N Terminus of CtIP Is Critical for Homologous Recombination-mediated Double-strand Break Repair*

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DNA double-strand breaks (DSBs) represent one of the most lethal types of DNA damage cells encounter. CtIP (also known as RBBP8) acts together with the MRN (MRE11-RAD50-NBS1) complex to promote DNA end resection and the generation of single-stranded DNA, which is critically important for homologous recombination repair. However, it is not yet clear exactly how CtIP participates in this process. Here, we demonstrate that besides the known conserved C terminus, the N terminus of CtIP protein is also required in DSB end resection and DNA damage-induced G$_2$/M checkpoint control. We further show that both termini of CtIP can interact with the MRN complex and that the N terminus of CtIP, especially residues 22–45, binds to MRN and plays a critical role in targeting CtIP to sites of DNA breaks. Collectively, our results highlight the importance of the N terminus of CtIP in directing its localization and function in DSB repair.

To protect the genome, all types of genotoxic lesions should be properly detected and repaired. Cells are equipped with an intricate network to ensure the maintenance and faithful transfer of genetic materials in response to DNA damage (1). DNA double-strand break (DSB)$^2$ is the most detrimental form of DNA damage (2). There are two major pathways to repair DSBs, the non-homologous end-joining pathway and the homologous recombination (HR) pathway (3). It is believed that during HR, the DNA ends are first resected in the 5′–3′ direction by nucleases. The resulting single-stranded DNA (ssDNA) is rapidly bound by replication protein A (RPA). Subsequently, RAD51, a key recombinase enzyme, displaces RPA’s ssDNA complexes with the help of its accessory factors to form a helical nucleoprotein filament that permits strand invasion and homology search. At the same time, the ssDNA-bound RPA can also recruit ATR, which phosphorylates CHK1 to trigger and activate cell cycle checkpoints (4). Therefore, the conversion of DNA double-stranded ends to ssDNA regions is considered as a key step that controls not only DNA repair but also DNA damage checkpoints.

The MRN complex, consisting of MRE11, RAD50, and NBS1, has long been implicated in the detection of DSBs and DNA end resection (5, 6), recombination (7), and S or G$_2$/M checkpoint control (8–10). More recently, the nuclear protein CtIP has been suggested to operate with the MRN complex. CtIP (also known as RBBP8) was originally identified as a protein that interacts with the transcriptional repressor CtBP (11), the retinoblastoma protein RB (12), and the tumor suppressor BRCA1 (13, 14). CtIP can be recruited to DNA damage sites and has been shown to bind to the BRCT domains of BRCA1 to control the DNA damage-induced G$_2$/M checkpoint (15–17). More recently, a role of CtIP in DNA repair has been unveiled. CtIP functions with the MRN complex to process DSB ends and generate ssDNA regions (18, 19). Furthermore, the recently identified CtIP homologs in other species, including Com1/Sae2 and Ctp1, also act with their corresponding MRE11 complexes to process DSB ends and form ssDNAs (18, 20–24). Together, these data support a conserved function of CtIP in DSB end resection, which is a critical step in initiating HR repair (25).

The C-terminal Sae2-like domain of CtIP is required for CtIP function (18, 19, 26), but the roles of other parts of CtIP protein in DNA damage and repair remain unknown. In this study, we report that the N terminus of CtIP, especially residues 22–45, binds to MRN, plays a critical role in targeting CtIP to sites of DNA breaks, and is required for damage-induced G$_2$/M checkpoint control.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies against γ-H2AX and RAD51 were described previously (17, 27, 28). Anti-Myc and anti-CHK1 antibodies were obtained from Santa Cruz Biotechnology. Anti-phospho-CHK1 (Ser$^{317}$) antibody was purchased from Cell Signaling. Anti-RPA2 antibody was obtained from Abcam. Anti-γ-tubulin and anti-FLAG (M2) antibodies were obtained from Sigma. Dr. Richard Baer (Columbia University, New York) provided mouse anti-CtIP monoclonal antibody.

