Cell Cycle-dependent Complex Formation of BRCA1-CtIP-MRN Is Important for DNA Double-strand Break Repair*

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BRCA1 plays an important role in the homologous recombination (HR)-mediated DNA double-strand break (DSB) repair, but the mechanism is not clear. Here we describe that BRCA1 forms a complex with CtIP and MRN (Mre11/Rad50/Nbs1) in a cell cycle-dependent manner. Significantly, the complex formation, especially the ionizing radiation-enhanced association of BRCA1 with MRN, requires cyclin-dependent kinase activity. CtIP directly interacts with Nbs1. The in vivo association of BRCA1 with MRN is largely dependent on the association of CtIP with the BRCT domains at the C terminus of BRCA1, whereas the N terminus of BRCA1 also contributes to its association with MRN. CtIP, as well as the interaction of BRCA1 with CtIP and MRN, is critical for IR-induced single-stranded DNA formation and cellular resistance to radiation. Consistently, CtIP itself is required for efficient HR-mediated DSB repair, like BRCA1 and MRN. These studies suggest that the complex formation of BRCA1-CtIP-MRN is important for facilitating DSB resection to generate single-stranded DNA that is needed for HR-mediated DSB repair. Because cyclin-dependent kinase is important for establishing IR-enhanced interaction of MRN with BRCA1, we propose that the cell cycle-dependent complex formation of BRCA1, CtIP, and MRN contributes to the activation of HR-mediated DSB repair in the S and G2 phases of the cell cycle.

Individuals carrying germ line mutations in BRCA1 have an extremely high risk of developing breast or ovarian cancer (1). Compelling evidence suggests that BRCA1 plays important roles in the maintenance of genome stability (2, 3). Although BRCA1 possesses multiple cellular functions including checkpoint control, transcription regulation, ubiquitination, apoptosis, and DNA repair (3, 4), its role in DNA repair, especially in DSB repair, is perhaps the most critical mechanism to protect the genome and to maintain genome stability. In BRCA1-deficient cells, HR-mediated DSB repair is significantly reduced (5–8). However, the molecular mechanisms by which BRCA1 participates in DSB repair are still unclear.

BRCA1 interacts with MRN (9–11), a complex essential for HR-mediated DSB repair in multiple organisms (12–14). Mre11 carries nuclease activities (15) and is important for DSB end resection to generate ssDNA tracts, the intermediates for HR-mediated repair process (16–18). Two distinct BRCA1 super complexes have been identified, and one of them contains BRCA1, MRN, and the BRCA1 association protein CtIP (19). CtIP was originally discovered as an interacting protein of CtBP (20) and was also found to be associated with Rb family members (21, 22). CtIP interacts with BRCA1 through the BRCT domains of BRCA1, and clinically relevant mutations within the BRCT domains of BRCA1, and clinically relevant mutations within the BRCT domains of BRCA1 abolish their interaction (23–25). Interestingly, the interaction of BRCA1 with CtIP requires phosphorylation of CtIP at Ser327 by CDK, which occurs in the late S and G2 phases of the cell cycle (26).

DSBs can be repaired by both homologous recombination (HR)3 and nonhomologous end joining (27). The cell cycle stage is a major factor to determine which repair pathways are used (28). Nonhomologous end joining is primarily used in G1, whereas HR becomes a choice for DSB repair in S and G2 when sister chromatids are present (29–31). Because homologous chromosomes are the only available homologous templates in G1, use of HR at this cell cycle stage would lead to a loss of heterozygosity. Therefore, choosing an appropriate repair pathway during the different stages of the cell cycle is important for preserving genetic integrity.

Given the importance of cell cycle-regulated selection of repair pathways, it is tempting to understand how HR is activated as the cell cycle proceeds. It has been shown in yeast that CDKs are directly involved in the selection of HR to repair DSBs in S/G2 (32–34). In mammalian cells, damage-induced Rad51 foci formation occurs predominantly in S/G2 (35, 36), and RPA-coated ssDNA formation is suppressed by the CDK inhibitor roscovitine (16). However, the detailed mechanisms by which CDKs regulate HR are still unknown.

