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Characterization of the mechanisms of ATM activation by the MRN complex and DNA

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Characterization of the mechanisms of ATM activation by the MRN complex and DNA

by

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Dedication

I dedicate this dissertation to my wife Hyunmin, my lovely daughter Hanna, and my parents and in-laws for all their years of love and support.

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Characterization of the mechanisms of ATM activation by the MRN complex and DNA

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The complex containing the Mre11, Rad50, and Nbs1 proteins (MRN) is essential for the cellular response to DNA double-strand breaks, integrating DNA repair with the activation of checkpoint signaling through the protein kinase ATM (ataxia Telangiectasia mutated). The ATM kinase signals the presence of DNA double-strand breaks in mammalian cells by phosphorylating proteins which initiate cell cycle arrest or apoptosis. We demonstrate that MRN stimulates the kinase activity of ATM in vitro toward its substrates p53, Chk2, and histone H2AX. We also show that the MRN complex acts as the double-strand break sensor for ATM and recruits ATM to broken DNA molecules. Inactive ATM dimers can be activated in vitro with DNA in the presence of MRN, leading to phosphorylation of downstream targets p53 and Chk2. ATM dimers are dissociated into monomers by MRN in a process that does not require ATM autophosphorylation. Unwinding of DNA ends by MRN is required for ATM stimulation, consistent with the central role of single-stranded DNA as an evolutionary conserved signal for DNA damage.

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CHAPTER 1 : INTRODUCTION

DNA Damage

Maintaining genetic content is essential for cells to exhibit normal function. Genomic instability can be caused by errors during DNA synthesis, DNA-damaging agents, and several kinds of radiation. Endogenous DNA-damaging agents can be produced during normal cellular metabolism and include superoxide ions, hydroxyl radicals, and hydrogen peroxide. DNA can be also damaged by exogenous chemicals, such as antitumor agent bleomycin, cross-linking agents, and alkylating agents, including methyl methanesulfonate (MMS). Ultraviolet radiation and ionizing radiation (IR) can also cause DNA damage.

One of the most cytotoxic forms of DNA lesion is the double-strand break (DSB). DNA DSBs are generated when the complementary strands of the DNA double helix are broken on either side of the helix. Although DSBs are a most dangerous form of DNA damage in cells, they are sometimes generated during normal cellular processes, for instance, meiotic recombination, and immunoglobulin gene rearrangement (reviewed in Khanna and Jackson 2001; reviewed in Jackson 2002). Ionizing radiation can also cause DSBs. If the DSB is not repaired, it may lead to the loss of genetic information and/or cell death. On the other hand, if DSBs are repaired improperly, mutations and chromosomal rearrangements can occur, ultimately leading to the development of cancer in multicellular organisms. Therefore, cells must detect and repair DNA damage to maintain genomic stability. (reviewed in Hoeijmakers 2001; reviewed in Shiloh 2003).

Mechanisms of DNA Double-Strand Break Repair

Cells can repair DSBs by two major pathways - homologous recombination (HR) and nonhomologous end joining (NHEJ). During G_1 and early S-phase, NHEJ is the more predominant repair pathway while HR is used primarily during late S-phase and G2. NHEJ is also known to be the preferred method for repairing DSBs in dividing mammalian cells as opposed to *S.cerevisiae* which prefers HR (Takata *et al.* 1998).

HR repairs DSBs using the undamaged sister chromatid or homologous chromosome as a template, removing damage in an error-free process. HR is performed by the Rad52 epistasis group of proteins, a large family of proteins including RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11, and XRS2. The first step of HR is recognition of the damaged DNA ends, followed by nucleolytic processing of the DNA, which produces a single-stranded 3' overhang at each broken end. After end processing, Rad51 is recruited to the RPA-coated single stranded DNA. Rad51, aided by Rad52, Rad54, Rad55, and Rad57, promotes strand exchange followed by branch migration and Holliday junction resolution (reviewed in Haber 2000; reviewed in Symington 2002)

In contrast to HR, NHEJ is an error-prone repair mechanism which does not use a homologous template to couple the DNA ends generated in DSBs. First, broken ends are processed to make them compatible and then rejoined and ligated together (reviewed in van Gent *et al.* 2001). The initial step of NHEJ is started with binding of the Ku protein to the broken ends of the DNA, followed by recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs phosphorylates many repair proteins, including Artemis, WRN, and XRCC4 (Anderson and Lees-Miller 1992; Leber *et al.* 1998; Yannone *et al.* 2001). After DNA end processing, gaps are filled with polymerases

and ligated together by a complex that consists of DNA ligase IV and Xrcc4 (reviewed in van Gent *et al.* 2001).

The cellular response to DSBs

The cellular response to DNA damage involves cell cycle arrest followed by DNA repair. In some cell types, unrepairable DNA damage triggers apoptosis to prevent the propagation of damaged DNA to daughter cells. Failure to trigger apoptosis by these damaged cells can lead to the improper segregation of the damaged DNA and genomic instability.

The cellular response to DNA damage requires sensor proteins to detect the DNA damage and elicit a signal, which can be relayed to effectors through the activity of intermediates called transducers. Transducers control DNA repair, cell cycle arrest, and apoptosis through the posttranslational modification of effectors directly or indirectly (reviewed in Shiloh 2003).

Several candidate proteins, such as breast cancer 1 (BRCA1), p53 binding protein 1 (53BP1), mediator of DNA damage checkpoint 1 (MDC1), the Mre11/Rad50/Nbs1 complex (MRN), Rad17-replication factor C (RFC), and Rad9/Rad1/Hus1(9-1-1), have been implicated as sensor proteins. However, the mode of DNA damage recognition are not well understood. It could be possible that the sensor proteins are triggered upon the presence of single-stranded DNA (ssDNA) or breaks in DNA (reviewed in Friedberg 1995). More recently, sensor proteins have been implicated in recognizing changes in chromatin structure that occur following DNA damage (Bakkenist and Kastan 2003). One initial change that happens to chromatin after a DSB is the phosphorylation of the C-terminal tail of the histone H2A variant H2AX. This suggests that it is possible that the

phosphorylated H2AX (γ-H2AX) may recruit sensor proteins to DSBs to initiate checkpoint pathways (Paull *et al.* 2000; Celeste *et al.* 2002).

The role of transducers in the DNA damage response is to transmit the signal of DNA damage to downstream effectors. Several mammalian transducers have been reported, such as ATM, ATR, and DNA-dependent protein kinase(DNA-PK), kinases that belong to PI3K-like protein kinases (PIKKs). These kinases are activated by different stimuli. ATM and DNA-PK are involved in the response to DSBs and ATR is involved in the response to UV, stalled replication forks and hypoxia. It is also known that ATR is activated in the later stages of the DSB response, although ATM is mainly responsible for sensing DSB during the initial phases of the damage response (reviewed in Shiloh 2003).

Ataxia Telangiectasia Mutated (ATM)

ATM is a serine-threonine kinase that is activated when cells are exposed to DNA DSBs (Kim *et al.* 2002; Bakkenist and Kastan 2003). The importance of ATM in response to DSBs was first observed in the genomic instability syndrome Ataxia-Telangiectasia (A-T), which is caused by mutations in the ATM gene.

Ataxia-Telangiectasia

A-T is a rare autosomal recessive disorder with a complex phenotype. It is characterized by cerebellar degeneration, immunodeficiency, hypersensitivity to DSB-inducing agents, thymic and gonadal atrophy, telangiectasia, and an increased risk of cancer (Crawford 1998). The severe phenotypes in cells from A-T patients also

emphasize the importance of ATM in response to DSBs. A-T cells show hypersensitivity after treatment to IR or radiomimetic agents although they show normal response to other types of genotoxic stimuli, such as UV radiation, alkylating agents, and replication blocks (McKinnon 1987; Shiloh 1997). A-T cells also exhibit defects in cell cycle checkpoint in response to IR. A-T cells fail to arrest at the G1/S and G2/M transitions after exposure to IR (Beamish *et al.* 1996; Morgan *et al.* 1997; reviewed in Petrini 2000). Individuals with A-T have mutations in the ATM gene that results in its loss or inactivation. More than 300 mutations in the ATM gene have been reported (http://www.vmmc.org/vmrc/atm.htm). Approximately 70% of mutations in ATM gene produce truncated and usually unstable products while 30% of mutations are missense mutations and small in-frame deletions/insertions, mostly clustered at the 3' end. Most of the patients are compound heterozygotes for null mutations of ATM and, in many populations, there is a strong founder effect (Khanna 2000).

Structure of ATM

ATM belongs to a superfamily of PI3K-related kinases (PIKK), each of which contains C-terminal kinase domain that is structurally related to PI-3 lipid kinases (Fig. 1.1A). This family is conserved from fungi to humans, and includes: Mec1 and Tel1 in *Saccharomyces cerevisiae*, Rad3 and Tel1 in *Saccharomyces pombe*, Mei-41 in *Drosophila melanogaster*, DNA-Pkcs, ATM, ATR, and mammalian target of Rapamycin (mTOR), also known as FKBP12 and rapamycin associated protein (FRAP), in mammals (reviewed in Khanna *et al.* 2001). Most of the PIKK family have been identified as protein kinases involved in the DNA damage except FRAP, which functions in response to nutrient levels and mitogenic stimuli (reviewed in Proud 2002; Shiloh 2003). The

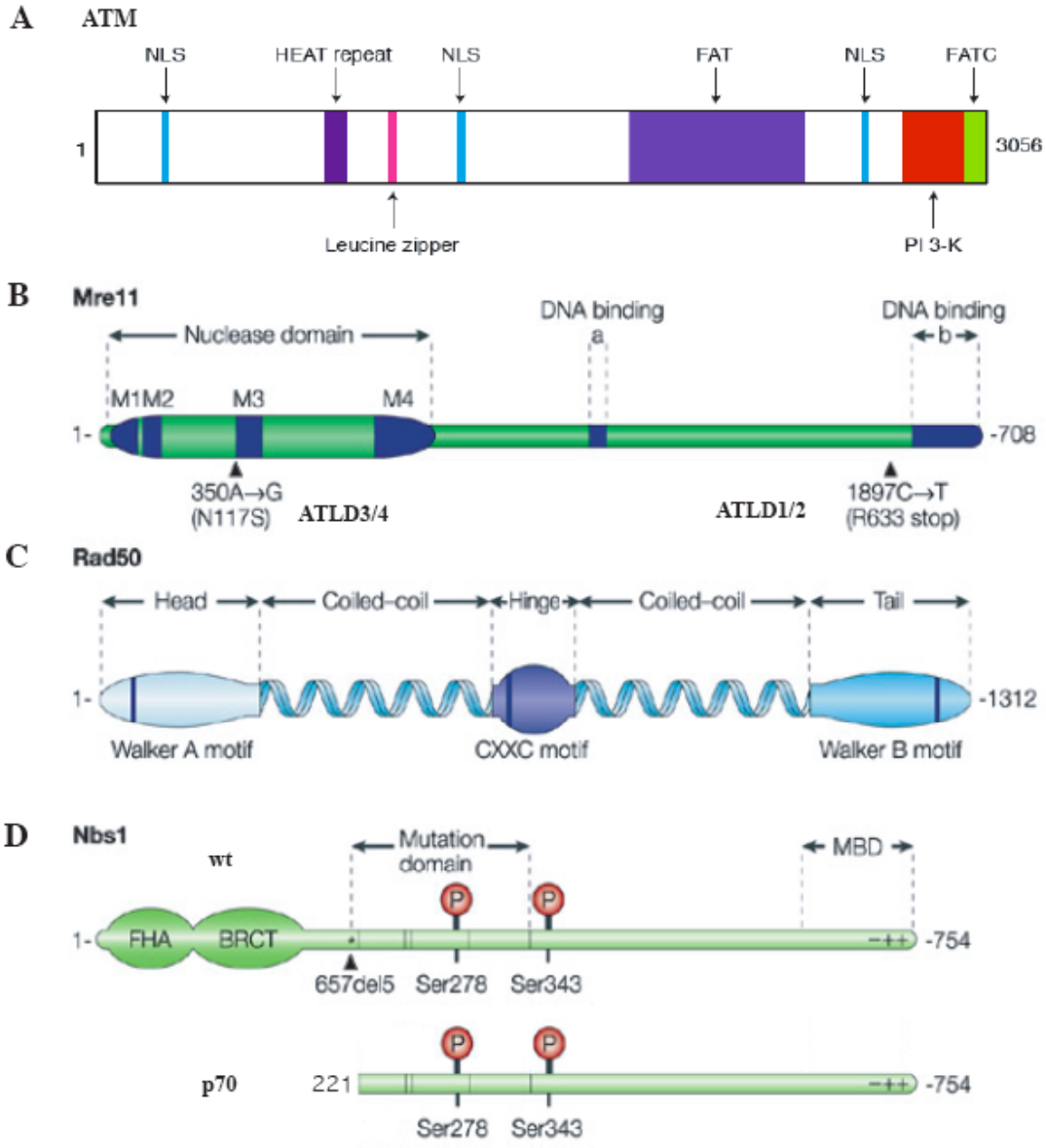

Figure 1.1 Schematic diagram of ATM, Mre11, Rad50, and Nbs1.

A, The ATM protein. The domains are as follows: NLS, nuclear localization signal; HEAT, sequence element common to the Huntington protein (H), elongation factor 3 (EF3) (E), the regulatory (A) subunit of protein phosphatase 2a (A), and the Tor 1p protein (T); FAT, a domain found in FRAP (F), ATM (A) and TRRAP (T) proteins; PI 3-K, a region containing signatures of the catalytic subunit of phosphatidylinositol 3-

Continued on the next page

kinase; FATC, a C-terminal domain common to the PI 3-K-related kinases. Abbreviations: FATC, extreme C-termini of FAT proteins with a highly conserved 30 residue tail. **B**, Mre11 protein. The blue regions that are labeled M1 to M4 above Mre11 represent the relative positions of the four conserved phosphoesterase motif. The triangle below Mre11 indicate the positions of the residues that are affected by mutations in patients with ataxia-telangiectasia-like disorder (ATLD). **C**, Rad50 protein. Rad50 is made up of N- and C-terminal catalytic domains, a long coiled-coil, and a hook domain at the center of the coiled-coil. The hook domain enables the formation of an anti-parallel coiled-coil to bring the N- and the C-terminal domains together to form the catalytic site. **D**, wild-type and mutant Nbs1 proteins. Shown are locations of the forkhead associated (FHA), BRCA1 carboxyl-terminal (BRCT) and Mre11-binding domain (MBD), and phosphorylation sites on Ser-278 and Ser-343. The vertical lines on Nbs1 represent the positions of residues affected by mutations that occur in NBS patients, whereas the star highlights the residue that is affected by the most common mutation (657del5). A is adapted by permission from Cambridge University Press: Expert Reviews in Molecular Medicine 5: 1-21; copyright 2003. B-D are adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology 3: 317-327; copyright 2002.

catalytic domain contains a PI3K motif and is located in the carboxyl-terminal regions of PIKKs. PIKKs also have a conserved domains named FAT (FRAP, ATM, and TRRAP) and FATC (FRAP, ATM, and TRRAP C-terminal). While the significance of the FAT and FATC domains are still unknown, the PI3K kinase domain is responsible for phosphorylating serine and threonine residues followed by glutamine in target substrates. Despite the relationship with lipid kinases, the damage-induced PIKKs exclusively phosphorylate protein substrates (reviewed in Shiloh 2003).

ATM also contains another predicted functional domain containing HEAT repeat (Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A, and TOR1) (Perry and Kleckner 2003). It has been demonstrated that HEAT repeats are constructed from an A and a B helix connected by a short turn, thereby forming superhelical scaffolding matices that can interact with other proteins, such as protein containing FXFG motif (reviewed in Bayliss *et al.* 2000; Perry and Kleckner 2003). Recently, You *et al.* showed that Tel1, an ATM homolog in fission yeast, interacts with an FXF/Y motif at the C-terminus of Nbs1, suggesting that HEAT repeat domain may mediate Nbs1 binding to ATM (You *et al.* 2005).

Catalytic activity of ATM

The importance of ATM kinase activity in response to IR was first suggested by Banin *et al.* and Canman *et al.* In 1998, these two groups showed that p53 is phosphorylated *in vivo* at Ser15 after DNA damage and this activity was markedly enhanced within minutes after treatment with IR or a radiomimetic drug. They also showed that immunoprecipitated ATM phosphorylates at Ser-15 of p53 *in vitro* (Banin *et al.* 1998; Canman *et al.* 1998). Canman *et al.* also showed that ATM kinase activity

toward p53 is not increased after treatment with UV. Many groups have shown that ATM phosphorylates a number of target substrates, including BRCA1, Nbs1 and Chk2, after exposure to IR but not to UV (Cortez *et al.* 1999; Gatei *et al.* 2000a; Gatei *et al.* 2000b; Matsuoka *et al.* 2000). ATM can also be activated after treatment with DSB-inducing agents, such as neocarzinostatin (NCS), a radiomimetic agent.

Several groups have shown that the kinase activities of both ATM and ATR were dependent on manganese *in vitro* (Banin *et al.* 1998; Canman *et al.* 1998; Chan *et al.* 2000). So far, most experiments investigating ATM kinase activity were performed using both manganese and magnesium *in vitro*. Like other PIKKs such as DNA-Pkcs and ATR, the kinase activity of ATM can be inhibited by wortmannin, a fungal metabolite (Banin *et al.* 1998; Sarkaria *et al.* 1998). Caffeine is also known to be an inhibitor of ATM and ATR (Blasina *et al.* 1999; Sarkaria *et al.* 1999; Zhou and Elledge 2000). Like ATR, ATM preferentially phosphorylates serine or threonine residues followed by glutamine (S/TQ motif) (Kim *et al.* 1999).

ATM checkpoint functions

Functions of ATM in the G1/S checkpoint

It has been shown that the stabilization of the tumor suppressor protein p53 is critical for the damage-induced G1/S checkpoint. Activated p53 can stimulate transcription of target genes, such as the cyclin-dependent kinase (CDK) inhibitor p21, and Gadd45. p21 arrests cells in G1 and blocks S phase entry by directly interacting with G1/S cyclin/CDK complexes and proliferating cell nuclear antigen (PCNA). Gadd45 also binds PCNA, causing inhibition of DNA replication (Abraham 2001). p53 has a high turnover in undamaged cells but it is stabilized after DNA damage. Many studies show

that there are number of post-translationally modified sites on p53 and modification of these sites is required for its stability.

ATM phosphorylates the tumor suppressor p53 on Ser-15 directly and Ser-20 indirectly through Chk2 (Banin *et al.* 1998; Canman *et al.* 1998; Chehab *et al.* 2000; Melchionna *et al.* 2000). Phosphorylation of p53 on Ser-15 does not affect its degradation or its association with MDM2, which is its negative regulator (Chehab *et al.* 1999; Dumaz *et al.* 2001). However, Dumaz and Meek showed that phosphorylation of Ser15 stimulates p53-dependent transactivation, which occurs through an increased binding to the p300 coactivator protein (Dumaz and Meek 1999). Another phosphorylation site on p53 regulated by ATM is Ser-20, which is regulated by Chk2. ATM phosphorylates Thr-68 on Chk2, which stimulates Chk2 autophosphorylation and activation of Chk2 kinase function. Several studies showed that phosphorylation of Ser-20 on p53 disrupts interaction between p53 and MDM2, thereby attenuating degradation of p53 (Chehab *et al.* 1999; Melchionna *et al.* 2000; Dumaz *et al.* 2001). Therefore, ATM not only regulates phosphorylation of p53 directly on Ser-15 to enhance its transcriptional activity, but also contributes to its stabilization by activating Chk2 (Abraham 2001). Recently, Fabbro *et al.* showed that the BRCA1-BARD1 complex is also required for ATM-dependent p53 phosphorylation on Ser-15, and that repressing this phosphorylation by depletion of BRCA1-BARD1 compromises G1/S checkpoint arrest in response to IR (Fabbro *et al.* 2004).

ATM also stabilizes p53 by phosphorylating MDM2. MDM2 is a negative regulator that promotes ubiquitin-mediated degradation of p53. Interestingly, in response to IR, but not UV, MDM2 is phosphorylated rapidly in an ATM-dependent manner, resulting in p53 accumulation (Khosravi *et al.* 1999). Another group showed

that MDM2 phosphorylated on Ser-395 by ATM *in vitro* and *in vivo* and that Mdm2 S395D was impaired in promoting p53 degradation. They also showed that S395D Mdm2 is markedly less able to promote p53 cytoplasmic export, suggesting its reduced ability to promote p53 degradation (Maya *et al.* 2001).

ATM is also required for phosphorylating Rad17 at Ser-635 and 645 and Rad9 at Ser-272. These phosphorylation events are essential for G1/S phase arrest after exposure to IR (Chen *et al.* 2001b; Post *et al.* 2001). Bao *et al.* showed that overexpression of mutant Rad17 proteins containing S635A and S645A mutations in ATM phosphorylation sites abrogated the G2 checkpoint after DNA damage, suggesting that phosphorylation of both sites are required for G2 checkpoint (Bao *et al.* 2001)

Functions of ATM in the S phase checkpoint

After DNA damage, ATM blocks entry into S phase through a series of phosphorylation events, whose final target is Cdc25A, a tyrosine phosphatase. Normal cell cycle progression requires Cdc25A dephosphorylation of Cdk 2, which activates the cyclin A/Cdk 2 complex, which is required for the initiation of DNA replication at G1/S transition (Abraham 2001). After DNA damage, ATM phosphorylates Chk2 and activated Chk2 phosphorylates Cdc25A on Ser-123 after exposure to IR. Phosphorylated Cdc25A is degraded by ubiquitination-mediated proteolysis, thereby inactivating cyclin A/Cdk 2 complexes (Mailand *et al.* 2000; Falck *et al.* 2001).

Recent studies have suggested that there is another branch of the S phase checkpoint pathway mediated by cohesin complexes that is distinct from the ATM/Chk2/CDC25A branch. Two groups showed that ATM phosphorylates SMC1 on Ser-957 and 966 *in vitro* and *in vivo*, and that mutations in these sites abrogates the S

phase cell cycle checkpoint after exposure to IR (Kim *et al.* 2002; Yazdi *et al.* 2002). Yazdi *et al.* showed that the phosphorylation of NBS1 by ATM is required for the phosphorylation of SMC1.

Recent studies also showed that breast cancer susceptibility protein (BRCA1) and FANCD2 (one of the Fanconi anemia proteins), which are phosphorylated by ATM after IR treatments, are also related to the S phase checkpoint. The phosphorylation of FANCD2 on Ser-222 is required for the S phase checkpoint activation (Taniguchi *et al.* 2002). Xu *et al.* showed that phosphorylation of BRCA1 on Ser-1387 is required for the ATM-mediated S phase checkpoint in response to IR (Xu *et al.* 2002b). However, the mechanisms of the activation of S phase checkpoint through the phosphorylation of these sites are still unclear.

Functions of ATM in the G2/M checkpoint

Progression from G2 to M phase requires Cdc25C which dephosphorylates inhibitory sites on Cdc2 (Abraham 2001). A-T cells are deficient in IR-induced rapid inhibition of Cdc2/cyclin B (Beamish *et al.* 1996), which is required for the G2/M transition. Chk2 null cells are also defective in the maintenance of checkpoint-induced G2 arrest (Hirao *et al.* 2000). Moreover, several groups have shown that Chk2 phosphorylates Cdc25C at Ser-216 *in vitro*, which inhibits its catalytic activity (Matsuoka *et al.* 1998; Brown *et al.* 1999). Therefore, an ATM/Chk2/Cdc25C/Cdc2 pathway has been proposed as the mechanism of ATM-dependent G2/M checkpoint arrest.

Another mechanism to control the G2/M checkpoint through ATM is mediated by p53. Cdc2 activity is regulated by 14-3-3, p21, and Gadd45, which are proteins induced

by p53. Cdc2 is inhibited by p21 directly, anchored in the cytoplasm by 14-3-3, and dissociated from cyclin B by Gadd 45 (Taylor and Stark 2001).

Like the S phase checkpoint, Xu *et al.* suggested that the G2/M checkpoint also requires phosphorylation of BRCA1 (Xu *et al.* 2001). They showed that phosphorylation at Ser-1423 on BRCA1 depends on ATM and that it is required for the immediate arrest of G2 cells in response to IR. Another group suggested that ATM can also control G2/M checkpoint by phosphorylating the BRCA1-associated protein CtIP, which is a transcriptional repressor of the BRCA1 gene. They showed that mutations in the ATM-dependent phosphorylation site in CtIP abrogates the dissociation of BRCA1 from CtIP, resulting in constant repression of BRCA1-dependent induction of Gadd45 after exposure to IR (Li *et al.* 2000).

Other target substrates of ATM

ATM is responsible for the phosphorylation of numerous proteins in response to DSBs. It phosphorylates substrates in every phase of the cell cycle, leading to cell cycle arrest, transcription, DNA repair, and apoptosis. Some of the common ATM substrates, such as p53, Chk2, BRCA1, MDM2, SMC1, FANCD2, and CtIP, are already discussed in the previous sections in detail about the role of ATM in the cell cycle.

H2AX is phosphorylated at Ser-139 by ATM following DSB and occurs very early after DNA damage. Thus, γ-H2AX, the phosphorylated form of H2AX, is thought to be one of the initial proteins at DSB sites. Burma *et al.* showed that H2AX phosphorylation and nuclear focus formation are normal in DNA-PKcs$^{-/-}$ cells and is severely compromised in ATM$^{-/-}$ cells in response to IR. This suggests that ATM is the primary kinase that phosphorylates H2AX in response to DSB. They also showed that ATM$^{-/-}$ cells

showed poor γ-H2AX focus formation and this minimal H2AX phosphorylation is abolished by wortmainin suggesting that DNA-PK, rather than ATR, is responsible for this phosphorylation in the absence of ATM (Burma *et al.* 2001). However, another group showed that γ-H2AX formation is suppressed in ATR deficient cells and markedly decreased in DNA-PK deficient cells but is not abrogated in ataxia telangiectasia cells at the sites of replication-mediated DNA double-strand breaks induced by mammalian DNA Topoisomerase I cleavage (Furuta *et al.* 2003). It is likely that all of the DNA damage-induced PIKKs contribute to γ-H2AX formation after DSBs.

Mdc1 and 53BP1 are also reported to be hyperphosphorylated in response to IR in an ATM-dependent manner. Both proteins colocalize with γ-H2AX and the MRN complex in response to IR (Anderson *et al.* 2001b; Rappold *et al.* 2001; Xu and Stern 2003a). However, the exact phosphorylation sites on both proteins are still not known. The functions of these proteins in ATM signaling will be discussed in more detail in the Chapter 5, conclusions and future directions.

E2F1 is phosphorylated at Ser-31 by ATM. This phosphorylation is required for the stabilization of E2F1 (Lin *et al.* 2001). It has been shown that one mechanism of E2F1-mediated apoptosis is through p53, and that this signaling is partially mediated by p14ARF, which interacts with MDM2 and prevents it from targeting p53 for degradation. Thus the stabilized p53 can induce the expression of genes involved in cell cycle arrest and apoptosis (reviewed in Sherr and Weber 2000). However, a recent study shows that p14ARF is dispensable for apoptosis induced by the ectopic expression of E2F1, suggesting that p14ARF is not an essential mediator of E2F1-induced apoptosis (Russell *et al.* 2002; Powers *et al.* 2004).

The tyrosine kinase c-Abl is phosphorylated by ATM on Ser-465 in response to IR (Baskaran *et al.* 1997; Shafman *et al.* 1997). c-Abl regulates Rad51 and Rad52, the homologous recombination repair proteins, by phosphorylating them on tyrosine 54 and on tyrosine 104 respectively in response to IR (Yuan *et al.* 1998; Kitao and Yuan 2002). Both Rad51 and Rad52 phosphorylation is important for assembly of the Rad51/Rad52 complex and subsequent DSB repair via HR after DNA damage (Chen *et al.* 1999; Kitao and Yuan 2002). These findings suggest that ATM may regulate the Rad51/Rad52 complex through c-Abl. This possibility can be supported by recent study showing that Rad51 and focus formation is defective in both ATM-/- and c-Abl-/- cells after exposure to IR (Yuan *et al.* 2003).

BLM is a helicase that is mutated in Bloom's syndrome, which is characterized by a broad range of symptoms, including immunodeficiency, telangiectatic erythema, growth retardation, and chromosome aberrations, and predisposition to several kinds of cancers (reviewed in Amor-Gueret 2005). Some of these characteristics are shared with A-T. These findings suggest that ATM and BLM are in the same pathway. In addition, BLM is phosphorylated on Thr 99 and 122 by ATM after exposure to IR (Bender *et al.* 2002). A recent study showed that phosphorylation of BLM by ATR is required for correct relocalization of the MRN complex at sites of stalled replication forks after treatment with hydroxyurea but not after IR (Franchitto and Pichierri 2002). However, the functional relationship between ATM and BLM is still unclear. BLM is thought to interact with many proteins involved in replication, recombination, and repair, suggesting that ATM may regulate these functions of BLM.

Pin2/TRF1, a telomeric protein that negatively regulates telomere elongation, is phosphorylated by ATM on Ser-219 after IR-induced DNA damage *in vivo* and *in vitro*

(Kishi *et al.* 2001b). Previous studies showed that overexpression of Pin2/TRF1 can induce entry into mitosis and apoptosis (Shen *et al.* 1997; Kishi *et al.* 2001a). Kishi *et al.* showed that Pin2 (S219A) mutant induces mitotic entry and apoptosis but Pin2 (S219D and S219E) mutant completely failed to induce apoptosis. This suggests that phosphorylation of Pin2/TRF1 on Ser-219 inhibits Pin2/TRF1 function, resulting in G2/M arrest and suppression of apoptosis.

