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Ser1778 of 53BP1 Plays a Role in DNA Double-strand Break Repairs

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53BP1 is an important genome stability regulator, which protects cells against double-strand breaks. Following DNA damage, 53BP1 is rapidly recruited to sites of DNA breakage, along with other DNA damage response proteins, including γ-H2AX, MDC1, and BRCA1. The recruitment of 53BP1 requires a tandem Tudor fold which associates with methylated histones H3 and H4. It has already been determined that the majority of DNA damage response proteins are phosphorylated by ATM and/or ATR after DNA damage, and then recruited to the break sites. 53BP1 is also phosphorylated at several sites, like other proteins after DNA damage, but this phosphorylation is not critically relevant to recruitment or repair processes. In this study, we evaluated the functions of phosphor-53BP1 and the role of the BRCT domain of 53BP1 in DNA repair. From our data, we were able to detect differences in the phosphorylation patterns in Ser25 and Ser1778 of 53BP1 after neocarzinostatin-induced DNA damage. Furthermore, the foci formation patterns in both phosphorylation sites of 53BP1 also evidenced sizeable differences following DNA damage. From our results, we concluded that each phosphoryaltion site of 53BP1 performs different roles, and Ser1778 is more important than Ser25 in the process of DNA repair.

Key Words: 53BP1, Phosphorylation, Nuclear foci, DSBs, DNA repair

INTRODUCTION

DNA damage recognition and repair are fundamental processes in the maintenance of genome fidelity. The disruption of these pathways is profoundly involved in the development of human disease, tumorigenesis, and cellular aging. Among the many types of DNA damage that can occur within the cell, the most dangerous is the DNA double-strand break (DSB); as little as one unrepaired DSB can be sufficient to kill a cell. These DSBs result from exposure to exogenous agents including ionizing radiation (IR) and certain chemotherapeutic drugs, from endogenously generated reactive oxygen species, and from mechanical stress on the chromosomes. When DSBs occur, a complex cellular response is elicited which promotes DNA repair with the least amount of collateral damage to genome integrity (Jeggo and Lobrich, 2007). The failure to repair DSBs, or misrepair, can result in cell death or large-scale chromosome changes, including deletions, translocations, and chromosome fusions which enhance genome instability, and are hallmarks of cancer cells. Cells have evolved groups of proteins that function in signaling networks that sense DSBs or other DNA damage, arrest the cell cycle, and activate DNA repair pathways.

During the very earliest stages of checkpoint activation, DNA damage sensors relay information to members of a family of phosphoinositide 3 kinase-related kinases. In mammalian cells, two PIKK family members--ataxia-telangiectasis mutated (ATM) and ATM and Rad 3-related (ATR) (Shiloh, 2003; Bakkenist and Kastan, 2004)--perform crucial functions in early signal transmission through cell cycle checkpoints. Once activated, ATR and ATM amplify the damage signal via the phosphorylation of a variety of substrates. Both kinases share the same minimal essential phosphorylation consensus sequence (Kim et al., 1999); substrate selection may be based on spatiotemporal interactions. Generally, it has been determined that ATM responds to DSBs, whereas ATR responds to almost all types of DNA damage, and also to the stalling of replisomes. ATM and ATR are believed to be activated via interaction with DNA damage sites, allowing them to phosphorylate multiple target proteins (Shiloh, 2003; Traven and Heierhorst, 2005). Both kinases translocate rapidly to DNA damage sites, and can directly phosphorylate other proteins associated with these sites, including the core histone variant H2AX, BRCA1, the MRN complex, MDC1/NFBD1, and 53BP1 (Burma et al., 2001; Ward and Chen, 2001; Stiff et al., 2004).

