Molecular Mechanism of the Recruitment of NBS1/hMRE11/hRAD50 Complex to DNA Double-strand Breaks: NBS1 Binds to \(\gamma\)-H2AX through FHA/BRCT Domain

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NBS1/\(\gamma\)-H2AX/Recognition of DNA damage.

DNA double-strand breaks represent the most potentially serious damage to a genome, and hence, many repair proteins are recruited to DNA damage sites by as yet poorly characterized sensor mechanisms. We clarified that NBS1 physically interacts with \(\gamma\)-H2AX to form nuclear foci at DNA damage sites. The fork-head associated (FHA) and the BRCA1 C-terminal domains (BRCT) of NBS1 are essential for this physical interaction and focus formation of NBS1 in response to DNA damage. The inhibition of this interaction by introduction of anti-\(\gamma\)-H2AX antibody into cells abolishes NBS1 foci formation in response to DNA damage. Consequently, the FHA/BRCT domain is likely to have a crucial role for both binding to histone and for re-localization of the NBS1/hMRE11/hRAD50 complex to the vicinity of DNA damage. Moreover, the foci formation of DNA repair-related proteins containing BRCT domain, such as BRCA1, requires the interaction with \(\gamma\)-H2AX in response to DNA damage. These findings indicate that the physical interaction between \(\gamma\)-H2AX and DNA repair-related proteins is indispensable for the recruitment of these proteins. Further, it was recently reported that the NBS1/hMRE11/hRAD50 complex has a crucial role for both the recruitment of ATM to DNA damage sites and the subsequent activation of ATM. Therefore, both \(\gamma\)-H2AX and the NBS1/hMRE11/hRAD50 complex might function for the initial recognition of DNA damage.

INTRODUCTION

DNA double-strand breaks (DSBs) must be recognized and able to promote the recruitment of several nuclear proteins to the damaged sites in order to initiate repair and checkpoint controls. So far, several DNA repair-related proteins, such as NBS1, BRCA1, MDC1 (Mediator of DNA damage Checkpoint) and 53BP1, are known to re-localize and form nuclear foci at DNA damage sites induced by exposure to ionizing radiation (IR).\(^{1-4}\) *NBS1* is the gene defective in Nijmegen breakage syndrome (NBS) known as a radiation hyper-sensitive disease.\(^5\) The cells from NBS patients show radiation hyper-sensitivity, radio-resistant DNA synthesis (RDS), chromosome instability and a defect in cell cycle checkpoints,\(^6\) which suggests that NBS1 functions for both cell cycle checkpoints and DNA repair in response to IR. The human NBS1 protein consists of 754 a.a., and includes some functional domains; a fork head-associated (FHA) domain and a BRCA1 C-terminus (BRCT) domain at the N-terminus and an hMRE11-binding domain at the C-terminus (Fig. 1). NBS1 forms a complex with hMRE11 and hRAD50 through the hMRE11-binding domain, and this complex rapidly re-localizes to DSB sites and forms nuclear foci.\(^{7-9}\) This complex also shows DNA nuclease activity *in vitro* and can function in DNA repair of DSBs. The DT40 cells, deficient in the NBS1 or MRE11 genes, shows a dramatic reduction in mitomycin C-induced sister chromatid exchange (SCE).\(^9\) Radiation-induced DSBs are repaired by at least two pathways: non-homologous end joining (NHEJ) and homologous recombination (HR) repair.\(^{9,10}\) This SCE reduction in Nbs1 mutant cells is comparable to that in *Rad54* and *Rad51* paralog knockout chicken cells,\(^{11,12}\) which are typical HR-deficient cells. These findings indicate that the NBS1/hMRE11/hRAD50 (N/M/R) complex functions for DNA repair by HR. Although the N/M/R complex is recruited to DSB sites for this DNA repair function in normal cells, NBS cells, lacking normal NBS1 protein, cannot re-locate the hMRE11/hRAD50 complex to DSB sites and this complex is confined to the cytoplasm after IR.\(^{1,13}\) Therefore, NBS1 is suggested to function for the recruitment of the N/M/R complex to DSB sites for DNA

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BRCA1, 53BP1, TOPBP1 and MDC1 contain BRCT domain. MRE11-binding region at the C-terminus (665–693 a.a.). (B) residues at serine278 and serine343 in a central region, and three functional regions: FHA domain (24–108 a.a.) and BRCT domain. (A) Structure of NBS1 protein. NBS1 consists of Fig. 1.