The Mre11/Rad50/Nbs1 (MRN) complex

The MRN complex, which is composed of three proteins, Mre11, Rad50, and Nbs1, is essential for DNA double strand break repair in mammals. The MRN complex mediates diverse functions in the DNA damage response, including homologous recombination, non-homologous end joining, and telomere maintenance (reviewed in Haber 1998; reviewed in D'Amours and Jackson 2002).

The MRN complex was first recognized in *Saccharomyces cerevisiae*. Rad50 and Xrs2 were identified in 1974 and 1992, respectively, in genetic screen for mutants sensitive to ionizing radiation (Game and Mortimer 1974; Ivanov *et al.* 1992). Xrs2 is the yeast ortholog of the vertebrate protein Nbs1. The Mre11 gene was identified in a genetic screen for mutants deficient in meiotic recombination (Ajimura *et al.* 1993). Mre11, Rad50, and Xrs2 deletion strains exhibit identical phenotypes and later, these proteins were found to form a complex (Usui *et al.* 1998).

The role in DNA repair

The inactivation of Mre11, Rad50, or Xrs2 affects a number of DNA metabolic processes, including HR and NHEJ in yeast, suggesting that the MRX complex has a role in both HR and NHEJ (reviewed in van den Bosch *et al.* 2002). Mre11, Rad50 and Xrs2 are classified under the Rad52 epistasis group of proteins, which are essential for the HR pathway of DNA repair. During meiosis, the MRX complex is thought to be involved in the initial formation of the DSB at genomic hotspots, which are regions with unusually high levels of recombination, for the purpose of genetic recombination. The MRX complex is then involved in the subsequent resection of the breaks to produce 3' overhangs that serve as a substrate for the DNA repair machinery (Cao *et al.* 1990).

Several studies suggested that the MRX complex may be involved in the HR pathway since nuclease-deficient MRX complexes exhibit defective processing of DSB ends *in vitro* (Paull and Gellert 1998; Tsubouchi and Ogawa 1998; Moreau *et al.* 1999). However, the role of the MRX complex in DSB repair by HR is controversial since deletion of Mre11, Rad50, or Xrs2 only delay but do not prevent processing of HO-endonuclease induced DSBs (Ivanov *et al.* 1994). Several groups have also shown that cells that contain nuclease-deficient Mre11 can perform mating type switching, IR-induced sister chromatid recombination and interhomologue recombination, suggesting that the nuclease activity of Mre11 is dispensable during vegetative growth (Bressan *et al.* 1999; Moreau *et al.* 1999). The MRX complex may also function in holding the ends of the break together to facilitate repair by the cellular repair machinery (Hartsuiker *et al.* 2001).

Mutants of MRX showed a severe defect in end joining *in vivo*, thus MRX is also involved in NHEJ pathway of DNA repair (Schiestl *et al.* 1994; Milne *et al.* 1996; Moore

and Haber 1996; Tsukamoto *et al.* 1996; Boulton and Jackson 1998). The MRX complex might play a structural role in NHEJ as an end-bridging factor rather than a catalytic role since nuclease-deficient mutants of Mre11 have no effect on the NHEJ pathway *in vivo* (Moreau *et al.* 1999; Zhang and Paull 2005) or *in vitro* (Chen *et al.* 2001a).

The role in telomere maintenance

The MRN(X) complex is required for the maintenance of telomere length in eukaryotes. In *S.cerevisiae*, the MRX complex recognizes and binds to shortened telomeres, facilitates loading of scCdc13, an end-protecting protein, and recruits telomerase (Nugent *et al.* 1998; Diede and Gottschling 2001). However, the nuclease activity of Mre11 is not required for telomere maintenance (Moreau *et al.* 1999; Tsukamoto *et al.* 2001). In mammals, it was shown that the MRN complex interacts directly with the telomere repeat binding factors TRF1 and TRF2 (Wu *et al.* 2000a; Zhu *et al.* 2000). Zhu *et al.* also showed that the MRN complex is associated with TRF2, which is suggested to play a role in the formation of T-loops during S phase. This suggests that the MRN complex may be involved in modulating TRF2 activity in T-loop formation. Another study showed that NBS cells have shortened telomeres and that this phenotype can be complemented by expression of wild-type Nbs1 or the catalytic subunit of telomere, suggesting that the MRN complex is also involved in recruitment of telomerase in mammalian cells (Ranganathan *et al.* 2001).

Components of the MRN complex : 1. The Mre11 protein

The Mre11 protein is an essential catalytic component of the MRN complex. Mre11 exhibits manganese-dependent 3'to 5' double-stranded exonuclease activity and single-

stranded and double-stranded endonuclease activities (Paull and Gellert 1998). The presence of Rad50 stimulates both the exonuclease and endonuclease activities of human Mre11, whereas for yeast Mre11, only the endonuclease activity can be enhanced by Rad50 in an ATP-dependent manner (Trujillo *et al.* 1998). In the presence of Nbs1, the Mre11 and Rad50 complex exhibits additional activities including partial unwinding of a DNA duplex and efficient cleavage of fully paired hairpins, which are also enhanced by the presence of ATP (Paull and Gellert 1998; Paull and Gellert 1999; Paull and Gellert 2000). Desai-Mehta *et al.* showed that N-terminal region of Mre11 is required for interacting with C-terminal region of Nbs1 *in vitro* (Fig. 1.1B)(Desai-Mehta *et al.* 2001).

Ataxia-Telangiectasia-Like Disorder (ATLD)

Mutations in the Mre11 gene are responsible for the chromosome instability syndrome, Ataxia-Telangiectasia-Like Disorder (ATLD). ATLD patients exhibit identical clinical phenotypes to that of A-T patients, including progressive cerebellar degeneration. The cellular phenotype of ATLD is also very similar to that of A-T and NBS, including radiosensitivity, chromosome fragility, radioresistant DNA synthesis, and loss of all damage-induced checkpoints (reviewed in Petrini 2000).

The 4 original ATLD patients were divided into two groups, ATLD1/2 and ATLD3/4, based on the Mre11 mutation (Fig. 1.1B). ATLD1/2 patients express a truncated Mre11 protein (633R→Stop) that forms complexes with Rad50 and Nbs1 but is present at low levels compared to wild-type Mre11. ATLD3/4 patients contain a missense mutaion (N117S) in the Mre11 nuclease domain, which results in a decreased affinity for Nbs1 (Stewart *et al.* 1999; Lee *et al.* 2003a).

Components of the MRN complex : 2. The Rad50 protein

Rad50 has Walker A and Walker B ATP-binding motifs at its N- and C-terminal regions, separated by a very long coiled-coil region (Fig. 1.1C). Recent studies show that the coiled-coil region of Rad50 folds back intramolecularly on itself and dimerizes with another Rad50 through a Zn^{2+}-binding hook (Hopfner *et al.* 2002). It was also shown that a functional ATP-binding cassette (ABC)-type ATPase domain is formed at the opposite end of the hook and is hypothesized to contribute to DNA end binding (de Jager *et al.* 2001; Hopfner *et al.* 2002). Mre11 was shown to bind to the coiled-coil region near the ATPase domain of Rad50, suggesting that exonuclease acitivity of Mre11 could be controlled by the ATP-driven conformational changes in Rad50 (Hopfner *et al.* 2001).

Components of the MRN complex : 3. The Nbs1 protein

The Nbs1 protein was identified as the interaction partner of Mre11 and Rad50 by Petrini and colleagues in 1998 (Carney *et al.* 1998). A parallel study also identified the Nbs1 gene as the source of mutations that cause Nijmegen Breakage Syndrome (NBS). Nbs1 is required for the localization of the MRN complex in the nucleus since expression of C-terminal fragment of Nbs1, which contains 354 amino acids with the Mre11 binding domain, is sufficient for the nuclear localization of Mre11 and Rad50 in NBS cells (Desai-Mehta *et al.* 2001).

Nijmegen Breakage Syndrome (NBS)

NBS is a rare autosomal recessive disorder characterized by microcephaly, bird-like facial features, growth retardation, immunodeficiency, hypersensitivity to IR, and predisposition to lymphoreticular malignancies (reviewed in Shiloh 1997). These clinical

phenotypes are similar to those exhibited by A-T patients, although NBS patients do not exhibit the cerebellar degeneration and telangiectasia characteristics of A-T patients. (reviewed in Weemaes *et al.* 1994; reviewed in Petrini 2000).

Cells from NBS patients show almost identical phenotypes with A-T cells, including radiosensitivity, chromosome breakage, telomere shortening, radioresistant DNA synthesis, and defects in S-phase checkpoint arrest, suggesting that Nbs1 functions in the same pathway as ATM after DNA damage (reviewed in Digweed *et al.* 1999; reviewed in Petrini 2000). However, NBS cells exhibit normal G1/S and G2/M checkpoint responses in contrast to A-T cells.

The majority of NBS patients carry a frameshift mutation, 657del5, which causes premature termination at codon 219 and produces 26-kD N-terminal Nbs1 protein (p26). This mutation also produces a 70-kD N-terminally truncated form of Nbs1 (p70) by internal translation initiation upstream of the deletion site (Fig. 1.1D). Both truncated forms of Nbs1 fragments are very weakly expressed (Maser *et al.* 2001). All other mutations in NBS also cause premature truncation of Nbs1 in the region downstream of the FHA and BRCT domains (Digweed *et al.* 1999). Maser *et al.* also showed that p70 is capable of forming a complex with Mre11 and Rad50 (M/R/N(p70)), although this mutant complex is present at very low levels in NBS cells. Thus, this hypomorphic mutant of Nbs1 may be responsible for the viability of NBS patients because it was already shown that deletions of the Mre11, Rad50, and Nbs1 genes in the mouse lead to early embryonic lethality (Xiao and Weaver 1997; Luo *et al.* 1999; Zhu *et al.* 2001).

Interestingly, NBS and ATLD cells show almost no gross defects in DSB repair (Kraakman-van der Zwet *et al.* 1999; Stewart *et al.* 1999). NBS and ATLD cells harbor hypomorphic mutations and have some residual functions of the MRN complex (Stewart

et al. 1999; Maser *et al.* 2001). The MRN(p70) and ATLD MRN complexes also exhibit normal catalytic activities *in vitro* (Lee *et al.* 2003a). This suggests that the MRN hypomorphic mutant complexes are mainly deficient in checkpoint functions rather than in DSB repair functions.

The structure of Nbs1

Nbs1 contains a fork-head-associated (FHA) domain (aa 24-108) and a breast cancer carboxyl terminal (BRCT) domain (aa 108-196) in its N-terminal region (Fig. 1.1D)(reviewed in Tauchi *et al.* 2002). FHA and BRCT domains are known to mediate phosphorylation-dependent protein-protein interactions (Durocher *et al.* 1999; reviewed in Glover *et al.* 2004). Both domains on Nbs1 have been shown to be required for both chromatin association and IR-induced responses of the MRN complex (Tauchi *et al.* 2001; Kobayashi *et al.* 2002; Zhao *et al.* 2002). A recent study also showed that C-terminus domain of Nbs1 is essential for Mre11 binding (Desai-Mehta *et al.* 2001).

The role in cell cycle checkpoint control

The G1/S checkpoint

Yamazaki *et al.* showed that NBS cells showed a G1/S checkpoint arrest comparable to that seen in normal cells although the kinetics was slower by 2hr in NBS cells (Yamazaki *et al.* 1998). ATLD cells also showed mild defects in the G1/S checkpoint and this deficiency was intermediate between normal and A-T cells (Theunissen *et al.* 2003). These findings suggest that NBS and ATLD have a mild defect in G1/S cell cycle arrest.

S phase checkpoint

Cells from NBS patients continue to replicate their DNA in the presence of DSBs caused by IR or radiomimetic drugs, suggesting that NBS cells cannot activate the intra-S phase checkpoint (Taalman *et al.* 1983). In murine models of NBS, the S phase checkpoint is also partially defective (reviewed in Stracker *et al.* 2004). Recently, Nussenzweig *et al.* showed that B cells with conditional deletions of Nbs1 exhibits radioresistant DNA synthesis, confirming the S phase checkpoint defect (Difilippantonio *et al.* 2005; Reina-San-Martin *et al.* 2005). ATLD cells also showed defects in the S phase checkpoint because DNA synthesis in ATLD cells was not greatly reduced in comparison to normal cells after IR, suggesting that intact Mre11 is required for the checkpoint control (Theunissen *et al.* 2003).

G2/M phase checkpoint

Defects in the G2/M checkpoint in NBS cells are controversial. It is known that NBS cells are capable of arresting cell cycle progression at the G2 phase after irradiation and that the early G2/M checkpoint is independent of Nbs1, suggesting that Nbs1 is dispensable for G2/M arrest (Yamazaki *et al.* 1998; Xu *et al.* 2001; Xu *et al.* 2002a). However, other reports have shown that NBS cells have a defective G2/M transition immediately after a low dose of IR (Buscemi *et al.* 2001; Williams *et al.* 2002). A recent study also showed that the number of $Nbs1^{\Delta/-}$ B cells containing phosphorylated histone H3, an indicator of mitotic progression, was not decreased after IR, similar to that of A-T cells. Interestingly, $Nbs1^{657\Delta5}$ B cells showed mild defects in the G2/M checkpoint, suggesting that interaction of N-terminal truncated Nbs1 with Mre11 partially restored this defects (Difilippantonio *et al.* 2005)**.** ATLD cells also showed defects in the G2/M

checkpoint because mitotic index of ATLD cells was not significantly reduced after IR and this deficiency was present between normal cell and A-T cells (Theunissen *et al.* 2003).

Phosphorylation of the component of MRN after DNA damage

Nbs1 is phosphorylated at Ser-278 and Ser-343 in an ATM-dependent manner following exposure to IR (Gatei *et al.* 2000b; Lim *et al.* 2000; Wu *et al.* 2000b; Zhao *et al.* 2000; Kim *et al.* 2002; Lee *et al.* 2003b). Gatei *et al.* showed that Nbs1 phosphorylation by ATM is not required for the MRN foci formation following treatment with IR. This is a controversial result, however since Zhao *et al.* showed that mutation of Ser-278 and Ser-343 to alanine caused a four-fold reduction in MRN focus formation. Mutations of these phosphorylation sites abolished all cell cycle checkpoint functions of Nbs1 after DNA damage (Zhao *et al.* 2000). Furthermore, the checkpoint defects in NBS cells could be complemented by the introduction of wild-type Nbs1, whereas the introduction of S343A Nbs1 mutant did not restore checkpoint defects (Buscemi *et al.* 2001). This suggests that phosphorylation of Nbs1 by ATM is required for transducing the signal to downstream targets of ATM.

Mre11 is also phosphorylated in an ATM-dependent manner after exposure to IR (Stewart *et al.* 2001). Dong *et al.* showed that hyperphosphorylation of Mre11 was abrogated in NBS cells that have undetectable levels of Nbs1 after IR (Dong *et al.* 1999). This suggests that Nbs1 is required for Mre11 phosphorylation. Recently ATM-dependent phosphorylation sites on Mre11 were identified (Jean-Yves Masson and Martin Lavin, personal communication). However, the biological function of ATM-dependent Mre11 phosphorylation is still unknown.

Mre11 and Nbs1 are also phosphorylated after treatment with UV, MMS, or HU (Dong *et al.* 1999; Wu *et al.* 2000b; Zhao *et al.* 2000). Zhao *et al.* showed that Nbs1 phosphorylation is not abolished in A-T cells after treatment with UV and HU, suggesting that Nbs1 can be phosphorylated after DNA damage independently of ATM. However, the consequence of ATM-independent phosphorylation of Mre11 or Nbs1 is still unknown.

IR-induced nuclear foci (IRIF) formation

The MRN complex localizes into distinct nuclear foci shortly after exposure to IR (Maser *et al.* 1997; Carney *et al.* 1998; Nelms *et al.* 1998). These foci are likely associated with DSB sites since DSB-inducing agents, such as IR and radiomimetic drugs, but not UV, normally induce MRN foci. NBS and ATLD cells do not show MRN IRIF formation, suggesting that normal interaction between Nbs1 and Mre11 is required for IRIF formation (Maser *et al.* 1997; Carney *et al.* 1998). Further studies suggested that nuclear localization of the MRN complex by itself is not sufficient for IRIF formation because expression of C-terminal fragment of Nbs1, which can induce the localization of the MR complex to the nucleus in NBS cells, did not restore MRN foci formation (Desai-Mehta *et al.* 2001; Tauchi *et al.* 2001; Zhao *et al.* 2002). Interestingly, A-T cells also showed reduced number of MRN foci, which is thought to be related to defects in IR-induced phosphorylation of Nbs1 and/or Mre11 (Zhao *et al.* 2000; Mirzoeva and Petrini 2001). This suggests that direct interaction between these proteins as well as ATM-dependent phosphorylation of these proteins is required for the localization of the MRN complex into nuclear foci.

One of the most rapid cellular responses after a DNA DSB is the phosphorylation of the histone H2A variant, H2AX. H2AX was shown to be phosphorylated within 1 minute and reaches a threshold within 10 minutes after exposure to IR (Rogakou *et al.* 1998). All of the three damage-induced PI-3 kinases, ATM, ATR, and DNA-PK, contribute to the phosphorylation of H2AX after DSBs. It was shown that the MRN complex localizes into nuclear foci containing phosphorylated H2AX (γ-H2AX) at later time points following formation of γ-H2AX, suggesting that γ-H2AX recruits the MRN complex (Paull *et al.* 2000). A recent study showed that Nbs1 IRIF were impaired in $H2AX^{-/-}$ B cells and that both FHA and BRCT domains of Nbs1 are required for the interaction between Nbs1 and γ-H2AX (Celeste *et al.* 2002; Kobayashi *et al.* 2002; Zhao *et al.* 2002). These studies clearly suggest that the MRN complex interacts with γ-H2AX, but the mode of interaction is still unclear. One possible mode of interaction is through the Mdc1 protein since recent studies show that Mdc1 interacts with γ-H2AX but not with unphosphorylated form of H2AX (Stewart *et al.* 2003). Moreover, MDC1 also associates with the MRN complex (Goldberg *et al.* 2003). Interestingly, when ChIP assays were performed in yeast, γ-H2AX is shown to exist over a widespread region surrounding the breaks (up to 60kb from the breaks) (Shroff *et al.* 2004; Unal *et al.* 2004). However, very little γ-H2AX was detected in regions around the break site where high levels of the MRN complex were found (within 1-2kb regions from the break site) (Shroff *et al.* 2004). These findings suggest that γ-H2AX is located away from the break and that the MRN complex exists both at foci and also at breaks.

Mechanisms of ATM activation

Autophosphorylation

After DSBs are formed, the mechanisms of ATM activation are unclear. Several groups showed that the total amount of ATM remains unchanged even after exposure to IR (Banin *et al.* 1998; Gately *et al.* 1998). Thus, one possible mechanism may be that ATM is posttranslationally modified after DSBs without any increase in the endogenous levels of ATM. A recent study from Bakkenist and Kastan supported this idea because they showed that ATM is rapidly autophosphorylated after exposure to IR. They analyzed formaldehyde cross-linked ATM from irradiated and unirradiated cells and observed that ATM from irradiated cells migrated in SDS-PAGE gels at its expected molecular weight while ATM from unirradiated cells migrated at a position consistent with a dimer form. Hence, they proposed that ATM exists as an inert dimer with its kinase domain bound to an internal domain of a second ATM, at its autophosphorylation site, under normal physiological conditions. DSBs cause the dissociation of the previously inert dimer into active monomers by autophosphorylation at the Ser-1981 residue (Bakkenist and Kastan 2003). As a monomer, ATM is activated and is capable of phosphorylating downstream substrates (Fig. 1.2)

DNA damage

ATM localizes at sites of DNA damage, suggesting an interaction between ATM and DNA. Immediately after localization at damage sites, ATM autophosphorylates itself at residue Ser-1981, which is suggested to activate its kinase activity (Bakkenist and Kastan 2003). These findings suggest that ATM is closely linked to DNA and ATM may

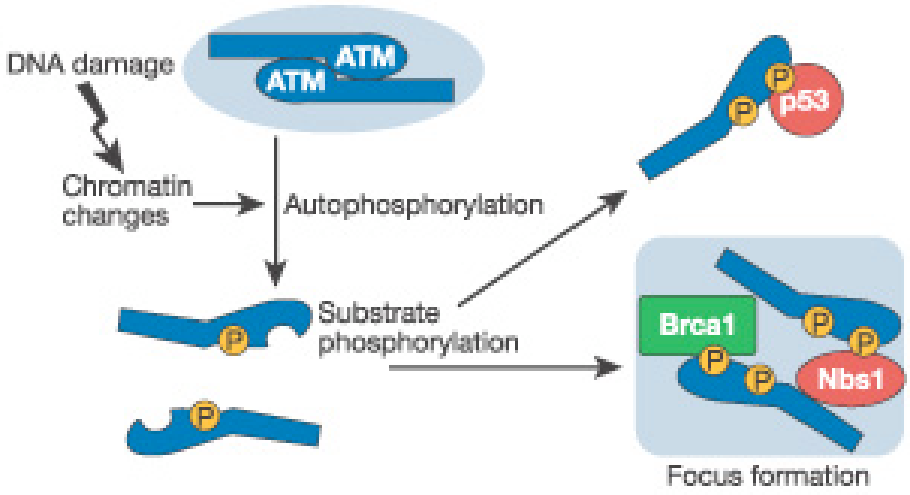

Figure 1.2 Schematic model of ATM activation after irradiation.

DNA strand breaks are proposed to lead to an alteration of chromatin structure that induce intermolecular autophosphorylation of ATM on Ser-1981, and dissociation of the previously inert dimer. Active ATM monomers are then free to migrate to and phosphorylate substrates such as Nbs1 and p53. Adapted by permission from Macmillan Publishers Ltd: Nature 421: 499-506; copyright 2003.

be activated by double-strand breaks. DNA-dependent ATM activation has been a topic of debate. Smith *et al.* showed that the addition of sheared DNA to ATM increased p53 phosphorylation *in vitro* (Smith and Jackson 1999). A modest stimulation of RPA phosphorylation was also observed (Chan *et al.* 2000). However, another study showed that the addition of supercoiled or linear DNA to ATM with p53 did not alter the activation of ATM (Kim *et al.* 1999). Another group has also reported that the addition of calf thymus DNA did not enhance the ATM-induced phosphorylation of PHAS-I, p53, or Chk2 *in vitro* (Chan *et al.* 2000).

Regulation by other factors

Another possibility may be the existence of a co-activator. The MRN complex, which is essential for DNA double strand break repair in mammals, is a well-known candidate because cells containing mutations in the Mre11 or Nbs1 genes show deficiencies in ATM-dependent phosphorylation events similar to that found in A-T cells (Girard *et al.* 2002; Kim *et al.* 2002; Gatei *et al.* 2003). Recent studies also show that Nbs1 promotes ATM-dependent phosphorylation events *in vivo* (Buscemi *et al.* 2001; Nakanishi *et al.* 2002; Yazdi *et al.* 2002; Lee *et al.* 2003b). These findings suggest that the MRN complex could function in the same pathway as the ATM and possibly participate in the activation of ATM kinase activity.

The role of the MRN complex in ATM activation

Since both NBS and ATLD shows similar clinical and cellular phenotypes compared to A-T, including chromosomal instability, radiation sensitivity, and defects in cell cycle checkpoints, it is likely that the MRN complex is in the same pathway with

ATM. Later, several groups showed that a direct interaction between ATM and Nbs1with ATM-dependent phosphorylation of Nbs1 on Ser-343 in response to IR (Gatei *et al.* 2000a; Lim *et al.* 2000; Wu *et al.* 2000b). Lim *et al.* also showed that expression of Nbs1 (S343A) abrogated S phase checkpoint induced by IR in normal cells and does not completely compensate radiosensitivity in NBS cells.

More recently, several groups have shown that many ATM-dependent events are also dependent on the MRN complex. For instance, Buscemi *et al.* showed that Nbs1 is required for the ATM-dependent activation of Chk2 in response to IR by showing that expression of S343A Nbs1 cannot complement Chk2 phosphorylation and activation in NBS cells whereas wild-type Nbs1 can (Buscemi *et al.* 2001). Further studies showed that phosphorylation of FANCD2 and SMC1, mediated by ATM, was dependent on Nbs1 (Nakanishi *et al.* 2002; Yazdi *et al.* 2002). Later, Girard *et al.* showed that phosphorylation of p53 on Ser-15 was impaired in NBS cells following exposure to low dose of IR although ATM from irradiated NBS cells was able to function normally as a protein kinase *in vitro*, suggesting that Nbs1 may be required for ATM-dependent phosphorylation of p53 (Girard *et al.* 2002).

Several studies also showed that expression of a carboxy-terminal deletion mutant of Nbs1, which cannot form a complex with Mre11 and Rad50, cannot complement the deficiency in Chk2 phosphorylation in NBS cells, suggesting that the MRN complex may be required for ATM-dependent phosphorylation events (Buscemi *et al.* 2001; Lee *et al.* 2003b). In support of this possibility, Uziel *et al.* showed that ATM-dependent phosphorylation of p53, Chk2, and Mdm2 was defective in ATLD cells after treatment with neocarzinostatin (NCS), a radiomimetic agent (Uziel *et al.* 2003). They also showed that expression of mutant Mre11 lacking nuclease activity failed to fully

restore activation by ATM in ATLD cells. Another study suggested that interaction between Mre11 and Nbs1 is required for ATM activation since addition of C-terminal Nbs1 fragment containing both Mre11- and ATM-binding domains can restore the ATM autophosphorylation in Nbs1-depleted *Xenopus* egg extract while a C-terminal Nbs1 fragment containing only ATM binding domain cannot activate ATM (You *et al.* 2005). These findings suggest that the functional MRN complex is required for ATM activation after DNA damage.

The functional relationship between ATM and the MRN complex has also been demonstrated using virus-infected cells. By studying the ATM signaling pathway after infection with adenovirus, Weitzman *et al.* suggested that the MRN complex functions upstream of ATM in mammalian cells. They showed that the cellular DNA damage response through ATM and ATR can be blocked by wild-type adenovirus, which relocalizes and degrades the MRN complex through the action of the viral proteins E1b55K/E4orf6 and E4orf3. As a result, ATM autophosphorylation and chromatin retention after treatment with NCS is abrogated (Carson *et al.* 2003; Araujo *et al.* 2005). These findings suggest that, after virus infection, the MRN complex is down-regulated by viral proteins for the replication of their DNA because the MRN complex has functions in DNA repair and ATM-mediated checkpoint control. In support of this, Wu *et al.* also showed that SV40 large T antigen can interact with Nbs1 and that this interaction disrupts Nbs1-mediated suppression of viral DNA replication (Wu *et al.* 2004). Recently, another group also showed that Herpes Simplex Virus (HSV) infection induced an ATM- and MRN- dependent DNA damage response, including ATM autophosphorylation and phosphorylation of ATM target substrates, such as Nbs1, p53, and Chk2, which were not

observed in A-T cells and were delayed in NBS cells. This finding also suggests that the functional MRN complex is required for efficient ATM activation (Shirata *et al.* 2005).

Hypothesis and goals

Several studies have shown that ATLD and NBS cells exhibit defects in ATM-dependent phosphorylation events, suggesting that the MRN complex is required for ATM-dependent signaling. ATLD and NBS cells not only contain mutant forms of the MRN complexes but also have greatly reduced levels of these complexes compared to the MRN complex in normal cells. These observations were further confirmed when the MRN complex was disrupted by conditional knock-out of the NBS1 gene (Difilippantonio *et al.* 2005; Reina-San-Martin *et al.* 2005). Moreover, phosphorylation of Nbs1 is required for the ATM kinase activity since the expression of MRN (S343A), containing a mutation of the critical ATM phosphorylation site in Nbs1, does not complement NBS cells for ATM signaling through Chk2 or SMC1. ATM has been assumed to act as a sensor for DSBs, since it gets activated soon after breaks are formed. However, the nature of ATM interactions at break sites are still unclear. Although several studies were performed to address this issue, the mechanism of ATM activation through DNA breaks has remained a subject of considerable debate. These findings raised several questions about the function of the MRN complex in ATM signaling: a) What is the role of the MRN complex in the ATM signaling pathway? b) Does ATM interact directly with the MRN complex or is the interaction mediated through other protein(s)? c) Does the MRN complex act upstream of ATM in the signaling pathway? d) what is the function of Nbs1 phosphorylation?

Based on the data from cellular experiments, we hypothesize that the MRN complex is required to activate ATM kinase activity. Furthermore, since the MRN complex directly binds DNA, we hypothesize that the MRN complex mediates the interaction between ATM and DNA and that ATM is activated in the presence of both the MRN complex and DNA ends. To test these hypotheses, we investigated whether the MRN complexes could activate ATM kinase activity *in vitro* using purified components. In addition, we also investigated ATM kinase activity with the mutant MRN complexes, including MRN(ATLD), MRN(p70), and MRN(S343A), in order to check whether ATM-dependent phosphorylation is defective. Since Rad50 exhibits ATP-dependent functions, we also determined if a Rad50 mutant deficient in catalytic functions affects ATM kinase activity. These results indicate that MRN complex acts as a double-strand break sensor for ATM and stimulates ATM kinase activity by facilitating the stable binding of substrate.

