RECONSTITUTION OF A HUMAN ATR-MEDIATED DNA DAMAGE CHECKPOINT PRESPONE

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ABSTRACT

Jun-Hyuk Choi: Reconstitution of a Human ATR-mediated DNA Damage Checkpoint Response

(Under the direction of Aziz Sancar)

The genomes of eukaryotic cells are constantly under assault by exogenous and endogenous forms of DNA damage. In response to DNA damage, cells activate the DNA damage checkpoint response which delays cell cycle progression. Accumulating evidence indicates that genetic checkpoint defects lead to pronounced predisposition to cancer. ATR is a key regulator of the UV-induced DNA damage checkpoint response and activates the downstream Chk1 protein kinase, which in turn affects cell cycle regulatory proteins. Despite recent progress in characterizing the molecular components of the ATR-mediated checkpoint pathway, how the components work coordinately in response to DNA damage remains ill-defined.

To understand the molecular mechanism of this checkpoint response, it is necessary to develop an *in vitro* checkpoint system reconstituted from purified proteins. Therefore, I describe here an *in vitro* system with purified human checkpoint proteins that recapitulates key elements of the ATR-mediated DNA damage checkpoint. The data from this system show that damaged DNA specifically stimulates TopBP1-dependent activation of ATR kinase activity toward its substrates, Chk1 and p53. Moreover, the *in vitro* system provides a useful tool for studying the DNA binding and ATR stimulatory activities of TopBP1 and indicates a cooperative activation mechanism for ATR

activation. Finally, I demonstrate *in vitro* evidence indicating the stimulatory effect of RPA-coated ssDNA on TopBP1- dependent ATR activation.

I have established a useful *in vitro* system for studying stimulation of ATR kinase activity by other checkpoint components and provide insight into the molecular mechanism of the ATR-mediated checkpoint response. Moreover, my work will eventually contribute to the reconstitution of the complete *in vitro* checkpoint response encompassing all identified checkpoint components.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
ATRIP	ATR-interacting protein
BPDE	benzo[a]pyrene diol epoxide
BRCA	breast cancer susceptibility
Cdc	cell division cycle
Cdk	cyclin-dependent kinase
Chk1	checkpoint kjnase 1
Chk2	checkpoint kinase 2
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
E. coli	*Escherichia coli*
53BP1	p53 binding protein 1
GST	glutathione-s-transferase
His	6x histidine epitope tag
H2AX	H2A histone family, member X
IP	immunoprecipitate
kDa	kilodaltons
kd	kinase-dead
L	liter
LB	Luria broth
MCM	minichromosome maintenance
MDC1	mediator of mammalian DNA damage checkpoint 1
mg	milligram
µg	microgram

min	minute
ml	milliliter
µl	microliter
mM	millimolar
µM	micromolar
µmole	micromole
Mre11	meiotic recombination 11
MRN	Mre11, Rad50, Nbs1 complex
N-Aco-AAF	*N*-acetoxy-2-acetylaminofluorene
Nbs1	Nijmegen breakage syndrome 1
PAGE	polyacrylamide gel electrophoresis
PIKK	phosphatidylinositol 3-kinase-related protein kinase
RPA	replication protein A
SDS	sodium dodecyl sulfate
sec	second
ss	single strand
TopBP1	topoisomerase IIβ binding protein 1
UV	ultraviolet

CHAPTER 1

INTRODUCTION

DNA damage response

The genomes of eukaryotic cells are constantly subjected to various forms of DNA damage. DNA damage can be caused by exogenous agents, including UV light, ionizing radiation, and reactive chemicals. In addition, byproducts of normal cellular metabolism, such as reactive oxygen species (ROS), free radicals, and replication errors, can be endogenous sources of DNA damage (Abraham, 2001). DNA damage includes base-pair mismatches which can be repaired by mismatch repair pathways, and covalent modifications in DNA structure, such as base lesions, DNA strand breaks, cross-links, and abnormal DNA structures, that can be processed by base excision repair, nucleotide excision repair, and recombination pathways (Sancar et al., 2004). If not repaired, DNA damage can perturb DNA metabolism, resulting in chromosome instability, aneuploidy, and tumorigenesis (Sancar et al., 2004).

Upon DNA damage, mammalian cells trigger several cellular responses that are known as the DNA damage response, including DNA repair, transcriptional responses, DNA damage checkpoints, and apoptosis (Sancar et al., 2004; Zhou and Elledge, 2000) (Figure 1.1). Cells activate DNA repair pathways to remove the damage directly, transcriptional responses to repress or induce transcriptional processes which may affect the repair of DNA damage, DNA damage checkpoint responses to arrest cell cycle progression in the presence of DNA damage, and apoptosis to eliminate seriously damaged cells (Sancar et al., 2004; Zhou and Elledge, 2000). A failure in any of these pathways can cause premature aging, predisposition to cancer, and inherited diseases in humans (Aguilera and Gomez-Gonzalez, 2008; Hartwell and Kastan, 1994; Kastan and Bartek, 2004; Sancar et al., 2004).