IR.

possibly, the first protein that is phosphorylated by ATM, proteins, including the N/M/R complex.

H2AX has a C-terminal region longer than histone H2A1 or protein and is ubiquitously distributed over chromatin. Histone H2AX, identified in the 1980s, constitutes approximately 10% of human H2A protein and is ubiquitously distributed over chromatin. Histone H2AX has a C-terminal region longer than histone H2A1 or H2A2, and this region contains an SQ-motif, which can be phosphorylated by ATM, ATR, and DNA-PK, and is highly conserved from yeast to humans. ATM is the defective gene in the human genetic disorder, ataxia telangiectasia (AT) syndrome and the cells from AT patients show a similar phenotype to NBS cells. The ATM protein is activated in response to DSBs and can phosphorylate many DNA repair and cell cycle checkpoint-related protein. Bakkenist and Kastan showed that generation of DSBs by IR results in intermolecular modification within ATM dimers that leads to their activation via autophosphorylation of serine1981. This intermolecular phosphorylation subsequently triggers dimer dissociation, and the free monomers can phosphorylate several DNA damage response proteins, including the N/M/R complex. Histone H2AX is, possibly, the first protein that is phosphorylated by ATM, being rapidly phosphorylated within a few minutes after IR. This phosphorylated H2AX (γ-H2AX) forms discrete foci at DSB sites immediately after irradiation and co-localizes with DNA repair related-proteins such as NBS1, BRCA1, MDC1 and 53BP1. H2ax (−/−) mouse cells are unable to form BRCA1, 53BP1 and MDC1 foci after exposure to radiation. Therefore, γ-H2AX is likely to be important for the recruitment of these DNA repair-related proteins to the DSB sites. However, it has been unclear how γ-H2AX mediates these recruitment. Hence, we review that the function of histone H2AX for the recruitment of the N/M/R complex (foci formation) to DSB sites

NBS1 PHYSICALLY INTERACTS WITH γ-H2AX THROUGH THE FHA/BRCT DOMAIN

Since γ-H2AX co-localizes with some DNA repair-related proteins at DSB sites, we examined the co-localization between γ-H2AX and the N/M/R complex after exposure to γ-ray in HeLa cells. Although γ-H2AX foci formation occurred rapidly (within 5 minutes after IR), most γ-H2AX foci co-localized with the foci of N/M/R complex within 2 hrs after irradiation. In the case of NBS cells, lacking normal NBS1 protein, hMRE11 protein was confined to the cytoplasm and did not form nuclear foci, while γ-H2AX foci were detected. As this co-localization suggests a physical interaction between γ-H2AX and the N/M/R complex, we tested these potential protein interactions using immunoprecipitation analysis by anti-γ-H2AX antibody. Although a small amount of NBS1 was detected in immunoprecipitates using anti-γ-H2AX antibody in non-irradiated normal cells, this amount increased dramatically after irradiation with 10 Gy. Since a small number of spontaneous DSBs is generated during S phase without IR, NBS1 might interact with γ-H2AX at these DSB sites in S phase cells. Similarly, γ-H2AX was co-immunoprecipitated using anti-NBS1 antibody after irradiation with 10 Gy. hMRE11 was also present in this NBS1/γ-H2AX immuno-complex, while the amount of hMRE11 did not parallel that of NBS1, probably due to the direct binding of hMRE11 to DNA. However, when NBS cells were irradiated, hMRE11 was not detected in immunoprecipitates obtained using anti-γ-H2AX antibody, indicating that hMRE11/hRAD50 cannot interact with γ-H2AX in the absence of NBS1. Moreover, the addition of ethidium bromide to these immunoprecipitation reaction did not disturb the interaction of NBS1 with γ-H2AX, which suggests that this interaction is most likely mediated by direct binding of NBS1 to γ-H2AX without DNA ends.