Cell Culture, Transfection, and Small Interfering RNAs—HeLa, 293T, and U2OS cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. The sequence of RAD51 small interfering RNA (siRNA) was CUAUCAUGUGUAGCUAUAU; the sequence of NBS1 siRNA was CCAACUA-

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2 The abbreviations used are: DSB, double-strand break; HR, homologous recombination; ssDNA, single-stranded DNA; RPA, replication protein A; siRNA, small interfering RNA; Gy, grays; GFP, green fluorescent protein; SBP, streptavidin-binding peptide.
AUGGCCAAGUAUU; the sequence of MRE11 siRNA was GGAGGUACGUCGUUUCAGAdTdT; and the sequence of RAD50 siRNA was ACAAGGAUCUGGAUAUUUAUU. The siRNA for CtIP and siRNA-resistant wild-type CtIP constructs were described previously (16). siRNA transfection was performed using Oligofectamine (Invitrogen) following the manufacturer's instruction.

Plasmid Constructs—All cDNAs were subcloned into pDONR201 (Invitrogen) as entry clones and subsequently transferred to Gateway-compatible destination vectors for N-terminal FLAG- or Myc-tagged fusion protein expression. All point or deletion mutants were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing.

Immunoblotting—Cells were lysed with 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40 on ice for 30 min. Cleared cell lysates were then collected and boiled in 2x Laemmli buffer and subjected to SDS-PAGE. Membranes were blocked in 5% milk in Tris-buffered saline/Tween and then probed with antibodies as indicated.

Immunostaining—Cells cultured on coverslips were treated with IR radiation, followed by recovery. Cells were then washed with phosphate-buffered saline, pre-extracted with 0.5% Triton solution for 5 min, and fixed with 3% paraformaldehyde for 12 min. Coverslips were washed with phosphate-buffered saline and then immunostained with primary antibodies in 5% goat serum for 60 min. Coverslips were washed and incubated with secondary antibodies conjugated to rhodamine or fluorescein.
isothiocyanate for 60 min. Cells were then stained with 4′,6-diamidino-2-phenylindole to visualize nuclear DNA. The coverslips were mounted onto glass slides with antifade solution and visualized using a Nikon ECLIPSE E800 fluorescence microscope with a Nikon Plan Fluor 40× oil objective lens (numerical aperture of 1.30) at room temperature. Cells were

FIGURE 2. Both the N and C termini of CtIP can interact with the MRN complex. A, CtIP can bind to all three components in the MRN complex. IP, immunoprecipitation. B, schematic representation of the Myc-tagged CtIP mutants used in this study. FL, full-length CtIP; CC, coiled-coil domain. C–E, N and C termini of CtIP can interact with NBS1, MRE11, or RAD50. 293T cells were transfected with plasmids encoding Myc-tagged wild-type CtIP (WT) or the N160, C166, N160C166, or 161–731 mutant of CtIP, together with plasmids encoding SBP-FLAG-tagged NBS1 (C), MRE11 (D), or RAD50 (E). Cells were lysed 24 h after transfection. Immunoprecipitation was carried out using S protein beads, and immunoblotting was performed using the antibodies indicated. F, N and C termini of CtIP can bind directly to MRN components. The in vitro binding assays described for A and F were performed using the baculovirus expression system. Sf9 cells were co-infected with baculoviruses expressing the indicated constructs. Forty-eight hours later, cells were collected and lysed, and pulldown experiments were performed using S protein beads or glutathione-agarose beads. Protein bound to the beads was eluted and resolved by SDS-PAGE and immunoblotting using the indicated antibodies.
photographed and analyzed using a SPOT camera (Diagnostic Instruments, Inc.) and Photoshop software (Adobe).

**G/M Checkpoint Assay**—G/M checkpoint assay was performed as described previously (29). Briefly, cells were treated with 2 grays (Gy) of IR radiation. One hour later, cells were fixed with 70% (v/v) ethanol overnight and then stained with anti-

**Retroviral Infection**—Wild-type or mutant CtIP was transfected into pEF1A-HA/FLAG retroviral vector using the Gateway system. Retroviral vectors were cotransfected with pCL-Ampho into BOSC23 packaging cells for virus production. Virus was collected 48 and 72 h after transfection and subsequently used to infect mouse embryonic fibroblasts or U2OS or HeLa cells. Two days after the last infection, cells were collected for experiments or selected in 2 μg/ml puromycin for establishing stable clones.