DNA damage checkpoint response

To deal with various forms of DNA damage, cells have evolved a surveillance mechanism, called the DNA damage checkpoint, to maintain genomic integrity. The DNA

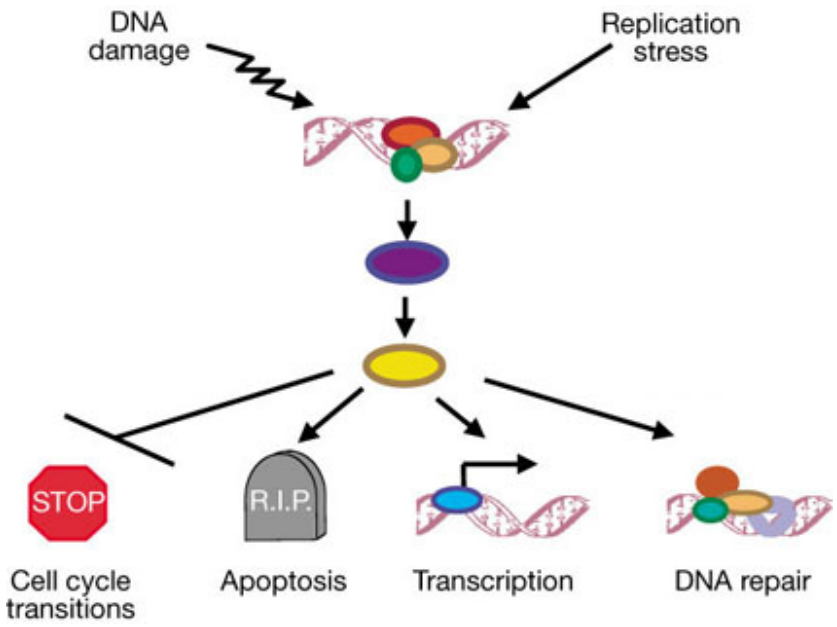

Figure 1.1. Several cellular responses induced by DNA damage in eukaryotic cells
Adapted from (Zhou and Elledge, 2000).

damage checkpoint is a signal transduction pathway that coordinates the cell cycle with other cellular responses and thus prevents the replication and segregation of damaged chromosomes. In response to DNA damage, cells activate the DNA damage checkpoint response which delays cell cycle progression to allow time for DNA repair before entering S phase or mitosis (Abraham, 2001). This cellular mechanism operates throughout the cell cycle and induces G1/S, intra-S, and G2/M checkpoints in the presence of DNA lesions.

Previous genetic studies have identified four groups of proteins as important to the DNA damage checkpoint response: damage sensors, mediators, signal transducers, and effectors (Sancar et al., 2004) (Figure 1.2). Through interactions with mediators, sensors recognize DNA damage sites and transmit signals to transducers to activate effector proteins, which eventually affect the cell cycle regulatory proteins.

Sensor proteins recognize DNA damage and initiate the signal transduction pathway. These sensors include ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3 related), and the Rad17-RFC/9-1-1 complexes. ATM and ATR are two key proteins that activate checkpoint signaling pathways in response to DNA damage and are members of the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs) (Abraham, 2001). Homologs of ATM and ATR are found in all eukaryotes, including budding and fission yeast (Abraham, 2001). ATM and ATR activate downstream proteins by phosphorylating at serine/threonine residues in the SQ/TQ sequence context (Abraham, 2001; Matsuoka et al., 2007). SQ/TQ-rich domains are often found in proteins implicated in DNA repair and checkpoint signaling pathways. Although there is some crosstalk, the ATM and ATR pathways are distinct. It is generally accepted that ATM responds primarily to double-strand breaks caused by ionizing radiation and radiomimetic agents, whereas ATR plays an important role in the response to incompletely replicated DNA and a broad range of DNA damage, including UV-damaged

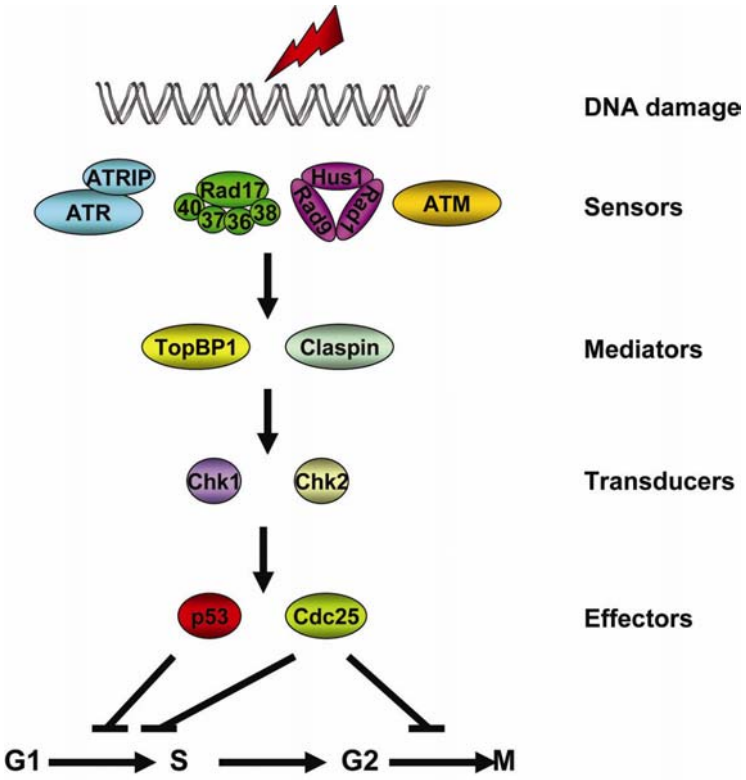

Figure 1.2. Components of the DNA damage checkpoints in human cells. The damage is detected by sensors that, with the aid of mediators, transduce the signal to transducers. The transducers, in turn, activate or inactivate other proteins (effectors) that directly participate in inhibiting the G1/S transition, S-phase progression, or the G2/M transition. Adapted from (Sancar et al., 2004)