We further investigated the H2AX-N/M/R interactions using recombinant proteins generated from E. coli. When a mixture of recombinant H2AX and recombinant full length NBS1 was immunoprecipitated with anti-H2A antibody (recognizes both H2AX and γ-H2AX), the precipitate did not contain recombinant NBS1. However, after phosphorylation of recombinant H2AX by ATM kinase, the immunoprecipitate using anti-H2A antibody contained recombinant
NBS1 and γ-H2AX. These findings demonstrate that NBS1 binds directly to γ-H2AX and that this binding is specific for the phosphorylated form of H2AX protein. Moreover, recombinant γ-H2AX showed no binding to a recombinant C-terminal NBS1 protein, but did bind to an N-terminal fragment of NBS1 containing the FHA/BRCT domain, which demonstrates that the FHA/BRCT domain of NBS1 is required for this direct binding. We also confirmed the requirement of the FHA/BRCT domain for this interaction by immunoprecipitation analysis from the NBS cells expressing several mutant forms of NBS1.13) Wild-type NBS1, an NBS1-S343A mutant (serine343 of NBS1 is phosphorylated by ATM and this phosphorylation is required for the intra-S phase checkpoint19,20) and the NBS1-R2 (truncation involving hMRE11-binding domain) mutant were co-immunoprecipitated with anti-γ-H2AX antibody after IR, but the deletion constructs of NBS1, lacking either or both of the FHA and BRCT domains were not immunoprecipitated by anti-γ-H2AX antibody, which is consistent with our results using recombinant proteins. Therefore this evidence indicates that the FHA/BRCT domain of NBS1 is essential to the interaction of the N/M/R complex with γ-H2AX in response to DNA damage.

THE FORMATION OF NBS1 FOCI IN RESPONSE TO IR REQUIRES THE INTERACTION WITH γ-H2AX

As H2AX is rapidly phosphorylated in the absence of NBS1,13,25) it is suggested that phosphorylation of H2AX is firstly recognized by NBS1 and followed by formation of NBS1 foci, in which hundreds to thousands of proteins are recruited and accumulated. Hence, we examined the γ-H2AX/NBS1 interaction and NBS1 foci formation after masking the epitope of γ-H2AX phosphoprotein,13) while it also might inhibit the consecutive conversion of H2AX to γ-H2AX. When anti-γ-H2AX antibody was introduced into normal cells, the immunoprecipitation of NBS1 with anti-γ-H2AX antibody and formation of NBS1 foci were strikingly inhibited, whereas both foci formation and NBS1/γ-H2AX interaction were not significantly affected by introduction of non-specific IgG. Moreover, the NBS cells expressing the mutated NBS1, which lacks the FHA/BRCT domain and cannot bind with γ-H2AX, do not show IR-induced NBS1 foci. However, the mutated NBS1, which lacks the hMRE11-binding domain and can bind with γ-H2AX, showed IR-induced NBS1 foci. Cerosaletti et al also obtained similar result that the mutated NBS1, containing disruptions to the conserved motifs in FHA or BRCT domains, cannot form nuclear foci after IR.26) Moreover, h2ax (+/−) mouse cells cannot form NBS1 foci in response to IR.19,20) H2AX is known to be phosphorylated in normal cells by ATM kinase shortly after IR, and hence, ATM-mutated AT cells exhibited delayed phosphorylation of H2AX after IR. When we examined the interaction between γ-H2AX and NBS1 by immunoprecipitation at early time after IR, this interaction in AT cells is less than that in HeLa cells.19) Moreover, rapid IR-induced foci formation of NBS1 was repressed in AT cells due to the low level of γ-H2AX. Taken together, this evidence indicates that the foci formation of the N/M/R complex requires the direct interaction of NBS1 with γ-H2AX through the FHA/BRCT domain of NBS1.