**Homologous Recombination Assay**—A U2OS cell clone stably expressing HR reporter DR-GFP was described previously (30). This reporter consists of two differentially mutated green fluorescent protein (GFP) genes oriented as direct repeats. Expression of I-SceI endonuclease will generate a site-specific DSB between the mutated GFP genes, which, when repaired by gene conversion, results in a functional GFP gene. Briefly, 2 days after transfection with siRNA, 1 × 10⁶ U2OS/DR-GFP cells were electroporated with 20 μg of pCBASce (an I-SceI expression vector). For HeLa cells stably expressing wild-type or mutant CtIP, 1 × 10⁶ HeLa cells were electroporated with 15 μg of pCBASce together with 15 μg of DR-GFP. Cells were harvested 2 days after electroporation and subjected to flow cytometry analysis to determine percentages of GFP-positive cells, which result from HR repair induced by DNA DSBs. Samples were analyzed in a BD Biosciences FACScan on a green (FL1) versus orange (FL2) fluorescence plot.

**RESULTS**

**Both Termini of CtIP Are Required for Damage-induced G/M Checkpoint Control and HR Repair**—Recent studies have demonstrated that CtIP promotes ATR activation and HR by mediating DSB resection (18, 19). Because the generation of ssDNAs, especially RPA-coated ssDNAs, is believed to be an intermediate step not only for HR repair (31, 32) but also for ATR/CHK1 activation and DNA damage checkpoint control (4, 10, 33), it is expected that all of these functions of CtIP should be linked. Indeed, in agreement with previous reports (16, 18), depletion of CtIP in human cells led to defective G/M checkpoint control (supplemental Fig. 1A), CHK1 activation (supplemental Fig. 1B), and RPA focus formation (supplemental Fig. 1C). To assess HR repair efficiency, we used the HR reporter assay (see “Experimental Procedures” for details) established by Dr. Maria Jasin and colleagues (30). Following siRNA-mediated knockdown of CtIP, we observed reduced HR repair efficiency compared with cells transfected with control siRNA (supplemental Fig. 1, D–G), confirming that CtIP is required for efficient HR repair.
We also evaluated the kinetics of the CtIP and RPA focus formation (supplemental Fig. 2, A and B). Although γ-H2AX foci emerged rapidly following DNA damage, CtIP focus formation was delayed and reached the peak at ~4 h. This was followed by RPA focus formation that peaked at ~8 h after DNA damage. The kinetics of these processes are consistent with the critical role of CtIP in processing DSB ends to form ssDNAs, which are required for subsequent RPA binding.

Although CtIP is required for G2/M checkpoint control and the generation of ssDNA regions upon DNA damage, we still do not know precisely how CtIP carries out these functions. To determine which regions of CtIP protein are critically required for its functions, we generated six internal deletion mutants (Fig. 1A). Each mutant was derived from a siRNA-resistant FLAG-tagged CtIP construct so that the mutant could be expressed when endogenous CtIP was depleted by siRNA. HeLa derivative cell lines stably expressing wild-type CtIP or each one of these internal deletion mutants (D1–D6) were generated. Following depletion of endogenous CtIP, we observed a G2/M checkpoint defect, which was rescued by the expression of siRNA-resistant wild-type CtIP (Fig. 1B). Interestingly, the CtIP mutants with deletion of either the N or C terminus (mutants D1 and D6) completely lost this G2/M checkpoint function (Fig. 1B). Consistently, the D1 and D6 mutants also failed to promote efficient CHK1 phosphorylation after IR radiation (Fig. 1C). In addition, we also observed diminished IR radiation-induced RPA focus formation (Fig. 1D) and reduced HR repair efficiency (Fig. 1E) in cells expressing only the D1 or D6 mutant. As control, we examined the D1 and D6 mutants and showed that their expression levels were similar to that of endogenous CtIP (supplemental Fig. 3). Together, these data indicate that both termini of CtIP are important for its functions in DNA damage response.