Recent studies identified homology of CtIP with the yeast proteins Sae2 and Ctp1 (37–40), suggesting that CtIP is likely a functional counterpart of Sae2 and Ctp1. Both Sae2 and Ctp1 function with the Mre11 complex to process DSB ends to generate ssDNA (37, 41, 42), and recent studies also reveal activities

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*This work was supported by National Institutes of Health Grant CA102361 and Ellison Medical Foundation New Scholar Award AG-NS-0251-04. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: HR, homologous recombination; DSB, double-strand break; IR, ionizing radiation; CDK, cyclin-dependent kinase; ssDNA, single-stranded DNA; shRNA, small hairpin RNA; RNAi, RNA interference; GST, glutathione S-transferase; GFP, green fluorescent protein; HA, hemagglutinin; Gy, gray(s).
of Sae2 to process hairpin DNA cooperatively with the Mre11 complex (43). In this report, we demonstrate that human CtIP is important for BRCA1 to interact with MRN. The complex formation of BRCA1-CtIP-MRN is cell cycle-dependent and requires CDK activity. We also show that CtIP, as well as the formation of BRCA1-CtIP-MRN complex, is needed for end resection activities at DSBs, thereby contributing to HR-mediated DSB repair. These findings suggest one important mechanism by which BRCA1 regulates DSB repair and also have significant implications for the cell cycle-dependent control of HR-mediated DSB repair.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Synchronization**—U2OS, T98G, BJ, and 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. HBL100 and HCC1937 cells were cultured in RPMI with 10% fetal bovine serum. Primary fibroblast cell line GM07166 (Nbs1-deficient) was immortalized by hTERT (44) and cultured as described (45). Insect cell line SF21 was cultured in Grace’s insect medium supplemented with 10% fetal bovine serum.

For cell cycle synchronization, T98G cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1% fetal bovine serum for 48 h and then released by adding 10% fetal bovine serum.

Plasmids, Mutagenesis, and shRNA/RNAi—Recombinant baculoviruses expressing FLAG-tagged Nbs1 and Mre11 or HA-tagged CtIP were generated by using Bac-to-Bac Baculovirus expression systems (Invitrogen). Myc-tagged BRCA1 full-length and deletion mutant (248-end) were subcloned into a mammalian expression vector, pcDNA3β, containing the sequence encoding the Myc epitope (46). Mutations in Nbs1 and CtIP were generated by site-directed mutagenesis (Stratagene). GST-fused CtIP fragments were constructed by cloning PCR products into pGEX4T-1 (GE Healthcare).

Silencing of endogenous CtIP in U2OS and BJ cells was achieved by retroviral infection using pMKO vector to produce shRNAs (47), or RNAi transfection. shRNA or RNAi target sequences are GGACCTTTGGACAAAACTAAA and GCTA-AAAACAGGAACGAATCTTT designed according to previous reports (26). shRNA-resistant wild type CtIP and CtIP-S327A were constructed by mutagenizing four nucleotides at the shRNA targeting sequences.

**Immunoprecipitation, in Vitro Binding, and Immunofluorescence**—Immunoprecipitation and Western blot analysis were performed as described (46). FLAG-Nbs1, FLAG-Mre11, or HA-CtIP was expressed in insect cells. FLAG-Nbs1 was purified using anti-FLAG M2 agarose (Sigma) and eluted with 3× FLAG peptide (Sigma). GST-fused CtIP fragments were expressed in *Escherichia coli* and purified using glutathione-Sepharose (GE Healthcare). In vitro binding was performed in NETN buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40).

For immunostaining, the cells were fixed by 70% methanol and 30% acetone at −20 °C overnight, followed by immunostaining analysis as described (48).

**Anti-body**—The polyclonal antibodies against Nbs1 (D29), Mre11 (D27), and the monoclonal antibody against Nbs1 (EE15) were described previously (49, 50). BRCA1 polyclonal antibodies were generated by immunizing rabbits with GST-fused BRCA1 fragments 1–324 or 1314–1863 and were affinity-purified. monoclonal anti-CtIP antibody was generously provided by Richard Baer (51). The other antibodies used were purchased from Oncogene (RPA2), Upstate (Rad50), Cell Signaling Technology (Chkl-pS317), R & D Systems (Chk1), CalBiochem (monoclonal anti-BRCA1, MS110), Sigma (M2), Covance (HA 11), and Santa Cruz Biotechnology (Ku70).