CHAPTER 2 : MATERIALS AND METHODS

Expression Constructs

wt MRN, wt MR, and Mre11 were expressed in a baculovirus system from the transfer vectors pTP11 (Rad50), pTP17 (Mre11), and pTP36 (Nbs1) as described (Paull and Gellert 1998; Paull and Gellert 1999). Construction of the ATLD1/2, ATLD3/4, and p70 versions of Mre11 and Nbs1 were described (Lee *et al.* 2003a). The S343A version of Nbs1 was made by quickchange mutagenesis (Stratagene) from pTP36 to generate pTP101, and a bacmid form, pTP102, which was used to make recombinant baculovirus (Invitrogen). The Nbs1 purified separately from Mre11 and Rad50 was made from pTP272, which contains an N-terminal Flag tag fused in frame with the full-length Nbs1 gene. The bacmid version of pTP272 was made, pTP328, which was used to make recombinant baculovirus. The S1202R mutant version of hRad50 was expressed from the transfer vector pTP140, made from the pFastBac1 baculovirus expression plasmid (Invitrogen) as described previously (Lee *et al.* 2003a).

The GST-p53 substrate (pTP317) was derived from a full-length GST-p53 construct (J. Huibregtse) (Scheffner *et al.* 1993) by truncation of the gene at amino acid 102. The full-length Chk2 substrate was expressed from pTP152, containing the gene fused to a C-terminal 6 X histidine tag, with a K249R mutation that inactivates the kinase domain of Chk2 (Brown *et al.* 1999). The Chk2 gene was amplified from a Chk2 expression vector (J. Chung) (Brown *et al.* 1999). The GST-Chk2 substrate was made with pGex4T-1 (Amersham), using a gene fragment containing amino acids 2 to 107 of Chk2, to generate pTP385. GST-H2AX was expressed from pTP365, which contains

amino acids 133 to 143 of histone H2AX fused to GST in pGEX4T-1. Cloning details and primer sequences are available upon request.

Expression constructs for Flag-tagged wild-type and S1981A alleles of ATM were gifts from Michael Kastan and were used as described previously (Canman *et al.* 1998). The wild-type version of HA-tagged ATM was a gift from R. Abraham, and the S1981A version was made in this construct using Quikchange mutagenesis (Stratagene).

Protein purification

wt MRN was purified as described (Paull and Gellert 1998), except with a Superose 6 (Amersham) column used for the final gel filtration step instead of a Superdex200 column. Mutant forms of MRN were purified identically to wt MRN, except that in some cases (ATLD1/2 and p70), 2-fold larger volumes of insect cell culture were used to express the proteins.

Recombinant monomeric ATM was made by transient transfection of expression constructs into 293T cells using calcium phosphate, as described (Canman *et al.* 1998), except that the Flag-tagged ATM was isolated on a 1 ml column of anti-Flag M2 agarose (Sigma), instead of by immunoprecipitation. The column was run in TGN buffer: 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% tween 20, and 1 mM dithiothreitol (DTT), and washed with TGN containing 0.5 M lithium chloride. The column was washed into buffer A: 25 mM Tris (pH 8.0), 100 mM NaCl, 10% glycerol, and 1 mM DTT, and Flag-tagged ATM was eluted with 5 ml buffer A containing 0.1 mg/ml Flag peptide. Fractions containing ATM were dialyzed against buffer A prior to storage in small aliquots at -80°C.

Dimeric ATM was made by transient transfection of expression constructs into 293T cells using calcium phosphate as described previously, except that no detergents were included in the lysis or purification buffers, and the anti-Flag antibody column was not washed with 0.5 M lithium chloride. At least 10 dishes (245x245x20 mm) of confluent 293T cells were used for each purification of recombinant dimeric ATM. The Flag peptide eluate from the anti-Flag column was loaded directly onto a 1 ml column containing anti-HA antibody conjugated to agarose beads (Bethyl Laboratories) in buffer A: 25 mM Tris (pH 8.0), 100 mM NaCl, and 10% glycerol. After extensive washing with buffer A, the ATM was eluted with 2.5 mls of HA peptide (0.4 mg/ml, Anaspec, Inc.). Fractions containing ATM were stored in small aliquots at -80°C.

All of the GST fusion proteins were expressed in the E. coli strain BL21 Codonplus (Stratagene). Cells were subcultured in LB at 30°C and induced with 1 mM IPTG for 4 hours before harvesting. The GST-fusion proteins were purified identically to the GST-Brca1 fragments previously described (Paull *et al.* 2001). The GST-p53 and GST-Chk2 proteins were further purified by separation on a Superdex 200 gel filtration column (Amersham) in buffer A.

Protein concentrations were determined by Bradford assay (Pierce) and by quantitation of protein preparations with standards on colloidal Coomassie-stained SDSPAGE gels using the Odyssey system (LiCor). When comparing MRN mutant complexes, levels of ATM stimulation correlated very consistently with the level of Rad50 protein, so the complexes were normalized on the basis of Rad50 concentration. When individual components of the MRN complex were tested in comparison to the complete complex, the amount of each individual component was equivalent to the amount of that component present in the MRN complex.

Reaction conditions used in Chapter 3 for monomeric ATM

ATM kinase assays were done in kinase buffer: 50 mM HEPES, pH 7.5, 50 mM potassium chloride, 10 mM magnesium chloride, 10 mM manganese chloride, 10% glycerol, and 1 mM dithiothreitol (DTT). Assays were performed in three stages: ATM was first incubated alone at 30°C for 30 min., then MRN was added and incubated at 30°C for 30 min., and then the substrate (120 nM Chk2, or 300 nM GSTp53) was added and incubated at 30°C for 60 min. Additional kinase buffer was added at each stage to maintain the concentrations of all of the components.

For the gel filtration experiments, the first 2 stages of the kinase assay were performed, then the samples were directly loaded on a Superose 6 PC 3.2/30 column (Amersham) in a volume of 50 μl. 50 μl fractions were obtained from the column, and analyzed by western blotting.

Kinase reactions with GST-H2AX contained kinase buffer except that the final ATP concentrations were 232 μM (cold), and 0.67 μM (γ-^{32}[P]). ATM and MRN were first incubated separately with 500 μM and 100 μM ATP, respectively, at 30°C for 30 min., and then incubated together at 30°C for 30 min. GST-H2AX was pre-incubated at 30°C for 60 min. in kinase buffer containing 1 mM ATP, and then combined with ATM, MRN, and γ-^{32}[P] ATP for an additional 60 min at 30°C.

For the pull-down assays, ATM (75 ng) was incubated in kinase buffer at 30°C for 30 min. before the addition of 1050 ng MRN in kinase buffer and further incubation at 30°C for 30 min. Anti-Flag M2 agarose beads (30 μl, Sigma) were added and incubated with rotation at 4°C for 60 min. The beads were washed three times with buffer A. GST-p53 (600 ng) or GST-Chk2 (1200 ng) was added to the beads and incubated with rotation at 4°C for 30 min. The beads were then washed three times with buffer A, and

ATM was eluted with 50 μl Flag peptide in buffer A at 0.1 mg/ml. The eluates were analyzed by western blotting with antibody to GST.

Reaction conditions used in Chapter 4 for dimeric ATM

All ATM kinase assays except for the reactions shown in Fig. 4.1A were performed in kinase buffer: 50 mM HEPES, pH 7.5, 50 mM potassium chloride, 5 mM magnesium chloride, 10% glycerol, 1 mM ATP, and 1 mM dithiothreitol (DTT) for 90 min. at 30°C in a volume of 20 μl (GST-Chk2) or 40 μl (GST-p53). 10 ng DNA was used in kinase assays with GST-p53; 2.5 ng were used with GST-Chk2. The kinase assay with monomeric ATM shown in Fig. 4.1A also contained 10 mM manganese chloride.

DNA substrates

The linear DNA used in the kinase assays shown in Fig. 4.1, Fig. 4.4, Fig. 4.5, and Fig. 4.6A was 1 kb ladder (Invitrogen). The plasmid DNA used in Fig. 4.3A was pTP179, which consists of a 60 bp insert in the topo-blunt II vector (Invitrogen) and was purified as previously described (Cunningham *et al.* 1980) by sucrose gradient sedimentation. The DNA substrates of varying length used in Fig. 4.3B were each generated by PCR. The 384 bp product was made with TP150 (5'-TCACCAACTAAATTGCCAAG-3') and TP162 (5'CTTCTATGGCCACATCATCC-3'), which amplify a product within the NBS1 open reading frame using pTP36 (2) as a template. The larger products each were amplified using TP347 (5'-TATTCCGGATTATTCATACCGTCCC-3') and TP474 (5'GATCCTCTAGTACTTCTC-3') which amplify inserts in pFastBacI (Invitrogen). The 689 bp, 1.1 kb, and 2.3 kb PCR products were amplified using these primers from pTP466, pTP29, and pTP36, respectively. Sequences of the plasmids are available upon

request. All PCR products were gel-purified (Qiagen). The 415 bp and 2.3 kb PCR products used for the DNA-binding assays were made using pTP36 as a template, with primers TP168 (5'CTCCAAAAGTAATACCATCCCCCGACTTCAAAGTTCGGGAAAAGCCATTCTGC-3') and TP1087 for the 415 bp product and TP474 and TP1087 for the 2.3 kb product. TP1087 is equivalent to TP347 except with biotin TEG at the 5' end.

The "unpaired ends" substrate used in Fig. 4.6 was made with two 2.7 kb PCR products. The first PCR product was made with primers TP270 (5'GATTAAAGACAACAACTCCAGGACCAAGCCTTGCACAAGGCGTGTCAGTTGA TGAAAAAC-3') and TP728 (5'-CGGTGCTACAGAGTTCTTGAAG-3') using pTP179 as a template. The second PCR product was made with primers TP307 (5'CCATTGGGACATGAAGTTAACCACAGTTGGGAAACAAGCATAGAAATGGAA GAAAGTGAAC-3') and TP728 using pTP180 as a template, which is identical to pTP179 except has a different 60 bp insert in the topo-blunt II vector. Equal amounts of each vector were mixed together, boiled for 5 minutes and cooled slowly over 30 minutes to 90°C, followed by gradual cooling to room temperature. The DNA was separated in a 1.1% agarose gel and electrophoresed at 5.7 V/cm for 7 hours. The boiled mixture of DNA molecules appeared as a doublet in the gel at 2.7 kb; the slower-migrating band was gel-purified and used as the "unpaired ends" product, while the same mixture of products without boiling was gel-purified and used as the "paired ends" product. The higher band in the doublet was not seen with only one species of DNA in the boiled mixture.

The DNA substrate with hairpin ends was generated by RAG1/2 protein cleavage from pDVG54. Cleavage reactions were performed as previously described (6) except that the manganese was omitted and XbaI was added to the reaction to remove uncut

circular plasmid which migrates at a similar position in the agarose gel as the doublehairpin product. The product was gel-purified by electroelution and concentrated in a Ultrafree microconcentrator MWCO 30,000 (Millipore) before use. pDVG54 was also cut with BamHI and SalI in parallel to generate a linear fragment of the same approximate size as the hairpin product (2.7 kb), and was purified the same way. The BamHI/SalI fragment was used as the "cut ends" substrate. The substrates were tested by Mung Bean nuclease digestion and denaturing agarose gel electrophoresis for the appropriate sized products before use in the ATM kinase assay.

DNA binding assays

PCR products were attached to streptavidin-coated magnetic beads according to the manufacturer's instructions (Dynal). DNA-bound beads were incubated with ATM and/or MRN in kinase buffer while rotating at 30°C for 90 min., then washed 3 times with buffer A. Bound proteins were eluted by boiling the beads in SDS loading buffer, and analyzed by SDS-PAGE and western blotting.

Glycerol gradient sedimentation

10% to 20% glycerol gradients were prepared in a volume of 12 mls per gradient, and also contained 25 mM Tris pH 8.0, 100 mM sodium chloride, and 2 mM dithiothreitol. 400 ml of ATM (1.1 nM) was layered on top in the same buffer except containing 5% glycerol. The tubes were centrifuged for 20 hours at 4°C at 29,000 RPM in a SW41 Ti rotor. 450 ml fractions were removed from the top of the gradient. 200 ml from each fraction was applied to PVDF membrane using a dot-blot apparatus and probed with anti-ATM antibody. Quantitation of ATM on the dot-blot was performed

using the LiCor imaging software (see below). ATM concentrations in fractions 3 through 27 are shown in the graph in Fig. 1. Molecular weight markers aldolase (158 kD), catalase (232 kD), ferritin (440 kD), and thyroglobulin (669 kD) from the High Molecular Weight Gel Filtration Calibration Kit (G.E. Amersham Biosciences) were also separated on a similar gradient in parallel and analyzed by SDS-PAGE and Coomassie staining.

ATM dimer dissociation assays

Dimeric ATM (4.3 fmoles) was bound to agarose beads conjugated with anti-Flag antibody (Sigma) in buffer A. After washing the beads 3 times with buffer A, the ATM-bound beads were incubated with MRN (0.7 pmoles) and/or linear DNA (20 ng) in kinase buffer for 90 min. at 30°C in a total volume of 80 ul. The supernatant was removed from the beads and analyzed by SDS-PAGE and western blotting.

Immunoblotting and quantitation

All of the proteins were separated on Tris-glycine SDS-PAGE gels and blotted onto PVDF-FL membrane (Millipore) using standard blotting techniques. The primary antibodies used were anti-ATM (Genetex), anti-Nbs1 (Genetex), anti-Rad50 (Genetex), anti-Mre11 (Genetex), anti-p53 phospho-serine15 (Oncogene), and anti-Chk2 phospho-threonine68 (Cell Signaling). AlexaFluor680 antirabbit and IRdye800 anti-mouse secondary antibodies were obtained from Invitrogen and Rockland Immunochemicals, respectively. The use of infrared fluorescent dyes on the secondary antibodies allows direct quantitation of the secondary antibodies using the Odyssey system (LiCor), and therefore no chemiluminescence techniques were used for any of the experiments. The membranes were scanned and quantified using the Odyssey system and associated

software. For determination of fold increases, the levels of phosphorylated product were determined in each lane relative to the background fluorescence on the blot, and the ratio between these values was calculated as the fold increase. If no signal was detectable in a lane, the amount of product was considered to be equal than the background level of fluorescence. In this case, the absolute fluorescence of the comparison band was calculated relative to the background level of fluorescence, and the fold increase was taken as being this ratio or greater. For instance, there was no detectable substrate phosphorylation by ATM alone in a reaction containing 12.5 nM GST-p53, shown in Fig. 4.1G, but the ATM plus MRN plus DNA signal is 60 times higher than the background signal. Thus the fold increase attributable to DNA is at least 60-fold.

CHAPTER 3 : Stimulation of ATM kinase activity by the Mre11/Rad50/Nbs1 Complex

INTRODUCTION

Eukaryotic cells respond to DNA damage with a rapid activation of signaling cascades that initiate from the ATR and ATM protein kinases. The response to DNA double-strand breaks (DSBs) occurs primarily through ATM and leads to phosphorylation of many targets critical for checkpoint activation, apoptosis, and DNA repair (Shiloh 2003). One of the targets of ATM phosphorylation is the Nbs1 (nibrin) protein, which associates with the conserved DSB repair factors Mre11 and Rad50 (Carney *et al.* 1998). ATM phosphorylates Nbs1 on Ser-343 and other residues, modifications necessary for S-phase checkpoint activation and for survival of ionizing-radiation exposure (Gatei *et al.* 2000b; Lim *et al.* 2000; Wu *et al.* 2000b; Zhao *et al.* 2000; Kim *et al.* 2002; Lee *et al.* 2003b).

The Nbs1 protein is not just a substrate of ATM, but also affects activation of ATM in response to DNA damage. The gene encoding Nbs1 is mutated in patients with the radiation sensitivity disorder Nijmegen breakage syndrome (NBS), characterized by chromosomal instability, radio-resistant DNA synthesis, and clinical phenotypes that include immunodeficiency and cancer (Varon *et al.* 1998). Cells from NBS patients do not produce full-length Nbs1 protein, and phosphorylation of Chk2, SMC1, and FANCD2 by ATM is reduced or absent in these cells (Buscemi *et al.* 2001; Girard *et al.* 2002; Kim *et al.* 2002; Nakanishi *et al.* 2002; Yazdi *et al.* 2002; Gatei *et al.* 2003; Lee *et al.* 2003b).

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Thus, the MRN complex may participate in activating ATM to phosphorylate multiple other downstream substrates.

RESULTS AND DISCUSSION

The MRN complex stimulates ATM phosphorylation of Chk2 on Thr-68

To test whether the MRN complex participate in activating ATM to phosphorylate multiple downstream substrate, we measured the effects of MRN on ATM phosphorylation of Chk2, a protein kinase that activates S phase and mitotic checkpoints. ATM phosphorylates Chk2 on Thr-68 in response to DNA damage, which activates Chk2 to phosphorylate substrates including p53 and Cdc25C (McGowan 2002). Using a phospho-specific antibody specific for Chk2 Thr-68, we found that the addition of MRN to ATM stimulated Chk2 phosphorylation up to 15-fold (Fig. 3.1A). The activating effect of MRN was dependent on the absolute concentration of ATM in the reaction (Fig. 3.1B). Addition of Mre11/Rad50 (MR), lacking the Nbs1 protein, stimulated ATM phosphorylation of Chk2 only partially (Fig. 3.1C) indicating that Nbs1 is important for Chk2 activation.

Phosphorylation of Nbs1 on Ser-343 is required for ATM stimulation

Nbs1 phosphorylation on Ser-343 is required for ATM phosphorylation of substrates including Chk2, SMC1, and FANCD2 *in vivo* (Buscemi *et al.* 2001; Nakanishi *et al.* 2002; Yazdi *et al.* 2002). To test the importance of Nbs1 phosphorylation for ATM stimulation, we used an S343A mutant version of Nbs1. The MRN(S343A) complex

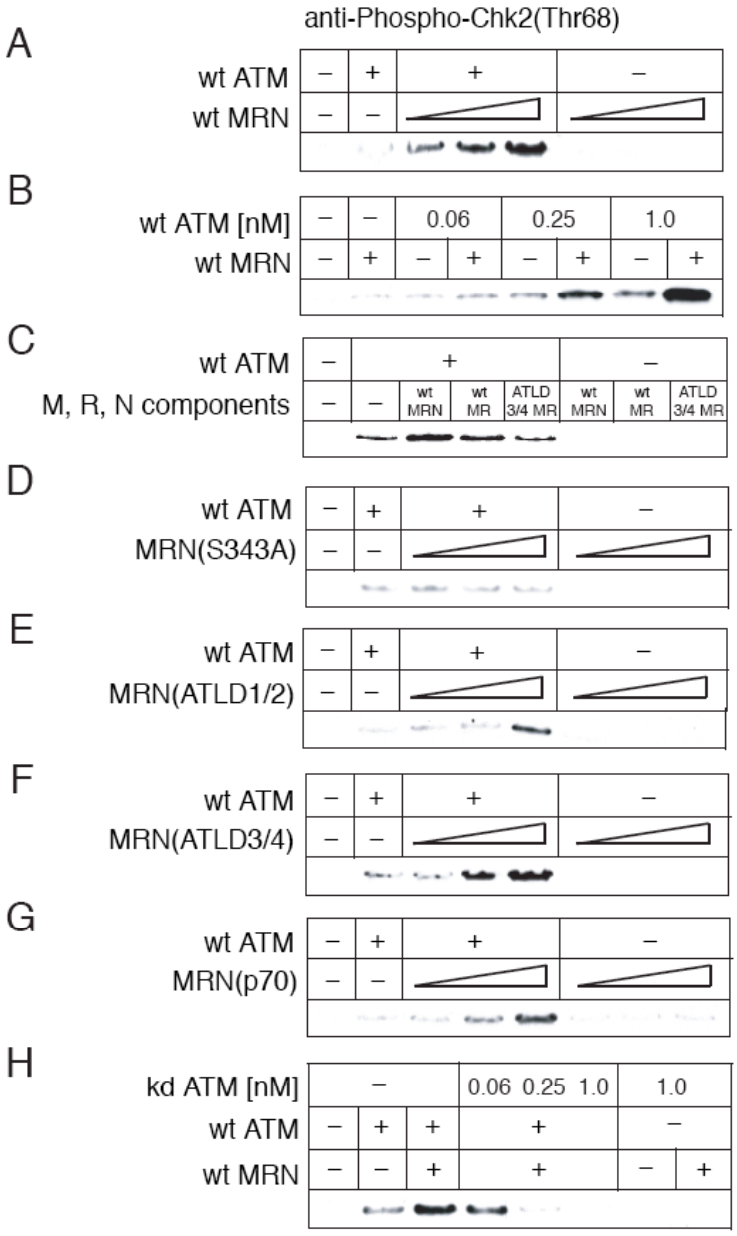

Figure 3.1 Stimulation of ATM phosphorylation of Chk2 on Thr-68 by MRN.

(**A**) Kinase assays contained Flag-tagged wt ATM and full-length histidine-tagged kinase-deficient Chk2, with MRN as indicated. Phosphorylation was visualized with anti-Chk2 phospho–Thr-68. Concentration of MRN = 1.9, 3.75, and 7.5 nM in lanes 3 to 5, respectively; concentration of ATM = 0.25 nM. (**B**) Kinase assays as in (A) except with varying ATM amounts. Concentration of MRN = 7.5 nM. (**C**) Kinase assays as in (A) except with wt MRN, wt MR, or MR(ATLD3/4) added at 7.5 nM. (**D** to **G**) Kinase assays as in (A) except with MRN(S343A), MRN(ATLD1/2), MRN(ATLD3/4), or MRN(p70) added instead of wt MRN. (**H**) Kinase assays as in (A) with 7.5 nM wt MRN, 0.25 nM wt ATM, and kd ATM added in lanes 4 to 6.

forms similarly to the wild-type (wt) complex and exhibits DNA binding and nuclease activities identical to those of the wt enzyme (Unpublished Data). The MRN(S343A) mutant complex did not stimulate ATM activity toward Chk2 *in vitro* (Fig. 3.1D). The presence of Nbs1 and phosphorylation of Ser-343 by ATM is therefore essential for MRN stimulation of ATM activity on Chk2. The complete absence of ATM stimulation by MRN(S343A) suggests that the presence of Nbs1 is inhibitory when Nbs1 cannot be phosphorylated.

The MRN(ATLD1/2) and MRN(p70) complexes stimulate Chk2 phosphorylation only partially

Ataxia telangiectasia (A-T) patients lack functional ATM protein and exhibit radiation sensitivity, genomic instability, and deficiencies in G1-S, S, and G2-M checkpoint responses. A-T–like disorder (ATLD) patients also have an A-T phenotype despite having wt alleles of the ATM gene (Stewart *et al.* 1999). ATLD patients carry mutations in the Mre11 gene that cause a truncation of the C terminus (ATLD1/2) or a N117S missense mutation in the nuclease domain (ATLD3/4). ATLD3/4 Mre11 exhibits variability in Nbs1 association, forming complexes either with [MRN(ATLD3/4)] or without [MR(ATLD3/4)] Nbs1 (Lee *et al.* 2003a). NBS cells express a truncated C-terminal polypeptide of Nbs1 (p70) that binds to Mre11 and forms a mutant MRN(p70) complex (Maser *et al.* 2001). MRN(p70), MRN(ATLD1/2), and MRN(ATLD3/4) exhibit DNA binding and nuclease activities similar to those of the wt MRN complex (Lee *et al.* 2003a).

We tested the ability of the ATLD and p70 forms of the MRN complex to stimulate ATM phosphorylation of Chk2 (Fig. 3.1). The MR(ATLD3/4) complex did not stimulate

ATM activity (Fig. 3.1C), but the MRN(ATLD3/4) form of the complex showed stimulation equivalent to that of wt MRN (Fig. 3.1F). The MRN(ATLD1/2) and MRN(p70) complexes stimulated Chk2 phosphorylation only partially (Fig. 3.1, E and G). The decreased ability of ATLD and p70 versions of MRN to stimulate ATM phosphorylation of Chk2 is consistent with the checkpoint deficiencies observed with cells from ATLD and NBS patients (Petrini 2000). The low levels of Chk2 phosphorylation observed in ATLD and NBS cell lines (Uziel *et al.* 2003) are likely due to a combination of the mutations in Mre11 and Nbs1 as well as the overall reductions in intranuclear MRN levels observed in these cells (Carney *et al.* 1998; Stewart *et al.* 1999; Uziel *et al.* 2003).

Kinase-deficient ATM inhibits phosphorylation of Chk2 by wt ATM

Some missense mutations in the human ATM gene are associated with higher rates of malignancy in heterozygotes (Gatti *et al.* 1999), and a missense allele of ATM acts as a dominant negative and increases rates of tumor formation in mice (Spring *et al.* 2002). We therefore tested the effects of catalytically inactive (kd) ATM (Canman *et al.* 1998) in reactions containing MRN and wt ATM (Fig. 3.1H). The kd ATM inhibited phosphorylation of Chk2 by wt ATM, indicating that kd ATM can exert dominant-negative inhibition of wt ATM activity.

The MRN complex stimulates ATM phosphorylation of p53 on Ser-15

We also tested the effects of MRN on ATM phosphorylation of the tumor suppressor p53, using amino acids 1 to 102 of p53 coupled to glutathione *S*-transferase as a substrate (GST-p53), and an antibody to phosphorylated Ser-15. Phosphorylation of

p53 on Ser-15 is required for p53 stabilization and transactivation (Shieh *et al.* 1997; Siliciano *et al.* 1997). Addition of MRN to ATM increased p53 phosphorylation up to 12-fold over phosphorylation by ATM alone (Fig. 3.2A). As with Chk2, the effect of MRN on p53 phosphorylation by ATM was very sensitive to the concentration of ATM in the reaction (Fig. 3.2B).

The MR complex is sufficient to stimulate ATM kinase activity toward p53

Subcomplexes of MRN were also tested for stimulation of ATM phosphorylation activity, showing that Mre11 and Nbs1, either alone or added together, were insufficient to induce p53 phosphorylation (Fig. 3.2C). However, Nbs1 appeared not to be required for p53 phosphorylation, because a complex of Mre11 and Rad50 (MR) stimulated ATM activity to the same extent as did the complete MRN complex (Fig. 3.2D). The MRN(S343A) complex exhibited nearly wt levels of activity in this assay (Fig. 3.2E), as did the MRN(ATLD1/2), MRN(ATLD3/4), and MRN(p70) complexes (Fig. 3.2, F to H). The ability of the mutant complexes to stimulate ATM phosphorylation of p53 is consistent with the normal p53 response in cell lines derived from ATLD and NBS patients (Stewart *et al.* 1999).

The MRN complex stimulates ATM phosphorylation of H2AX

One of the first targets of ATM *in vivo* is the C-terminal tail of histone H2AX, which is phosphorylated rapidly at the sites of chromosomal DSBs (Rogakou *et al.* 1998; Paull *et al.* 2000). As with Chk2 and p53, ATM phosphorylation of a GST-H2AX substrate was also stimulated fivefold by the addition of MRN (Fig. 3.2I).

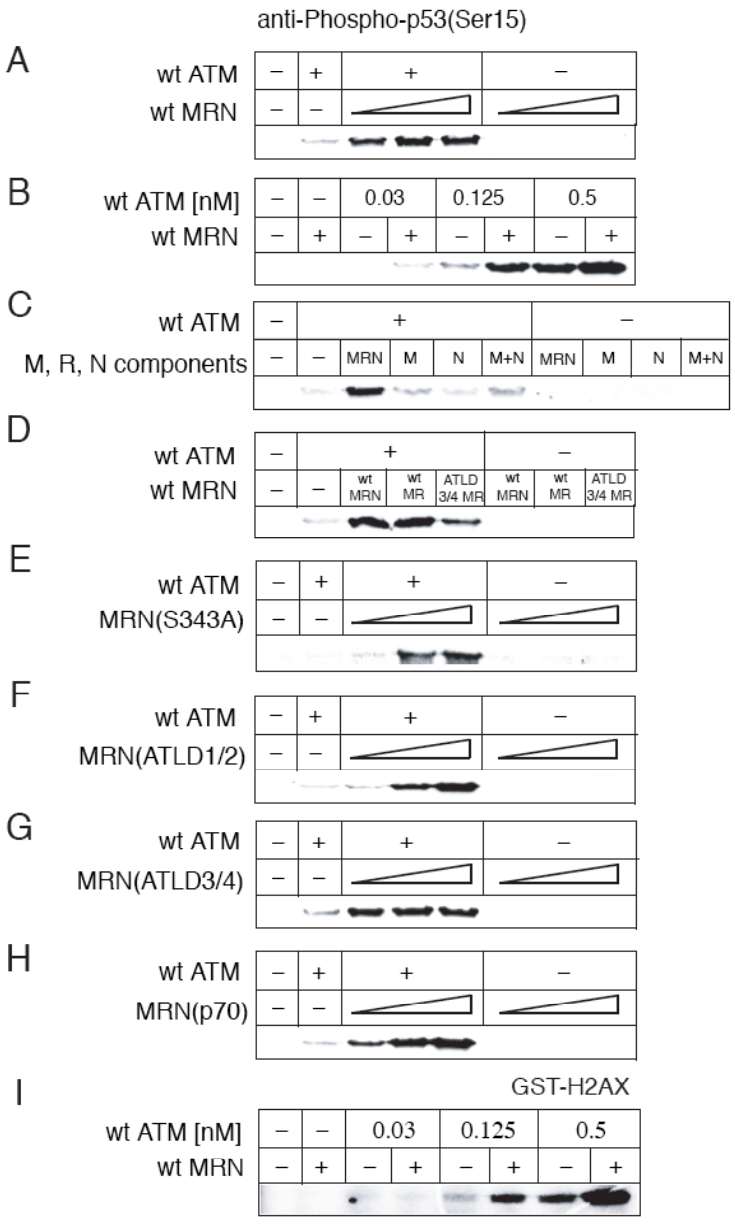

Figure 3.2 Stimulation of ATM phosphorylation of p53 on Ser-15 by MRN.

