53BP1 and NFBDI/MDC1-Nbs1 Function in Parallel Interacting Pathways Activating Ataxia-Telangiectasia Mutated (ATM) in Response to DNA Damage

Tamara A. Mochan,1,2 Monica Venere,1,2 Richard A. DiTullio, Jr.,1,2 and Thanos D. Halazonetis1,3

1The Wistar Institute, Philadelphia, Pennsylvania; 2Cell and Molecular Biology Graduate Group, Biomedical Graduate Studies, and 3Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Abstract

53BP1 and NFBDI/MDC1 are recruited rapidly to sites of DNA double-strand breaks (DSBs), where they are hypothesized to function downstream of the ataxia-telangiectasia mutated (ATM) checkpoint kinase as “mediators” of DNA DSB signaling. To test this hypothesis, we suppressed 53BP1 and NFBDI/MDC1 expression by small interference RNA and monitored ATM autophosphorylation at Ser1981 as a marker for ATM activation. Suppression of NFBDI/MDC1 led to decreased ATM activation and phosphorylation of ATM substrates. This phenotype was identical to that observed in cells with defective Nbs1 function and is consistent with recent observations identifying NFBDI/MDC1 as a component of the Mre11–Rad50–Nbs1 protein complex. In cells with wild-type Nbs1, suppression of 53BP1 expression had no effect on ATM activation but was associated with increased recruitment of NFBDI/MDC1 and Nbs1 to sites of DNA breaks, suggesting that decreased 53BP1 function might be compensated for by increased recruitment of NFBDI/MDC1 and Nbs1 activity. Indeed, in cells with mutant Nbs1, suppression of 53BP1 led to decreased ATM activation and phosphorylation of ATM substrates. We conclude that DNA DSBs activate ATM through at least two independent pathways involving 53BP1 and NFBDI/MDC1-Nbs1, respectively.

Introduction

DNA double-strand break (DSB) signaling in higher eukaryotes is mediated by protein kinases, such as ataxia-telangiectasia mutated (ATM) and Chk2, as well as by many other proteins whose functions are less well-defined (1–3). Several proteins in the latter category, such as 53BP1, NFBDI/MDC1, and BRCA1, contain COOH-terminal BRCT repeats. The proteins 53BP1 and NFBDI/MDC1 have been referred to as “mediators,” an ill-defined term for proteins that are thought to function downstream of ATM in transducing the DNA damage signal (4–7). Although their function has not been well defined, 53BP1 and NFBDI/MDC1 may recruit substrates to ATM (4–14).

Nbs1 is another protein that fits the definition of mediator, because suppression of its function also compromises DNA DSB signaling, leading to decreased phosphorylation of ATM substrates (15–18). The role of Nbs1 in ATM signaling was described well before the term mediator was introduced, and Nbs1 had been referred to as an “adaptor,” a term describing its suggested role in recruiting substrates to ATM. The evidence that Nbs1 functions downstream of ATM is compelling; ATM phosphorylates Nbs1 on Ser145, and substitution of this residue with alanine compromises the ability of Nbs1 to facilitate phosphorylation of ATM substrates (15–20).

Although it is clear that the adaptors/mediators described above participate in DNA DSB signaling, the phenotype observed when their function is suppressed is also consistent with a function upstream of ATM because decreased ATM activation will lead to decreased phosphorylation of ATM substrates. In fact, Nbs1 was recently shown to function upstream of ATM in addition to its well-established role downstream of ATM (21, 22). For 53BP1 and NFBDI/MDC1, there is no experimental evidence to indicate that these proteins function exclusively downstream of ATM, as suggested by the term mediator. Here we reevaluate their role in ATM activation, using a recently described phosphospecific antibody that can monitor ATM activation with high sensitivity (23). Our experiments were designed under the assumption that the so-called adaptors/mediators may be components of independent pathways leading to ATM activation, in which case inhibition of multiple adaptors/mediators would be required to inhibit ATM activation.

Materials and Methods

Cell Lines. Cell lines used were obtained from the following sources: U2OS and HeLa cells were from American Type Culture Collection (Manassas, VA); AT5B1 was from Coriell (Camen, NJ); normal human dermal fibroblasts (NHFds) were from Clonetech (San Diego, CA); NBS-ILB1 LXIN [Nijmegen breakage syndrome (NBS) cells]; NBS-ILB1 NBS1 (NBS+; nbs1wt), and NBS-ILB1 S343A (NBS+; nbs1A343) were from P. Conchon (Virginia Mason Research Center, Seattle, WA); Refs. 15, 24); and XPC-EN12 [xeroderma pigmentosum complementation group C (XPC) cells], an XPC human fibroblast cell line immortalized with SV40 and EBNA, was from E. Friedberg (University of Texas Southwestern Medical School, Dallas, TX).