**FIGURE 4.** Residues 22–45 of CtIP are required for NBS1 binding and damage-induced G2/M checkpoint control. A, residues 22–45 of CtIP are required for binding between the N terminus of CtIP and NBS1. 293T cells were transfected with plasmids encoding Myc-tagged wild-type CtIP (WT) or N160 and N160 (d22–45) CtIP together with plasmids encoding SBP-FLAG-tagged NBS1. Cells were lysed 24 h after transfection. Immunoprecipitation (IP) was carried out using S protein beads, and immunoblotting was performed using the antibodies indicated. B, residues 22–45 of CtIP are required for CtIP function in G2/M checkpoint control. HeLa cells stably expressing siRNA-resistant wild-type CtIP and deletion mutants were generated. These cells were transfected twice with CtIP siRNA, and G2/M checkpoint assays were performed as described under "Experimental Procedures." The percentage of phosphohistone H3 (pH3)-positive cells was determined by fluorescence-activated cell sorter analysis. Means ± S.D. (error bars) were obtained from three independent experiments. C, the stable cells shown in B were transfected with control (siCtrl) or CtIP (siCtIP) siRNA. Forty-eight hours later, cells were treated with IR radiation (10 Gy) and allowed to recover for 1 h. Cells were collected and lysed. Immunoblotting was performed using anti-phospho-CHK1 (Ser317), anti-CHK1, or anti-FLAG antibody. D, proposed model of CtIP function in DNA damage repair.
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The N Terminus of CtIP Is Essential for Its Focus Formation following DNA Damage—To investigate which region of CtIP is responsible for its IR radiation-induced focus formation, we used the HeLa cell lines that stably produced wild-type or internal deletion mutants (D1–D6) (see Fig. 1) of FLAG-tagged CtIP. Stably expressed FLAG-CtIP readily formed foci in a damage dose-dependent manner (supplemental Fig. 4, A and B). We found that although other mutants could form foci similar to wild-type CtIP following IR treatment, the D1 mutant exhibited uniform nuclear localization before or after IR treatment (Fig. 3A and data not shown). The D1 mutant of CtIP lost residues 18–159 of CtIP (as shown in Fig. 1A). Further mini-deletion analysis revealed that residues 22–45 at the N terminus of CtIP were required for its focus formation upon DNA damage (Fig. 3, B and C).

Residues 22–45 of CtIP Are Required for NBS1 Binding and Damage-induced G2/M Checkpoint Control—We have shown that the N terminus of CtIP could bind to the MRN complex (Fig. 3). The results that residues 22–45 are required for damage-induced CtIP focus formation prompt us to ask whether residues 22–45 would also be required for MRN binding, which is indeed the case (Fig. 4A). This is entirely consistent with the observation that deletion of any component in the MRN complex led to a reduction of CtIP focus formation (supplemental Fig. 4, C and D). In addition, we observed that residues 22–45 of CtIP were required for the function of CtIP in damage-induced G2/M checkpoint control (Fig. 4, B and C).

DISCUSSION

Besides the known functions in DNA damage checkpoint control, recent studies have put the MRN-CtIP complex at the center stage of DSB repair mediated by HR. CtIP is believed to function with the MRN complex to process DSB ends and generate ssDNA regions (18, 19). Consistent with recent studies (15–19), we have shown that CtIP is involved in DSB end resection and DNA damage-induced G2/M checkpoint control. We further demonstrated that both the N and C termini of CtIP protein are required for these functions. Our current hypothesis is that these two termini of CtIP have separate functions.

The importance of the C terminus of CtIP has been recognized previously because it is highly conserved and required for CtIP function in human cells (18, 19, 26). However, the role of the N terminus of CtIP was not known until this study. Our current hypothesis is that the N terminus of CtIP binds to MRN and targets CtIP to DNA breaks, and therefore, it is equally important for CtIP function in DNA damage response.

In an attempt to determine the key residues within this region (residues 22–45), we aligned the N terminus of CtIP from different species and identified several highly conserved residues within this region of CtIP (supplemental Fig. 5A). However, none of the four individual point mutations we generated (H31A, V35A, K41A, and L45A) could abolish CtIP focus formation (supplemental Fig. 5B). Additional mutagenesis and functional studies are needed to elucidate the requirements for CtIP focus formation and its interaction with the MRN complex.

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