53BP1 was originally identified via yeast two-hybrid

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ABBREVIATIONS: 53BP1, TP53-binding protein 1; NCS, neocarzinostatin; DSBs, double-strand breaks; ATM, ataxia-telangiectasis mutated; ATR, ATM and Rad 3-related.

screening using the p53 tumor suppressor as bait, and was subsequently characterized as an activator of p53-dependent gene transcription (Iwabuchi et al., 1998). The C-terminal end of 53BP1 harbors two BRCT domains--a protein interaction module contained in a number of proteins that have been implicated in various aspects of cell cycle control, recombination, and DNA reapir (Anderson et al., 2001; Rappold et al., 2001; Manke et al., 2003; Yu et al., 2003). Multiple S/T-Q motifs have been detected in the N-terminal region of 53BP1, and some of these S/T-Q motifs have been shown to be ATM phosphorylation sites (Jowsey et al., 2007). It has been proposed that 53BP1 functions as an adaptor protein that is responsible for the recruitment and assembly of various proteins in DNA damage responses (Wang et al., 2002). However, the molecular functions of 53BP1 in DNA damage responses have yet to be thoroughly elucidated.

In this study, we assessed the functions of phosphor-53BP1 in DNA damage response via a comparison of two phosphorylation sites on 53BP1; Ser25 is located in a S/T-Q motif and Ser1778 lies within the BRCT domain. The BRCT domain consists of ~95 amino acids and has been detected in a number of proteins involved in the DNA damage response (Bork et al., 1997). The BRCT domain mediates protein-protein interactions and, in some cases, binds specifically to phosphorylated targets (Dulic et al., 2001; Manke et al., 2003; Yu et al., 2003). Although the BRCT domain performs important roles in a variety of pathways, the importance of the BRCT domain of 53BP1 remains to be clearly elucidated. Thus, in this study we assessed the roles of Ser25 and Ser1778 on 53BP1, which are located in different functional domains.

METHODS

Cells lines and drug treatment

U2OS cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 10 μg/ml of streptomycin, and 10 U/ml of penicillin at 37°C in the presence of 5% CO_2. To induce DNA damage, exponentially growing cells were treated with 200 ng/ml of neocarzinostatin (NCS; Sigma) and harvested at different timepoints (0 h, 1 h, 3 h, 6 h, 12 h, and 24 h) after treatment.

Antibodies and immunoblotting

Harvested NCS-treated cells were lysed with M-PER (Pierce) containing proteinase inhibitors (Complete Mini, Roche) and separated via SDS-PAGE. The separated proteins were then transferred to PVDF membranes (Millipore), and the membranes were blocked for 1 h with 5% nonfat dry milk in TBS-T (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20). The membranes were then incubated with primary antibodies against phospho-53BP1 (S1778) (Cell Signaling Technology), phospho-53BP1 (S25) (Novus), 53BP1 (BD Biosciences and Santa Cruz Biotechnology), and α-tubulin (NeoMarkers). After incubation with primary antibody, the membranes were incubated with the corresponding secondary antibodies and visualized via chemiluminescence (Intron).

Immunofluorescence (IF) analysis

Standard immunofluorescence procedures were conducted in accordance with the methods described by Nakamura et al. (2006). Cells grown on cover slides were briefly rinsed in PBS and then fixed for 15 min with freshly prepared 3.7% paraformaldehyde in PBS. The slides were then either directly processed or exposed to 70% ethanol prior to use at 4°C. After washing in PBS, the cover slides were blocked for 1 h in 5% BSA in PBS at room temperature, and then incubated overnight at 4°C with the following primary antibodies: mouse monoclonal γ-H2AX (S139) (1 : 200; Upstate Biotechnology) or rabbit polyclonal 53BP1 (S25 and S1778) (1 : 100; Novus and Cell Signaling Technology). Secondary antibodies labeled with Alexa 488 or Alexa 594 (Molecular Probes) were added at 1 : 200 and incubated for 1 h at room temperature. The slides were mounted with a mounting solution containing DAPI (Vector Laboratories). Images were acquired with a Nikon ELIPSE 80i microscope.