Cell Transfection. Flag-tagged Chk2 mutants were expressed in U2OS cells by transient transfection using calcium phosphate, as described previously (25). Sixteen h after transfection, the cells were washed, and fresh medium was added. Cells were analyzed after an additional 24 h.

Small Interfering RNA (siRNA) Transfection. All siRNA transfections were performed with Oligofectamine reagent (Invitrogen, Carlsbad, CA). Cells seeded in 60-mm plates were incubated with mixtures of Oligofectamine and 400 pmol control (luciferase) or one of the following siRNAs (Dharmacon, Lafayette, CO): atm (SMART pool), 53bp1 (GAACGAGGAGACG-GUAUUATdTd), nbs1 (GCGAGUCAGCUCCAAAGAGCtTdT), or nfd1/ndc1 (UCUCGAGACCCUUAGGUUUUdTdT). Cells were analyzed 72 h after siRNA treatment.

Immunofluorescence Analysis. NHDF and XPC cells were seeded in 100-mm plates. Immunofluorescence microscopy and image acquisition were performed as described previously (26) with antibodies specific for 53BP1 (26), NFBDI/MDC1 (a gift from J. Chen, Mayo Clinic, Rochester, MN; Ref. 6), or Nbs1 (Calbiochem, La Jolla, CA).

Cell Extracts and Immunoblotting. Whole-cell extracts were prepared and analyzed by immunoblotting as described previously (25) with antibodies specific for Chk2, Chk2 phosphorylated at Thr68, Chk2 phosphorylated at Thr383, and Chk2 phosphorylated at Thr68.
Ser33/35 (Cell Signaling Technology, Beverly, MA), ATM (MAT3-mouse ATM #3; a gift from Y. Shiloh, Tel Aviv University, Ramat Aviv, Israel), ATM phosphorylated at Ser1981 (a gift from M. Kastan, St. Jude Children’s Research Hospital, Memphis, TN; Ref. 23), Nbs1 (Calbiochem, La Jolla, CA), 53BP1 (26), SMC1, SMC1 phosphorylated at Ser657 (Bethyl Laboratories, Montgomery, TX), NFB1/MDC1 (a gift from S. Jackson, University of Cambridge, Cambridge, UK; Ref. 5), or Flag epitope (M5; Sigma, St. Louis, MO).

Results

53BP1 Suppresses NFB1/MDC1 Recruitment to Sites of DNA DSBs. One property shared by various adaptors/mediators is recruitment to sites of DNA DSBs, a term used to describe not only the DSB lesion itself, but also long stretches of chromatin adjacent to the DSB lesion that undergo DNA damage-induced modifications, such as phosphorylation of histone H2AX (27, 28). Interestingly, recruitment of adaptors/mediators to sites of DNA DSBs is hierarchical. For example, recruitment of Nbs1 is dependent on NFB1/MDC1, a finding consistent with these two proteins associating with each other as part of a larger protein complex that also contains Mre11 and Rad50 (5). Recruitment dependency of one adaptor/mediator on another could indicate that the two adaptors/mediators function in the same pathway. We were particularly interested in the recruitment dependency between 53BP1 and NFB1/MDC1 because recruitment of 53BP1 to sites of DNA DSBs has been reported to be NFB1/MDC1-dependent by one laboratory (7) and NFB1/MDC1-independent by another (5).

