ku/DNA-PK in an error-free end-joining repair pathway. As shown in Fig. 3A, in MDC1 siRNA transfected cells, significantly more end-joining occurred using the alternative pathway, similar to the cells transfected with DNA-PK siRNA or that in DNA-PK−/− cells (V3) (Fig. 3A). These results suggest that MDC1 is involved in DNA-PKcs/Ku dependent error-free end-joining. The repair defect observed in cells transfected with MDC1 siRNA is not due to any change in cell cycle progression, since we do not observed any detectable difference in cell cycle profile with or without MDC1 siRNA treatment (data not shown). To further confirm our observations, we next used random plasmid integration assay, another assay for DNA-PK-dependent end-joining activity (18, 19). Linearized DNA with selection marker is used to transfect cells, and the efficiency of random chromosomal integration of the plasmid DNA is measured by colony formation in selection media. Consistent with previous findings (18, 19), the integration frequency of the purmoycin-resistant gene is significantly lower in DNA-PK−/− cells than that observed in the wild-type parental cell line (Fig. 3B). Knock-down MDC1 using siRNA resulted in a significant decrease of integration frequency in DNA-PK-proficient cells but only a minor reduction in DNA-PK-deficient cells (Fig. 3B). The integration frequency was also significantly lower in HeLa cells transfected with MDC1 siRNA or DNA-PKcs siRNA, compared with that in cells transfected with control siRNA (Fig. 3C). Taken together, these results suggest a role of MDC1 in DNA-PK-dependent DSB repair.

Since MDC1 has multiple binding partners, depletion of MDC1 might have an indirect effect on DSB repair. To demonstrate that the interaction between DNA-PKcs/Ku and MDC1 is important for DNA-PKcs phosphorylation and DNA end-joining, we generated constructs encoding siRNA-resistant full-length MDC1 (MDC1*), or MDC1 with a deletion of the repeat region (MDC1delR*). Reconstituting MDC1 knock-down cells with full-length (MDC1*), but not deletion mutation of MDC1 (MDC1delR*), restored pT2609DNA-PK foci formation, suggesting that the interaction of MDC1 and DNA-PKs/Ku is important for DNA-PK phosphorylation (supplementary Fig. S4). Consistent with DNA-PK phosphorylation, reconstitution of full-length MDC1 re-established normal DSB joining (Fig. 3D) and plasmid integration (Fig. 3E), while reconstitution of MDC1delR* failed to do so. As a control, we evaluated NBS1 foci formation in cells reconstituted with MDC1* or MDC1delR*. In agreement with an early study suggesting that NBS1 interacts with MDC1 FHA domain (3), reconstitution of either full-length MDC1* or MDC1delR* restored NBS1 foci formation in response to IR (supplementary Fig. S5). Furthermore, reconstitution of either full-length MDC1* or MDC1delR* restored the intra-S checkpoint in response to IR (data not shown), suggesting that MDC1delR* has intact
checkpoint function. These data support that the specific interaction between MDC1 and DNA-PKcs/Ku plays an important role in DNA end-joining.

We report here that in addition to the FHA and BRCT domain, the repeat region of MDC1 is involved in protein-protein interactions. It directly binds Ku and regulates DNA-PKcs autophosphorylation. Previously, DNA-PKcs autophosphorylation has been shown to be Ku-dependent, presumably facilitated by oligomerization of DNA-PKcs/Ku at DNA ends (9). Currently, we could not evaluate the effect of MDC1 on the recruitment of DNA-PKcs/Ku to DNA ends, since we have not been able to detect DNA-PKcs or Ku foci formation in response to IR nor could we detect increased binding of DNA-PKs/Ku on chromatin (data not shown). Since Ku binds DNA ends with very high affinity, we speculate that MDC1 may not be required for initial binding of Ku and DNA-PKcs to DNA ends. Instead, MDC1 might help the oligomerization and stabilization of DNA-PKcs/Ku complexes at the sites of DNA damage. A similar role for MDC1 has been suggested for the sustained binding of NBS1/MRE11/Rad50 to DNA ends (20). An emerging theme in DNA damage signaling is that mediators such as NBS1 and MDC1 are important for ATM phosphorylation and activation by facilitating the concentration of ATM at the sites of DNA damage (21–23). We propose that MDC1 may play a similar role in regulating the autophosphorylation of DNA-PKcs following DNA damage.

DNA-PKcs phosphorylation is important for DSB repair (9, 15, 16). Mutation of DNA-PKcs autophosphorylation sites affects cell survival following radiation and also error-free end-joining. However, DNA-PKcs with mutations at these phosphorylation sites could still support DNA end-joining using an alternative pathway that leads to mutations and deletions (15, 16). Consistent with this notion, cells depleted of MDC1 show a mild defect in overall DSB joining when analyzed by pulse field gel electrophoresis, suggesting that overall DSB joining activity does not require MDC1. Interestingly, cells depleted of MDC1 show significant decrease in the frequency of plasmid integration into chromosomes (Fig. 3C), which has been shown to be dependent on DNA-PK (18, 19). In addition, using a plasmid-based assay, we show that cells depleted of MDC1 often use alternation error-prone pathway to repair DSBs (Fig. 3A), suggesting that MDC1 is involved in a DNA-PK-mediated error-free end-joining pathway.

In summary, we have shown for the first time that the repeat region of MDC1 is a protein-protein interaction domain and binds directly to Ku/DNA-PK. We also demonstrate that MDC1 is critical for the efficient IR-induced autophosphorylation of DNA-PK, which in turn facilitates DSB repair. These data suggest that in addition to be a mediator of DNA damage checkpoint, MDC1 also contributes to DNA damage repair.

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