**Homologous Recombination Assay**—U2OS cells were transfected with DR-GFP (a generous gift from Andrew Pierce and Maria Jasin) (52), and stable clones carrying a single copy of DR-GFP were identified by Southern blot analysis. To determine the efficiency of HR-mediated DSB repair in CtIP-proficient or -deficient cells, U2OS (DR-GFP) cells were infected with retroviruses expressing two different shRNAs against CtIP or empty vector. Subsequently, the cells were transfected with I-Sce1 expression vector (52, 53) using FuGene HD transfection reagent (Roche Applied Science). 48 h after I-Sce1 transfection, flow cytometric analysis was used to determine GFP-positive cells.

**RESULTS**

CDK Activity Is Required for Efficient Interaction of BRCA1 with MRN before and after DNA Damage—BRCA1 and MRN were colocalized at damage-induced foci, and their interaction was enhanced following IR treatment (9, 11, 19). CtIP was identified in a BRCA1- and MRN-containing complex (19). To understand the biological function of the BRCA1-CtIP-MRN complex, we further analyzed the interaction of BRCA1 with MRN during the cell cycle and upon DNA damage.

The interaction of BRCA1 and CtIP is cell cycle-dependent through the binding of the BRCT domain of BRCA1 with CtIP (26). We synchronized T98G cells by serum starvation and found that MRN was coimmunoprecipitated with BRCA1 when cells enter the S and G2 phases of the cell cycle in a similar manner as the association of BRCA1 with CtIP (Fig. 1A and data not shown). These data suggest that BRCA1, CtIP, and MRN form a protein complex when cells enter the S and G2 phases of the cell cycle. The interaction of BRCA1 and Nbs1 is stimulated by IR treatment when cells are in S/G2 (Fig. 1B), similar to the results from asynchronized cells (11, 19).

The interaction of BRCA1 and CtIP depends on CDK-mediated phosphorylation of Ser327 on CtIP (26). To examine whether the BRCA1 and MRN interaction is also CDK-dependent, we treated the human breast carcinoma cell line HBL-100 with the CDK inhibitor roscovitine. As shown in Fig. 1C, the interaction of BRCA1 and MRN is diminished after roscovitine treatment (compare immunoprecipitation samples without IR treatment with or without roscovitine). More strikingly, inhibition of CDK activities also abolished IR-enhanced interaction of BRCA1 and MRN. Similar results were obtained when T98G and U2OS cells were used (data not shown). These data suggest that CDK plays a critical role in the regulation of the interaction of BRCA1 with MRN.

CtIP Directly Interacts with Nbs1 and Is Required for Efficient Interaction of BRCA1 with MRN—The similar binding patterns of BRCA1 with CtIP and with MRN prompted us to test...
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Both the N Terminus and C Terminus of BRCA1 Are Important for Stable Complex Formation of BRCA1, CtIP, and MRN—Because both BRCA1 and MRN participate in DSB repair, association of CtIP with BRCA1 and MRN in vivo, we examined whether the C-terminal BRCA1 fragment is sufficient to bind MRN. The GST-BRCA1 C-terminal fragment (residues 1314–1863) was incubated with cell lysates prepared from U2OS cells. Intriguingly, this fragment does not sufficiently pull down MRN from mammalian cell lysates, although it strongly interacts with CtIP (Fig. 2F). These data suggest that although CtIP is important for the interaction of BRCA1 with MRN in vivo, it is not sufficient to bridge the interaction of MRN with the BRCA1 C-terminal fragment alone. Other parts of BRCA1 in addition to the CtIP binding domain (the BRCT repeats) may be required for BRCA1 to form a stable complex with MRN.

It was described previously that the N-terminal fragment of BRCA1 interacts with MRN in vivo (11). We further demonstrated that the GST-BRCA1 N-terminal fragment (residues 1–324) interacts with FLAG-Nbs1 expressed in insect cells (Fig. 2G). These data suggest that the N terminus of BRCA1 also directly contacts with Nbs1, and this interaction may contribute to stable complex formation of BRCA1-CtIP-MRN. Consistently, a BRCA1 N-terminal truncation mutant deleted for the first 247 residues is significantly impaired in its interaction with endogenous MRN (Fig. 2H). Collectively, our studies suggest that BRCA1 interacts with MRN through both the N and C termini of BRCA1 and CtIP bridges the C terminus interaction between BRCA1 and Nbs1 (Fig. 2F). Both N- and C-terminal interactions of BRCA1 are important for stable complex formation of BRCA1, CtIP, and MRN in vivo.