To study the recruitment dependency between 53BP1 and NFB1/MDC1, expression of one of these proteins was suppressed by siRNA in NHDFs and recruitment of the other protein to sites of DNA DSBs was monitored by immunofluorescence. In nonirradiated cells, neither 53BP1 nor NFB1/MDC1 localized to discreet nuclear foci. After exposure to relatively high doses of ionizing radiation [5–9 Gy of ionizing radiation (IR)] both proteins colocalized to IR-induced foci and suppression of the expression of either of them by siRNA did not affect recruitment of the other protein to the sites of DNA DSBs (data not shown). Exposure of NHDFs to a relatively low dose of IR (1 Gy) led to recruitment of 53BP1, but not NFB1/MDC1, to IR-induced foci. At this dose, suppression of 53BP1 expression by siRNA facilitated recruitment of NFB1/MDC1 to IR-induced foci, whereas suppression of NFB1/MDC1 expression by siRNA had no effect on 53BP1-induced focus formation (Fig. 1A). Similar results were observed with fibroblasts derived from an individual with XPC, in which suppression of 53BP1 expression led to increased localization of NFB1/MDC1 and of Nbs1 to sites of DNA DSBs (Fig. 1B). The increased localization of Nbs1 to sites of DNA DSBs in response to suppression of 53BP1 expression was accompanied by increased phosphorylation at Ser343 (Fig. 1C), suggesting that in the absence of 53BP1 a greater pool of Nbs1 participates in ATM signaling and becomes phosphorylated by ATM. We conclude that 53BP1 and NFB1/MDC1-Nbs1 are recruited to sites of DNA DSBs independently of each other, although there is some cross-talk because suppression of 53BP1 expression in cells exposed to low doses of IR is accompanied by increased recruitment of NFB1/MDC1 and Nbs1 to sites of DNA DSBs. If 53BP1 and NFB1/MDC1 perform similar functions in DNA DSB signaling, then their independent recruitment...
Defective Chk2 Ser^{33/35} Phosphorylation and ATM Activation in NBS Cells. NFB1/MDC1 associates with Nbs1 (5), raising the possibility that loss of NFB1/MDC1 and Nbs1 function may have similar phenotypes. Therefore, as a first step in exploring whether NFB1/MDC1 is required for ATM activation, we examined cells from patients with NBS, in which the nbs1 gene is mutated. Previous studies indicate that NBS cells exposed to IR show decreased phosphorylation of ATM substrates. Expression of wild-type Nbs1 reverses these defects, but NBS cells expressing Nbs1, in which Ser^{343} has been substituted with Ala, still show at least a partial defect in phosphorylation of ATM substrates (15–18). A recent report further indicates that ATM activation is defective in NBS cells and that the defect can be reconstituted with wild-type Nbs1 (21). However, the ability of the Nbs1 Ala^{343} mutant to reconstitute ATM activation was not examined.

Using NBS cells that were infected with a control retrovirus or infected with retroviruses expressing wild-type Nbs1 or Nbs1, in which Ser^{343} was substituted with alanine, we observed a delay in the electrophoretic mobility shift of Chk2 after irradiation in the control NBS cells and, to a lesser extent, in the NBS cells expressing the Ala^{343} mutant (Fig. 3A). Chk2 phosphorylation at Thr^{68} was evident in all cells, but Chk2 phosphorylation at Ser^{33/35} was absent in the control NBS cells and the NBS cells expressing the Ala^{343} mutant (Fig. 3A). We also examined phosphorylation of SMC1 at Ser^{345}, which Ser343 was substituted with alanine, we observed a delay in the electrophoretic mobility shift of Chk2 after irradiation in the control NBS cells and, to a lesser extent, in the NBS cells expressing the Nbs1 Ala^{343} mutant (Fig. 3B).

Interestingly, the effect of loss of Nbs1 function on Chk2 phosphorylation at Thr^{68} and Ser^{33/35} exactly paralleled the effect of partial loss of ATM function by siRNA in NHDfs and HeLa cells (Fig. 2D). We therefore examined whether ATM activation, as monitored by autophosphorylation at Ser^{1981}, was defective in irradiated NBS cells. The control NBS cells showed almost complete absence of ATM phosphorylation when exposed to 1 Gy of IR and modest ATM phosphorylation when exposed to 9 Gy of IR, whereas the NBS cells reconstituted with wild-type Nbs1 or, interestingly, with the Nbs1 Ala^{343} mutant showed robust ATM phosphorylation after exposure to both doses of IR (Fig. 3C). Consistent with a recent report (21), these findings suggest that Nbs1 is required for ATM activation. Furthermore, the Nbs1 Ala^{343} mutant dissociates the function of Nbs1 required for ATM activation from its adaptor/mediator function downstream of ATM.

Defective Chk2 Ser^{33/35} Phosphorylation and ATM Activation after Suppression of NFB1/MDC1. Because NFB1/MDC1 is thought to function in the same complex as Nbs1, we next sought to
examine whether it also plays a role in ATM activation. We first suppressed NFBD1/MDC1 expression by siRNA in NHDFs. When these primary cells were exposed to 9 Gy of IR, suppression of NFBD1/MDC1 led to diminished phosphorylation of Chk2 at Ser\(^{33/35}\) but phosphorylation at Thr\(^{68}\) was unaffected (Fig. 4A). This phenotype, which is similar to that observed in NBS cells, was also observed when Nbs1 expression was suppressed by siRNA in NHDFs (Fig. 4A).

The effect of suppressing NFBD1/MDC1 on ATM autophosphorylation at Ser\(^{1981}\) was examined in two cell lines in which we could achieve very efficient suppression of NFBD1/MDC1 protein levels with siRNA. In HeLa cells exposed to 1 Gy of IR, suppression of NFBD1/MDC1 led to decreased ATM phosphorylation at Ser\(^{1981}\) (Fig. 4B). Similarly, in an immortalized fibroblast cell line derived from a patient with XPC, which we were studying in the context of another research program, suppression of NFBD1/MDC1 led to decreased ATM autophosphorylation at Ser\(^{1981}\) after exposure of the cells to either 1 or 9 Gy of IR (Fig. 4C).