(**A**) Kinase assays as in Figure 3.1 except with GST-p53 (amino acids 1 to 102) as substrate and anti-p53 phospho–Ser-15. Concentration of ATM = 0.125 nM. (**B**) Kinase assays as in (A) except with varying ATM amounts, with or without MRN (7.5 nM). (**C**) Kinase assays as in (A) with MRN (7.5 nM), Mre11 (M), Nbs1 (N), or Mre11 and Nbs1 (M + N). Mre11 and Nbs1 concentrations were equivalent to the amounts present in wt MRN. (**D**) Kinase assays as in (A) except with wt MRN, wt MR, or MR(ATLD3/4) added at 7.5 nM. (**E** to **H**) Kinase assays as in (A) except with MRN(S343A), MRN(ATLD1/2), MRN(ATLD3/4N), or MRN(p70) added instead of wt MRN. (**I**) Kinase assays with wt ATM (0.25 nM) and wt MRN (0.75 nM) on a GST-H2AX (amino acids 133 to 143) substrate with [-32P]ATP, visualized by PhosphorImager.

ATM associates with the MRN complex *in vitro*

To investigate the mechanism underlying MRN stimulation of ATM activity, we analyzed associations between the recombinant purified proteins by gel filtration (Fig. 3.3). Incubation of ATM with MRN caused a subset of ATM to elute with the MRN complex, indicating an interaction between the factors (Fig. 3.3A). kd ATM also appeared to interact with MRN. Unlike wt ATM, however, kd ATM induced a change in the mobility of the MRN components into a range of smaller complexes, suggesting dissociation of the complex. This effect on MRN may contribute to the dominant-negative activity of kd ATM.

The MRN(ATLD1/2) and MRN(p70) mutant complexes also associated with ATM (Fig. 3.3, B and C), although the percentage of ATM observed in the larger complex was less than that observed with similar amounts of wt MRN, particularly with the p70 complex. In contrast, ATM associated with the MRN(S343A) complex as it did with the wt complex (Fig. 3.3D), indicating that an interaction between the complexes occurs even in the absence of Nbs1 phosphorylation.

The stimulation of p53 phosphorylation by MR (Fig. 3.2) suggests that ATM must make contacts with the MRN complex that are completely independent of Nbs1. Consistent with this result, gel filtration showed that a subset of ATM comigrated with MR even in the absence of Nbs1 (Fig. 3.3E). ATM also associated directly with Nbs1 in the absence of MR, however (Fig. 3.3F), indicating that ATM makes multiple direct contacts with the MRN complex.

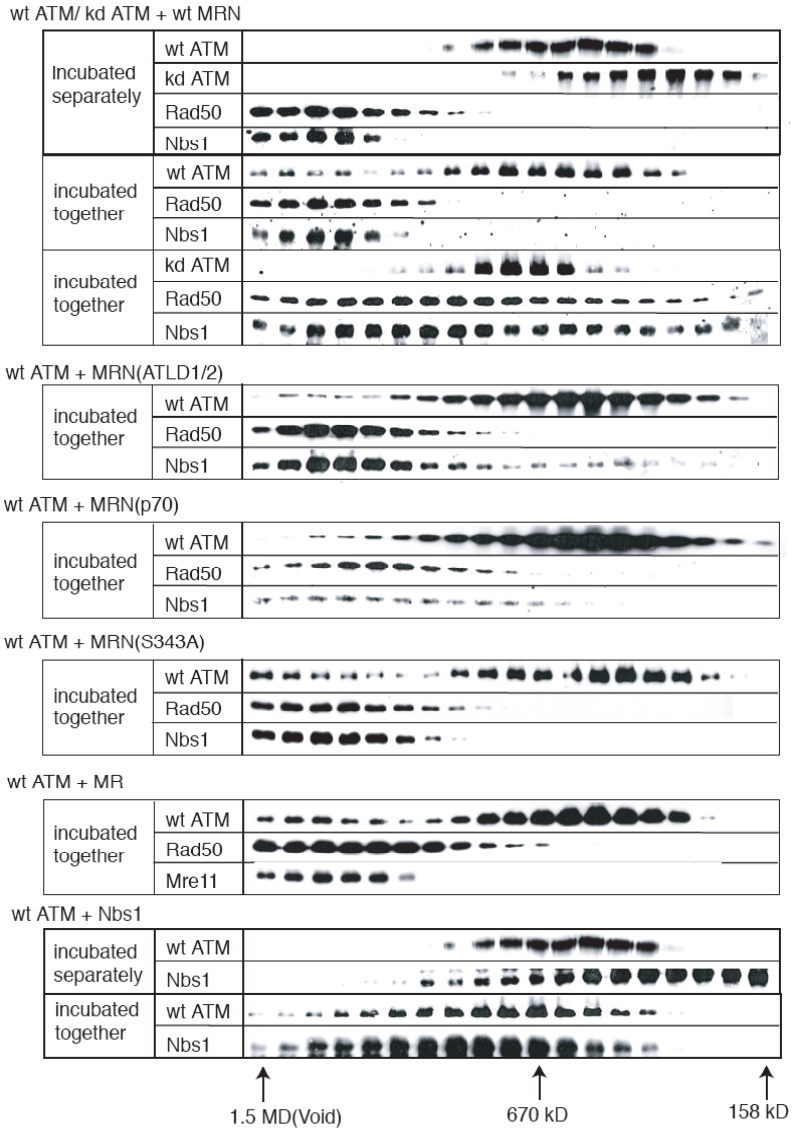

Figure 3.3 Multiple direct associations between ATM and the MRN complex.

(**A**) wt MRN was incubated with wt ATM or kd ATM either separately (top), or together (bottom) for 30 min before gel filtration (Superose 6), SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting with antibodies to ATM, Rad50, and Nbs1. Molecular mass: ATM = 3.5 x 10^5 daltons, MRN = 1.2 x 10^6 daltons (*18*). (**B** to **E**) wt ATM incubated with MRN(ATLD1/2) complex (B), MRN(p70) complex (C), MRN(S343A) complex (D), or wt MR (E) and analyzed as in (A). (**F**) wt ATM and Nbs1 protein incubated separately (top) or together (bottom), separated by gel filtration, and analyzed as in (A) with antibodies to ATM or Nbs1.

The MRN complex stimulates binding of substrates to ATM

To determine the mechanism of MRN stimulation of ATM kinase activity, we examined the effect of the complex on the binding of specific substrates by ATM. ATM was incubated with or without MRN, then isolated by immunoprecipitation with anti-Flag beads. GST-Chk2 or GST-p53 substrates were then incubated with the beads, and bound proteins were detected with an antibody to GST. The presence of MRN during the first incubation stimulated binding of both p53 and Chk2 to ATM during the second incubation (Fig. 3.4, A to D). The MRN(S343A), MRN(ATLD1/2), and MRN(p70) complexes showed lower stimulation of GST-Chk2 association (Fig. 3.4B), consistent with reduced ability of the complexes in stimulating kinase activity. Mre11 did not increase association of GST-p53 with ATM, whereas MRN stimulated association by more than fivefold and MR showed an intermediate level of stimulation. These effects on substrate binding were not seen when adenosine 5'-triphosphate (ATP) and divalent cations were present during the second incubation (Unpublished Data), suggesting that substrates are released from ATM after phosphorylation. We propose that MRN induces a conformational change in ATM that increases its affinity for its substrates (Fig. 3.4E).

SUMMARY AND CONCLUSION

The addition of DNA to ATM kinase assays, with or without MRN, did not stimulate phosphorylation (Unpublished data), similar to findings of other reports (Bakkenist and Kastan 2003). Our experiments were performed in the absence of DNA and indicate that DNA is not absolutely required for the associations between ATM,

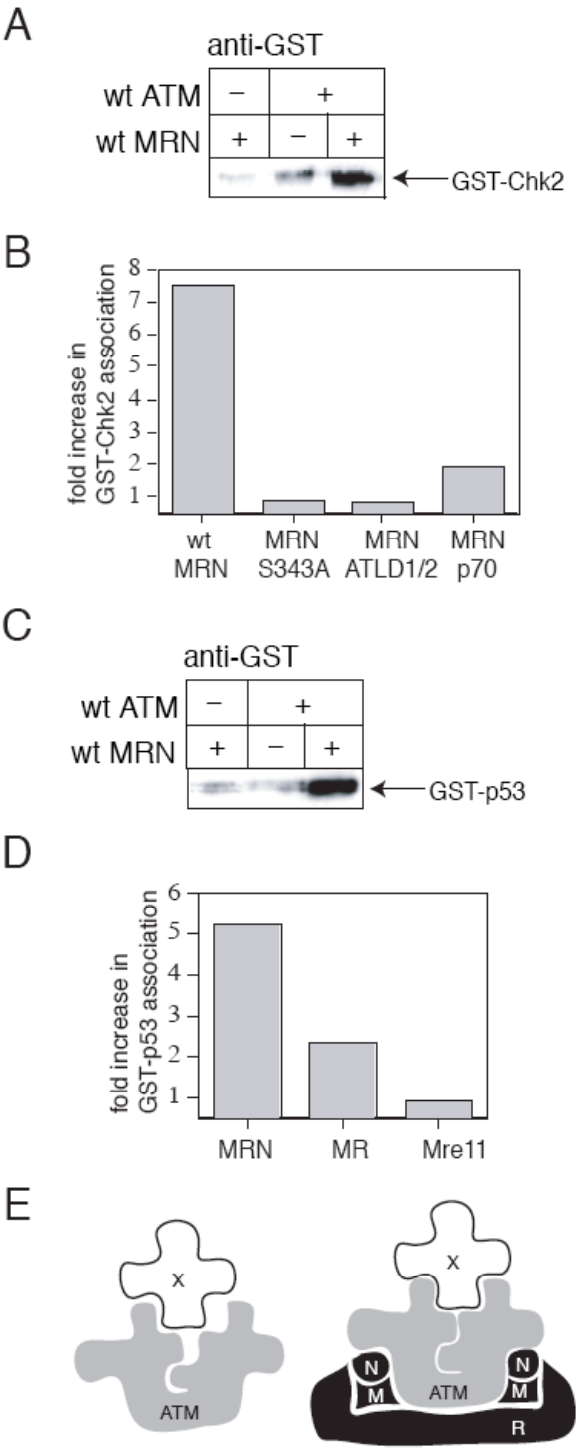

Figure 3.4 MRN stimulates binding of substrates to ATM.

(**A** and **C**) ATM was incubated with or without MRN in kinase buffer, and ATM complexes were isolated using beads conjugated to anti-Flag (recombinant ATM contains N-terminal Flag tag). Complexes were incubated with GST-Chk2 (amino acids 2 to 107) (A) or GST-p53 (amino acids 1 to 102) (C), without ATP, and ATM was isolated again with anti-Flag beads. Associated material was eluted from the beads, separated by SDS-PAGE, and analyzed with anti-GST. (**B**) Association of GST-Chk2 with ATM stimulated by wt MRN, MRN(S343A), MRN(ATLD1/2), and MRN(p70) determined as in (A) and represented as the fold increase in GST-Chk2 association. (**D**) Association of GST-p53 with ATM stimulated by wt MRN, wt MR, and Mre11 determined as in (A) and represented as the fold increase in GST-p53 association. (**E**) Schematic model of MRN stimulation of ATM kinase activity. ATM is postulated to bind substrates in low-affinity mode (left), or in high-affinity mode (right) when bound to MRN.

MRN, and ATM substrates. The requirement for MRN in ATM activation shown in this study may explain many aspects of the NBS and ATLD phenotypes, and it illustrates how a single complex can facilitate spatial and temporal coordination of DNA repair with signaling pathways that mediate checkpoint activation.

CHAPTER 4 : DNA-dependent ATM activation through the Mre11/Rad50/Nbs1 complex

INTRODUCTION

In mammalian cells, DNA double-strand breaks trigger activation of the ataxia-telangiectasia mutated (ATM) protein kinase, which phosphorylates downstream targets that initiate cell-cycle arrest, DNA repair, or apoptosis. Several of these targets, including p53, Chk2, Brca1, and H2AX, function as tumor suppressors *in vivo*, and the phosphorylation of these factors is critical for their function after DNA damage.

The Nbs1 (nibrin) protein is also a substrate for ATM, and abrogation of Nbs1 phosphorylation inhibits checkpoint signaling during the S phase (chromosome replication) of the cell cycle (Stracker *et al.* 2004). Nbs1 is part of the Mre11-Rad50-Nbs1 (MRN) complex, which is essential for DNA double-strand–break repair and genomic stability. Cells from patients with Nijmegen breakage syndrome (NBS) or ataxia telangiectasia–like disorder (ATLD) express mutant forms of the Nbs1 or Mre11 protein, respectively, and exhibit decreased levels of ATM substrate phosphorylation, particularly on Chk2 (Buscemi *et al.* 2001; Girard *et al.* 2002; Lee *et al.* 2003b; Uziel *et al.* 2003) and Smc1 (Yazdi *et al.* 2002; Kitagawa *et al.* 2004), despite the presence of wild-type ATM. Thus, the MRN complex may not only be a downstream effector of ATM but also may function in activating ATM to initiate phosphorylation of cellular substrates.

** Portions of this chapter have been published previously in the Journal* ***Science*** *(Lee and Paull 2005)*

RESULTS AND DISCUSSION

Monomeric ATM is not stimulated by the addition of DNA in the presence of the MRN complex

MRN stimulated ATM activity *in vitro* toward p53, Chk2, and histone H2AX in a kinase assay with purified recombinant components (Lee and Paull 2004). MRN and ATM associated through multiple protein-protein interactions, and MRN contributed to ATM kinase activity by increasing the affinity of ATM for its substrates. In this *in vitro* assay, however, there was no effect of DNA on ATM, either with or without MRN (Fig. 4.1A).

Purification of dimeric ATM

ATM exists *in vivo* as an inactive multimer that dissociates into active monomers after DNA damage or other forms of cellular stress (Bakkenist and Kastan 2003). Thus, the ATM we studied previously may have been monomeric, either already present as a monomer in cells or converted into monomers during purification. To study the multimeric form of ATM specifically, we transfected human 293T cells with two ATM expression constructs encoding Flag- and hemagglutinin (HA)–epitope–tagged ATM, and we modified our purification procedure to preserve multimeric interactions. Sequential purification with antibodies directed against the Flag and HA epitopes yielded ATM complexes (Fig. 4.1B). Glycerol gradient sedimentation analysis of complexes containing both epitope-tagged forms of ATM showed that the majority of this protein fractionated as a dimer, whereas the previously purified form of ATM fractionated as a monomer (Fig. 4.1C).

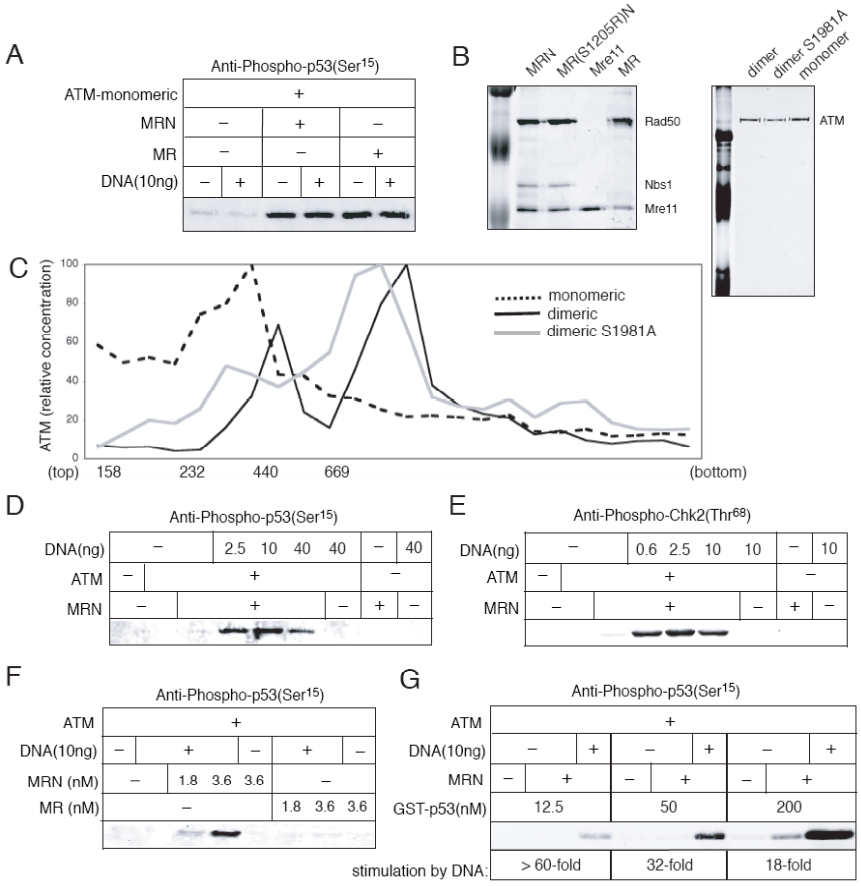

Figure 4.1 Requirement of DNA and MRN for activity of dimeric ATM.

(**A**) Kinase assays contained 0.125 nM monomeric ATM, 7.5 nM MRN, 7.5 nM MR, 50 nM GST-p53 (amino acids 1 to 102), and 10 ng of linear DNA. Western blots were probed with antibody to phosphoserine 15 of p53. (**B**) Coomassie-stained SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of MRN, MR(S1202R)N, MR, Mre11, Flag-tagged monomeric ATM (monomer), Flag-ATM–HA-ATM dimeric complex (dimer), and S1981A dimeric complex (dimer S1981A). (**C**) Glycerol gradient sedimentation analysis of monomeric ATM, dimeric ATM, and S1981A dimeric ATM, with positions of molecular size markers as indicated. ATM concentrations are shown relative to the most concentrated fraction within each gradient. (**D**) Kinase assays with 0.2 nM dimeric ATM, 3.6 nM MRN, 50 nM GST-p53 substrate, and linear DNA, probed with antibody to phosphoserine 15 of p53. (**E**) Kinase assays with 0.2 nM dimeric ATM, 4.8 nM MRN, 200 nM GST-Chk2 substrate, and linear DNA, probed with antibody to phosphothreonine 68 of Chk2. (**F**) Kinase assays as in (D) with indicated amounts of MRN or MR (1.8 and 3.6 nM, respectively). (**G**) Kinase assays as in (D) with indicated amounts of GST-p53.

Dimeric ATM requires both the MRN complex and DNA for activity

Unlike monomeric ATM, dimeric ATM required both the MRN complex and DNA for activity. Dimeric ATM was tested for kinase activity with a glutathione S-transferase (GST) fusion protein containing residues 1 to 102 of p53, and phosphorylation was detected with a phosphospecific antibody directed against p53 protein phosphorylated on Ser-15 (S15). Minimal activity was seen with ATM alone, or with ATM with MRN, whereas substantial phosphorylation was seen with ATM, MRN, and linear DNA incubated together in the kinase reaction (Fig. 4.1D).

Similar results were seen with dimeric ATM and a GST-Chk2 substrate when an antibody that is specific for phosphothreonine 68 was used (Fig. 4.1E). In each case, MRN increased the amount of phosphorylated product by only two- to fivefold, whereas DNA plus MRN yielded an increase in phosphorylated product of 80- to 200-fold over ATM alone. The addition of DNA to dimeric ATM in the absence of MRN did not stimulate kinase activity (Fig. 4.1, D and E). Dimeric ATM also required the complete MRN complex and was not stimulated by MR complex, which lacks Nbs1 (Fig. 4.1F), unlike monomeric ATM, which is stimulated by both the MR and MRN (Fig. 4.1A). Identical results were observed with full-length Chk2 and p53 (Fig. 4.2).

The relationship between ATM kinase activity and substrate concentration

The level of ATM stimulation by DNA was modulated by the substrate concentration (Fig. 4.1G). The increase in phosphorylation induced by DNA over that by MRN alone was greater than 60-fold with 12.5 nM GST-p53 substrate and 32-fold with 50 nM substrate, but only 18-fold with 200 nM substrate. Therefore, DNA and MRN may act on dimeric ATM by stimulating substrate recruitment, in similar fashion to how they

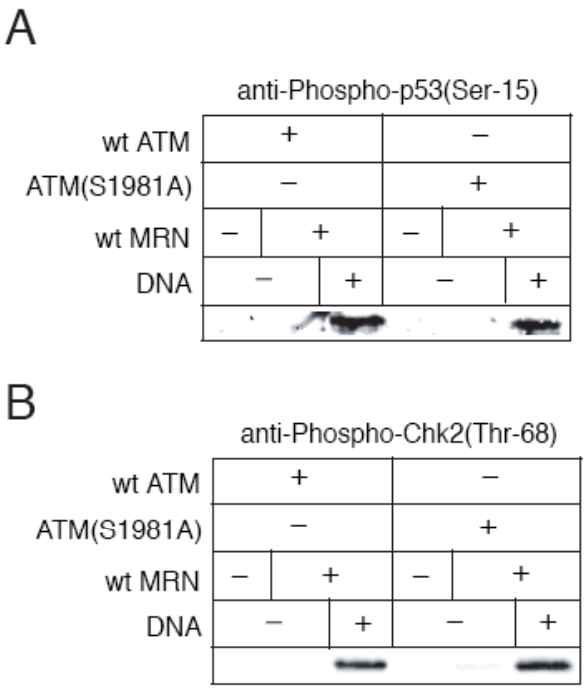

Figure 4.2 Requirement of DNA and MRN for activity of dimeric ATM toward full-length p53 and full-length Chk2.

(**A**) and (**B**) ATM kinase assay as in Figure 4.1, D and E, except with full-length p53(GST fusion) and full-length Chk2 instead of truncated p53 and Chk2 GST fusion substrates.

act on monomeric ATM (Lee and Paull 2004).

DNA ends are required for dimeric ATM activation through the MRN complex

In vivo, ATM is activated by DNA double-strand breaks. To test whether DNA ends are required with dimeric ATM, we added closed circular plasmid DNA instead of linear DNA fragments. The circular DNA stimulated ATM fivefold in the presence of MRN, but when restriction enzymes were also included in the kinase reaction, the phosphorylation of p53 and Chk2 was increased by 13- to 25-fold (Fig. 4.3A). We did not observe any DNA sequence specificity (Unpublished Data); however, optimal stimulation of dimeric ATM required that the length of the DNA fragment be at least 1 to 2 kb (Fig 4.3B).

The MRN complex is required for the stable association of ATM with DNA

To determine which proteins are required for DNA binding, we attached a 2.3-kb DNA fragment to magnetic beads through a biotin-streptavidin interaction. The DNA-bound beads were incubated with recombinant ATM and MRN, and we identified the bound proteins by Western blotting. MRN bound to the DNA-containing beads irrespective of the presence of ATM, whereas ATM was associated with the beads only when MRN was present (Fig. 4.3C). Thus, *in vitro*, MRN is required for the stable association of ATM with DNA. The MR complex, which lacks Nbs1, also stimulated ATM binding to DNA (Fig. 4.3D), but Mre11 alone did not (Fig. 4.3E), suggesting that the interaction between MR and ATM (Lee and Paull 2004) is through the Rad50 component of the complex.

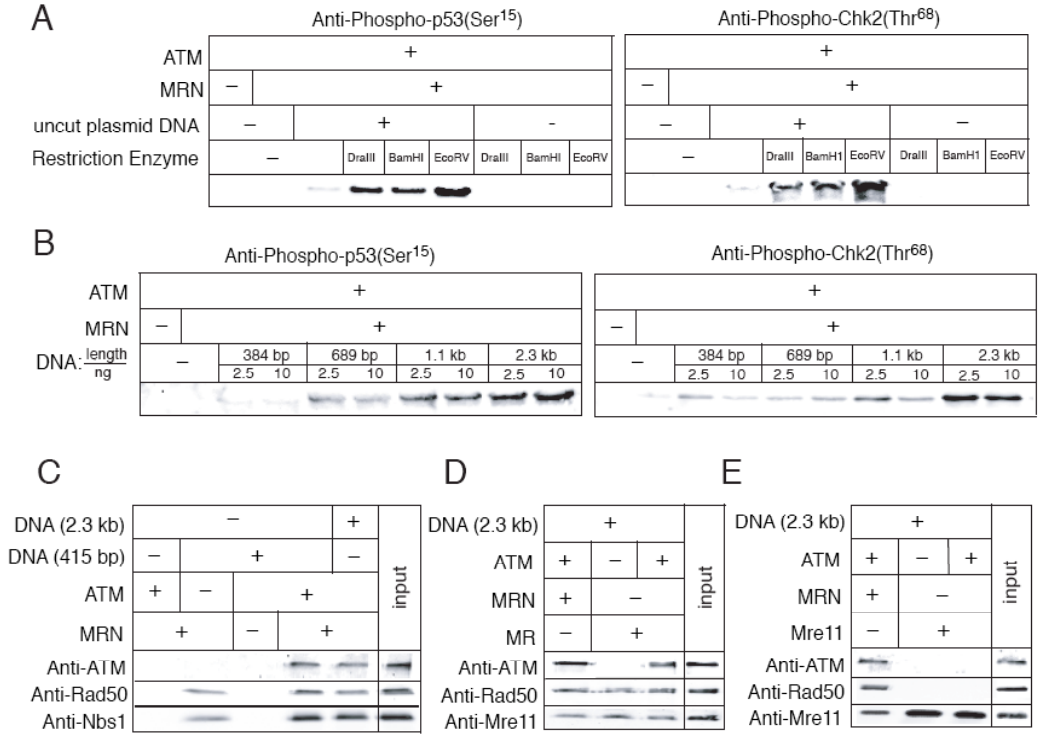

Figure 4.3 Importance of DNA ends for ATM activation through MRN.

(**A**) Kinase assays as in Fig. 1 are shown except with closed circular relaxed plasmid DNA (uncut plasmid DNA) instead of linear DNA fragments. One unit of Dra III, Bam HI, or Eco RV restriction enzyme was added as indicated. Each enzyme cuts a unique site in the plasmid. (**B**) Kinase assays with linear DNA of various lengths. (**C**) Biotinylated 415-bp and 2.3-kb DNA fragments were attached to streptavidin-coated magnetic beads and incubated with MRN and ATM. Associated MRN and ATM were detected by Western blotting. The input lane has one-10th of ATM and one-third of MRN used in the reaction. (**D** and **E**) Binding assays as in (C), except with MR or Mre11 compared to MRN.

Both the MRN complex and the larger DNA fragments are required for ATM stimulation

Both MRN and MR recruited ATM to DNA (Fig. 4.3D), yet only MRN stimulated the kinase activity of ATM (Fig. 4.1F). Similarly, ATM bound equally well to 415–base pair (bp) and 2.3-kb DNA fragments in the presence of MRN (Fig. 4.3C), yet was only efficiently activated by the larger fragments (Fig. 4.3B). Thus, ATM recruitment to DNA does not appear to be sufficient for activation.

Autophosphorylation on Ser-1981 is not essential for ATM stimulation in the presence of both MRN and DNA, *in vitro*

ATM autophosphorylation of Ser1981 (S1981) was shown to be required for the activation of ATM activity and for the damage-induced conversion of ATM dimers to monomers (Bakkenist and Kastan 2003). A small amount of ATM S1981 autophosphorylation in the *in vitro* assay was detected with a phosphospecific antibody, and it increased threefold in the presence of MRN and DNA (Fig. 4.4A).

To test whether autophosphorylation of ATM on S1981 is necessary for the DNA stimulation of dimeric ATM *in vitro*, we cotransfected Flag- and HA-tagged ATM S1981A mutant (where Ser1981 is replaced by Ala) expression constructs and purified complexes with antibodies to Flag (anti-Flag) and anti-HA (Fig. 4.1B). This S1981A protein also migrated as a dimer in the glycerol gradient (Fig. 4.1C). The S1981A dimeric ATM, as well as the wild-type dimeric protein, responded similarly to DNA and MRN, exhibiting greater than 200-fold stimulation of both p53 and Chk2 phosphorylation by DNA and MRN (Fig. 4.4, A and B). Thus, *in vitro*, S1981 autophosphorylation is not essential for MRN-dependent stimulation of dimeric ATM by DNA.

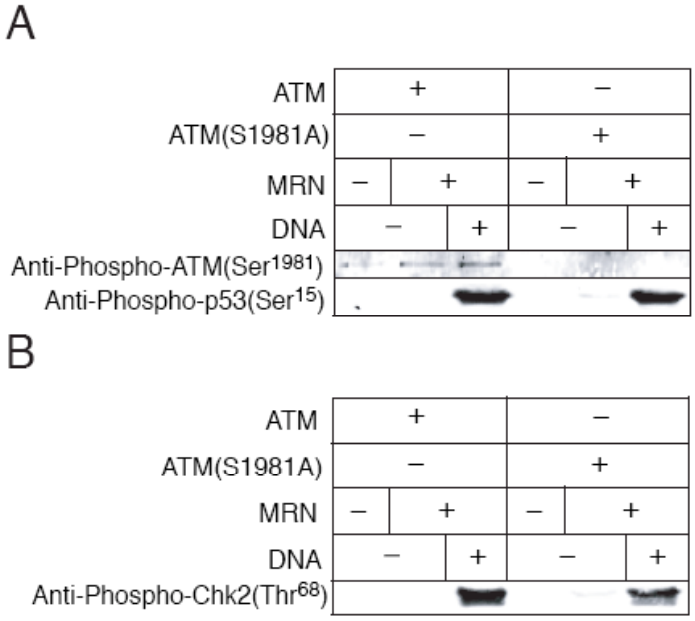

Figure 4.4 ATM autophosphorylation on Ser-1981.