DNA (Abraham, 2001). ATR was identified as a large protein kinase (301 kDa) (Cimprich et al., 1996), and forms a complex with a critical binding partner, called ATRIP (ATR-interacting protein, 87 kDa) that is essential for checkpoint functions (Cortez et al., 2001). The ATR gene is essential as knockout in embryos results in embryonic lethality and cells cultured from these mice are also not viable (Brown and Baltimore, 2000). In response to various types of DNA damage or stalled replication, ATR phosphorylates its downstream targets, including Chk1 (Abraham, 2001; Liu et al., 2000; Zhao and Piwnica-Worms, 2001).

ATM was identified as the product of the human gene responsible for the recessive disease ataxia-telangiectasia (A-T) when mutated (Shiloh, 2003). Cells cultured from patients with ataxia-telangiectasia are broadly defective in responding to double-strand breaks (Shiloh, 2003). ATM is a 351 kDa protein kinase, which is rapidly activated in response to DNA double-strand breaks. ATM exists as inactive dimers in unperturbed cells. In response to DNA double-strand breaks, the ATM dimers are converted into highly active monomers (Bakkenist and Kastan, 2003). During this process, ATM undergoes autophosphorylation on Ser1981 and is recruited to sites of DNA dmage and phosphorylates multiple DNA damage checkpoint proteins, including Chk2 (Bakkenist and Kastan, 2003; Sancar et al., 2004).

The Rad17-RFC and 9-1-1 complexes associate with damaged DNA independently of ATM or ATR, suggesting these complexes may also sense the sites of DNA damage (Zou et al., 2002). The Rad17-RFC complex is a structural homolog of the replication factor, RFC. The replication factor C (RFC) complex is an ATPase composed of four small subunits (p40, p38, p37, and p36) and one large subunit (p140) (Bell and Dutta, 2002). The Rad17-RFC complex, in which the p140 subunit is replaced by the 75 kDa Rad17 protein, is one of the known RFC-like complexes (Green et al., 2000; Lindsey-Boltz et al., 2001). The 9-1-1 complex consists of Rad9, Hus1, and Rad1 which

form a ring structure similar to PCNA (Griffith et al., 2002; Venclovas and Thelen, 2000). . In a manner similar to PCNA loading by RFC, the Rad17-RFC complex loads the 9-1-1 complex onto DNA in both humans and *S. cerevisiae* (Bermudez et al., 2003; Ellison and Stillman, 2003; Majka and Burgers, 2003; Zou et al., 2003).

The sensor proteins depend upon additional proteins which serve as mediators of the ATR-checkpoint response to phosphorylate their downstream substrates. Mediators increase the efficiency and specificity of signal transduction by communicating with damage sensors, transducers, and even effectors. The mediator proteins include Claspin and several proteins containing BRCA1 carboxy-terminal (BRCT) domains (Kumagai and Dunphy, 2000; Sancar et al., 2004).

Claspin was first discovered as a protein that associates with Chk1 and is essential for ATR-mediated Chk1 phosphorylation in *Xenopus* egg extracts (Kumagai and Dunphy, 2000). Claspin is a 151 kDa protein which shares slight sequence homology with Mrc1 in budding and fission yeast (Abraham, 2001). Claspin was shown to bind to branched DNA structures and to associate with checkpoint and replication proteins, suggesting Claspin has a role in checkpoint control at the stalled replication forks (Chini and Chen, 2004; Sar et al., 2004). Claspin is phosphorylated in a manner dependent on ATR upon DNA damage although it is not clear which kinase is responsible for Claspin phosphorylation (Chini and Chen, 2004). The phosphorylation of Claspin at these residues is required for the interaction with Chk1 and its subsequent activation (Kumagai and Dunphy, 2003).

Human TopBP1 was first identified as a topoisomerase IIβ binding protein in two-hybrid studies (Yamane et al., 1997). TopBP1 is a 170 kDa protein containing eight BRCT (carboxyl-terminus of BRCA1) regions, which are often found in proteins involved in DNA repair and checkpoint signaling (Garcia et al., 2005; Yamane et al., 1997). TopBP1 interacts with DNA polymerase ε and is required for DNA replication (Makiniemi

et al., 2001). In response to DNA damage, TopBP1 is phosphorylated and colocalizes with other checkpoint proteins (Makiniemi et al., 2001; Yamane et al., 2002). Moreover, recent studies have shown the important role of TopBP1 in stimulation of ATR kinase activity (Ball et al., 2007; Hashimoto et al., 2006; Kumagai et al., 2006).