THE INTERACTION WITH γ-H2AX IS REQUIRED FOR THE FOCI FORMATION OF DNA REPAIR-RELATED PROTEINS

DNA repair-related proteins such as BRCA1, 53BP1 and MDC1/NFBD1 also contain the BRCT domains (Fig. 1). As these proteins form nuclear foci in response to IR and co-localize with γ-H2AX, we investigated whether the interaction with γ-H2AX is essential to these IR-induced foci formation. When we investigated the interaction between γ-H2AX and BRCA1 using HeLa and HCC1937 cells, normal BRCA1 can bind to γ-H2AX but mutated BRCA1 expressed in HCC1937 cells, which lacks the tandem BRCT domain, failed to do so.13) Moreover, this mutated BRCA1 cannot form nuclear foci in response to IR. These findings suggest that the interaction with γ-H2AX is indispensable for IR-induced foci formation of BRCA1. MDC1 and 53BP1 are also known to interact with γ-H2AX by immunoprecipitation analysis and the mutated MDC1 or TOPBP1, which lack the BRCT domain, fail to form foci after IR.3,27,28) Further, h2ax (−/−) mouse cells are also unable to form BRCA1, 53BP1 and MDC1 foci in response to DSBs.3,19,20) These findings suggest that the physical interaction with γ-H2AX through the BRCT domain is required for foci formation of these proteins at DSB sites. This is further supported by the recent report on an SQ motif-binding protein, PTIP (Pax transactivation domain-interacting protein), which was identified by using an ATM/ATR motif library.29) PTIP contains a tandem repeat of BRCT, and this domain interacts with proteins containing a phosphorylated SQ motif. PTIP foci form after IR and co-localize with γ-H2AX, and mutated PTIP (lacking the tandem BRCT domain) fails to form such foci. These findings suggest that PTIP interacts with the phospho-SQ-motif of γ-H2AX through its BRCT domain, and that PTIP forms foci at DSB sites. Thus, the interaction of DNA repair-related proteins with γ-H2AX through BRCT domains is important for the recruitment of these molecular repair machinery to DSBs.

THE FUNCTION OF γ-H2AX AND N/M/R COMPLEX FOR THE RECOGNITION OF DNA DAMAGE

Recently, it was reported that γ-H2AX is not required for
the initial recognition of DSBs by DNA repair-related proteins under certain conditions.\(^{30}\) γ-H2AX and several DNA repair-related proteins, such as NBS1, 53BP1 and BRCA1, are rapidly (within minutes) recruited to DNA damage sites irradiated by laser microbeam in H2AX (+/+ ) cells.\(^{30–32}\) Unexpectedly, h2ax (−/−) mouse cells display similar rapid recruitment of these proteins after microbeam irradiation. These findings suggest that γ-H2AX is not essential to this recruitment. However, laser micro-irradiation can induce the recruitment of KU70, SMC1 and ATM to the irradiated sites, although these proteins cannot form foci after exposure to ionizing radiation. Hence, DNA damage sites generated by laser microbeam irradiation may contain structures different from those induced by radiation, or those presenting as more densely clustered DSB. Therefore, these DNA repair-related proteins may be able to recognize DNA damage sites containing clusters of DSBs in the absence of γ-H2AX, while other DNA repair-related proteins might require γ-H2AX for the accurate recognition of a small number or low density of DSBs. The dose-dependent requirement of H2AX is supported by the evidence that h2ax (−/−) cells show an obvious defect in G2 checkpoint only after exposure to low dose of radiation,\(^{19,33}\) and that ATM and NBS1 are not required for G2 arrest and CHK2 phosphorylation in cells exposed to high dose of radiation.\(^{34}\)