Comet assay

The single cell gel electrophoresis assay was conducted as described previously, with some modifications (Chowdhury et al., 2005). The cells were treated with 200 ng/ml of NCS, trypsinized, and resuspended in PBS. Aliquots of the cell suspension (20 μl, 1×10^5 cells) were transferred to 1.5 ml tubes, mixed with 200 μl of low-melting agarose, and distributed onto conventional microscope slides. The slides were precoated with normal melting agarose (0.5% in PBS) and dried at room temperature. The agarose was then solidified for 10 min at 4°C. The slides were subsequently immersed in lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris-HCl (pH 10), and freshly added 1% Triton X-100 and 10% DMSO) for 1 hr at 4°C, then placed into a horizontal electrophoresis apparatus filled with freshly made buffer (1 mmol/l Na_2EDTA and 300 mmol/l NaOH (pH > 13)). Electrophoresis was conducted for 30 min at 300 mA. After electrophoresis, the slides were washed in 70% ethanol, stained with 30~50 μl of ethidium bromide (40 μg/ml) for 1 h, and maintained in a moist chamber in darkness at 4°C until analysis. The cells were analyzed 24 h after staining at a magnification of 400× using a fluorescence microscope (Zeiss) equipped with a 50-W mercury lamp. The microscope images revealed circular shapes indicating undamaged DNA and comet-like shapes indicating that the DNA had migrated out from the head to form a tail (damaged DNA). The extension of each comet was analyzed using a computerized image analysis system (Komet5.5, Andor Technology) which provided a "tail moment," which is defined as the product of DNA in the tail and the mean distance of its migration in the tail, and is considered to be the variable that is related most directly to DNA damage.

RESULTS

53BP1 phosphorylation after NCS-induced DNA damage

Several phosphorylation sites have been identified in 53BP1 (Fig. 1). Among those sites, eight are basal phosphorylation sites (Ser294, Ser380, Ser523, Ser552, Ser831, Ser1028, Ser1086, and Ser1114) and the others are phosphorylated by ATM and ATR after DNA damage (Ser13, Ser25, Ser166, Ser176/178, Thr302, Ser452, Ser523, Thr543, Ser831, Thr1171, Ser1219, and Ser1778). However, there is currently no information regarding the biological func-

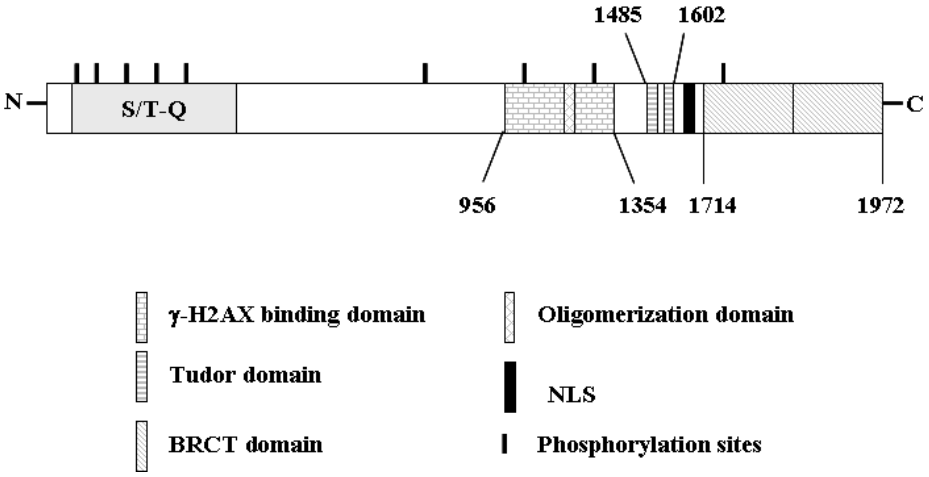

Fig. 1. Schematic diagram of 53BP1. Human 53BP1 is composed of 1,972 amino acids and contains several noteworthy structural features. 53BP1 possesses a number of clustered PIK phosphorylation sites (S/T-Q motif) and two repeated C-terminal BRCT domains. Additionally, 53BP1 harbors a tandem Tudor domain, a stretch rich in glycine and arginine residues.

tions of these sites after DNA injury, even including Ser25 and Ser1778, which have been associated with some commercially available antibodies.