Chk1 (54 kDa) and Chk2 (61 kDa) are transducer proteins, which were identified as essential components of the DNA damage checkpoint (Melo and Toczyski, 2002). These proteins are serine/threonine protein kinases, which are activated by the upstream checkpoint proteins in the presence of DNA damage. Although there is some cross-talk, Chk1 is phosphorylated and activated in response to UV damage, and Chk2 is phosphorylated and activated in response to double-strand breaks (Abraham, 2001; Hirao et al., 2000; Shiloh, 2003; Zhao and Piwnica-Worms, 2001). The activated transducer proteins, in turn, relay the signal of DNA damage to the effector proteins that directly affect the cell cycle regulatory proteins. These effector proteins include the three tyrosine phosphatases Cdc25A, B, and C, and the transcription factor p53. The transducers phosphorylate and inactivate the tyrosine phosphatases, which normally promote cell cycle progression by dephosphorylating cyclin-dependent kinases (CDKs) (Furnari et al., 1997; Sanchez et al., 1997; Zeng et al., 1998). Phosphorylated p53 induces transcription of p21 which in turn, inhibits CDK2 activity and arrests the cell cycle (Bartek and Lukas, 2001).

ATR activation

The ATR-mediated DNA damage checkpoint response is a critical pathway that responds to various forms of damaged and incompletely replicated DNA. Recently, some critical discoveries have provided significant insight into the mechanism of activation of ATR in response to DNA damage. However, a major question with respect to ATR activation remains to be answered. What is the initial signal for ATR activation? Some previous studies have indicated that a common DNA structure, RPA-coated

single-stranded DNA (ssDNA) generated by processing of damage sites is the initial signal to activate checkpoint responses (Zou, 2007). However, it is likely that more than one type of DNA/protein structure may constitute a signal for checkpoint activation *in vivo*. Our lab previously reported that primary DNA lesions induced by *N*-Aco-AAF and UV light can be a signal for checkpoint activation *in vivo* and checkpoint proteins bind to these lesions directly *in vitro* (Jiang and Sancar, 2006; Unsal-Kacmaz et al., 2002). Although there is some conflicting data, a number of studies have shown that RPA-coated single-stranded DNA and primer/template junctions are implicated in the activation of the ATR checkpoint (MacDougall et al., 2007; Michael et al., 2000; You et al., 2002; Zou, 2007; Zou and Elledge, 2003). It was reported that RPA is sufficient to recruit ATR to ssDNA through interaction with ATRIP in both human and budding yeast system (Zou and Elledge, 2003). Therefore, it has been proposed that RPA-coated ssDNA interacts with ATRIP and recruits the ATR-ATRIP complex to the sites of damage.

Previous studies indicate that optimal ATR activation requires the other checkpoint components, Rad17-RFC and 9-1-1 (Zou et al., 2002). The Rad17 complex loads the 9-1-1 complex onto DNA structures containing primer/template junctions and this loading is stimulated by RPA (Ellison and Stillman, 2003; Majka and Burgers, 2003; Zou et al., 2003). Interestingly, although the Rad17-dependent loading of the 9-1-1 complex is required for proper ATR activation, recruitment of Rad17 and 9-1-1 onto chromatin is independent of ATR-ATRIP (Zou et al., 2002). Therefore, it has been proposed that Rad17 is recruited to the primer/template junctions independently of ATR-ATRIP through interaction with RPA-coated ssDNA and consequently the 9-1-1 complex is loaded onto sites of DNA damage.

In the budding yeast system, it was shown that the 9-1-1 complex loaded onto DNA directly stimulates the kinase activity of Mec1, the yeast ortholog of ATR (Majka et

al., 2006). However, there is no evidence of such direct activation in other model systems. Importantly, recent work using *Xenopus* egg extracts has shown that TopBP1 is a direct activator of ATR-ATRIP and strongly stimulates the kinase activity of ATR in the absence of RPA or DNA (Kumagai et al., 2006). These authors also identified the domain of TopBP1 that is responsible for ATR activation and found that TopBP1 interacts with ATR through the ATR activating domain, located between the sixth and seventh BRCT repeats (Kumagai et al., 2006). It has been also reported that the Rad9 subunit of 9-1-1 interacts with TopBP1, and this interaction is required for ATR activation, suggesting that the primary role of the 9-1-1 complex is to recruit TopBP1 to ATR (Delacroix et al., 2007; Lee et al., 2007). These studies found that through direct interaction between the phosphorylated C-terminal region of Rad9 and the first two BRCT repeats of TopBP1, the chromatin-bound 9-1-1 complex locates TopBP1 to the ATR kinase, triggering ATR activation (Delacroix et al., 2007; Lee et al., 2007). Activated ATR can phosphorylate a number of its substrates, including Rad17, which is phosphorylated on at least two residues, and this phosphorylation is reported to be essential for Rad17 function in response to DNA damage (Bao et al., 2001). In addition, Claspin interacts with Chk1 In response to DNA damage, and phosphorylation of Claspin is essential for the interaction (Kumagai and Dunphy, 2003). Interestingly, phosphorylated Rad17 interacts with Claspin and this interaction promotes phosphorylation of Claspin (Wang et al., 2006). Therefore, it has been proposed that ATR phosphorylates Rad17 and phosphorylated Rad17 interacts with Claspin, which in turn promotes ATR-mediated Chk1 phosphorylation. Chk1 is phosphorylated on serine 317 and 345 by ATR in response to DNA damage and propagates the signals to downstream targets (Abraham, 2001; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). After its phosphorylation, Chk1 is released from chromatin to promote the DNA damage checkpoint response by phosphorylating its substrates (Smits et al., 2006).