(**A** and **B**) Kinase assays as in Fig. 1D, with S1981A dimeric ATM compared with wild-type dimeric ATM, with 3.6 nM MRN and linear DNA as indicated. (**C**) The Flag-ATM–HA-ATM dimer was bound to anti-Flag conjugated to agarose beads. Dissociation of ATM was monitored by Western blotting of the supernatant with anti-HA and antibodies to Flag, Rad50, and Nbs1. The input lane has one-third of the ATM and one-10th of the MRN used in the reaction. (**D**) Dissociation of the S1981A dimeric ATM complex as in (C).

The MRN complex is required for dissociation of dimeric ATM complex

The conversion of ATM dimers into monomers occurs in human cells after DNA damage and correlates with ATM activation (Bakkenist and Kastan 2003). To assay the dimer-to-monomer transition *in vitro*, we bound the Flag-ATM–HA-ATM dimer preparation to anti-Flag conjugated to agarose beads. After washing the beads, we added MRN and DNA, isolated the beads again, and analyzed the supernatant to look for dissociated ATM proteins (Fig. 4.5A). Flag-ATM was not observed in the supernatant because it was still bound to the beads, but one-third of the total HA-ATM used in the reaction was found in the supernatant when MRN was added to the dimeric ATM. This dissociation of HA-tagged ATM from the Flag-tagged ATM on the beads was not dependent on DNA and occurred similarly with S1981A dimeric ATM (Fig. 4.5B). Neither Mre11 alone nor a nonspecific protein had any effect on ATM dimer dissociation (Fig. 4.5C).

ATP binding and DNA unwinding activities of Rad50 are required for ATM stimulation

The Mre11 protein exhibits manganese-dependent nuclease activity *in vitro* (Paull and Gellert 1998). However, the nuclease activity of Mre11 is not active in the kinase assays shown here, because all of the reactions were performed in magnesium only, conditions which do not allow Mre11 nuclease activity (Paull and Gellert 1998) but support ATM activity in the presence of MRN.

The MRN complex also exhibits DNA binding and DNA unwinding activities that are dependent on both adenine nucleotides and Nbs1 (Paull and Gellert 1999; Lee *et al.* 2003a). We tested the MR(S1202R)N mutant complex (where Ser1202 of Rad50 is

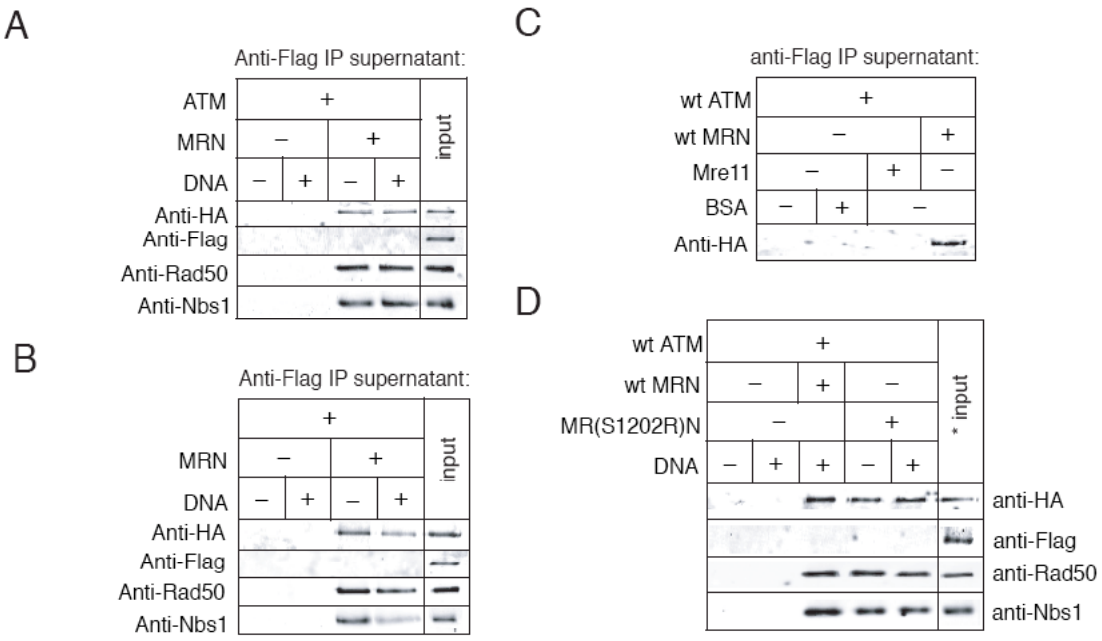

Figure 4.5 Dissociation of dimeric ATM.

(A) The Flag-ATM–HA-ATM dimer was bound to anti-Flag conjugated to agarose beads. Dissociation of ATM was monitored by Western blotting of the supernatant with anti-HA and antibodies to Flag, Rad50, and Nbs1. The input lane has one-third of the ATM and one-10th of the MRN used in the reaction. (**B**) Dissociation of the S1981A dimeric ATM complex as in (A). (**C**) ATM dissociation assay as in (A) except with only a 5 minute incubation with MRN, and additional controls of BSA and Mre11. (**D**) ATM dissociation assay as in (A) except with MR(S1202R)N compared with wild-type MRN. Input lane:1/3 of ATM, 1/10 of MRN used in the reaction.

replaced by Arg), which is specifically deficient in the adenosine triphosphate (ATP)–dependent functions of MRN (Moncalian *et al.* 2004), for stimulation of ATM activity. This mutant complex did not stimulate ATM activity (Fig. 4.6A), which indicates that at least one of the ATP-dependent activities of Rad50 is required for ATM stimulation. The deficiency of the mutant complex does not seem to be in DNA binding, because the MR(S1202R)N complex bound to DNA in equal amounts as wild-type MRN and also recruited ATM to DNA (Fig. 4.6B). The mutant complex also dissociated the ATM dimer similarly to the wild-type protein (Fig. 4.5D).

To test the importance of DNA unwinding for ATM stimulation, we prepared a DNA substrate with closed hairpins on each end. This substrate did not stimulate ATM activity, which indicates that opening of the DNA helix is required for MRN stimulation of dimeric ATM (Fig. 4.6C). We then prepared a substrate containing 60 noncomplementary base pairs at one end to mimic an unwound DNA molecule. This DNA ("unpaired ends") complemented the MR(S1202R)N mutant for ATM stimulation (Fig. 4.6D); thus, the MRN-specific role of ATP in this reaction may be to stimulate DNA unwinding.

SUMMARY AND CONCLUSION

In this study, we reconstituted DNA damage signaling to ATM with recombinant purified components. The accumulating evidence indicates that DNA breaks are sensed directly by the MRN complex, which binds DNA, unwinds the ends, recruits ATM, and dissociates the ATM dimer. These results are consistent with recent studies in budding

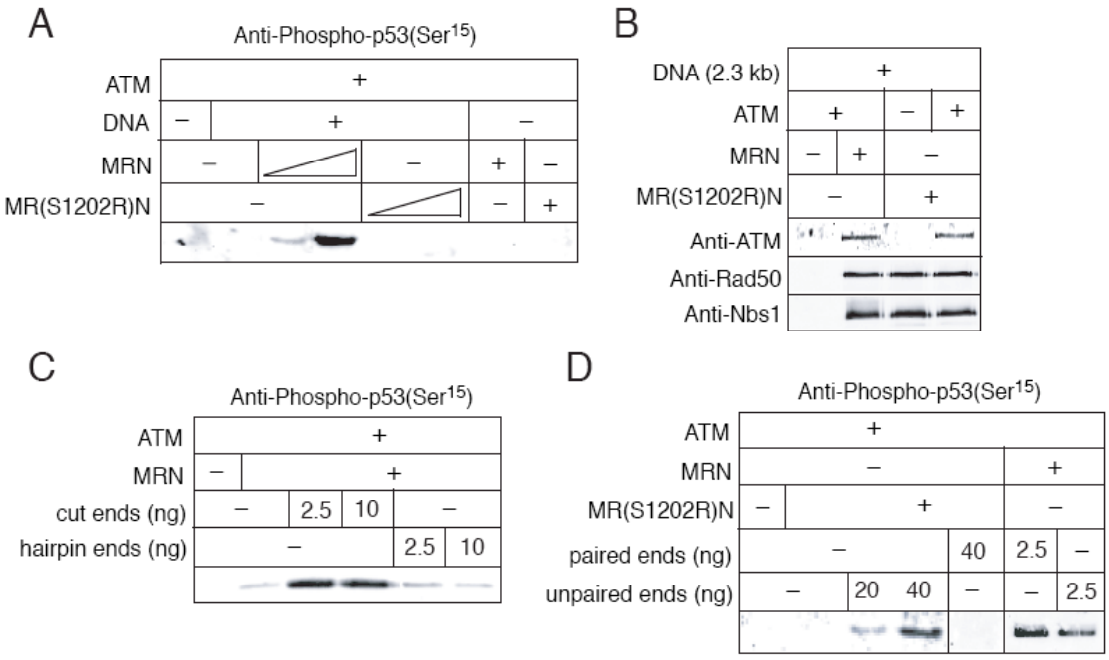

Figure 4.6 Requirement of Rad50 ATP binding and DNA unwinding for DNA-dependent stimulation of ATM.

(**A**) Kinase assays as in Fig. 1D with MR(S1202R)N complexes or wild-type MRN assayed with GST-p53. (**B**) DNA binding assays as in Fig. 2C, except with MR(S1202R)N mutant complexes compared with wild-type MRN. (**C**) Kinase assays with MRN, ATM, and DNA containing either normal cut ends or closed hairpin ends. (**D**) Kinase assays comparing 2.7-kb linear DNA (paired ends) with 2.7-kb DNA containing 60 bp of heterology on one end (unpaired ends).

yeast, which show that the Mre11-Rad50-Xrs2 complex localizes to DNA breaks very rapidly *in vivo* (Lisby *et al.* 2004; Shroff *et al.* 2004) and recruits the ATM homolog Tel1 to DNA breaks (Nakada *et al.* 2003). However, our experiments do not recapitulate the requirement for ATM autophosphorylation that is observed in human cells (Bakkenist and Kastan 2003). Effects of other factors, including protein phosphatases 5 (Ali *et al.* 2004) and 2A (Goodarzi *et al.* 2004) and chromatin remodeling complexes, may be needed to reconstitute the effects of autophosphorylation that are seen *in vivo*. Our results in this study define in mechanistic detail the pathway for ATM activation after the formation of a DNA double-strand break and provide a biochemical foundation for the characterization of other factors that influence the activity of ATM in cells.

CHAPTER 5 : CONCLUSIONS AND FUTURE DIRECTIONS

Characterization of monomeric ATM

Kinase activity of monomeric ATM

Based on our results with monomeric ATM, we conclude that the MRN complex is required for ATM-dependent phosphorylation events. We showed that the MRN complex strongly stimulated ATM kinase activity *in vitro* on p53, Chk2, and histone H2AX.

MRN (ATLD1/2) and MRN (p70) showed defects in stimulation of ATM kinase activity; however MRN (ATLD3/4) showed stimulation of ATM kinase activity similar to wild-type MRN complex. Phosphorylation of Nbs1 was critical for the MRN to stimulate the ATM kinase activity on Chk2, but not for p53 phosphorylation by ATM. These results are in agreement with previous studies using murine fibroblasts expressing Mre11(ATLD1), which shows that no phosphorylated Chk2 was observed after 5Gy of IR (Theunissen *et al.* 2003; Uziel *et al.* 2003). In another study, IR-induced Chk2 phosphorylation was not observed in NBS cells that express only the p70 form of Nbs1. In addition, the expression of S343A Nbs1 cannot complement Chk2 phosphorylation and activation in NBS cells, whereas wild-type Nbs1 can complement both Chk2 phosphorylation and activation, suggesting that Nbs1 and its phosphorylation are necessary for Chk2 phosphorylation by ATM (Buscemi *et al.* 2001; McGowan 2002). In contrast, several studies have shown that NBS and ATLD cells show normal p53 phosphorylation levels (Gatei *et al.* 2000b; Girard *et al.* 2002; Gatei *et al.* 2003; Theunissen *et al.* 2003), consistent with our results showing that the MR complex was sufficient to stimulate monomeric ATM kinase activity towards p53.

Our experiments also showed that kinase-deficient ATM inhibited wild-type ATM phosphorylation of Chk2. This result is consistent with the dominant-negative effect of kinase-deficient (kd) ATM *in vivo* since Lim *et al.* showed that overexpression of kd form of ATM inhibited the ATM-dependent phosphorylation of Nbs1 (Lim *et al.* 2000). Some missense alleles of ATM have been found to be linked to early-onset breast cancer in heterozygote carriers, also consistent with the idea of dominant-negative activity of ATM mutants (Gatti *et al.* 1999; Khanna 2000).

Interaction of monomeric ATM with MRN

Using gel filtration analysis, we showed that ATM interacts directly with the MRN complex. When wild-type ATM and MRN were loaded on to a Superose 6 column, it was observed that both fractionate close to their predicted molecular weights of 350 kDa and 1.2MDa respectively (Lee *et al.* 2003a). To determine if there was any interaction between ATM and the different mutant MRN complexes, ATM was incubated with wild-type MRN, MRN (S343A) or wild-type MR after which the reaction mixture was passed through a Superose 6 gel filtration column. Our results showed that ATM fractionated with these complexes. However, when ATM was incubated with MRN (ATLD1/2) or MRN (p70), association of ATM with the mutant MRN complexes was observed only at a low level. Taken together, these results suggest that Chk2 activation by ATM requires a strong association of ATM with the MRN complex while p53 activation does not.

ATM is associated with the MR complex even in the absence of Nbs1, although the Nbs1 protein also directly interacts with ATM in the absence of MR. Recently, Falck *et al.* suggested that there is a conserved ATM binding domain within the C-terminus of

Nbs1 (Falck *et al.* 2005). These findings suggest that ATM makes multiple direct contacts with the MRN complex – one through Nbs1 and the other through MR.

Model of monomeric ATM activation by the MRN complex

Based on the results from immunoprecipitation assays, we showed that the presence of the MRN complex stimulated binding of both p53 and Chk2 to ATM, suggesting that the MRN complex facilitates stable binding of the substrates to ATM. Therefore, we propose that the MRN complex induces a conformational change in ATM in a manner that facilitates an increase in affinity of ATM towards its substrates.

The metal ion specificity of ATM activity

ATM kinase activity was previously shown to be manganese-dependent (Chan *et al.* 2000). So far, most experiments (including our results with monomeric ATM) investigating ATM kinase activity were performed in the presence of both magnesium and manganese. However, since manganese concentrations under physiological conditions are very low, we wanted to determine if ATM is indeed manganese-dependent. We observed that ATM kinase activity is slightly higher in the presence of both manganese and magnesium when compared to the kinase activity in the presence of magnesium alone. However, manganese is not required for the activity of ATM in the presence of MRN complex since our experiments showed that when MRN was present, ATM was active irrespective of the presence of manganese. Therefore, our kinase assays with dimeric ATM are performed with magnesium alone, which resembles *in vivo* conditions (Fig. 5.1).

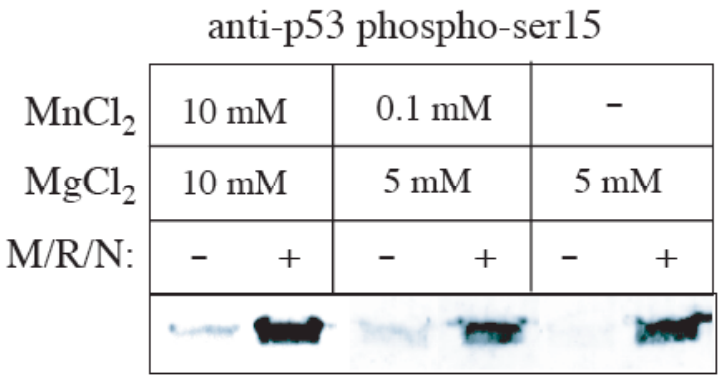

Figure 5.1 Requirement of MRN for activity of monomeric ATM in physiological magnesium.

Kinase assays contained Flag-tagged wt ATM and GST-p53 (amino acids 1 to 102), with MRN as indicated. Phosphorylation was visualized with anti-p53 phospho–Ser-15.

Characterization of dimeric ATM

Kinase activity of dimeric ATM

Bakkenist and Kastan previously showed that inert ATM dimers are dissociated into active monomers after DNA damage by autophosphorylation of ATM at the Ser-1981 residue (Fig. 1.2). From this result, we hypothesized that DNA is required for the conversion of inert dimer to active monomers. In order to determine if this is true, we attempted to purify the inert form of ATM by co-expressing ATM-Flag and ATM-HA followed by the affinity purification using both anti-Flag and anti-HA columns. Using glycerol gradient sedimentation analysis, we verified that this ATM was dimeric and also showed that the previously purified ATM was in monomeric form.

Based on the results from dimeric ATM experiments, we conclude that the MRN complex acts as a double-strand break sensor for ATM and recruits it to broken DNA ends. In the presence of DNA and the MRN complex, inactive ATM dimers are activated *in vitro,* and can then phosphorylate downstream cellular targets such as p53 and Chk2. This DNA dependence was not observed with monomeric ATM. Interestingly, dimeric ATM was not stimulated by the MR complex, suggesting that it requires the complete MRN complex containing the Nbs1 protein. We also showed that dimeric ATM requires DNA ends with a minimum length requirement of 1kb for its activation in the presence of the MRN complex.

The mode of dimeric ATM activation by the MRN complex and DNA

We showed that the MRN complex is required for the stable association of ATM with DNA. Interestingly, the MR complex also stimulated ATM binding in the presence of long or short DNA fragments with equal affinity (2.3kb and 415-bp), although the MR

complex and short DNA failed to stimulate the kinase activity of dimeric ATM. This suggests that ATM recruitment to DNA does not appear to be sufficient for its activation.

Our *in vitro* assays also suggested that the conversion of ATM from its dimeric form to the monomeric form requires the presence of the MRN complex. More importantly, this conversion does not require the autophosphorylation of ATM, conflicting with the earlier study by Kastan *et al.* Interestingly, the conversion of the dimeric form of ATM to its monomeric form does not require the presence of DNA. Lastly, we showed that the unwinding of DNA ends by MRN was essential for the stimulation of ATM which is consistent with the central role of single-stranded DNA as an evolutionarily conserved signal for DNA damage.

Does ATM kinase activity require its autophosphorylation?

It is suggested that ATM autophosphorylation is an indicator of its kinase activity since ATM was autophosphorylated *in vivo* after treatment of IR or NCS, a radiomimetic agent (Bakkenist and Kastan 2003; Ali *et al.* 2004; Buscemi *et al.* 2004; Goodarzi *et al.* 2004). Recently, Kastan *et al.* also suggested that ATM exists as an inactive monomer in normal state and becomes an active dimer by the autophosphorylation at Ser-1981 after DNA damage (Bakkenist and Kastan 2003). These findings prompted them to suggest that ATM autophosphorylation is necessary for its kinase activity. However, a small amount of our dimeric ATM was autophosphorylated by itself in our *in vitro* kinase reactions, which only produced a 3-fold increase in the stimulation after the addition of either DNA or the MRN complex (Fig. 4.4A). In Chapter 4, we also showed that ATM kinase activity was increased 80- to 200-fold in the presence of both DNA and the MRN complex. These findings suggest that autophosphorylation of ATM does not correlate

with its kinase activity. In support of this possibility, we also showed that the S1981A dimeric ATM responded similarly to DNA and the MRN complex, exhibiting greater than 200-fold stimulation of both p53 and Chk2 phosphorylation by DNA and the MRN complex (Fig. 4.4, A and B). It could be possible that there is a missing link between the *in vivo* assays and our *in vitro* assays.

Is there another protein required for ATM autophosphorylation?

It could be possible that another activator or inactivator protein is required for ATM autophosphorylation and that this is required for ATM signaling pathway *in vivo*. Recent articles show that two phosphatases, protein phosphatase 2A (PP2A) and protein phosphatase 5 (PP5), both affect ATM activity *in vivo* (Ali *et al.* 2004; Goodarzi *et al.* 2004). PP2A is a member of the protein serine/threonine phosphatase family and composed of a catalytic (C) subunit, a scaffolding A subunit and a regulatory B subunit. Guo *et al.* showed that ATM is required for the dissociation of the B regulatory subunit from PP2A complex in response to IR, suggesting that ATM directly associates with PP2A (reference; (Guo *et al.* 2002)). Recently, another group showed that ATM interacted directly with the A subunit and that the A and C subunits co-immunoprecipitated with ATM in unirradiated cells but dissociated after IR (Goodarzi *et al.* 2004). They also showed that overexpression of dominant-negative C subunit induced autophosphorylation of ATM on Ser-1981 in the absence of IR. This suggests that autophosphorylation of ATM is regulated by PP2A.

PP5 is also a member of the serine/threonine phosphatase family. Ali *et al.* showed that PP5 also co-immunoprecipitates with ATM from human cells and this interaction increases after exposure to either the radiomimetic chemical

neocarzinostatin (NCS) or IR. They also showed that the disruption of PP5 activity resulted in defects in ATM activation and radioresistant DNA synthesis (Ali *et al.* 2004). In addition, the phosphatase activity of PP5 was shown to be required for ATM autophosphorylation and activation (Ali *et al.* 2004). These findings suggest that PP5 might control ATM kinase activity by dephosphorylating ATM, the MRN complex, or other factors involved in ATM activation.

There are several possible mechanisms of PP5 function with ATM. One possibility is that ATM is phosphorylated under normal circumstances and is dephosphorylated by PP5 after DNA damage. In order to check this possibility, we tested for ATM kinase activity in the presence of wild-type PP5 or a PP5 containing mutations in the catalytic site. ATM kinase activity was not affected by the addition of wild-type or mutant PP5 in the presence of MRN and DNA (data not shown). This suggests that there is no inhibitory phosphorylation site on ATM that can be dephosphorylated by PP5. An alternate possibility is that PP5 could act in conjunction with another yet to be identified protein factor to activate ATM by dephosphorylating it. It is known that the A subunit of PP2A interacts with PP5, suggesting that PP5 requires A subunit of PP2A for its function (Lubert *et al.* 2001). A third possibility is that PP5 dephosphorylates PP2A, as a result activating ATM. Further experiments are required to determine which, if any, of these possibilities are correct.

Other putative co-activators include the mediator of DNA damage checkpoint protein 1 (MDC1) and p53 binding protein 1 (53BP1), which have BRCT repeats, similar to Nbs1. 53BP1 and MDC1 co-localize with γ-H2AX following treatment with agents that cause DNA DSBs, such as IR and etoposide (Schultz *et al.* 2000; Anderson *et al.* 2001b; Rappold *et al.* 2001; Xia *et al.* 2001; Shang *et al.* 2003). Both proteins can interact with

each other through their C-terminal BRCT repeats and are found to associate only with the phosphorylated form of H2AX (γ-H2AX) (Ward *et al.* 2003; Xu and Stern 2003b). MDC1, through its FHA domain, can also bind to phosphorylated Chk2 at threonine 68 after DNA damage (Goldberg *et al.* 2003; Lou *et al.* 2003; Peng and Chen 2003; Xu and Stern 2003a). 53BP1 has been shown to interact with methylated lysine 79 of Histone H3 and this interaction was inhibited by the suppression of methylation, suggesting that 53BP1 senses DSBs indirectly through changes in higher-order chromatin structure that expose methylated lysine 79 (Huyen *et al.* 2004). Recently, Mochan *et al.* showed that suppression of MDC1 causes decreased ATM activation and phosphorylation of ATM substrates. They also showed that phosphorylation of various ATM substrates was reduced when 53BP1 function was suppressed either by siRNA or by the knockout of the 53BP1 gene (Mochan *et al.* 2003; Mochan *et al.* 2004). These results suggested that MDC1 and 53BP1may act as co-activators or mediators for ATM function.

Recent studies showed that BRCA1 is also important in the ATM-dependent signaling pathway. It is known that BRCA1 is phosphorylated on Ser-1387 and Ser-1423 and that phosphorylation at these sites is important for the S phase and the G2/M checkpoint in response to IR, respectively (Xu *et al.* 2001; Xu *et al.* 2002b). Moreover, Fabboro *et al.* suggested that BRCA1-BARD1 complex acts as an adaptor protein between p53 and ATM and that BRCA1 needs to be phosphorylated at Ser-1423 or Ser-1524 to perform this function. They also showed that BRCA1 is dispensable for the phosphorylation of Chk2, Chk1, c-jun, and H2AX after DNA damage (Fabbro *et al.* 2004). However, this is controversial because phosphorylation of Chk2, CtIP, Nbs1, p53 and c-Jun by ATM or ATR was shown to be affected by BRCA1 in another study (Foray *et al.* 2003). Another recent report suggests that BRCA1 may have a role in ATM-

dependent phosphorylation with several other proteins since both Nbs1 and BRCA1 are required for the phosphorylation of SMC1 by ATM in response to IR (Kitagawa *et al.* 2004). Although there are conflicting results, it is clear that BRCA1 likely plays a role in the ATM signal transduction pathway.

Is the Ser-1981 the only site required for ATM activation?

It is possible that there could be other sites on ATM that are important for its activation. The autophosphorylation site on ATM, Ser-1981, is not highly conserved among different organisms, suggesting that ATM autophosphorylation on this site is not absolutely essential for ATM activation. Recent work suggests that there is an alternate autophosphorylation site on ATM (Martin Lavin, personal communication). However, the significance of this phosphorylation site is not clear since mutation of this site does not affect ATM kinase activity *in vitro* (unpublished data).

It has also come to our attention that a highly conserved cysteine residue is present close to the autophosphorylation (S1981) site. It could be possible that a disulfide bond is involved in the stabilization of the ATM dimer and the autophosphorylation contributes to the breakage of the cysteine linkage, thus activating ATM. We are currently testing this hypothesis by mutating this conserved cysteine and by preparing dimeric forms of ATM in the absence of reducing agents.

Schematic Model of ATM activation by DNA DSBs mediated by the MRN complex

Based on our results, we proposed a schematic model for ATM activation after DNA damage (Fig. 5.2). According to this model, we propose that an additional factor

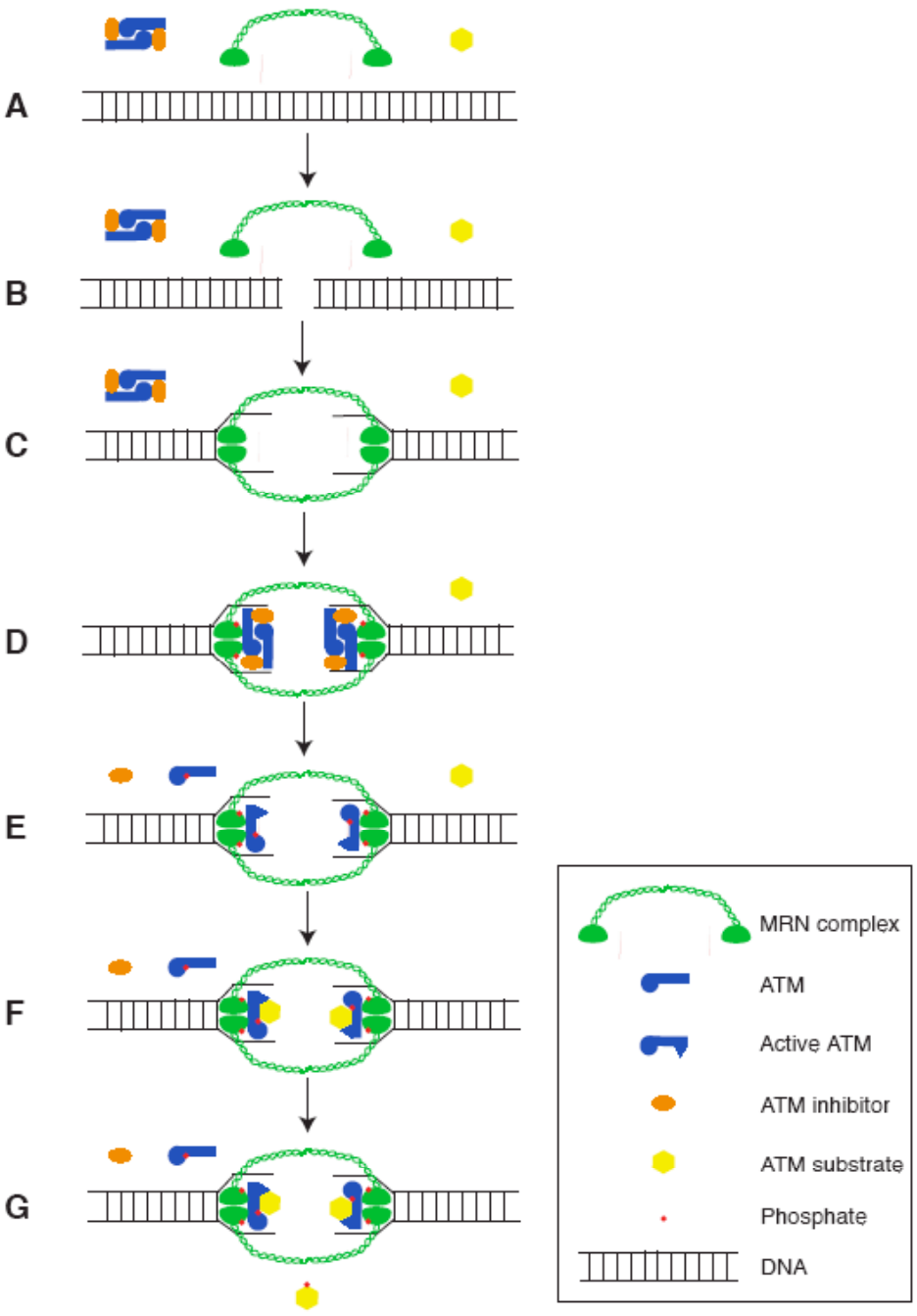

Figure 5.2 Schematic model of ATM activation by DNA double-strand breaks mediated by MRN.