Suppression of CtIP Expression Leads to a Defect in DSB Repair—Because both BRCA1 and MRN participate in DSB repair, association of CtIP with BRCA1 and MRN prompted us to investigate whether CtIP is also important for DSB repair. We inhibited the expression of endogenous CtIP in U2OS by retroviral infection of two short hairpin RNAs directed against different sites in CtIP mRNA (Fig. 3A, top panel), using retroviral shRNA vector MKO (47). Suppression of CtIP expression led to defective Chk1 phosphorylation after IR as described previously (Ref. 26 and Fig. 3B). We did not detect a significant difference in cell

whether the interaction of BRCA1 with MRN depends on CtIP. U2OS and T98G cells were infected with retroviruses expressing shRNAs against CtIP or the vector control MKO (47). The association of Nbs1 with BRCA1 was significantly reduced when the expression of CtIP was suppressed (Fig. 2A and data not shown). Consistently, a BRCA1 C-terminal truncation mutant deleted for the last 11 residues (Tyr1853 to stop) exhibits much weaker interaction with MRN compared with wild type BRCA1 (Fig. 2B, left panel). This BRCA1 truncation mutant destabilizes the BRCT folding (54, 55) and is impaired in binding with CtIP (24). Moreover, as shown in Fig. 2B (right panel; and data not shown), the interaction of BRCA1 with MRN is much weaker in the BRCA1 mutant cell line HCC1937 than in its derivative cell line reconstituted with wild type BRCA1 (56). HCC 1937 expresses a BRCA1 truncation mutant deleted for the last 11 residues (Tyr1853 to stop) exhibits much weaker interaction with MRN than in wild type BRCA1 (Fig. 2E). These data suggest that forming the MRN complex is important for an efficient association of MRN with CtIP in vitro, although Nbs1 can directly interact with CtIP. Because BRCA1 is present in vivo, this optimal interaction of MRN with CtIP may be due to a stable complex formation of BRCA1-CtIP-MRN.

Although Nbs1 can directly interact with CtIP in vitro, the in vivo interaction of Nbs1 with CtIP is significantly strengthened when Nbs1 forms a complex with Mre11 and Rad50. Mutation of two conserved lysine residues (Lys686 and Lys690) to alanine residues completely abolished the interaction of Nbs1 with Mre11/Rad50 (data not shown). We transfected 293T cells with Myc-tagged Nbs1 and the Nbs1 mutant (K686A,K690A) along with HA-tagged CtIP. The association of the Nbs1 (K686A,K690A) mutant with CtIP is much weaker than that of wild type Nbs1 (Fig. 2E). Therefore, Nbs1 can directly interact with CtIP in the absence of BRCA1 in vitro and raises a possibility that CtIP bridges the interaction of BRCA1 with MRN.

To test whether CtIP might directly interact with MRN, we infected insect cells with baculoviruses expressing FLAG-tagged Nbs1 and Mre11 as well as HA-tagged CtIP. Immunoprecipitation reveals that Nbs1, but not Mre11, directly interacts with CtIP (Fig. 2C). We also examined the interaction of FLAG-tagged Nbs1 expressed and purified from insect cells with a series of GST-tagged CtIP fragments produced and purified from bacteria. Both N- and C-terminal CtIP fragments efficiently interact with Nbs1 (Fig. 2D). Therefore, Nbs1 can directly interact with CtIP in the absence of BRCA1 in vitro and

FIGURE 1. BRCA1 interacts with MRN in a cell cycle-dependent manner, and the interaction requires CDK activities. A, T98G cells were synchronized by serum starvation, and BRCA1 was immunoprecipitated (IP) at different time points after releasing. Western blot analysis was performed using indicated antibodies. The cell cycle profile was determined by fluorescence-activated cell sorter analysis. B, 24 h after releasing from serum starvation, T98G cells were treated with IR (20 Gy). Anti-BRCA1 immunoprecipitation was performed before or 1 h after IR, followed by immunoblotting using indicated antibodies. RoM, rabbit anti-mouse IgG. C, HBL100 cells were incubated with roscovitine (50 μm) for 3 h or left untreated, followed by IR treatment (20 Gy). The interaction of BRCA1 with MRN was examined by anti-BRCA1 immunoprecipitation before or 1 h after IR and subsequent Western blot analysis using indicated antibodies.