Altogether, these observations have led to the model shown in Figure 1.3. Upon DNA damage, ssDNA can be generated by processing during nucleotide excision repair or by uncoupling of MCM helicase and DNA polymerase activities at stalled replication forks. RPA rapidly accumulates on the exposed ssDNA regions and consequently, RPA-ssDNA complexes recruit the ATR-ATRIP complex and the Rad17-RFC complex. The 9-1-1 complex is then loaded onto primer/template junctions by the Rad17-RFC complex and recruits TopBP1 in the proximity of ATR to activate the kinase activity. Activated ATR then phosphorylates Rad17, which then interacts with Claspin to facilitate ATR-mediated Chk1 phosphorylation and activation.

Conclusion

The importance of the checkpoint response has emerged from studies of cancer predisposition syndromes and human cancers resulting from mutations in DNA damage checkpoint genes. For example, the human genetic disorder ataxia-telangiectasia (A-T), which is characterized by neurodegeneration, radiation sensitivity, immunodeficiency and cancer predisposition, results from mutations in ATM (Shiloh, 1997; Shiloh, 2003). Missense mutations in the human ATR gene are associated with the human autosomal recessive disorder Seckel's syndrome (O'Driscoll et al., 2003). Although recent genetic studies have begun to reveal the molecular details of the DNA damage checkpoint response, the biochemical mechanism of the response still remains largely unknown. Therefore, the goal of my work has been elucidate the molecular mechanism of the ATR-mediated DNA damage checkpoint response. To understand the molecular mechanism of this complex checkpoint network, it is necessary to develop an *in vitro* checkpoint system reconstituted from purified proteins. However, much of the previous *in vitro* studies for the DNA damage checkpoint response have used cell-free systems, which have certain limitations for mechanistic studies of the DNA damage checkpoint

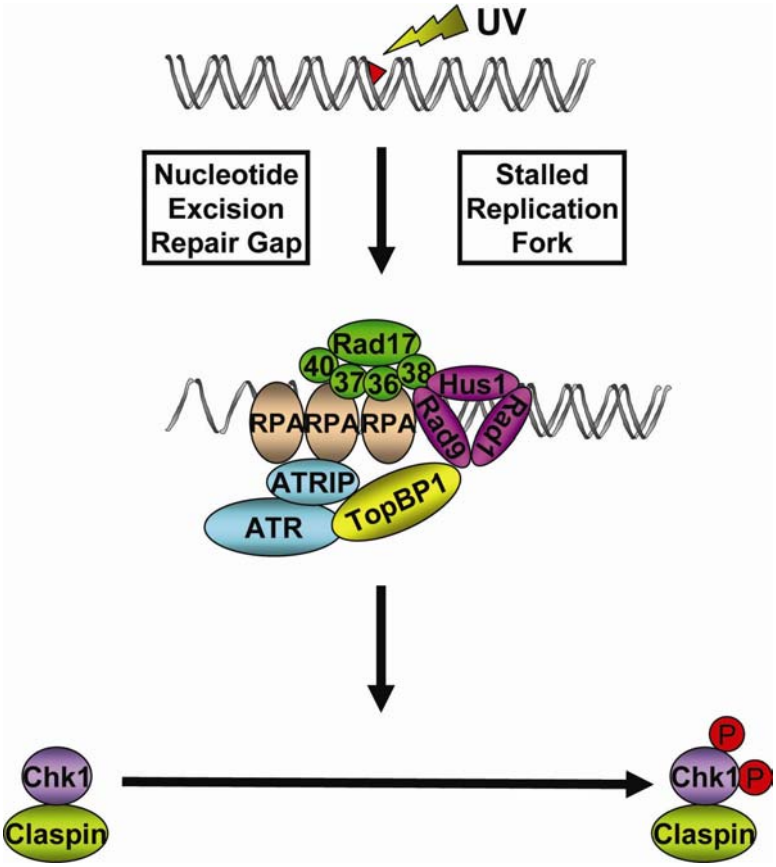

Figure 1.3. A current model of ATR activation. Upon DNA damage, RPA-coated ssDNA regions are generated by processing of nucleotide excision repair or by blocking replication forks. RPA-ssDNA complexes recruits the ATR-ATRIP complex and the Rad17-RFC/9-1-1 complexes. The 9-1-1 complex then recruits TopBP1 in the proximity of ATR to activate the kinase activity. Activated ATR results in Chk1 phosphorylation, which is facilitated by Claspin.

response (Clarke and Clarke, 2005; Guo et al., 2000; Hashimoto et al., 2006; Kumagai et al., 1998; MacDougall et al., 2007; Michael et al., 2000; Yan et al., 2006; Yoshioka et al., 2006). Therefore, the focus of my work has been to reconstitute a human ATR-mediated DNA damage checkpoint *in vitro* with purified proteins and to use this system for studying of checkpoint activation induced by DNA damage.

Chapter 2 describes a human *in vitro* checkpoint system in which damaged DNA specifically stimulates TopBP1-depedent activation of ATR kinase activity toward its substrate, Chk1. Chapter 3 presents characterization of the DNA binding and ATR stimulatory activities of TopBP1 and ATR activation by a cooperative activation mechanism. Chpater 4 demonstrates *in vitro* evidence indicating the stimulatory effect of RPA-coated ssDNA on TopBP1- dependent ATR activation.