To assess the functional roles of phosphor-53BP1, we induced DNA DSBs in U2OS cells via NCS treatment (200 ng/ml) and then harvested the cells in a time-dependent fashion (0 h, 1 h, 3 h, 6 h, 12 h, and 24 h). First, we assessed the phosphorylation patterns of Ser25 and Ser1778 of 53BP1 after NCS-induced DNA damage by Western blotting using commercially available antibodies; there are only two commercial antibodies (Ser25 and Ser1778) for phosphor-53BP1. Interestingly, we detected distinct patterns for both 53BP1 sites (Fig. 2). Whereas the Ser25 of 53BP1 was phosphorylated rapidly within 1 hour and remained phosphorylated to almost the same degree from 1 hour until 24 hours, phosphorylation at Ser1778 peaked at 1 hour and then decreased gradually to 24 hours (Fig. 2A). This result can be observed in Fig. 2B. The total level of 53BP1 did not change after NCS-induced DNA damage. These results suggest the possibility that the Ser25 and Ser1778 of 53BP1 may perform different and distinct functions in DNA damage responses.

Foci formation of both Ser25 and Ser1778 of 53BP1 after DNA damage

The formation of nuclear foci in the region surrounding DNA lesions is an early event occurring in response to DNA damage. The first proteins that relocalize to these nuclear foci are MDC1, 53BP1, and the Mre11-Rad50-NBS1 (MRN) complex. In an effort to evaluate the foci formation pattern of Ser25 and Ser1778 of 53BP1, we conducted immunofluorescent analysis in a time-course manner using each phosphor-antibody of 53BP1 in U2OS cells. Interestingly, the Ser25 and Ser1778 of 53BP1 also evidenced differences in foci formation patterns (Fig. 3). The number of foci at Ser25 had increased dramatically at 1 hour (174 per cell) and persisted until 24 hours (160 per cell), but the number of Ser1778 foci diminished in a time-dependent fashion (from 177 per cell to 36 per cell) (Fig. 3B). Additionally, the sizes of foci at Ser25 and Ser1778 also evidenced different patterns in the DNA repair process. Early on (from 1 hour to 3 hours) the focal sizes at both phosphor-53BP1 sites had the same pattern, with many small-sized foci.