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(A) Prior to DSB formation, dimeric ATM (blue) and MRN (green) are loosely bound to DNA. The ATM dimer is held together by the hypothetical ATM inhibitor (orange). After DSBs are introduced (B), MRN binds to the broken ends and unwinds the ends (C). The MRN/DNA complex recruits ATM (D) resulting in Nbs1 phosphorylation (red). The interaction of ATM with MRN induces release of the hypothetical inhibitor, ATM autophosphorylation, and monomerization (E), leaving at least a subset of activated monomeric ATM tightly associated with the MRN and DNA. Substrate molecules (yellow) are then recruited to the MRN/ATM/DNA complex with higher affinity due to a conformational change in ATM induced by interaction with the MRN/DNA complex (F). Phosphorylated substrates are released, facilitating further substrate interactions (G).

exists *in vivo*, which binds to ATM and prevents the dissociation of the dimers in the absence of DNA damage. We hypothesize that the release or inhibition of this factor requires the autophosphorylation and that in its absence the autophosphorylation is not required.

Is ATM activated without DNA breaks?

Bakkenist and Kastan showed that chromatin-modifying agents induced autophosphorylation of ATM on serine 1981 in the absence of DNA breaks (Bakkenist and Kastan 2003). This suggests that ATM can be activated without DNA breaks *in vivo*, and that it may be possible to model this DSB-independent activation process *in vitro*. To test for activation of ATM in the absence of DNA breaks and the role of the MRN complex in this process, ATM kinase activity will be tested with DNA substrates of varying topology. We have preliminary evidence that negatively supercoiled DNA can activate ATM in the presence of the MRN complex (unpublished data).

Does ATM interact with DNA without DNA breaks?

In dividing cells, ATM is found in the nucleus. After treatment with DNA damaging agents, a fraction of ATM adheres strongly to the chromatin but not in cells with MRN deficiencies. This observation is consistent with our results showing that ATM binds to DNA ends through the MRN complex. In detergent extracted cells, it was observed that very little amounts of ATM and MRN complex associate with chromatin in the absence of a DNA damaging agent (Mirzoeva and Petrini 2001; Uziel *et al.* 2003) . This suggests that interaction between ATM/MRN and undamaged DNA is very weak. It is possible that ATM and MRN stably bind DNA only after breaks are formed. In order to

determine if this is true, interaction of ATM with different forms of DNA using an IP assay will be performed.

Why are Mre11 and Nbs1 phosphorylated by ATM?

Nbs1 is phosphorylated in an ATM-dependent manner after DNA damage (Gatei *et al.* 2000b; Lim *et al.* 2000; Wu *et al.* 2000b; Zhao *et al.* 2000; Stewart *et al.* 2001; Kim *et al.* 2002; Lee *et al.* 2003b). Phosphorylation of Nbs1 is also shown to be required for the subsequent phosphorylation of Chk2, SMC1, and FANCD2 (Buscemi *et al.* 2001; Nakanishi *et al.* 2002; Yazdi *et al.* 2002). We also showed that the MRN complex containing mutant Nbs1 (S343A) failed to stimulate the phosphorylation of Chk2 by ATM.

Mre11 is also phosphorylated on at least two SQ sites by ATM *in vivo* (Jean-Yves Masson and Martin Lavin, personal communication). However, the consequences of Mre11 phosphorylation on ATM-dependent phosphorylation events are still not clear. One possibility for the requirement of phosphorylated Mre11 could be that certain ATM substrates might require Mre11 to be phosphorylated. We have found that mutation of the serine residues in Mre11 and Nbs1 to either alanine or aspartate results in destabilization of the complex, thus this issue has been difficult to address. It is also possible that phosphorylation of these residues *in vivo* results in the disassembly of the MRN complex, a question we are currently trying to address.

The role of MRN in ATR pathway

ATR is involved in the cell's response to treatment with UV light, stalled replication forks and hypoxia and it is responsible in regulating the later stages in the

DSB response, although ATM is primarily responsible during the initial stages of a DSB. Recently, ATR-Seckel syndrome was identified as a subclass of Seckel syndrome, and patients with this disorder have hypomorphic mutations in the ATR gene (O'Driscoll *et al.* 2003). The clinical features of ATR-Seckel patients overlap with those of NBS patients, including growth retardation, microcephaly, and a characteristic facial features (reviewed in O'Driscoll *et al.* 2004). ATM and ATR also share common substrates, including p53, Chk1, and BRCA1. These findings suggest that the MRN complex might have a role in the ATR-dependent signaling pathway. A recent study strongly supported this by showing that ATR-dependent phosphorylation of Chk1 and p53 but not H2AX are impaired after UV treatment in NBS and ATR-Seckel fibroblast cells (Stiff *et al.* 2005). Moreover, Zhong *et al.* also showed by RNA interference that the MRN complex regulates ATR-dependent phosphorylation of SMC1 and Chk1 but not H2AX after UV exposure (Zhong *et al.* 2005). However, the mechanism by which the MRN complex facilitates ATR-dependent phosphorylation is still unknown.

One possibility is that the MRN complex might activate ATR by processing DNA ends. It has been shown that association of ATR with replication protein A (RPA) and ssDNA is required for ATR kinase activity following ionizing radiation. These results suggest that broken DNA ends must be processed to single-stranded regions to activate ATR. A recent study suggested that the MRX complex functions in concert with exonuclease 1 (Exo1) to activate Mec1, the yeast homolog of human ATR, by generating long ssDNA tails and facilitating the association of Mec1 with ssDNA (Nakada *et al.* 2004).

There could be other possible ways that the MRN complex could stimulate ATR. One possible mode would be to facilitate stable binding of ATR with its substrates,

similar to that observed with ATM. A recent study suggested that the ATR-ATRIP complex might interact with DNA through either RPA-dependent or RPA-independent mechanisms since the RPA-depleted ATR-ATRIP complex from nuclear extracts can associate with ssDNA cellulose *in vitro*. However, purified ATR and ATRIP was unable to bind ssDNA *in vitro*. These findings suggest that another protein, such as the MRN complex, may mediate the binding of ATR-ATRIP to ssDNA (Bomgarden *et al.* 2004). If this is indeed true, it will be interesting to determine whether the MRN complex can substitute for RPA in ATR activation.

It has been shown that each of the damage-induced PI3K-like protein kinases (PIKKs) require other proteins to localize to DNA lesions. For example, ATM associates with DNA through the MRN complex, ATR requires ATRIP, and DNA-Pkcs requires the Ku70/80 complex (Falck *et al.* 2005). Each of these interactions has been shown to be mediated, at least a part, through conserved PIKK interaction motif in the C-termini of each DNA-binding component (Nbs1, ATRIP, and Ku80, respectively). In the MRN complex, the Nbs1 component contains a PIKK interaction motif and it could be possible that the same conserved C-terminal motif on Nbs1 can directly interact with ATR. We are currently investigating whether the MRN complex can activate ATR/ATRIP in the presence of RPA and ssDNA *in vitro* to test this possibility.

APPENDIX A

Effects on nucleotide-dependent DNA binding and association with Ataxia-Telangiectasia-Like Disorder mutant complexes

Abstract

The Mre11/Rad50 complex is a critical component of the cellular response to DNA double-strand breaks, in organisms ranging from archaebacteria to humans. In mammalian cells, Mre11/Rad50 (M/R) associates with a third component, Nbs1, that regulates its activities and is targeted by signaling pathways that initiate DNA damage-induced checkpoint responses. Mutations in the genes that encode Nbs1 and Mre11 are responsible for the human radiation sensitivity disorders Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder (ATLD), respectively, which are characterized by defective checkpoint responses and high levels of chromosomal abnormalities. Here we demonstrate nucleotide-dependent DNA binding by the human M/R complex that requires the Nbs1 protein and is specific for double-strand DNA

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duplexes. Efficient DNA binding is only observed with non-hydrolyzable analogs of ATP, suggesting that ATP hydrolysis normally effects DNA release. The alleles of MRE11 associated with ATLD and the C-terminal Nbs1 polypeptide associated with NBS were expressed with the other components and found to form triple complexes except in the case of ATLD 3/4, which exhibits variability in Nbs1 association. The ATLD 1/2, ATLD 3/4, and p70 M/R/N complexes exhibit nucleotide-dependent DNA binding and exonuclease activity equivalent to the wild-type enzyme, although the ATLD complexes both show reduced activity in endonuclease assays. Sedimentation equilibrium analysis of the recombinant human complexes indicates that Mre11 is a stable dimer, Mre11 and Nbs1 form a 1:1 complex, and both M/R and M/R/N form large multimeric assemblies of 1.2 MDa. Models of M/R/N stoichiometry in light of this and previous data are discussed.

Introduction

The *mre11* and *rad50* mutants in *Saccharomyces cerevisiae* were originally named for the deficiencies in meiotic recombination and ionizing radiation survival observed in mutant strains (Game and Mortimer 1974; Ajimura *et al.* 1993). We now know that the protein products of these two genes associate together with a third component, Xrs2, in budding yeast, and that the complex plays important roles in homologous recombination, non-homologous end-joining, telomere maintenance, S-phase checkpoint control, and meiotic recombination (Alani *et al.* 1990; Ivanov *et al.* 1992; Moore and Haber 1996; Kironmai and Muniyappa 1997; Boulton and Jackson 1998; Haber 1998; Bressan *et al.* 1999; Chen *et al.* 2001a; D'Amours and Jackson 2001; Grenon *et al.* 2001; D'Amours and Jackson 2002; Lobachev *et al.* 2002; Gonzalez-

Barrera *et al.* 2003). Homologs of Mre11 and Rad50 have been identified in every organism that has been genetically characterized, as well as some bacteriophage, indicating that the functions of the complex are fundamental to DNA transactions. Proteins analogous to the Xrs2 component have not been identified in prokaryotes or archaebacteria, however, suggesting that this part of the complex is unique to eukaryotic cells. In mammals, the Mre11 and Rad50 proteins associate with a protein called Nbs1 (also nibrin and p95) (Carney *et al.* 1998) into a large complex, Mre11/Rad50/Nbs1 (M/R/N).

Nbs1 has little if any sequence similarity to Xrs2, but has clinical significance because mutations in the NBS1 gene have been identified as causal factors in the human autosomal recessive genetic disorder Nijmegen breakage syndrome (NBS) (Varon *et al.* 1998). Patients with NBS exhibit radiation sensitivity, immune system deficiency, and a high rate of malignancy (Shiloh 1997). At the cellular level, abnormalities include a defective S-phase checkpoint response and an elevated rate of chromosomal breakage and translocations, although few overt deficiencies in DNA repair are found. It was recently demonstrated that the most common NBS allele, 657del5, can generate a C-terminal polypeptide through the use of an internal ribosome entry site upstream of the 5-nucleotide deletion (Maser *et al.* 2001). This translation product (p70) is capable of binding Mre11; thus, the 657del5 NBS allele is very likely a hypomorphic allele that can supply a subset of the normal functions of Nbs1.

The clinical presentation of NBS patients constitutes a subset of the phenotype of patients with ataxia-telangiectasia (A-T), a related radiation sensitivity disorder. A-T is caused by mutations in the A-T-mutated gene (ATM), which encodes a large protein kinase that initiates DNA damage signaling in response to DNA double-strand breaks in

eukaryotic cells. A biochemical connection between the two disorders was established with the demonstration that the ATM protein kinase phosphorylates the Nbs1 protein, in addition to many other targets, and that Nbs1 phosphorylation by ATM is essential for a normal S-phase checkpoint response (Gatei *et al.* 2000b; Lim *et al.* 2000; Wu *et al.* 2000b; Zhao *et al.* 2000). An additional connection between M/R/N and ATM arose with the identification of two families with A-T-like disorder (ATLD), clinically identical to A-T, yet caused by mutations in the MRE11 gene (Stewart *et al.* 1999). The two known ATLD mutations are quite different; the ATLD 1/2 allele contains a premature stop codon resulting in a truncation of the C-terminal domain, whereas the ATLD 3/4 allele contains a missense mutation within the nuclease domain in the N terminus. The biochemical basis of the abnormalities seen in ATLD patients has not yet been elucidated.

Using a recombinant baculovirus system, we have expressed the human Mre11, Rad50, and Nbs1 proteins together and found that they form a large protein complex that exhibits several distinct enzymatic activities on DNA substrates. The Mre11 protein contains highly conserved phosphoesterase motifs that are responsible for the manganese-dependent nuclease activities of M/R/N. By itself and in association with Rad50 and Nbs1, Mre11 exhibits a distributive, 3' to 5' exonuclease activity on blunt and 3' recessed ends (Paull and Gellert 1998; Trujillo *et al.* 1998), as well as a weak endonuclease activity on distorted DNA substrates such as hairpin structures. Association of Nbs1 with Mre11 stimulates the endonuclease function to act on hairpin structures and on 3' overhangs (Paull and Gellert 1999), although Nbs1 itself has no apparent enzymatic activities.

The Rad50 protein contains conserved Walker A and Walker B ATPase motifs that

are closely related to the ABC transporter family of membrane-associated ATPases. A long coiled-coil region separates the N-terminal and C-terminal ATP binding domains, which are thought to associate with each other via intramolecular, antiparallel association of the coiled-coil region along its length, a hypothesis supported by microscopy data and by analogy with the SMC family of related coiled-coil proteins (de Jager *et al.* 2001; Haering *et al.* 2002; Hirano and Hirano 2002). M/R can catalyze a limited DNA unwinding reaction on DNA ends that is stimulated by ATP and also requires Nbs1 (Paull and Gellert 1999).

Crystal structures of the catalytic cores of Mre11 and Rad50 homologs from the archaebacterium *Pyrococcus furiosus* illuminate the essential components of the active sites for both the phosphoesterase and the ATPase (Hopfner *et al.* 2000a; Hopfner *et al.* 2000b). Biochemical studies with the *P. furiosus* Rad50 protein also indicated that ATP binding induced dimerization of the Walker A/Walker B catalytic unit, and that this dimerization interface promoted ATP-dependent DNA binding by the protein. *S. cerevisiae* Rad50 had previously been shown to exhibit nucleotide-dependent binding to DNA as well (Raymond and Kleckner 1993). Mutations in the ATP-binding motifs of Rad50 in budding yeast have been shown to be equivalent to null mutations with respect to meiotic recombination, mitotic recombination, and growth rate (Alani *et al.* 1990), so ATP binding (and likely also hydrolysis) is essential to Rad50 function.

In this study, we have investigated the nucleotide-dependent biochemical properties of the human M/R/N complex, focusing on the role of the Nbs1 protein. Nbs1 stimulates nucleotide-dependent DNA binding by Mre11/Rad50 (M/R), and these complexes are promoted and stabilized by the presence of non-hydrolyzable ATP analogs. Nucleotide-bound M/R/N binds specifically to double-stranded DNA duplexes,

but does not show an obvious preference for DNA ends. Association of Nbs1 with the rest of the complex is destabilized in one of the ATLD M/R/N complexes (ATLD 3/4) but not in the other (ATLD 1/2), but both ATLD complexes show reduced levels of endonuclease activity compared with the wild-type enzyme. The M/R/N(p70) complex exhibits enzymatic activities essentially equivalent to the wild-type enzyme. Finally, sedimentation equilibrium analysis of the complex shows that Mre11 is clearly a dimer in solution, whereas both M/R and M/R/N are extremely large protein assemblies of 1.2 MDa.

Experimental procedures

Plasmid Expression Constructs—Baculovirus expression constructs for human Mre11, Rad50, and Nbs1 have been described previously (Paull and Gellert 1998; Paull and Gellert 1999): pTP17, pTP11, and pTP36, respectively. The ATLD 1/2 allele of Mre11 was generated by PCR from pTP17, generating a 6-histidine tag and stop codon at arginine 633, to form pTP219. A bacmid was made from this plasmid using the Bac-to-Bac system (Invitrogen), to form pTP221. The ATLD 3/4 allele of Mre11 was generated using the QuikChange system (Stratagene), introducing the N117S mutation into pTP17 to make pTP131 and subsequently the bacmid form, pTP133. The S1202R version of Rad50 was also generated using QuikChange in the hRad50 gene in pFastBac1 to make pTP140, and the bacmid version, pTP141. The p70 version of Nbs1 was constructed in two steps. First, a 5-nucleotide deletion was made at position 657 in the Nbs1 gene using QuikChange to re-create the 657del5 Nbs1 allele. Second, an N-terminal truncated version of this allele was generated by PCR, using the ATG at position 602 of the original gene as the initiator codon. This version of Nbs1 was cloned into pFastBac1 (Invitrogen)

without an affinity tag, generating pTP270 and the bacmid form, pTP271. GST-Nbs1 was constructed by insertion of the glutathione *S*-transferase gene from pGEX-4T-1 (Amersham Biosciences) into pTP36 (Paull and Gellert 1999) to create a fusion protein with GST at the N terminus of Nbs1. All of the alleles generated by PCR were sequenced in their entirety. Each of the bacmids was used to make recombinant baculovirus according to the instructions from the manufacturer.

Substrate DNA—The substrate in the gel mobility shift assays and in the 3' overhang cutting assay consisted of TP423 (CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGACAGGCCAGATC) annealed to TP424 (CACAGTGCTACAGACTGGAACAAAAACCCTGCAGTACTCTACTCATCTC). TP423 was labeled with 32P at the 5' end for the gel mobility shift assays, or at the 3' end for the 3' overhang cutting assay. Labeling was performed with T4 polynucleotide kinase on the 5' end (New England Biolabs) using [-32P]ATP, or with terminal deoxynucleotidyltransferase on the 3' end (Roche Molecular Biochemicals) using [-32P]cordycepin. The single-stranded DNA used in Fig. A.2A consisted of labeled TP423 only. The plasmid DNA used as a competitor in Fig. A.2 was a derivative of the topo 2.1 vector (Invitrogen), unrelated in sequence to the substrate oligonucleotides. The substrate used in Fig. A6A for the exonuclease assay consisted of TP74 annealed to TP124 (Paull and Gellert 2000), with TP74 labeled with 32P at the 5' end. The hairpin substrate consisted of TP355 (CATCCATGCCTACCTGAGTACCAGTAGCTACTGGTACTCAGGTAGGCATGGATGCCAG ATCGAC), labeled with 32P at the 5' end.

Proteins—M/R/N complexes were purified as previously described (Paull and Gellert

1998). The mutant M/R/N complexes were purified using the same protocol as with the wild-type complex. All of the Mre11 proteins and Rad50 contain a C-terminal histidine tag. Nbs1 (wild-type and p70) expressed with Mre11 or with both Mre11 and Rad50 did not contain an affinity tag. GST-Nbs1 was expressed by itself in the baculovirus system and purified over a glutathione-Sepharose column, followed by ion exchange chromatography on Q Sepharose (Amersham Biosciences). GST-Nbs1 eluted from the Q Sepharose at 0.2 M NaCl. Protein concentrations were determined by Bradford assay (Pierce) and confirmed by comparison with protein standards in Coomassie-stained SDS-PAGE gels. Western blotting of Mre11, Rad50, and Nbs1 was performed with antibodies PC388 (Oncogene), MS-RAD10 (Genetex), and MS-NBS10 (Genetex), respectively, on polyvinylidene difluoride membrane (Millipore) using standard immunoblotting techniques.

Assay Conditions—Gel mobility shift assays were performed in a volume of 10 μl with 25 mM MOPS, pH 7.0, 50 mM NaCl, 1 mM dithiothreitol, 0.1% Tween 20, 100 μg/ml bovine serum albumin, 5 mM magnesium chloride, 0.5 mM AMP-PNP or ATP as indicated, and 1 nM oligonucleotide substrate, with M/R/N complex added as indicated in the figure legends (between 20 and 120 ng, which is 1.7–10 nM assuming 1.2×10^6 g/mol for M/R/N). In Fig. A.2 (B and C), unlabeled plasmid DNA or single-stranded DNA (TP423) was added to the reaction before the addition of protein. M/R/N was incubated with the DNA and other reaction components for 15 min at room temperature before the addition of 1 μl of 50% glycerol and separation on a 0.7% agarose, 0.5x Tris borate-EDTA (TBE) gel at 5.7 V/cm for 100 min. Gels were dried and analyzed using a phosphorimager (Amersham Biosciences). In the competitor assays, the amount of

complex formed by M/R/N in the absence of competitor was considered 100%, and the decreases in complex formation relative to that amount were determined by quantitative phosphorimager analysis.

Nuclease assays were performed in a volume of 10 μl with 25 mM MOPS (pH 7.0), 50 mM NaCl, 1 mM dithiothreitol, 1 mM manganese chloride, 1 nM oligonucleotide substrate, with M/R/N complex added as indicated at 20–25 nM (hairpin and 3' overhang assays) or 2.0–2.5 nM (exonuclease assays). M/R/N was incubated with the DNA and other reaction components at 37 °C for 90 min (hairpin and 3' overhang assays) or 15–30 min (exonuclease assays) before the addition of 1 μl of 2% SDS, 0.1 M EDTA. ATP (0.5 mM) was included in the 3' overhang nuclease assays as indicated in Fig. A.5C. Reactions were lyophilized, resuspended in 7 μl of formamide loading buffer, separated on a denaturing, 20% polyacrylamide sequencing gel, and analyzed by phosphorimager.

Analytical Ultracentrifugation—Mre11, Rad50, and Nbs1 protein complexes were dialyzed overnight against buffer A (50 mM NaCl, 25 mM Tris, pH 8.0, and 5 mM 2-mercaptoethanol) (Mre11) or buffer B (100 mM NaCl, 25 mM Tris, pH 8.0, 5 mM 2-mercaptoethanol, and 5% (v/v) glycerol) (Mre11/Rad50, Mre11 ATLD 1/2/Nbs1 and Mre11/Rad50/Nbs1) at 4 °C. Samples were loaded and studied at nominal loading concentrations of 0.35 (Mre11), 0.11 (Mre11/Rad50), 0.31 (Mre11 ATLD1/2/Nbs1), and 0.08–0.20 (Mre11/Rad50/Nbs1) *A*280. In the case of the double (Mre11/Rad50) and triple complexes loaded at 0.11 and 0.08 *A*280, respectively, data were also collected at shorter wavelengths of 230 and 225 nm.

Sedimentation equilibrium experiments were conducted at 4.0 °C on a Beckman Optima XL-A analytical ultracentrifuge. Mre11 samples were studied at 6,000, 8,000, and 10,000 rpm. Complexes of Mre11 with Rad50 and with both Nbs1 and Rad50 were studied at rotor speeds of 3,000, 3,500, and 4,000 rpm, whereas complexes of Mre11 ATLD1/2 with Nbs1 were studied at rotor speeds of 6,000, 7,000, and 8,000 rpm. Data were acquired as an average of eight absorbance measurements at 280 nm and a radial spacing of 0.001 cm. Scans were collected at 6-h intervals until equilibrium was reached. Data were analyzed in terms of a single ideal solute to obtain the buoyant molecular mass, $M1(1 - \upsilon)$, using the Optima XL-A data analysis software (Beckman, Microcal Origin 3.78). In the case of Mre11 ATLD1/2 with Nbs1, data were analyzed in terms of two non-interacting solutes. Residuals were calculated. A random distribution of the residuals around zero was obtained as a function of the radius. A global analysis in terms of a single ideal solute performed in Sigma Plot 2001 as described (Ghirlando *et al.* 1995) yields similar results. Values of the molecular mass, M, were obtained from the buoyant molecular mass, $M1(1 - \upsilon)$, and calculated using densities, , at 4.0 °C obtained from standard tables. Values of υ = 0.7303, 0.7326, and 0.7339 ml g^{-1} were calculated for Mre11, ATLD1/2, and Nbs1, respectively, based on the amino acid composition using consensus data for the partial specific molar volumes of amino acids (Perkins 1986). Otherwise, a consensus protein value of 0.73 ml g^{-1} was utilized.

Results

Nucleotide-dependent DNA Binding by M/R/N—The crystallographic structure of the *P. furiosus* Rad50 enzyme suggested that heterodimeric Rad50 catalytic domains further dimerize into a tetrameric unit in the presence of ATP (Hopfner *et al.* 2000b).

This change in multimeric state, stabilized by ATP, was hypothesized to facilitate DNA binding by the enzyme. Rad50 from *S. cerevisiae* has also been reported to bind DNA in an ATP-dependent manner (Raymond and Kleckner 1993). Despite this compelling evidence, we have not observed any significant ATP dependence in DNA binding by the human M/R/N enzyme (Fig. A.1A, lanes 4 and *5* and data not shown).

When we measured DNA binding by wild-type M/R/N in the presence of magnesium and the non-hydrolyzable ATP analog AMP-PNP, however, we observed 20–40-fold higher levels of DNA binding compared with reactions with ATP or without added nucleotides (Fig. A.1A, *lanes 6* and *7*). The protein concentrations used here are 2–3-fold lower than the concentrations previously used to demonstrate M/R/N binding of DNA in the absence of nucleotides (Paull and Gellert 1999). We have also observed a similar phenomenon with the non-hydrolyzable ATP analog ATPS, and in each case, magnesium is absolutely required for formation of the protein-DNA complex (data not shown).

We also tested subcomplexes of M/R/N for AMP-PNP-dependent DNA binding and found that Mre11/Rad50 (M/R) was incapable of binding DNA under these conditions, suggesting that the presence of Nbs1 in the complex is necessary for nucleotide-dependent DNA binding (Fig. A.1B). We confirmed this to be the case by performing DNA-binding assays with M/R plus varying amounts of GST-Nbs1, which was expressed and purified in the absence of Mre11 and Rad50. As shown in Fig. A.1C, the addition of GST-Nbs1 restored AMP-PNP-dependent DNA binding to the M/R complex (Fig. A.1C, lanes 5 and *6*), yet did not exhibit any DNA-binding capability alone (*lane 7*).

The Rad50 protein is the only component of the complex that contains ATP-

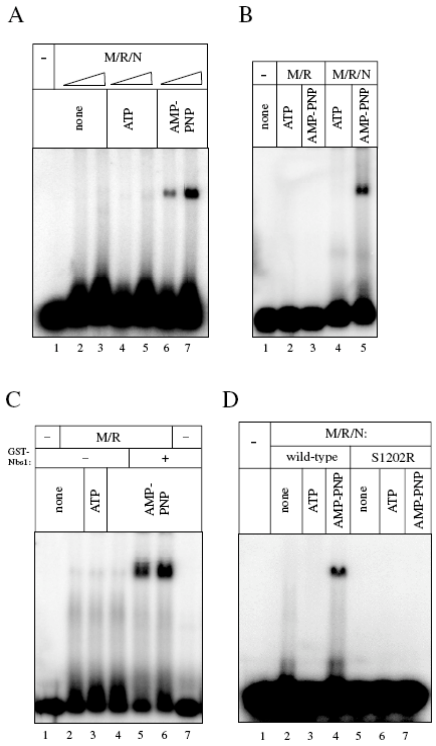

Figure A.1 Nbs1 alters the DNA binding properties of Mre11/Rad50.

A, gel mobility shift assays were performed with M/R/N in the presence of magnesium and either ATP or AMP-PNP, as indicated. Reactions in *lanes 2, 4*, and *6* contained 20 ng of M/R/N, and reactions in *lanes 3, 5*, and *7* contained 60 ng of M/R/N (1.7 and 5 nM, respectively). Proteins were mixed with a 32P-labeled double-stranded DNA substrate containing 3' overhangs at each end, and incubated for 15 min at room temperature before electrophoresis in a 0.7% 0.5x TBE-agarose gel. *B*, gel mobility shift assays were performed as in *A* with 60 ng of M/R/N or 120 ng of M/R, and ATP or AMP-PNP, as indicated. *C*, gel mobility shift assays were performed as in *A* with 180 ng of M/R, and ATP or AMP-PNP, as indicated. 150 ng of GST-Nbs1 protein was also included in reactions 5–7. In the reaction shown in *lane 5*, the GST-Nbs1 was pre-incubated on ice with the M/R complex for 10 min before the addition of the other reaction components. In the reactions shown in *lanes 6* and *7*, the GST-Nbs1 was added to M/R simultaneously with the labeled DNA and reaction mix. *D*, gel mobility shift assays were performed as in *A* with 60 ng of wild-type M/R/N or 60 ng of M/R(S1202R)/N, and ATP or AMP-PNP, as indicated.

binding motifs and is therefore the likely source of nucleotide-dependent DNA binding by M/R/N. To confirm this, we expressed a mutant form of Rad50 containing a single amino acid change (S1202R) that is equivalent to the mutation made in the *P. furiosus* enzyme (S793R), which was previously shown to abolish ATP-driven dimerization of the ATP-binding domain (Hopfner *et al.* 2000b). The S1202R Rad50 mutant in association with wild-type Mre11 and Nbs1 failed to bind DNA in the presence of AMP-PNP, verifying that the ATP-binding domain of the Rad50 protein is required for the protein-DNA complex observed with the wild-type enzyme (Fig. A.1D).