CHAPTER 2

RECONSTITUTION OF A HUMAN DNA DAMAGE CHECKPOINT RESPONSE *IN VITRO* WITH DAMAGED DNA AND PURIFIED PROTEINS

Abstract

The DNA damage checkpoint response delays cell cycle progression upon DNA damage and prevents genomic instability. Genetic analysis has identified sensor, mediator, signal transducer, and effector components of this global signal transduction pathway. Here we describe an *in vitro* system with purified human checkpoint proteins that recapitulates key elements of the DNA damage checkpoint. We show that the damage sensor ATR, in the presence of TopBP1 mediator/adaptor protein, phosphorylates the Chk1 signal transducing kinase in a reaction that is strongly dependent on the presence of DNA containing bulky base lesions. The dependence on damaged DNA requires DNA binding by TopBP1, and indeed, TopBP1 shows preferential binding to damaged DNA. This *in vitro* system provides a useful platform for mechanistic studies of the human DNA damage checkpoint response.

Introduction

Most of our knowledge about the DNA damage checkpoint response is based on genetic data from model organisms including budding and fission yeasts and humans and the *Xenopus* egg extract system. These studies have identified damage sensors, mediators, signal transducers, and effectors as key components of this signal transduction pathway (Abraham, 2001; Nyberg et al., 2002; Sancar et al., 2004). The phosphatidylinositol 3-kinase-related protein kinase (PIKK) family members have been shown to be key DNA damage sensors and signal transducers in the checkpoint response. Of these, ATM is mainly responsible for initiation of the checkpoint response elicited by double-strand breaks caused by ionizing radiation or radiomimetic agents. Some semi-defined systems for the ATM-mediated checkpoint response have been described recently (Bakkenist and Kastan, 2003; Costanzo et al., 2000; Dupre et al., 2006; Lee and Paull, 2004; Lee and Paull, 2005). Another PIKK family member, ATR, initiates the DNA damage checkpoint response caused by UV radiation and UV-mimetic agents that produce base damage such as *N*-acetoxy-2-acetylaminofluorene (*N*-Aco-AAF). Although this important signal transduction pathway has been characterized in some detail using *Xenopus* egg extracts (Guo et al., 2000; Hashimoto et al., 2006; Kumagai et al., 1998; Kumagai et al., 2006; MacDougall et al., 2007; Michael et al., 2000; Yan et al., 2006) and in human cell free systems (Clarke and Clarke, 2005; Yoshioka et al., 2006), only recently have partial *in vitro* systems been developed with a subset of either *Xenopus* (Hashimoto et al., 2006; Kumagai et al., 2006) or yeast checkpoint proteins (Majka et al., 2006). Currently, there is no well-defined system for ATR-mediated DNA damage checkpoint response in humans. Recently, it was shown that the multifunctional XtopBP1 protein, which is known to be required for the ATR-mediated checkpoint (Parrilla-Castellar and Karnitz, 2003), activated the ATR kinase on downstream targets, in particular the Chk1 signal transducing kinase, in the absence of

DNA or any other checkpoint protein except the ATR interacting protein, ATRIP (Kumagai et al., 2006). Here we describe a human *in vitro* system in which ATR phosphorylates Chk1 kinase in a reaction that is dependent on TopBP1 and strongly stimulated by DNA containing bulky DNA adducts. We believe this is a useful system for the ultimate development of an *in vitro* human checkpoint response dependent on all checkpoint proteins identified by genetic methods. Such a system would open new opportunities for mechanistic studies of the human DNA damage checkpoint response.

Materials and Methods

Antibodies and Checkpoint Proteins

Chk1 P345 antibody was purchased from Cell Signaling Technology. ATRIP, ATM, and DNA-PK antibodies were purchased from Novus Biologicals. Chk1 and ATR (N-19) antibodies were purchased from Santa Cruz Biotechnology. DNA-PKcs (Ab-4) antibody was purchased from NeoMarkers. GST-TopBP1-His and GST-TopBP1 (978-1192) fragment were induced in *Escherichia coli* BL21 RIPL (Stratagene) at 16°C for 5 hr. GST-TopBP1-His was purified with glutathione sepharose (GE Healthcare) and nickel-NTA agarose (Qiagen), and GST-TopBP1 (978-1192) fragment was purified with glutathione sepharose by standard procedures. His-Chk1-kd was produced by baculovirus infection of SF21 insect cells and purified with nickel-NTA agarose beads as described (Yoshioka et al., 2006). The p11-tRPA plasmid was obtained from M.S. Wold (University of Iowa), and RPA was purified as described (Henricksen et al., 1994).

Purification of ATR-ATRIP From HeLa Cell Free Extracts

Native ATR-ATRIP complex was isolated from 70L of HeLa cells. 80 ml (8.3 mg/ml) of HeLa cell free extracts were prepared as described (Manley et al., 1983) and applied onto a DE52 (Whatman) column. The FT was then loaded onto a CM Affi-Gel Blue (BioRad) column, eluted with 1M NaCl, and fractions were collected and analyzed by Western blotting and silver-staining. The ATR-containing fractions were pooled and dialyzed against Buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT) containing 100mM KCl. It should be noted that this column separates RPA from ATR-ATRIP quantitatively (Mu et al., 1996), and as a consequence our highly purified ATR-ATRIP was free of RPA. The dialyzed fractions were applied onto a 50 ml DEAE Sepharose (GE Healthcare) column, and the FT was collected and loaded onto a 20 ml Mono Q column (GE Healthcare) and then eluted with a 0.1-0.5 M KCl gradient. The ATR-containing fractions were pooled and dialyzed against Buffer D containing 25 mM

KCl. The dialyzed fractions were then loaded onto a 1 ml Mono S column (GE Healthcare) and eluted with a 25-400 mM KCl gradient. The peak of ATR-ATRIP complex eluted at 220 mM KCl. This fraction (0.4 ml, 44 µg) was free of DNA-PKcs and ATM as determined by Western blotting, contained approximately 4 µg of ATR-ATRIP, and was stored frozen in Buffer D containing 220 mM KCl.