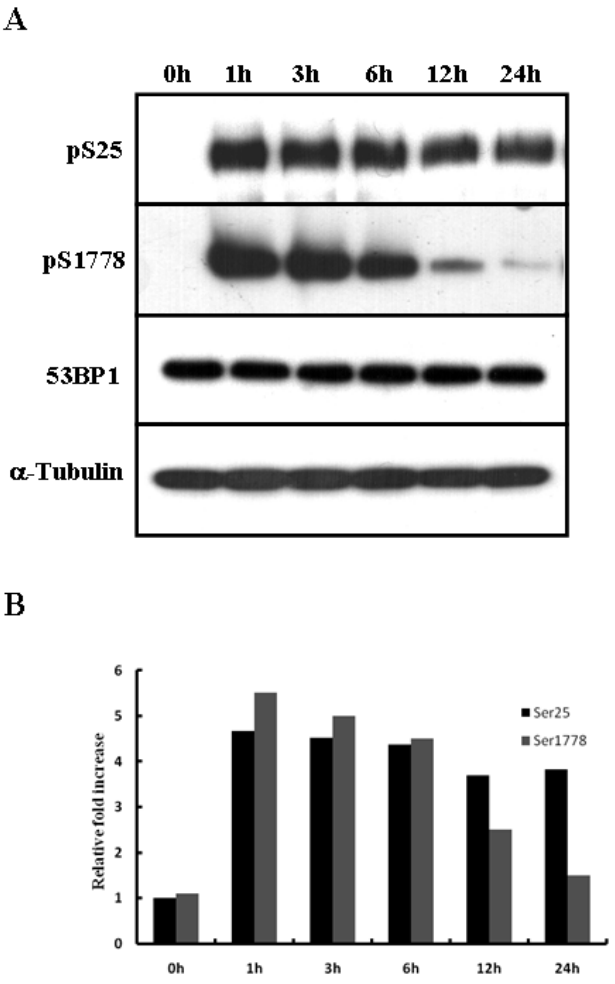

Fig. 2. Phosphorylation at Ser25 and Ser1778 of 53BP1 evidenced differing patterns after NCS-induced DNA damage. (A) U2OS cells were treated with NCS (200 ng/ml) for the indicated times, and then Western blot analysis was conducted to examine the phosphorylation patterns at Ser25 and Ser1778 of 53BP1. (B) Quantitative results showed the differences in the intensity between Ser25 and Ser1778 phosphorylation after DNA damage.