The AMP-PNP-dependent M/R/N complex is only formed on double-stranded DNA duplexes and not on single-stranded oligonucleotides, as shown in Fig. A.2A. Addition of increasing amounts of M/R/N to the labeled double-stranded DNA in the presence of magnesium and AMP-PNP actually generates two types of protein-DNA complex; the low mobility species previously indicated (complex I), and a higher mobility species that migrates close to the unbound DNA (complex II). Formation of each complex is highly cooperative, normally appearing at a threshold of 30–60 ng of M/R/N per 10-μl reaction (equivalent to 2.5–5nM, considering a molecular mass of 1.2 MDa). The relative amounts of labeled DNA in the two species do not change with increasing M/R/N concentration, although the mobility of both complexes decreases, suggesting that more protein can be incorporated into each complex with additional M/R/N. Under these conditions, essentially 100% of the DNA is incorporated into one or the other of these forms, with a majority of the DNA in complex II. Preliminary off-rate experiments suggest that complex I has an off-rate on the order of several minutes, whereas the off-rate of complex II is less than 30 s (data not shown).

Competition studies with M/R/N on DNA duplexes indicated that both linear and

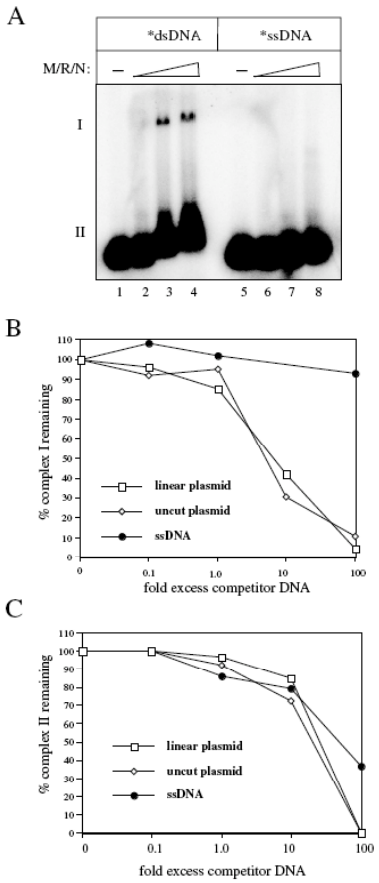

Figure A.2 The nucleotide-dependent protein-DNA complex formed by M/R/N is specific for double-stranded DNA.

A, gel mobility shift assays were performed as in Fig. 1 with varying amounts of wild-type M/R/N in the presence of AMP-PNP and a 32P-labeled double-stranded DNA substrate (**TP423/TP424*; *lanes 1–4*) or a 32P-labeled single-stranded DNA substrate (**TP423 only*; *lanes 5–8*). Reactions contained 30 ng (*lanes 2* and *6*), 60 ng (*lanes 3* and *7*), or 120 ng (*lanes 4* and *8*) of M/R/N, as indicated. *B*, gel mobility shift assays were performed as in Fig. 1 with 60 ng of M/R/N and varying amounts of unlabeled competitor DNA: linearized plasmid, uncut plasmid, or a single-stranded oligonucleotide. 0.1, 1, 10, or 100-fold excess (mol nucleotides) of competitor DNA was mixed with the 32P-labeled DNA before addition of the protein. The amount of low mobility complex (*I*) without competitor was quantified and set to 100%, and the decrease in the formation of this complex with increasing competitor was quantified and shown here. *C*, assays were performed with the same DNA competitors as in *B* except quantifying the higher mobility complex (*II*).

uncut plasmid DNA could compete effectively with short DNA duplexes for M/R/N binding in complex I (Fig. A.2B). Thus, DNA ends are not essential for nucleotide-dependent binding by M/R/N. Single-stranded oligonucleotide (Fig. A.2B) and tRNA (data not shown) did not compete for binding. In contrast, single-stranded DNA was able to partially compete with the double-stranded duplex in complex II (Fig. A.2C), indicating that complex II is less specific for duplex DNA. Mre11 protein alone is capable of forming a complex similar to complex II in gel mobility shift assays (Paull and Gellert 1999), also suggesting that this complex does not require functional Rad50 protein. We have not observed any sequence specificity of M/R/N binding to DNA substrates, although an exhaustive analysis of this question has not been performed.

ATLD Mutant Complexes—Mutations in Mre11 have been identified in human patients with the inherited genomic instability syndrome ATLD (Stewart *et al.* 1999). Two distinct mutations, ATLD 1/2 and ATLD 3/4, were identified in different family groups. The ATLD 1/2 allele contains a premature stop codon, truncating the protein at amino acid 633, and eliminating the last 76 amino acids. The ATLD 3/4 allele contains a missense mutation, N117S, but encodes a polypeptide of normal length. These alleles confer a clinical phenotype virtually identical to A-T.

We expressed the R633stop (ATLD1/2) and N117S (ATLD 3/4) alleles of Mre11 together with wild-type Nbs1 and Rad50 and isolated complexes with each protein (Fig. A.3A). The R633stop version of Mre11 purified with Nbs1 and Rad50 identically to wild type, yielding a triple complex of similar apparent stoichiometry to the wild-type complex. In contrast, the N117S Mre11 usually failed to bind Nbs1, but did form a complex with Rad50 (Fig. A.3A, lane 3, ATLD 3/*4*). The N117S Mre11 mutant was

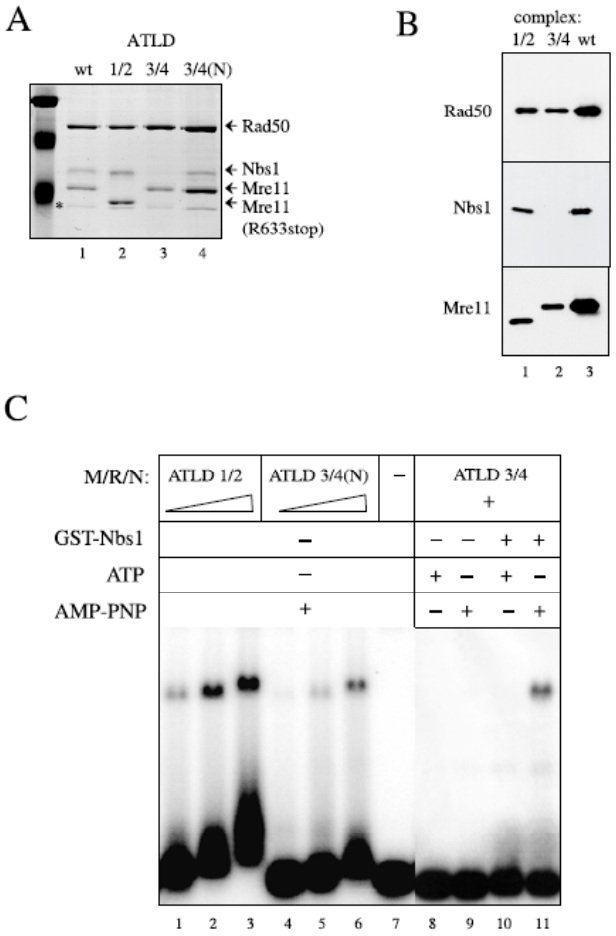

Figure A.3 ATLD M/R/N complexes bind DNA.

A, Coomassie-stained SDS-PAGE of ATLD M/R/N complexes. Mre11(R633stop)/R/N (ATLD 1/2) and Mre11(N117S)/R/N (ATLD 3/4) complexes are shown in comparison to the wild-type (*wt*) complex. The ATLD 3/4 allele of Mre11 associated with Rad50 and Nbs1 in one preparation (*3/4(N)*), but failed to associate in another preparation (*3/4*). The band marked with an *asterisk* is a degradation product of Rad50 that is found in all of the protein preparations. *B*, Western blot of ATLD M/R/N complexes. Blots containing wild-type, ATLD 1/2, and ATLD 3/4 complexes were probed with antibodies directed against Rad50, Nbs1, and Mre11, as indicated. *C*, gel mobility shift assays with ATLD complexes. Increasing amounts of ATLD M/R/N complexes were incubated with 32P-labeled duplex DNA and separated on an agarose gel as described in Fig. 1. Reactions contained 30 ng (*lanes 1* and *4*), 60 ng (*lanes 2* and *5*), or 120 ng (*lanes 3, 6*, and *8–10*) of M/R/N. GST-Nbs1 protein was also included in reactions shown in *lanes 10* and *11*.

unusual, however, in that it did retain some Nbs1 binding in some protein preparations, as shown in Fig. A.3A, lane 4 (*ATLD 3/4(N)*). At this point it is not clear what conditions during expression or during purification are responsible for retaining this association. In our experience, wild-type Mre11 has never exhibited this variability in Nbs1 binding. The identity of the R633stop protein and the absence of Nbs1 in the ATLD 3/4 complex was verified by Western blotting, as shown in Fig. A.3B.

In the nucleotide-dependent DNA-binding assay, the ATLD 1/2 M/R/N complex bound the DNA fragment similarly to wild type, and the ATLD 3/4 M/R complex did not bind (Fig. A.3C), consistent with the requirement for Nbs1 in this assay as demonstrated in Fig. A.1. When GST-Nbs1 was added to the ATLD 3/4 complex, however, it induced formation of a protein-DNA association similar to that observed with wild-type M/R in the presence of GST-Nbs1. The ATLD 3/4(N) complex also bound the DNA substrate, but at a reduced level compared with the wild-type complex (Fig. A.3C, *lanes 4–6*). The reduction in binding is likely the result of the lower levels of Nbs1 in this preparation relative to M/R. The ATLD 3/4 Mre11 mutant is thus capable of binding Nbs1 and forming nucleotide-dependent complexes with DNA, although it is apparently reduced in its affinity for Nbs1 such that it exhibits variability in Nbs1 association.

M/R/N(p70) Complex—A large majority of identified NBS patients are homozygous for an allele of the NBS1 gene that contains a 5-nucleotide deletion just downstream of the forkhead and BRCT domains (657del5) (Carney *et al.* 1998; Varon *et al.* 1998). This deletion causes a frameshift at codon 219 and a premature stop at codon 233. Petrini and colleagues (Maser *et al.* 2001) have observed that, in addition to this truncated product, lymphoblast cells from NBS patients also express a novel C-terminal

polypeptide that utilizes either of two alternative initiation codons just upstream of the deletion. This C-terminal protein, p70, is able to bind to Mre11 and was subsequently shown by the same group to support viability in the mouse, in contrast to the lethality caused by null alleles of NBS1 (Williams *et al.* 2002). All of the available evidence suggests that the 657del5 mutation creates a hypomorphic allele that can still fulfill the essential functions of the M/R/N complex but lacks other nonessential functions because of the missing N terminus of the protein.

To study the biochemical properties of the M/R/N(p70) complex, we expressed a 657del5 allele of NBS1 utilizing the first of the two alternative ATG codons upstream of the deletion (Maser *et al.* 2001). This construct generates a polypeptide of 553 amino acids, of which the C-terminal 535 residues are identical to the C terminus of wild-type Nbs1. Co-infection of insect cells with baculovirus containing the p70 construct and wild-type Mre11 and Rad50 yielded a triple complex of all three proteins (Fig. A.4A). The p70 band migrates in the SDS gel very close to a polypeptide present in the wild-type preparation that is likely a degradation product of Rad50 (data not shown), but the p70 band is clearly an Nbs1 product based on Western blotting with anti-Nbs1 antibody (Fig. A.4B).

M/R/N(p70) complexes are capable of binding DNA in the AMP-PNP-dependent assay, as shown in Fig. A.4C. The migration of the protein-DNA complex is very similar to the wild type in this assay (data not shown), suggesting that the overall mass of the p70 M/R/N complex when bound to DNA is similar to the normal complex and that the N terminus of Nbs1 is dispensable for stimulation of Rad50-mediated DNA binding.

Nuclease Activity of ATLD and NBS Complexes—The M/R/N complex exhibits

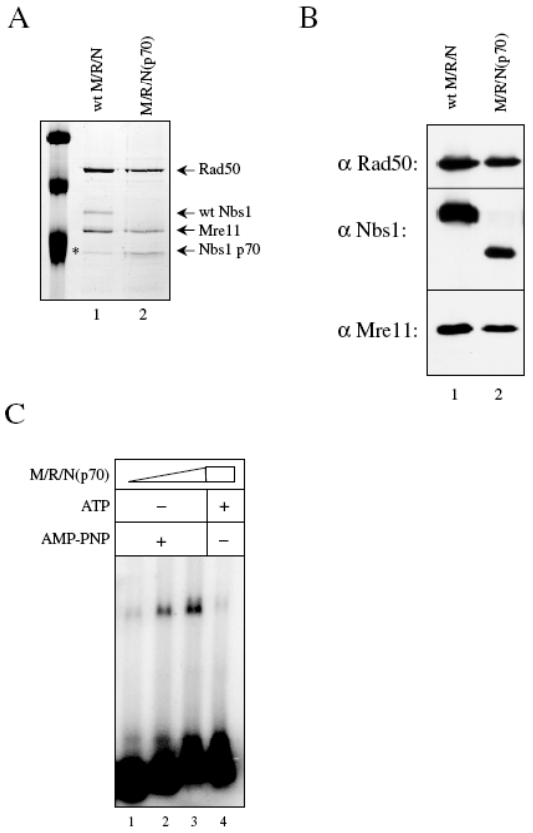

Figure A.4 M/R/N(p70) complexes bind DNA.

A, Coomassie-stained SDS-PAGE of M/R/N(p70) complex in comparison to the wild-type (*wt*) complex. The band marked with an *asterisk* is a degradation product of Rad50 that is found in all of the protein preparations. *B*, Western blot of the M/R/N(p70) complex in comparison to wild type. Blots containing wild-type and M/R/N(p70) complexes were probed with antibodies directed against Rad50, Nbs1, and Mre11, as indicated. *C*, gel mobility shift assays with M/R/N(p70) complexes. Increasing amounts of M/R/N(p70) complexes were incubated with 32P-labeled duplex DNA and separated on an agarose gel as described in Fig. 1. Reactions contained 30 ng (*lane 1*), 60 ng (*lane 2*), or 120 ng (*lanes 3* and *4*) of M/R/N(p70) and ATP or AMP-PNP as indicated.

exonuclease activity on blunt and 3'-recessed double-stranded DNA ends, and endonuclease activity on hairpins and 3' overhangs (Paull and Gellert 1998; Trujillo *et al.* 1998; Paull and Gellert 1999). The ATLD and p70 mutant M/R/N complexes were analyzed for exonuclease activity as shown in Fig. A.5A, and each of the mutant complexes exhibited steady-state levels of activity nearly indistinguishable from the wild-type enzyme. The initial rate of resection on a 50-bp DNA fragment containing a 4-nucleotide recessed 3' strand was examined for each of the complexes, and the ATLD 3/4 and ATLD 1/2 complexes exhibited rates within 5–10% of wild type, whereas the M/R/N(p70) complex was 20% reduced.

The ATLD and p70 mutant complexes were also tested for endonuclease activity on a hairpin substrate (Fig. A.5B) and a 3' overhang (Fig. A.5C). In comparison to the wild-type enzyme, the ATLD1/2 complex exhibited 50% activity on the 3' overhang specifically, whereas the ATLD 3/4 + N complex showed lower activity on both substrates. The lower activity of the ATLD 3/4(N) complex may very well be a result of the reduced binding of Nbs1, as Nbs1 is required for endonuclease activity (Paull and Gellert 1999). The preparation of ATLD 3/4 without Nbs1 shows no activity in these endonuclease assays, as expected (data not shown). The p70 complex exhibits endonuclease activity between 50 and 70% of the wild-type enzyme (Fig. A.5, B and C). Overall, the ATLD complexes show reduced, but not completely abrogated, levels of endonuclease activity. This defect appears to be specific to the endonuclease function, considering that the rate of exonucleolytic degradation seen with both of the ATLD mutant complexes was essentially identical to the wild-type enzyme.

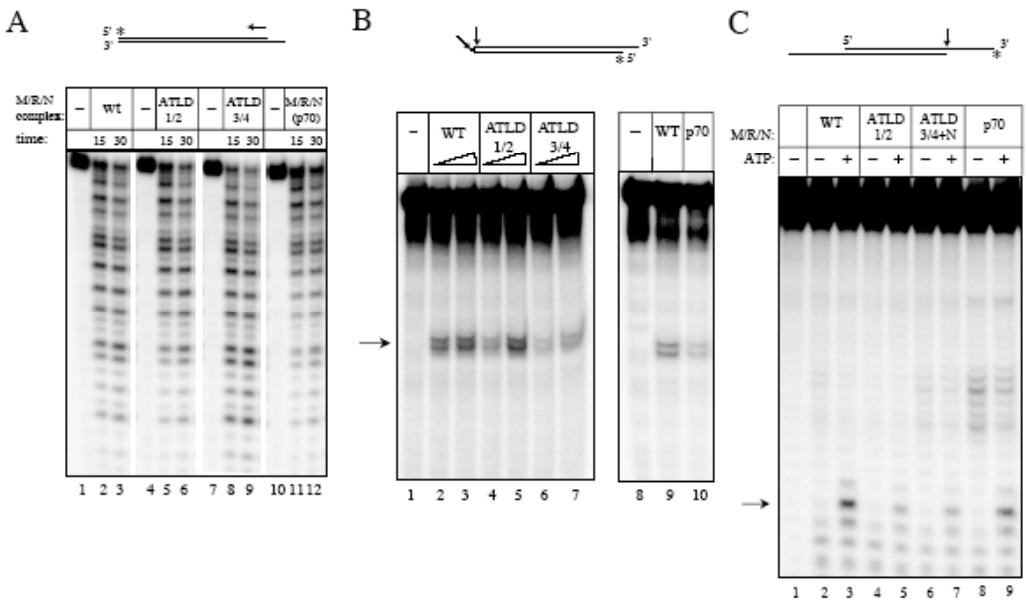

Figure A.5 Nuclease assays of ATLD and p70 M/R/N complexes.

A, exonuclease assays were performed with wild-type (*wt*), ATLD 1/2, ATLD 3/4, and M/R/N(p70) complexes on a 50-bp 32P-labeled oligonucleotide substrate containing a recessed 3' end (*diagram*). Exonuclease activity by M/R/N proceeds in the 3' to 5' direction (*arrow*). Reactions were incubated for 15 or 30 min at 37 °C, as indicated. *B*, endonuclease assays were performed with wild-type (*WT*), ATLD 1/2, ATLD 3/4(N), and M/R/N(p70) complexes on a 50-bp 32P-labeled oligonucleotide substrate containing a hairpin on one end and a 3' overhang on the other (*diagram*). The predominant sites of cleavage by M/R/N are 1 and 2 nt 3' of the hairpin tip (*arrows*). Reactions were incubated for 90 min. *C*, endonuclease assays were performed in the presence of ATP as indicated with wild-type (*WT*), ATLD 1/2, ATLD 3/4(N), and M/R/N(p70) complexes on a 50-bp 32P-labeled oligonucleotide substrate with 3' overhangs on each end (*diagram*). The predominant site of cleavage by M/R/N in the presence of ATP is at the single-strand/double-strand junction (*arrow*). Reactions were incubated for 90 min. All of the exo- and endonuclease assays were performed in the presence of 1 mM manganese, and the reaction products were separated on denaturing polyacrylamide gels. The location of the 32P label is indicated with an *asterisk* (*) in all of the *diagrams*.

Sedimentation Studies of the Mre11/Rad50/Nbs1 Complex— The larger of the protein-DNA complexes formed by M/R/N is significantly retarded in mobility compared with the free oligonucleotide DNA, even in the low percentage agarose gels used for the separation of the complexes. To obtain a better estimate of the size and stoichiometry of this complex, we analyzed wild-type M/R/N complexes, as well as Mre11, Mre11/Nbs1 (M/N), and Mre11/Rad50 (M/R) by analytical ultracentrifugation. Sedimentation equilibrium experiments on wild-type Mre11 resulted in the gradual loss of material as a result of sample precipitation; however, the Mre11 mutant H217Y (Paull and Gellert 2000) was found to have increased solubility. Studies of H217Y Mre11 show that the sample is monodisperse with an average buoyant molecular mass, $M(1 - \upsilon)$ of 40,360 ± 4,100 g mol^{-1} (Fig. A.6A). This corresponds to a measured molecular mass of 150,500 ± 15,300 g mol^{-1}, indicating that under these conditions the Mre11 is dimeric (n = 1.9 ± 0.2). Previously it was observed that the migration of human Mre11 in gel filtration experiments was more consistent with a multimeric stoichiometry (Paull and Gellert 1998), whereas measurements of yeast Mre11 using glycerol gradient sedimentation suggested that Mre11 is a monomer in solution (Anderson *et al.* 2001a). Our results in this work clearly indicate a dimer configuration, however, and at least one other measurement of yeast Rad50 is consistent with this analysis (Furuse *et al.* 1998). Gel filtration experiments with wild-type and H217Y Mre11 give identical separation profiles (data not shown); thus, the wild-type protein is very likely to also be a dimer in solution.

Mre11 and Rad50 interact to form a large macromolecular complex. Sedimentation equilibrium studies indicate that this complex is monodisperse with a buoyant molecular mass of 318,000 ± 17,000 g mol^{-1}. Based on a consensus partial

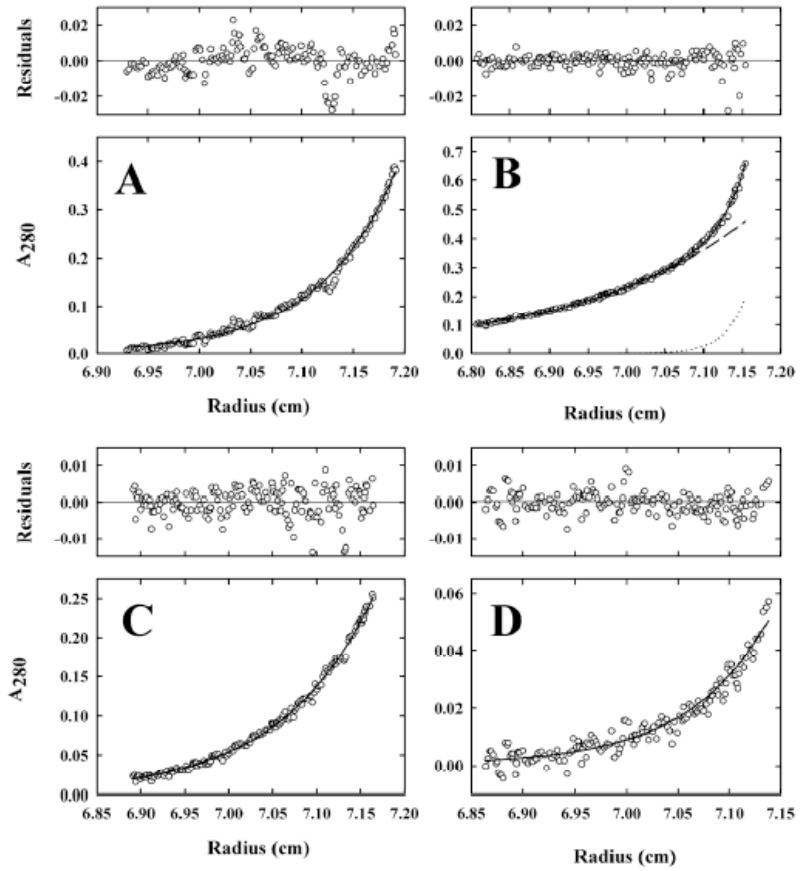

Figure A.6 Sedimentation equilibrium profiles for Mre11 (H217Y) (*A*), the Mre11(ATLD1/2)/Nbs1 complex (*B*), the Mre11/Rad50 complex (*C*), and the M/R/N triple complex (*D*) shown as a distribution of *A*280 at equilibrium.

A, data were collected at 10,000 rpm. The best single component fit for Mre11 is shown as a *solid line*, and the corresponding distribution of the residuals is shown *above* the plot. *B*, data were collected at 6,000 rpm and analyzed in terms of two non-interacting ideal solutes to account for the presence of the Mre11/Rad50/Nbs1 triple complex. The best fit is shown as a *solid line*. The contributions of the double (M/N) and triple (M/R/N) complexes are indicated as *dashed* and *dotted lines*, respectively. The corresponding distribution of the residuals is shown *above* the plot. *C*, data were collected at 3,000 rpm. The best single component fit for the Mre11/Rad50 complex is shown as a *solid line*, and the distribution of the residuals is shown *above* the plot. *D*, base-line corrected data for the triple M/R/N complex shown were collected at 3,500 rpm. The best single component data fit is shown as a *solid line*. The corresponding distribution of the residuals is shown *above*.

specific molar volume for proteins, this corresponds to a complex molecular mass of (1.24 ± 0.06) x 10^6 g mol^{-1}.

Sedimentation equilibrium studies on the triple complex formed between Mre11, Rad50, and Nbs1 show that these species interact to form a large, discrete, and slightly polydisperse complex. Data analysis in terms of a single ideal solute indicated rotor speed dependence for the buoyant molecular mass, with values ranging from 225,000 to 350,000 g mol^{-1} at speeds of 4,000 and 3,000 rpm, respectively. Average $M(1 - \upsilon)$ values of 293,000 ± 69,000 g mol^{-1} were calculated. Interestingly, global data analysis in terms of a single ideal solute yielded an excellent fit returning a similar $M(1 - \upsilon)$ value of 303,000 g mol^{-1} (Fig. A.6C). This corresponds to a complex molecular mass of (1.2 ± 0.2) x 10^6 g mol^{-1}. The mass observed for the Mre11/Rad50/Nbs1 complex is thus identical, within the precision of the method, to that measured for the Mre11/Rad50 complex. From these data, it is not possible to assign a clear stoichiometry to the M/R/N complex, and it is possible that the triple complex may in fact be a collection of complexes with varying stoichiometry.

We also tested wild-type M/N by sedimentation analysis, but found that the complex precipitated over the course of the experiment, similar to wild-type Mre11 alone. The ATLD1/2 form of Mre11 expressed very well in insect cells, however, so an M/N complex of ATLD1/2 Mre11 and Nbs1 was used for the sedimentation analysis and was found to be stable over long periods in solution. These results show that Mre11 ATLD1/2 and Nbs1 interact to form a discrete 1:1 complex. Sedimentation equilibrium studies show that a solution containing this complex has both low and high molecular mass species. Data analysis in terms of two single and non-interacting solutes return a buoyant molecular mass of 40,260 ± 2,000 g mol^{-1} for the low molecular mass complex.

This represents the predominant species (Fig. A.6B). The experimental buoyant mass appears to represent the 1:1 sum of the buoyant masses calculated for the Mre11 ATLD1/2 (18,546 g mol^{-1}) and Nbs1 (21,387 g mol^{-1}) components, indicating a 1:1 stoichiometry for the M/N complex. Based on the buoyant molecular mass, a complex mass of 159,740 ± 7,950 g mol^{-1} is calculated (n = 1.01 ± 0.05). Because ATLD1/2 M/N separates identically to wild-type M/N in gel filtration experiments (data not shown), we conclude that the complex of wild-type Mre11 with Nbs1 is very likely to have 1:1 stoichiometry as well. All of the sedimentation equilibrium results are summarized in Table A.1.

Sedimentation equilibrium analysis of ATLD1/2 M/N also indicated the presence of a small amount of a high molecular mass species. The preparation of the ATLD1/2 Mre11 complex with Nbs1 came from a preparation of the ATLD1/2 triple complex containing all three recombinant proteins. The M(ATLD1/2)/N was separated from the M(ATLD1/2)/R/N by gel filtration, and a small amount of ATLD1/2 M/R/N was present in the fraction used for the analytical ultracentrifugation (data not shown). The M/R/N in the fraction accounts for the minor species of large complex present, and has a buoyant molecular mass consistent with the presence of an Mre11, Rad50, and Nbs1 triple complex present in small amounts (Fig. A.6B).

Discussion

Nbs1 Regulates Rad50 Function—The results shown here suggest that Nbs1 plays an important role in modulating the DNA binding activity and specificity of the Rad50 protein. It was previously demonstrated that Nbs1 association alters the endonuclease specificity of Mre11 (Paull and Gellert 1999). Here we show that Nbs1 also alters Rad50

Table A.1

Estimated mass of Mre11/Rad50/Nbs1 complexes measured by sedimentation equilibrium analysis

protein	Mass	Stoichiometry [a]
	$g\ mol^{-1}$	
Mre11(H217Y)	150,500 ± 15,300	$(Mew11)_n$, $n = 1.9 \pm 0.2$
Mre11(ATLD1/2)/Nbs1	159,740 ± 7,950	$(Mre11\ Nbs1)_n$, $n = 1.01 \pm 0.05$
Mre11/Rad50	$1.24 \pm 0.06 \times 10^6$	See Fig. A.7
Mre11/Rad50/Nbs1	$1.2 \pm 0.2 \times 10^6$	See Fig. A.7

[a] Molecular weight of each polypeptide including C-terminal histidine tags on Mre11 and Rad50 ($g\ mol^{-1}$): Mre11, 81,963; Mre11(ATLD1/2), 73,343; Nbs1, 84,905; Rad50, 155,702

function, such that nucleotide-dependent DNA binding is strongly promoted when Nbs1 is present in the complex. This increased DNA binding activity may explain the previously reported requirement for Nbs1 in DNA unwinding by Mre11/Rad50, an activity that is stimulated by ATP (Paull and Gellert 1999).