Thus, NBS1 can rapidly recognize the high density of DNA damage sites without γ-H2AX, which suggests a further function for the N/M/R complex. Uziel et al reported that NBS cells and ATLD cells deficient in the NBS1 and hMRE11 genes respectively, showed a defect in the phosphorylation of ATM on serine1981 in response to DSBs.\(^{35}\) They also showed the abnormality of nuclear retention of ATM in response to DSBs in NBS and ATLD cells, although ATM was retained in HeLa cells. These findings suggest that both ATM binding to DSB sites and the subsequent activation of ATM require the functional N/M/R complex. This hypothesis is supported by the report that Tel1, a yeast homologue of ATM, binds to DNA damage sites with similar dependence on the C-terminus of Xrs2 the yeast counterpart of NBS1.\(^{36}\) Hence, ATM binding to DNA damage sites requires the functional MRN complex, which activates ATM kinase, resulting in phosphorylation of downstream substrates, including H2AX (Fig. 2).\(^{18,37}\) In turn, this H2AX phosphorylation recruits or retains additional NBS1 at DSB sites, and possibly leads to amplification of ATM activation by accumulation of the MRN complex at damaged sites. This amplification promotes an ATM/NBS1/hMRE11 chain reaction. Moreover, DNA damage response genes, such as BRCA1, 53BP1, TOPBP1 and MDC1, bind to γ-H2AX independently on the NBS1, and form nuclear foci at DSB sites. As a result, DNA repair or other cellular responses, such as cell cycle checkpoints or apoptosis, might be initiated. Thus, the N/M/R complex and H2AX have a pivotal role for high fidelity repair when cells are exposed to low dose of radiation, since human cells must detect and correctly repair a single DSB generated in a background of $3 \times 10^9$ bp of the genome. Therefore, the defects in these genes cause the abnormality of cellular response to low dose of IR in NBS, ATLD and h2ax (−/−) mouse cells.

**PERSPECTIVE**

To date it is unclear why mammalian cells can correctly detect a single DSB generated in a background of $3 \times 10^9$ bp of the genome. However, we demonstrated that γ-H2AX physically interacts with NBS1 and this interaction is indispensable for the foci formation of the N/M/R complex in response to DSBs. Moreover, we showed that the disruption of this interaction by introduction of anti-γ-H2AX antibody into cells repressed NBS1 foci formation in response to IR. Furthermore, we found that the interaction between γ-H2AX
and BRCA1 is important for IR-induced foci formation of BRCA1. Therefore, these findings indicate that the physical interaction between γ-H2AX and the DNA repair-related proteins, containing BRCT domain, is important for their recruitment to DSBs and γ-H2AX is indispensable for the recognition of DNA damage. Furthermore, evidence was provided that the N/M/R complex, as such, functions for the detection and recognition of DSBs. Recently, we obtained preliminary data showing that NBS1 can be recruited to highly clustered DSB sites without γ-H2AX and hMRE11 (Kobayashi et al., in preparation). These findings may suggest that NBS1 as well as γ-H2AX possesses the function for recognition of DSBs. Therefore, it is very important to clarify the detailed role of H2AX and the N/M/R complex in DNA damage recognition, since the oversight of a single DSB can induce genome instability and carcinogenesis.

ACKNOWLEDGEMENTS

This review article summarizes the experiments carried out with Dr. Kenshi Komatsu (Kyoto University), Dr. Shinya Matsuura (Hiroshima University), Dr. Hiroshi Tauchi (Ibaraki University) and the members of the Department of Radiation Biology, Research institute for Radiation Biology and Medicine, Hiroshima University. These experiments also have been done in collaboration with Dr. Katsuyuki Tamai and Toshiko Kobayashi (Medical and Biological Laboratory). I thank Dr David J. Chen, Dr. Benjamin P Chen and Dr Sandeep Burma (Lawrence Berkeley National Laboratory) for very helpful discussion. I also thank Dr Keiji Tanimoto and the members of Department of Oral and Maxillofacial Radiology, Graduate School of Biomedical Sciences, Hiroshima University for various assistances. A part of this work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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