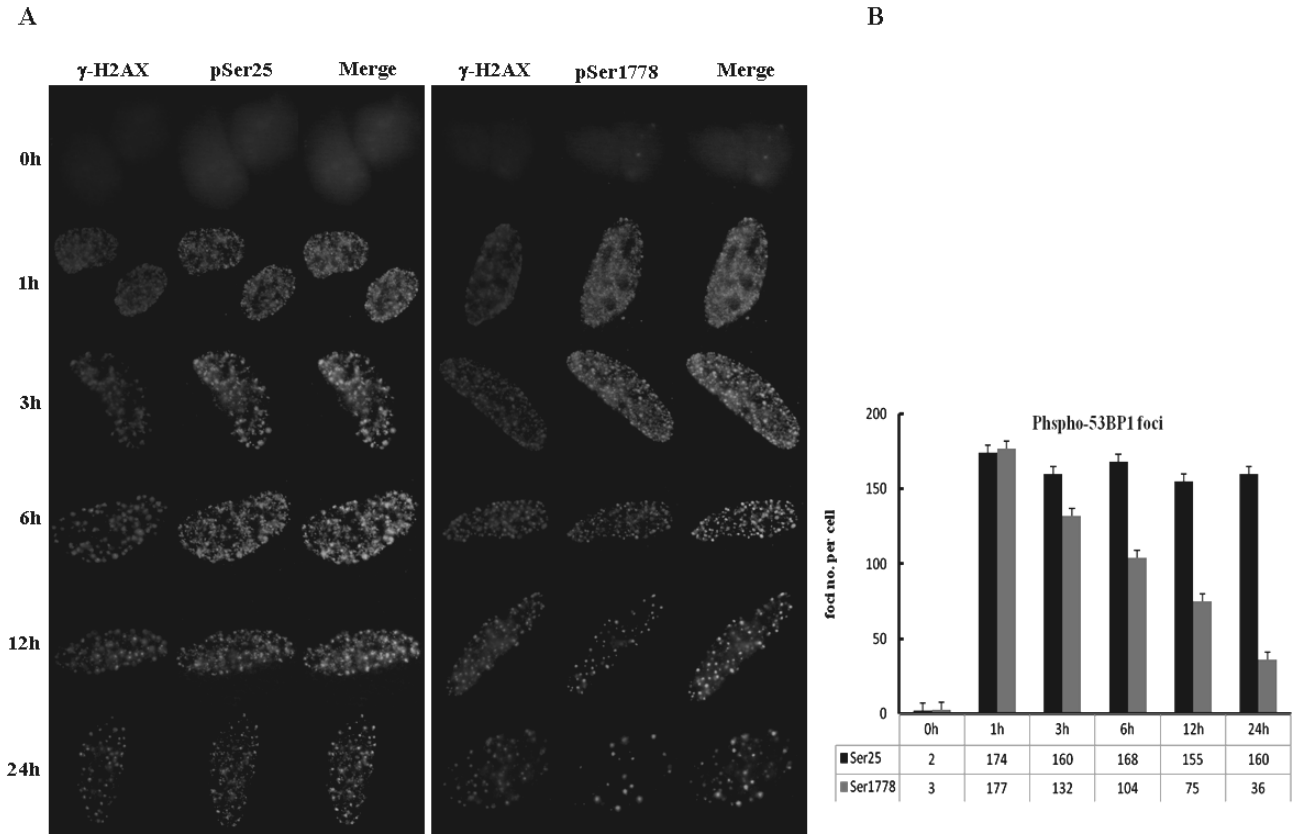

Fig. 3. Ser 25 and Ser1778 of 53BP1 differed in nuclear foci formation. (A) U2OS cells were grown on cover slides and then treated with NCS (200 ng/ml). At 0, 1, 3, 6, 12, and 24 hours after NCS treatment, the cells were fixed and immunostained with antibodies. (B) The graph shows the average number of Ser25 and Ser1778 foci based on ~100 nuclei per sample. The values are expressed as the means±S.D.

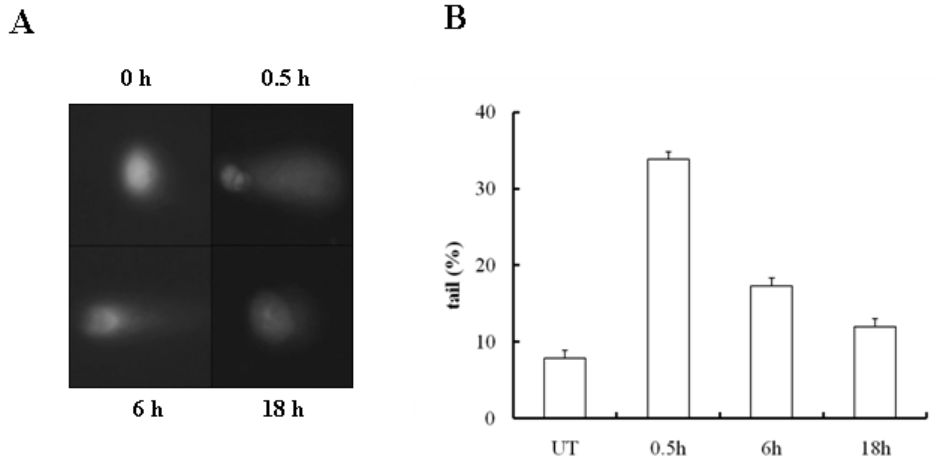

Fig. 4. DNA repair was finished within 24 hours after NCS treatment. (A) DNA fragmentation in U2OS cells was analyzed via a comet assay after NCS (200 ng/ml) treatment. DNA was stained with EtBr and all images were acquired at the indicated times. (B) DNA fragmentation was quantified by determining the tail moment of at least 100 comets per condition.

However, the sizes of the Ser1778 foci became bigger at later stages (12 hours and 24 hours), but the Ser25 foci maintained a similar pattern as was seen in the 1~3 hour stage. According to these data, we suggest that phosphorylation at Ser1778 may be more important than Ser25 in the DNA repair process.

Phosphorylation at the Ser1778 of 53BP1 is related closely to DNA repair

The comet assay is a versatile and sensitive method for measuring single- and double-strand DNA breaks. Thus, we

compared the relationship between the phosphorylation pattern of 53BP1 and DNA repair. As is shown in Fig. 4, we determined that the NCS-induced DNA breaks in U2OS cells were almost completely repaired at 18 hours. DNA damage peaked at 30 min after NCS treatment and then declined gradually over 18 hours. The percentage of tail DNA was also shortened from 34% (30 min) to 11% (18 hours). These repair patterns matched precisely with the phosphorylation patterns detected at the Ser1778 of 53BP1. These results indicate that phosphorylation at the Ser1778 of 53BP1 performs a critical function in the DNA repair process.