Crystallographic analysis of *P. furiosus* Rad50 suggested that binding of adenine nucleotides by Rad50 promotes dimerization of the protein and creates a binding site for a DNA helix (Hopfner *et al.* 2000b). The sedimentation equilibrium analysis we report here for the human M/R/N complex did not yield any evidence for nucleotide-induced dimerization, but DNA-binding assays *in vitro* revealed a high affinity protein-DNA complex formed by M/R/N in the presence of non-hydrolyzable ATP analogs. AMP-PNP-dependent binding occurs 20–40-fold more efficiently than in the absence of nucleotide, and is much less variable than complexes occasionally observed in the presence of ATP (data not shown). The most straightforward interpretation of these data is that non-hydrolyzable nucleotides facilitate DNA binding by M/R/N but prevent DNA release, thus locking all of the DNA-bound molecules into this state. The similarity between the conformations of the ATP and AMP-PNP-bound Rad50 crystal structures (Hopfner *et al.* 2000b) predicts that the AMP-PNP-bound state in M/R/N complexes is simply blocked at the hydrolysis step of catalysis, and is not a fundamentally different protein-DNA interaction compared with the ATP-bound state. The requirement for a non-hydrolyzable ATP analog instead of ATP also suggests that ATP hydrolysis by M/R/N must be very rapid.

Although M/R/N forms transient complexes with both single and double-stranded DNA, the nucleotide-dependent DNA binding shown in this work is specific for double-stranded duplexes. The effect of DNA end structure was not assessed in this work, but a

recent microscopy study of M/R also showed that AMP-PNP altered the specificity of DNA binding to favor 3' overhang structures (de Jager *et al.* 2002).

Nbs1 C Terminus Is Sufficient for Stimulation of M/R Enzymatic Functions—NBS homozygotes have no full-length Nbs1 protein (Carney *et al.* 1998), but some tissues in NBS patients contain a C-terminal Nbs1 protein generated from an alternative start site (Maser *et al.* 2001). This truncated Nbs1 protein, p70, is sufficient for association with Mre11, and the data presented here indicate that this region is also sufficient for the stimulation of Mre11 endonuclease activity as well as for the nucleotide-dependent binding by Rad50. Although the exact biochemical activities of M/R/N are still under investigation, our analysis shows that every enzymatic function we can measure *in vitro* is essentially unchanged in a M/R/N(p70) mutant compared with the wild-type complex. This observation is not unexpected, considering that NBS cells have no overt defects in DNA repair but primarily exhibit deficiencies in checkpoint-related DNA damage responses (Shiloh 1997). Other studies have also demonstrated that the N terminus of Nbs1 that includes the forkhead and BRCT domains is necessary and sufficient for focus formation at sites of DNA damage, but that the C terminus is required for survival of cells following ionizing radiation treatment (Desai-Mehta *et al.* 2001; Tauchi *et al.* 2001; Kobayashi *et al.* 2002; Zhao *et al.* 2002; Cerosaletti and Concannon 2003). Regulatory interactions between the C terminus of Nbs1 and Mre11/Rad50 are likely to be necessary for the DNA repair carried out by the complex and are therefore necessary for cell survival.

ATLD Mutant Complexes—The most obvious difference between the ATLD

mutant M/R/N complexes and the wild-type enzyme is seen with ATLD 3/4, which exhibits a high degree of variability in Nbs1 binding. As shown here and in previous work (Paull and Gellert 1999), the degree of Nbs1 association affects both nucleotide-dependent DNA binding as well as endonuclease activity of Mre11. Immunoprecipitations of the ATLD 3/4 Mre11 protein by Stewart *et al.* (Stewart *et al.* 1999) show that relatively little Nbs1 protein associates with ATLD 3/4 Mre11, and would thus suggest that the Nbs1-dependent enzymatic functions of the mutant complexes are likely to be compromised in these cells. In contrast to ATLD 3/4, the C-terminal truncation mutation in ATLD 1/2 has no apparent effect on Nbs1 binding in our recombinant expression system. Neither of the ATLD mutant M/R/N complexes shows any significant reduction in nucleotide-dependent DNA binding, although the ability of ATLD 3/4 to bind DNA was affected by the level of Nbs1 association. The exonuclease activities of the ATLD complexes are similarly unaffected compared with the wild-type enzyme, although both mutant complexes exhibit a significant reduction in endonuclease function.

ATLD cells, like A-T cells, do not show an overt defect in DNA repair (Stewart *et al.* 1999), although a partial loss in endonuclease function could result in repair deficiencies that would be difficult to assess using assays for whole-genome recovery. Indirect support for a repair deficiency in ATLD cells comes from a recent observation that ATLD3 cells are deficient in processing of adenovirus genome intermediates, an activity that is postulated to occur via Mre11 endonuclease activity (Stracker *et al.* 2002).

Stoichiometry of the M/R/N Complex—Mre11 shows the ability to interact with itself in two-hybrid assays (Johzuka and Ogawa 1995), suggesting that it forms a

multimeric complex *in vivo*. The sedimentation equilibrium analysis of a nuclease-deficient mutant of Mre11 (H217Y) performed in this study indicates that this mutant Mre11 is clearly a dimer in solution. Wild-type Mre11 was not soluble over the long time required for equilibrium centrifugation (several days), but separates identically to the H217Y protein during gel filtration over a Superdex 200 column; thus, wild-type Mre11 is very likely a stable dimer in solution.

Our sedimentation analysis of the Mre11(ATLD1/2) complex with Nbs1 indicates that M/N has a 1:1 stoichiometry in solution, because the mutant and wild-type M/N complexes also separate identically in gel filtration. Nbs1 binding to Mre11 is therefore mutually exclusive with Mre11 dimer formation, either because Nbs1 binding occurs via the same interface, or because Nbs1 causes a conformational change in Mre11 that inhibits dimer formation.

By analogy with the SMC family of coiled-coil ATPases (Haering *et al.* 2002; Hirano and Hirano 2002) and microscopy analysis of the human M/R complex (de Jager *et al.* 2001; Hopfner *et al.* 2002), it is likely that each Rad50 molecule folds back on itself to form an antiparallel coiled-coil within a monomer unit. The central region of the Rad50 coiled coil has recently been shown to form a zinc-mediated hook structure that can associate with another zinc hook (Hopfner *et al.* 2002); thus, Rad50 is predicted to form dimers connected between their hook domains. Mre11 binds to Rad50 at the base of the coiled-coil region (Anderson *et al.* 2001a; Hopfner *et al.* 2001), and thus the M/R complex would be expected to contain two Rad50 molecules and four Mre11 molecules (assuming a dimer of Mre11 bound to each Rad50 protein). Support for this type of configuration also comes from electron microscopy studies of yeast M/R (Anderson *et al.* 2001a) and *Escherichia coli* SbcC/SbcD (Connelly *et al.* 1998). All of these complexes

have been observed to form extended, dumbbell-like shapes with globular domains at the ends.

The mass of M/R in solution as determined in this work by equilibrium sedimentation was 1.24 ± 0.06 MDa, however, which is significantly larger than a M_4R_2 configuration. A stoichiometry of M_4R_2 would yield a complex of 630 kDa, approximately half of the observed value. One possibility is that the stoichiometry is simply $(M_4R_2)_2$, which would contain M_4R_2 complexes held together along the length of the coiled-coils, or associated at the globular domains with the zinc hooks on the outside (Fig. A.7B). Both of these configurations have been observed for human M/R by electron microscopy (Hopfner *et al.* 2002). A recent study of human M/R by scanning force microscopy also visualized complexes with a very large globular domain at the center with arms (coiled-coils) extending outward (de Jager *et al.* 2001), supporting the head-to-head model of Mre11 and Rad50 interactions.

Our expectation in analyzing the mass of M/R and M/R/N complexes was that the mass of M/R/N would be larger than that of M/R, and that the difference between the values would be a consequence of Nbs1 association. The unexpected conclusion from our studies, however, is that the mass of the triple complex of human M/R/N is not significantly larger than that of M/R within the limits of our assay, but exhibits a polydisperse character suggesting a heterogeneous collection of protein complexes. One explanation for this result is that varying numbers of Mre11 and Nbs1 are associated with each Rad50 dimer (or pair of dimers) such that M/R/N may in fact consist of a heterogeneous group of assemblies (two of the most plausible possibilities are shown in Fig. A.7C). Our finding of a 1:1 stoichiometry of Mre11 with Nbs1 would suggest that the

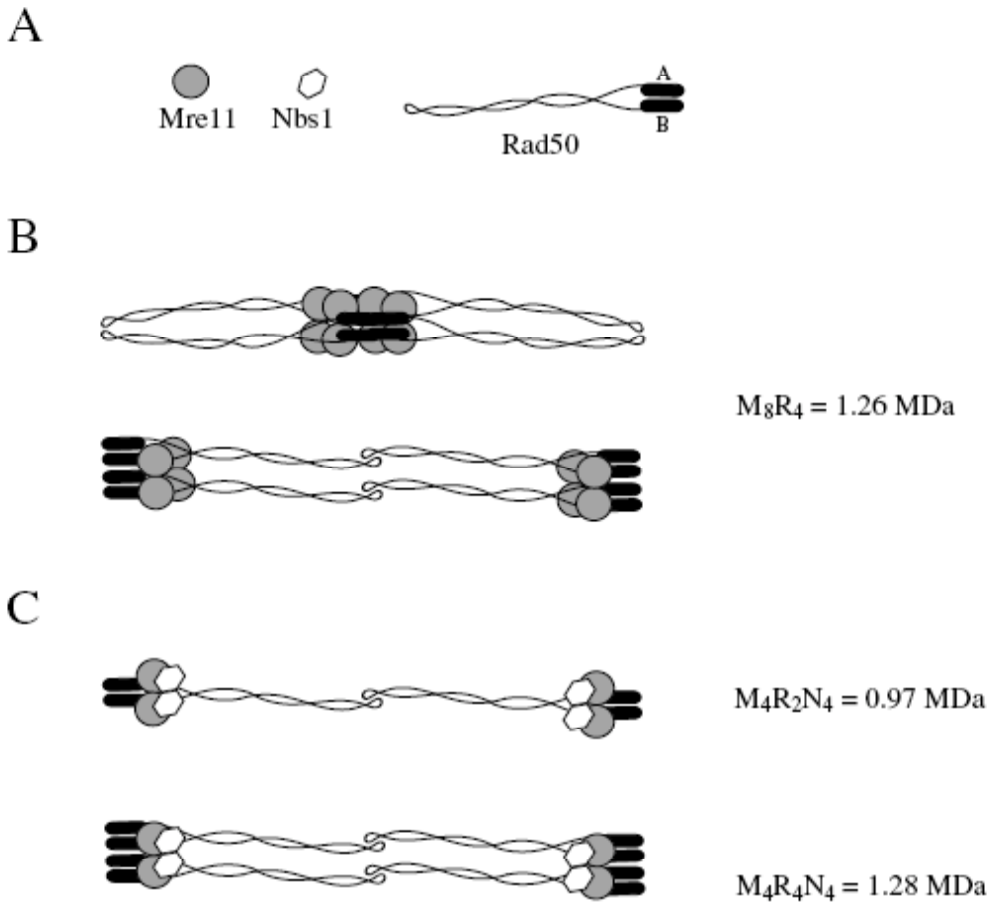

Figure A.7 Hypothetical models of M/R/N stoichiometry.

A, Mre11, Nbs1, and Rad50 are symbolized by a *circle*, a *hexagon*, and an intramolecular coiled coil with catalytic domains A and B, respectively. *B*, two possibilities for the configuration of M/R consistent with the observed mass of the complex are shown: head-to-head (*top*) and tail-to-tail (*bottom*). *C*, two possibilities for the configuration of M/R/N consistent with the observed mass of the complex are shown in a tail-to-tail configuration.

models that maintain a 1:1 ratio of these proteins would be more likely to be correct, assuming that M/N associates as a unit with Rad50 molecules. Further experiments are clearly necessary to determine which, if any, of the proposed models for the M/R/N triple complex are correct, and whether the stoichiometry is altered under different conditions.

APPENDIX B

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF ATM AND MRN

Ji-Hoon Lee and Tanya T. Paull

Methods in Enzymology, In Press

Abstract

Ataxia-Telangiectasia Mutated (ATM) is a serine-threonine kinase that is activated by DNA double-strand breaks (DSBs) to phosphorylate many cellular proteins involved in cell cycle regulation and DNA repair. We have previously shown that the activation of ATM can be reconstituted in an *in vitro* system using recombinant human ATM. In this system, ATM activity is dependent on the Mre11/Rad50/Nbs1 (MRN) complex and linear DNA, similar to requirements observed in human cells. This article describes methods used for the overexpression and purification of human ATM and MRN, as well as a protocol for *in vitro* kinase assays.

Many diverse cellular stress responses, including apoptosis, cell cycle arrest, and gene induction, are controlled by phosphorylation and dephosphorylation. Ataxia-Telangiectasia Mutated (ATM) is a serine-threonine kinase that is activated when cells are exposed to DNA double-strand breaks (DSBs) (Kim *et al.* 2002; Bakkenist and Kastan 2003). ATM phosphorylates a number of proteins involved in cell cycle checkpoint control, apoptotic responses and DNA repair, including p53, Chk2, Chk1, Brca1, RPAp34, H2AX, SMC1, Rad17, and Nbs1(Shiloh 2003). Phosphorylation of cell-cycle related proteins by ATM initiate a series of events that ultimately halt the growth of damaged cells and facilitate repair (Shiloh 1997; Abraham 2001). ATM-dependent phosphorylation events in cells require the MRN complex, an assembly of proteins that plays a critical role in DNA double-strand break repair, in addition to its role in ATM signaling (Carson *et al.* 2003; Uziel *et al.* 2003).

ATM exists as an inert dimer under normal physiological conditions, and dissociates into active monomers upon DSB formation (Bakkenist and Kastan 2003). We have investigated the kinase activity of recombinant human ATM (both monomeric and dimeric forms) in the presence of recombinant human MRN and DNA (Lee and Paull 2004; Lee and Paull 2005). These results were consistent with the observations made in human cells in that MRN and DNA were both required for the activation of ATM dimers by DNA double-strand breaks. This chapter describes methods for the transient expression of ATM in human cells, purification of recombinant ATM and MRN by chromatography, and kinase assays of ATM.

Purification of Dimeric ATM

Transfection

Solutions and Materials

293-T cells (ATCC, Manassas, VA)

DMEM medium : 1 X DMEM (Invitrogen Corporation, Grand Island, NY) plus 10% Fetal Bovine Serum or FetalPlex Animal serum complex (Gemini Bio-Products, Woodland, CA), and 3.7 g/liter sodium bicarbonate

Tissue Culture Dishes 245x245x25 (Nunclon, Roskilde, Denmark)

pcDNA-FLAG-ATM wt : N-terminal FLAG-tagged mammalian expression vector (Bakkenist and Kastan 2003)

HA-tagged ATMwt expression vector (Bakkenist and Kastan 2003)

2 X HEBS : 16 g/liter NaCl, 0.74 g/liter KCl, 0.198 g/liter $NaHPO_4$, 2 g/liter Dextrose, 10 g/liter HEPES, adjusted to pH 7.2 and filter sterilized

2.5 M $CaCl_2$ in 10 mM HEPES : 0.238 g/100 ml HEPES, 27.75 g/100 ml $CaCl_2$, adjusted to pH 7.2 and filter sterilized

1 X TE : 10 ml/liter Tris-HCl pH 7.5, 2 ml/liter EDTA pH 8.0, adjusted to pH 7.3 and filter sterilized

Procedure

293-T cells are transfected by the calcium phosphate coprecipitation technique, which is carried out as follows (this protocol is to transfect the cells in 100 ml media in a 245x245x25mm dish). We used 24 dishes for the purification of dimeric ATM by this method.

1. Split cells to about 40-50% confluency at least 12 hrs prior to transfection. This gives them enough time to be 70-75% confluent at the time of transfection.
2. In one tube, aliquot 100 μg of pcDNA-FLAG-ATM wt and 200 μg of HA-tagged ATM wt expression vectors, 500 μl of 2.5 M $CaCl_2$ in 10mM HEPES, and enough TE to bring the total volume to 5 ml. In another tube, aliquot 5 ml of 2 X HEBS.
3. Add the $CaCl_2$/DNA mix to the 2 X HEBS drop by drop. Do this while bubbling the mixture with a pipet. Fine opalescent precipitate should appear within 10 min.
4. Let the mixture stand at room temperature for 30 min.
5. Add the mixture directly to the surface of the media containing the cells (drop by drop) and swirl the plate gently to mix.
6. Incubate the cells at 37°C/5% CO_2 overnight.
7. Remove the $Ca_3(PO_4)_2$ containing medium and replace with fresh DMEM medium.
8. Incubate the cells at 37°C/5% CO_2 for 36-48 hrs, harvest the cells by gentle pipetting, and centrifuge (1,500 X g).
9. Remove supernatant, and freeze pellets with liquid nitrogen and store at -80°C.

Extraction Preparation

Solution

Lysis buffer : 25 mM Tris pH 8.0, 250 mM NaCl, 20 mM $MgCl_2$, 0.5 mM PMSF (phenylmethylsulfonylfluoride), 1 mM DTT, 20% Glycerol

Procedure

1. Resuspend the pellets with 50 ml of lysis buffer

2. Homogenize with 50 strokes using a Dounce homogenizer (A type).
3. Centrifuge the homogenate at 10,000 X g for 15 min at 4°C.
4. Transfer the supernatant into a 50 ml tube.

Anti-Flag Affinity Chromatography

Solutions and materials

Buffer A : 25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, 10% Glycerol

Flag Elution Buffer : 100 μg/ml Flag peptide (Sigma, Saint Louis, MO) in buffer A (5 mls)

Anti-Flag M2 affinity gel (Sigma, Saint Louis, MO) (1ml)

Procedure

1. Mix the prepared cell extracts with 1 ml of anti-Flag M2 affinity gel and incubate at 4°C for 1hr on a bench top rotator.
2. Centrifuge the beads at 500 X g for 1 min and discard supernatant.
3. Wash the beads with lysis buffer and centrifuge again. Repeat washes two more times.
4. Pack the beads into a small chromatography column.
5. Wash the column at a flow rate of 0.25 ml/min using buffer A until the OD_{280} reaches a constant baseline. Flow rate should not exceed 0.25 ml/min, as the agarose beads are sensitive to high pressure.
6. Elute the flag-tagged ATM with 5ml of flag elution buffer: after running one column volume of flag elution buffer, pause the flow for 15 min in order to facilitate the removal of the bound ATM by the flag peptide.

7. Continue to flow and collect the eluted ATM protein.
8. To clean the resin, wash the column with 5 ml of 0.1 mM glycine (pH 3.4) and then thoroughly wash with buffer A.

This protocol generates approximately 50 μg total protein in 5 ml from 24 dishes (245x245x25mm).

Anti-HA Affinity Chromatography

Solutions and materials

HA Elution Buffer : 0.4 mg/ml HA peptide (AnaSpec Inc., San Jose, CA) in buffer A (2.5 ml)

Agarose-immobilized goat anti-HA antibody (Bethyl, Montgomery, TX) (200 μl)

Bio-Gel A-1.5m Gel (Bio-Rad, Hercules, CA) or other inert resin (800 μl)

Procedure

1. Prepare a chromatography column with 0.2 ml of anti-HA agarose and 0.8 ml of Bio-Gel A-1.5m gel or other inert resin (or use 100% anti-HA agarose in 0.5-1.0 ml).
2. Equilibrate the column with buffer A.
3. Load the pooled fractions from the Flag column onto the HA column at 0.25 ml/min.
4. Wash the column at the same flow rate with buffer A until the OD_{280} reaches a constant baseline.

5. Elute HA-tagged ATM with 2.5 ml of HA elution buffer. After passing one column volume of HA elution buffer, pause the flow for 15 min in order to facilitate the removal of the bound ATM by the HA peptide.
6. Continue the flow and collect the eluted ATM protein.
7. To clean the resin, wash the column with 5 ml of 0.1 mM glycine (pH3.4) and then thoroughly wash with buffer A.
8. Store in aliquots at -80°C after flash freezing in liquid nitrogen.

This protocol generates approximately 1.5 to 2.5μg total protein in 1.5ml from 32 dishes (245x245x25mm).

Purification of Monomeric ATM

The protocol described above is specifically designed to purify dimeric forms of ATM; however it is also sometimes useful to prepare the monomeric form, particularly if ATM kinase assays are to be performed in the absence of the MRN complex (in the presence of manganese). Monomeric preparations generally require 10-fold smaller cell cultures.

Transfection

1. Prepare 20 to 25 100 mm dishes containing 293-T cells at 70-75% confluency, each containing 10ml of DMEM medium.
2. Transfect cells with 10 to 20 μg of plasmid DNA (flag-tagged ATM) per dish.
3. Use the same calcium phosphate transfection technique as described above, except reduce the amount of reagents accordingly (~10-fold less).

Anti-Flag Affinity Chromatography

Solutions

TGN buffer : 50 mM Tris pH 7.4, 150 mM NaCl, 1% tween 20, 0.3% NP 40, 2 mM DTT, 10% Glycerol

Lysis buffer : 0.5 mM PMSF, 1 mM NaF, and 5 mM DTT in TGN buffer

Washing buffer : 0.5 M LiCl in TGN buffer

Flag elution buffer : 2 μg/ml Flag peptide in buffer A, 1% tween-20

Procedure

1. Resuspend the pellets with 10 ml lysis buffer.
2. Homogenize with 50 strokes using a dounce homogenizer and then sonicate (3 times 20 s using microconicator tip at setting 2 to 3).
3. Centrifuge the homogenate at 50,000 X g for 30 min at 4°C.
4. Load the supernatant onto a 1 ml column containing anti-Flag M2 antibody conjugated to agarose beads at a flow rate of 0.25 ml/min.
5. Wash the column with at least 5 ml of washing buffer containing 0.5 M LiCl.
6. Wash the column with buffer A until the OD_{280} reaches a constant baseline.
7. Elute the flag-tagged ATM with 5 ml of flag elution buffer containing 1% tween. After passing one column volume of flag elution buffer, pause the flow for 15 min.
8. Continue the flow and collect the eluted protein.
9. To clean the resin, wash the column with 5 ml of 0.1 mM glycine (pH 3.4) and then thoroughly wash with buffer A.
10. Pool the concentrated fractions of ATM together and store in aliquots at 80°C after flash freezing in liquid nitrogen.

Purification of the MRN complex

Transfection and Extraction Preparation

Solutions and Materials

Sf21 (or Sf9) cells

High-titer baculovirus prepared from transfer vectors containing human Rad50, Mre11, and Nbs1. Corresponding vectors from the Paull lab are pTP11 (C-terminal His6-tagged Rad50), pTP17 (C-terminal His6-tagged Mre11), and pTP36 (Nbs1), respectively.

Nickel A buffer : 0.5 M KCl, 50 mM KH_2PO_4, 5 mM imidazole, 20 mM β-mercaptoethanol, 10% glycerol

Lysis buffer : 0.5% tween 20 and 2 mM PMSF in Nickel A buffer

Procedure

1. Infect 1.4 liter of Sf21 cells in suspension culture (or 2,000 cm^2 of Sf21 cells in adherent culture) at an MOI of ~10 with baculovirus expressing Rad50, Mre11, and Nbs1 in combination (use a ratio of 3:1:2 or 3:1:3)
2. After 48 hr, harvest the cells, wash the pellet with PBS, and freeze in liquid nitrogen.
3. To prepare the lysate, thaw and resuspend the cells in 100 ml of lysis buffer.
4. Resuspend the mixture briefly with a dounce homogenizer (type A) to remove lumps and then sonicate (3 times 20 s using microconicator tip at setting 2 to 3).
5. Centrifuge the homogenate at 100,000 X g for 1 hr at 4°C.
6. Transfer and keep the supernatant into a tube at 4°C.

Nickel Chromatography

Solutions and materials

Nickel A buffer : 0.5 M KCl, 50 mM KH_2PO_4, 5 mM imidazole, 20 mM β-mercaptoethanol, 10% glycerol

Nickel B buffer : 0.5 M KCl, 50 mM KH_2PO_4, 250 mM imidazole, 20 mM β-mercaptoethanol, 10% glycerol

Buffer A : 25 mM Tris pH 8.0, 100 mM NaCl, 0.1 mM DTT, 10% Glycerol

Nickel-NTA superflow resin (Qiagen, Valencia, CA)

Procedure

1. Load the prepared cell extracts onto an ~5 ml column of nickel-NTA superflow resin equilibrated in Nickel A buffer.
2. Wash the column with Nickel A buffer until the OD_{280} reaches a constant baseline.
3. Wash the column with 10% Nickel B buffer (~30 mM imidazole) until the OD_{280} reaches a constant baseline.
4. Elute the MRN complex with 50% Nickel B buffer (~130 mM imidazole).
5. To clean the resin, wash the column with 100% Nickel B buffer (250 mM imidazole).
6. Pool the fractions containing the MRN complex and dialyze into buffer A.

Ion-Exchange Chromatography

Solutions and materials

Buffer A : 25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, 10% Glycerol

Buffer B : 25 mM Tris pH 8.0, 1 M NaCl, 1 mM DTT, 10% Glycerol

HiTrap Q sepharose column (Amersham Biosciences, Piscataway, NJ)

Procedure

1. Load the pooled and dialyzed fractions from the Nickel column onto HiTrap Q sepharose column equilibrated in buffer A.
2. Wash the column with Nickel A buffer until the OD_{280} reaches a constant baseline.
3. Elute the MRN complex with 50% buffer B (~550 mM NaCl).
4. To clean the resin, wash the column with 100% buffer B (1 M NaCl).

Size-Exclusion Chromatography

Solutions and materials

Buffer A : 25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, 10% Glycerol

Superose 6 HR 10/30 column (Amersham Biosciences, Piscataway, NJ)

Procedure

1. Prepare loading sample containing 0.1% tween-20 with best fraction from Q column.
2. Load the sample onto superose 6 column equilibrated in buffer A.

3. Collect the eluted protein. The MRN complex is eluted in a sharp peak at ~1.2 Mda in comparison to protein standards.

This protocol generates approximately 100 μg total protein in 2 ml from 1.4 liter suspension culture or 2000 cm^2 adherent culture.

ATM Kinase Assay

In vivo, ATM is activated rapidly by DNA DSBs to phosphorylate many target proteins including p53 and Chk2 (Shiloh 2003). Recently, we have demonstrated that the DNA repair complex MRN stimulates monomeric ATM activity *in vitro* (Lee and Paull 2005) and also serves as a DSB sensor to activate dimeric ATM (Lee and Paull 2005). ATM kinase activity has also been shown by other groups to be manganese-dependent (Chan *et al.* 2000), and most experiments investigating ATM kinase activity have been performed using both magnesium and manganese. However, manganese concentration *in vivo* are negligible, thus we have tested ATM kinase activity in the presence of magnesium alone, which resembles *in vivo* conditions. Our recent reports showed that kinase activity of dimeric ATM is stimulated by MRN and DNA in the presence of magnesium alone (Lee and Paull 2005). Here, we describe the method for the ATM kinase assay with MRN and DNA using p53 and Chk2 as model ATM substrates.

Kinase Reaction

Solutions and Materials

Purified proteins : ATM, MRN, and ATM substrates (GST-p53, GST-Chk2 or full-length proteins)

2 X Kinase buffer : 100 mM HEPES pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 10% glycerol

Procedure

1. Mix 3 ng of dimeric ATM, 250 ng of MRN, 100 ng of p53 or 600 ng of Chk2, 10 ng of DNA for p53 or 2.5 ng of DNA for Chk2, and enough buffer A to bring the total volume to 20 μl.
2. Add 20 μl of 2 X kinase buffer.
3. Incubate the mixture at 30°C for 1.5 hr.

For kinase assays with monomeric ATM, 3 ng of dimeric ATM is substituted with 3 ng of monomeric ATM, keeping the rest of the protein components the same. However, the kinase assay with monomeric ATM is performed in a reaction containing both magnesium and manganese (10mM each, final concentration). Under these conditions, ATM is active without the MRN complex and DNA although the MRN complex still stimulates the kinase activity of monomeric ATM in the presence of manganese. Monomeric ATM does not require DNA (Lee and Paull 2004).

Western Blotting

Solutions and materials

Immobilon-FL PVDF (Millipore Corporation, Billerica, MA)

Odyssey Blocking Buffer (Li-Cor Biotechnology, Lincoln, NE)

Anti-p53, Phospho-Specific (Ser15) antibody (Ab-3) (Calciochem, San Diego, CA)

Phospho-Chk2 (Thr68) Antibody (Cell Signaling, Beverly, MA)

Alexa Fluor 680-conjugated goat anti-rabbit IgG (Invitrogen, Grand Island, NY)

1 X PBS : 8g/liter NaCl, 0.2g/liter KCl, 1.44g/liter Na_2HPO_4, 0.24g/liter KH_2PO_4, adjusted to pH 7.4

1 X PBST : 0.1% tween 20 in PBS

Odyssey Imaging System (Li-Cor Biotechnology, Lincoln, NE)

Procedure

1. Load kinase reactions onto a 10% SDS-PAGE gel and transfer the proteins to Immobilon-FL PVDF membrane.
2. Block in Odyssey blocking buffer for 1hr at room temperature. Do not use BSA or milk.
3. Add primary antibody to the blocking buffer (first time use of primary) or use a previously made primary antibody solution. Use primary antibody at 1:2,000 dilution.
4. Incubate for 2 hrs at room temperature or overnight at 4°C.
5. Wash with PBST several times.
6. Incubate the membrane with secondary antibody solution in Odyssey blocking buffer that also includes 0.01% SDS. Use Alexa Fluor 680 goat anti-rabbit IgG at 1:10,000 dilution.
7. Incubate at room temperature for 1 to 2 hrs and protect from light from this point onward.
8. Wash with PBST several times and perform the final wash with PBS.
9. Dry the membrane before scanning at room temperature and keep it protected from light.
10. Scan the membrane using Odyssey Imaging System.

Alternatively, one can also carry out the western blotting using traditional chemiluminescence technique. In this case, normal PVDF or nitrocellulose membrane can be used with 5% milk as a blocking solution and 1% BSA containing primary and secondary antibody solution.

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