whether the interaction of BRCA1 with MRN depends on CtIP. U2OS and T98G cells were infected with retroviruses expressing shRNAs against CtIP or the vector control MKO (47). The association of Nbs1 with BRCA1 was significantly reduced when the expression of CtIP was suppressed (Fig. 2A and data not shown). Consistently, a BRCA1 C-terminal truncation mutant deleted for the last 11 residues (Tyr1853 to stop) exhibits much weaker interaction with MRN compared with wild type BRCA1 (Fig. 2B, left panel). This BRCA1 truncation mutant destabilizes the BRCT folding (54, 55) and is impaired in binding with CtIP (24). Moreover, as shown in Fig. 2B (right panel; and data not shown), the interaction of BRCA1 with MRN is much weaker in the BRCA1 mutant cell line HCC1937 than in its derivative cell line reconstituted with wild type BRCA1 (56). HCC 1937 expresses a BRCA1 truncation mutant deleted for one of the C-terminal tandem BRCT repeats, and thus these data suggest that the C-terminal part of BRCA1 is important for BRCA1 to interact with MRN. Furthermore, because the BRCT domains are required for BRCA1 to interact with CtIP, these results further support the idea that the interaction of BRCA1 with CtIP is needed for efficient interaction of BRCA1 with MRN.
growth when the expression of CtIP was suppressed by shRNAs under the condition we used (data not shown). However, suppression of CtIP expression led to a significant increase of radiation sensitivity (Fig. 3C). These data suggest that CtIP may play an important role in DNA DSB repair.

To more directly examine the role of CtIP in DSB repair, we performed the well established DSB repair assay using I-Sce1 endonuclease to introduce chromosomal DSBs in mammalian cells (57). A homologous recombination reporter DR-GFP composed of two mutated GFP cassettes, SceGFP and iGFP, in a directed orientation, was introduced into U2OS cells (Ref. 52 and Fig. 3D, left panel). In this assay, transient expression of I-Sce1 endonuclease would create a DSB and when such a DSB is repaired by a gene conversion event, a functional GFP gene is restored. U2OS cell lines carrying the intact DR-GFP substrate were infected with retroviruses expressing shRNA-CtIP or vector MKO (47). Anti-BRCA1 immunoprecipitation (IP) was performed, followed by Western blot analysis using indicated antibodies. B, 293T cells were transfected with HA-tagged full-length BRCA1 (WT), a BRCA1 mutant with a deletion for the last 11 amino acids (1853stop), or vector (vec) pCDNA3 (left panel). The interaction of endogenous CtIP and Nbs1 with HA-tagged BRCA1 species was revealed by anti-HA immunoprecipitation and anti-CtIP and anti-Nbs1 Western blot analysis. Cell lysates were prepared from the HCC 1937 cell lines with (+ BRCA1) or without (−) reconstitution of wild type BRCA1 (right panel). Anti-BRCA1 immunoprecipitation was performed, followed by immunoblotting using indicated antibodies (right panel). C, baculoviruses expressing FLAG-tagged Nbs1 or Mre11 or HA-tagged CtIP were used to infect insect cells SF21 as indicated. Immunoprecipitation was performed using antibodies against HA or FLAG, followed by indicated immunoblotting (IB). D, FLAG-Nbs1 was expressed in SF21 insect cells and purified by anti-FLAG antibody M2. Pulldown experiments were performed after incubating purified FLAG-Nbs1 with GST-fused CtIP fragments containing residues as indicated. The input of GST-CtIP fragments is indicated by Coomassie Blue staining. The input of FLAG-Nbs1 is shown as 1/20 of the total amount used in the pulldown experiments. E, 293T cells were cotransfected with HA-CtIP and Myc-Nbs1 wild type (WT), Myc-Nbs1 mutant K686A/K690A (KK) defective in Mre11 binding, or vector. Anti-HA immunoprecipitation was performed, followed by anti-Myc Western blotting. F, pulldown experiments were performed by incubating 293T lysates with GST-BRCA1 fragment covering the C-terminal residues 1314–1863 (61). Coomassie Blue staining indicates the input of GST-BRCA1 (1314–1863) and GST. G, pulldown experiments were performed by incubating lysates prepared from insect cells infected with FLAG-Nbs1 baculoviruses with purified GST-BRCA1 fragment covering the N-terminal residues 1–324. Coomassie Blue staining indicates the input of GST-BRCA1 (residues 1–324) and GST. H, 293T cells were transfected with Myc tagged BRCA1 full-length (WT) and the BRCA1 N-terminal deletion mutant (248–1863). Anti-Myc immunoprecipitation was performed followed by anti-Nbs1 immunoblotting. The expression of Myc-tagged BRCA1 and the deletion mutant was shown by anti-Myc Western blot analysis. Vec., vector.
and B), these data suggest that the complex formation of BRCA1-CtIP-MRN is critical for DSB repair, and CDK plays an important role in this respect through regulating the complex formation.