Preparation of DNA substrates for Kinase Assays

Single-stranded or double-stranded pIBI25 plasmid was purified by standard procedures. For preparation of deca-primed DNA, ten 30-mer oligomers were hybridized to single-stranded pIBI25 DNA at regular intervals. The annealing reaction (100 µl) contained 10 mM Hepes, pH 7.9, 100 mM NaCl, 9 pmol each oligomer, and 5 pmol single-stranded pIBI25 DNA. The reaction was incubated at 90°C for 3 min and cooled gradually. For *N*-acetoxy-2-acetylaminofluorene (*N*-Aco-AAF)-damaged DNA, pUC19 plasmid was treated with *N*-Aco-AAF (NCI Chemical Carcinogen Reference Standard Repository, Midwest Research Institute) as described previously (Hess et al., 1996) to introduce approximately 20-30 adducts per plasmid. Briefly, pUC19 (50 µg/ml) was treated with 0.3 mM *N*-Aco-AAF in 2 mM sodium citrate, pH 7.0 at room temperature for 3 hr. The reaction was followed by ether extraction and ethanol precipitation to remove the non-reacted excess *N*-Aco-AAF. For UV irradiation, pUC19 plasmid was irradiated with 3,000 J/m^2 of UVC from a 254 nm germicidal lamp as determined with a UVX radiometer. After *N*-Aco-AAF treatment or UV irradiation, the DNA was analyzed on a 0.8% agarose gel to determine that no additional nicking was introduced by the treatment.

Kinase Assays

Kinase assay reactions contained 1 µM microcystin, 15 mM Hepes, pH 7.9, 35 mM KCl, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM ATP, 5 mM creatine phosphate, 8 µg/ml of creatine kinase, 0.5 mM DTT, 1% polyethylene glycol, and 5% glycerol in a 10 µl final

volume. Where indicated, the kinase assays were carried out with different salt concentrations. 1 nM purified ATR was pre-incubated for 15 min at 30°C with 1 nM GST-TopBP1-His and 0.62 - 5 nM DNA. After the pre-incubation, 20 nM Chk1-kd was added into the reaction, incubated for 20 min at 30°C, unless otherwise indicated, terminated by the addition of SDS-PAGE loading buffer, and separated by SDS-PAGE. Chk1 phosphorylation was detected by immunoblotting using Chk1 phospho-S345 antibody. For kinase assays with RPA, 5 nM DNA was first incubated with 0.24 - 1.92 μM RPA for 10 min on ice. The reaction was then followed by the procedures described above. For quantification purposes, levels of Chk1 phosphorylation were quantified using ImageQuant 5.2 software after scanning. The level of Chk1 phosphorylation in the control reaction (without TopBP1 and DNA) was quantitated and set equal to 1. The levels of Chk1 phosphorylation in the other lanes were determined relative to this value.

DNA Binding Assays

The procedure was a modification of a previously described method (Yamane and Tsuruo, 1999). pUC19 plasmid was linearized with BamHI and 5′-end labeled with [γ-^{32}P]ATP by T4 PNK. The labeled DNA was then mock-treated or treated with *N*-Aco-AAF as described above. Purified proteins (3-30 pmol) on glutathione beads were incubated at 37°C for 10 min with the DNA (1 ng) in Buffer B (10 mM Tris-Cl, pH7.7, 1 mM EDTA, 0.5% NP 40) containing 200 mM NaCl. After the incubation, the beads were washed three times with Buffer B, and bound DNA was eluted by incubation with 0.1 μg/μl proteinase K at 37°C for 15 min. The DNA was resolved in a 0.8% agarose gel, visualized by autoradiography, and quantitated using a PhosphorImager and ImageQuant 5.2 software.

DNA Excision Assays

The internally radiolabeled DNA substrate was a 148-bp duplex prepared by ligating a *N*-Aco-AAF-damaged oligomer with five other partially overlapping oligomers

as described previously (Reardon and Sancar, 2006). Human excision repair proteins were purified from various sources as described (Henricksen et al., 1994; Reardon and Sancar, 2006). 7.5 fmol substrate DNA was incubated at 30°C for 2 h with purified excision repair proteins (27 nM XPA, 9 nM XPC-HR23B, 100 nM RPA, 300 ng partially purified TFIIH, 4 nM XPF-ERCC1, and 5 nM XPG) or with purified checkpoint proteins (1 nM ATR-ATRIP, 1 nM GST-TopBP1-His, 20 nM His-Chk1-kd) in 12.5 µl of reaction buffer containing 32 mM Hepes, pH7.9, 64 mM KCl, 6.4 mM $MgCl_2$, 5% glycerol, 4 mM ATP, and 200 µg/ml BSA. After the incubation, the DNA was purified, resolved in a 10% denaturing polyacrylamide gel, and visualized by autoradiography.