DISCUSSION

In this report, we evaluated the potential importance of phosphorylation at Ser1778 on 53BP1 after NCS treatment in U2OS cells. Previous studies of 53BP1 have been focused principally on phosphorylation itself in response to DNA damage. In this study, we demonstrated that the phosphorylation status of 53BP1 is associated with DNA repair by comparing two phosphorylation sites in a time-dependent manner. Our results suggest that each phosphorylation site of 53BP1 (Ser25 and Ser1778) performs different functions in checkpoints and/or the repair process. According to the results of Western blotting and immunostaining analysis for Ser1778 of 53BP1, we could confirm the importance of the phosphorylation of Ser1778 of 53BP1. However, the role of Ser25 remains unclear.

It has been proposed that 53BP1 may be involved in DNA DSB repair, on the basis of the observed increased radiation sensitivity and impaired class switch recombination (CSR) in 53BP1-deficient mice (Morales et al., 2003; Ward et al., 2003; Ward et al., 2004). 53BP1 has been implicated in the regulation of the activation of the G2/M phase checkpoint (DiTullio et al., 2002; Wang et al., 2002), as well as the repair of DNA DSBs via non-homologous end joining (Difilippantonio et al., 2008). Moreover, 53BP1 has been shown to be required for the assembly of the Rif1 checkpoint protein at DNA breakage sites (Silverman et al., 2004), which also suggests a function for 53BP1 in the regulation of the intra-S phase checkpoint. Despite these observations, the precise molecular functions of 53BP1 and its role during the cellular response to DNA damage remains to be clearly elucidated.

Until several years ago, studies of 53BP1 phosphorylation have generally been limited to Ser25, and the roles of phosphor-53BP1 have not yet been well-characterized. The majority of DNA repair proteins are first phosphorylated by ATM after the formation of DNA DSBs, and then relocalized at DNA break sites. However, the recruitment of 53BP1 to DNA breakage sites does not require ATM activity (Schultz et al., 2000), but 53BP1 was rapidly hyperphosphorylated by ATM (Schultz et al., 2000; Rappold et al., 2001). The formation of 53BP1 after DNA injury is sufficient with the oligomerization domain and the tandem Tudor domain, as it recognizes methylated H3-K79 and H4-K20 (Huyen et al., 2004; Botuyan et al., 2006; Zgheib et al., 2009).

Furthermore, the BRCT domain of 53BP1 is not required for DNA repair (Ward et al., 2006). In recent years, several reports have been filed regarding 53BP1 phosphorylation after IR-induced DNA damage. First, Jowsey et al. (2007) identified new phosphorylation sites on 53BP1 (Thr302, Ser831, and Ser1219) using a mass spectrometer after IR-induced DNA damage. However, they also analyzed the phosphorylation patterns at just one time point. This year, the phosphorylation at Ser1219 on the γ-H2AX binding domain was characterized, and that site influenced the foci formation of MDC1 and γ-H2AX (Lee et al., 2009). Our finding that the Ser1778 of 53BP1 may perform an important function in DNA repair was strongly suggested by the results of our previous study (Kang et al., 2009). In our previous study, it was demonstrated that the hyper- and hypo-phosphorylation of Ser25 and Ser1778 strongly influenced the NHEJ activity in U2OS cells. In this study, however, we have analyzed the repair activities including homologous recombination (HR) and NHEJ after DNA DSBs. Using a comet assay, we were able to determine that the repair times were highly consistent with the dephosphorylation time of S1778.

In the present study, we demonstrated that the phosphorylation and foci formation of 53BP1 at Ser25 and Ser1778 evidenced a distinct pattern after DNA damage, and suggested that the phosphorylation at Ser1778 of 53BP1 is closely related to DNA repair. We anticipate that our results might be a clue to understanding the roles of phosphorylation and the BRCT domain of 53BP1 in the DNA damage response.

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