CtIP and the C Terminus of BRCA1 Are Important for Generating ssDNA after IR—Ctp1, the fission yeast homolog of CtIP, is required for HR, and resection of DSB ends to generate ssDNA (37). In mammalian cells, CtIP is required for ATR-mediated Chk1 phosphorylation that depends on ssDNA as activation signals (26). Moreover, CtIP is in a complex with MRN that carries DSB resection function (16, 18). To test whether CtIP plays a similar role in processing DSBs as Ctp in fission yeast, we examined whether IR-induced RPA foci formation requires CtIP. IR-induced RPA foci formation is impaired in HCC1937 compared with the wild type BRCA1 reconstituted cell line (Fig. 4A, right panel). Because the interaction of BRCA1 and MRN is impaired in HCC1937 (Fig. 2B, right panel), these data favor the idea that forming a functional complex of BRCA1-CtIP-MRN is important for facilitating DNA resection at DSB ends to generate ssDNA and thus is required for efficient checkpoint activation and HR-mediated DSB repair.

Because BRCA1 is also required for Chk1 activation and ssDNA formation (59, 60), we examined whether its interaction with CtIP and MRN are important for IR-induced ssDNA formation. We monitored RPA foci formation in HCC1937 and its derivative reconstituted with wild type BRCA1. Significantly, IR-induced RPA foci formation is impaired in HCC1937 compared with the wild type BRCA1 reconstituted cell line (Fig. 4A, right panel). Because the interaction of BRCA1 and MRN is impaired in HCC1937 (Fig. 2B, right panel), these data favor the idea that forming a functional complex of BRCA1-CtIP-MRN is important for facilitating DNA resection at DSB ends to generate ssDNA and thus is required for efficient checkpoint activation and HR-mediated DSB repair.

IR-induced Damage Foci Formation of CtIP and MRN Is Independent from Each Other—In fission yeast, recruitment of Ctp to DSBs depends on Mre11 (37). In mammalian cells, IR-induced CtIP foci are colocalized with that of Mre11 and Nbs1 (Fig. 4B). However, the foci formation of CtIP and MRN appears to be independent from each other. CtIP depletion does not influence Mre11 and Nbs1 foci formation (Fig. 4C). Similarly, CtIP forms similar levels of IR-induced foci in Nbs1-deficient cell line GM07166 and its derivative reconstituted...
with Nbs1 (Fig. 4D). These data suggest that although BRCA1, CtIP, and MRN form a complex that is stimulated by DNA damage, these proteins can be independently recruited to DSBs in mammalian cells.

DISCUSSION

While our manuscript was under preparation, Sartori et al. (40) published results consistent with our findings that CtIP interacts with MRN and promotes DNA end resection. In our study, we further elucidate that BRCA1 forms a complex with CtIP and MRN in a cell cycle-regulated manner. Our studies suggest that not only CtIP and MRN, but also formation of the complex BRCA1-CtIP-MRN are important for DSB end resection and HR-mediated DSB repair. These studies underline a critical mechanism for BRCA1 to participate in HR-mediated DSB repair.