**Defective Chk2 Phosphorylation and ATM Activation after Suppression of 53BP1 in NBS cells.** Like NFBD1/MDC1, 53BP1 has COOH-terminal BRCT repeats and has been implicated in ATM signaling pathways. Suppression of 53BP1 function by siRNA or gene knockout leads to modest cell cycle checkpoint defects and to decreased ATM-dependent substrate phosphorylation at sites of DNA DSBs, as detected by immunofluorescence. However, with the exception of one report, suppression of 53BP1 function has no effect on Chk2 phosphorylation (4, 11–14). We have also seen no effect on Chk2 phosphorylation at Thr\(^{68}\) or even at Ser\(^{33/35}\) when 53BP1 levels were suppressed by siRNA in NHDFs, HeLa, or U2OS osteosarcoma cells (data not shown). One possible explanation for this finding is that 53BP1 and the NFBD1/MDC1–Nbs1 complex have redundant functions. Accordingly, the effects of suppressing 53BP1 function on DNA damage signaling might be stronger in cells with defective NFBD1/MDC1–Nbs1 function. Indeed, in the control NBS cells, suppression of 53BP1 led to decreased phosphorylation of Chk2 at Thr\(^{68}\) decreased phosphorylation of SMC1 at Ser\(^{957}\), as well as decreased autophosphorylation of ATM at Ser\(^{1981}\) (Fig. 5A). In the NBS cells reconstituted with wild-type Nbs1, suppression of 53BP1 expression had no effect on the electrophoretic mobility supershift of Chk2 after irradiation or on the phosphorylation of Chk2 at Ser\(^{33/35}\), SMC1 at Ser\(^{957}\) and ATM at Ser\(^{1981}\) (Fig. 5B).

**Discussion**

The major obstacle toward establishing whether the adaptors/mediators 53BP1 and NFBD1/MDC1 function upstream or downstream...
of ATM had been the lack of a sensitive assay to monitor ATM activation. This obstacle was overcome recently by development of a phosophospecific antibody that monitors ATM autophosphorylation at Ser1981 (23). On the basis of our results obtained with this antibody, we propose that 53BP1 and NFBD1/MDC1 activate ATM through independent pathways. This hypothesis is further supported by the observation that suppression of 53BP1 leads to increased recruitment of NFBD1/MDC1 and Nbs1 to sites of DNA DSBs, as well as to increased phosphorylation of Nbs1 at Ser343. This also indicates that once one pathway, e.g., the 53BP1 pathway, is established to activate ATM, then the other redundant pathways are inhibited. As shown in Fig. 5C, we consider that the Mre11–Rad50–Nbs1 complex functions within the context of the NFBD1/MDC1 pathway because recruitment of Nbs1 to sites of DNA DSBs is dependent on NFBD1/MDC1 and because NFBD1/MDC1 was identified as the fourth component of a purified Mre11–Rad50–Nbs1–NFBD1/MDC1 complex (5). Our observations do not allow us to establish whether the 53BP1 and NFBD1/MDC1 pathways are the only pathways that can activate ATM. It is possible that additional pathways may also activate ATM, which would explain why suppression of 53BP1 in NBS cells did not lead to complete loss of ATM autophosphorylation at Ser1981 after irradiation.

Our studies do not address the molecular mechanism by which 53BP1 and NFBD1/MDC1 activate ATM. In the case of NFBD1/MDC1, the mechanism may involve recruitment of the Mre11–Rad50–Nbs1 complex to sites of DNA DSBs, which may then recruit and activate ATM. In support of this mechanism, in human cells retention of ATM on chromatin after DNA damage is dependent on the Mre11–Rad50–Nbs1 complex (21, 22), and in budding yeast Xrs2 recruits Tel1 to sites of DNA DSBs (30). A similar mechanism may underlie activation of ATM by 53BP1 because 53BP1 and ATM coimmunoprecipitate in irradiated, but not in control, cells (12). Irrespective of the mechanism, our findings are not inconsistent with a recent model proposing that ATM activation is initiated by DNA DSB-initiated changes in chromatin structure that lead to autophosphorylation of ATM at Ser1981 and dissociation of inactive ATM dimers into active ATM monomers (23). The DNA DSB-initiated change in chromatin structure may be the stimulus for recruitment of 53BP1 or NFBD1/MDC1 to megadaltons of DNA flanking the DSB and, subsequently, for ATM recruitment and activation.

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References

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