We demonstrate that BRCA1, CtIP, and MRN form a complex when cells enter the S and G2 phases of the cell cycle, and the complex formation requires CDK activity. CtIP directly interacts with Nbs1 in vitro, and the in vivo interaction of BRCA1 with MRN is largely dependent on the presence of CtIP. It has been described that the interaction of BRCA1 and CtIP requires the CDK-mediated phosphorylation at Ser327 (26). Here we show that the C-terminal BRCT domains, required for CtIP association, are also needed for efficient interaction of BRCA1 with MRN. Based on these studies, we favor the model that MRN is recruited by CtIP to form a complex with BRCA1 after CtIP is phosphorylated by CDK at least at Ser327 in late S and G2 phases of the cell cycle. Meanwhile, we showed that the N terminus of BRCA1 is also important for BRCA1 to interact with MRN in vivo. Thus, after MRN is recruited by CtIP to BRCA1 in S and G2, stable complex formation of BRCA1-CtIP-MRN requires both the N terminus and C terminus of BRCA1 (Fig. 2).

Significantly, our studies demonstrate that CDK is not only needed for BRCA1 to interact with MRN during the cell cycle but is also important for IR-stimulated association of BRCA1 with MRN. This regulation has important implications in the control of HR-mediated DSB repair in a cell cycle-dependent manner (see below). It has been described that checkpoint kinases contribute to the stimulation of the interaction of BRCA1 with MRN (19). We propose that CDK-dependent phosphorylation events initiate the complex formation of BRCA1-CtIP-MRN, whereas subsequent checkpoint-mediated phosphorylation events on each component of the complex may further strengthen the interaction and lead to more stable complex formation after IR. For instance, BRCA1 interacts with CtIP after it is phosphorylated by CDK at Ser327, recruiting
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MRN to form a complex with BRCA1, and this cell cycle-specific complex formation of BRCA1, CtIP, and MRN is a prerequisite for further checkpoint-mediated stimulation of the interactions among the subunits of the complex in response to DNA damage.

Consistent with the work by Sartori et al. (40), we showed that CtIP is important for DSB resection to generate ssDNA and HR-mediated DSB repair. CtIP is not only involved in end resection for camptothecin-induced DSBs in S phase (40) but is also needed when cells are treated with IR when DSBs are induced in S/G2 (this study). Our studies also further demonstrated that the BRCA1 C-terminal truncation mutants defective in CtIP and MRN association are significantly impaired in ssDNA formation at the early time points after IR. Moreover, CDK-mediated phosphorylation of CtIP at Ser327, a critical event to establish the association of BRCA1 with CtIP and thus the complex formation of BRCA1-CtIP-MRN, is important for cellular radiation resistance. Because CtIP-S327A impairs the association of BRCA1 with CtIP, but not the interaction of CtIP with MRN, our studies suggest a critical role of BRCA1 for HR-mediated DSB repair through its interaction with CtIP and MRN. It has been described that CtIP promotes the nuclease activity of Mre11 (40). It will be interesting to examine whether the association of BRCA1 with CtIP-MRN further modulates the nuclease activity of Mre11, thereby facilitating ssDNA formation at DSBs.

HR-mediated DSB repair is activated when cells enter the S and G2 phases of the cell cycle, and CDK activity is important for this control (28). However, the mechanisms underlying this regulation and the CDK targets that are directly involved in DSB repair are not clear. Our studies highlight a critical role for regulation and the CDK targets that are directly involved in HR-mediated DSB repair. CtIP is not only involved in end resection for camptothecin-induced DSBs in S phase (40) but is also needed when cells are treated with IR when DSBs are induced in S/G2 (this study). Our studies also further demonstrated that the BRCA1 C-terminal truncation mutants defective in CtIP and MRN association are significantly impaired in ssDNA formation at the early time points after IR. Moreover, CDK-mediated phosphorylation of CtIP at Ser327, a critical event to establish the association of BRCA1 with CtIP and thus the complex formation of BRCA1-CtIP-MRN, is important for cellular radiation resistance. Because CtIP-S327A impairs the association of BRCA1 with CtIP, but not the interaction of CtIP with MRN, our studies suggest a critical role of BRCA1 for HR-mediated DSB repair through its interaction with CtIP and MRN. It has been described that CtIP promotes the nuclease activity of Mre11 (40). It will be interesting to examine whether the association of BRCA1 with CtIP-MRN further modulates the nuclease activity of Mre11, thereby facilitating ssDNA formation at DSBs.

Acknowledgments—We thank Richard Baer, Junjie Chen, Maria Jasmin, Andrew Pierce, Roger Johnson, Sharon Cantor, and Roger Greenberg for valuable reagents and Paul Russell for helpful discussions.

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