Results

TopBP1-Dependent Stimulation of ATR Kinase Activity by DNA

We are interested in developing a human ATR-mediated checkpoint system *in vitro*. To this end, we purified human ATR-ATRIP, TopBP1, and Chk1 proteins. The ATR-ATRIP complex was purified from HeLa cell free extracts (Figure 2.1*A* and *B*) to a high degree of purity, free of other PIKK kinases which could possibly complicate the interpretation of the results. TopBP1 and Chk1 were purified from *E. coli* and baculovirus-infected insect cells, respectively (Figure 2.2) Fractions containing highly purified ATR-ATRIP were tested for the ability to phosphorylate Chk1-S345 in the presence and absence of TopBP1 (Figure 2.1*C*). We found that in reactions which were of low ionic strength, ATR phosphorylated Chk1 in a manner virtually dependent on TopBP1, which is in agreement with previous reports (Ball et al., 2007; Kumagai et al., 2006) (Figure 2.3, lane 3**)**. However, when the ionic strength was increased to more physiologically relevant levels, the stimulation of ATR kinase by TopBP1 was by-and-large eliminated (Figure 2.3, lanes 7 and 11**)**. We reasoned that under these conditions the checkpoint signaling reaction may be reestablished by including DNA in the reaction. Figure 2.3 shows the phosphorylation of Chk1 by ATR in the absence and presence of DNA with and without TopBP1. Under higher ionic strength reaction conditions, TopBP1 has no measurable effect on the kinase activity of ATR (lane 7). Significantly, however, the addition of DNA to this reaction resulted in a 7-fold stimulation of ATR activity (lane 8), restoring the Chk1 phosphorylation to levels observed in the low ionic strength buffer (lane 3).

Effects of Various DNA Substrates on TopBP1-Dependent ATR Kinase Activity

The dramatic TopBP1-dependent stimulation of ATR kinase activity that we observed in Figure 2.3 was achieved with double-stranded plasmid DNA. We reasoned that a more physiologically relevant form of DNA might result in further stimulation of ATR in our *in*

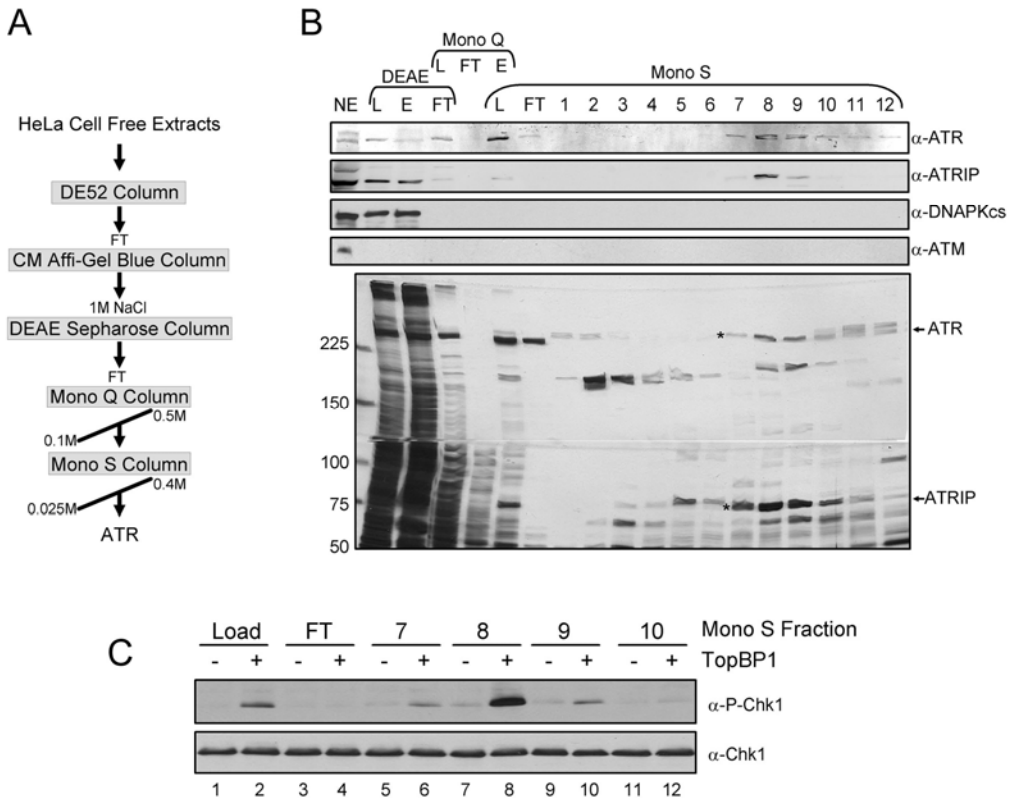

Figure 2.1. Purification of human ATR-ATRIP and activation of ATR kinase activity by TopBP1. (A) Scheme of ATR-ATRIP purification from HeLa cell free extracts. (B) Purification of ATR-ATRIP. Fractions from the Mono S column were separated on a 5/10% discontinuous SDS-polyacrylamide gel and analyzed by Western blotting using the indicated antibodies and by silver staining (Bottom). ATM and DNA-PK were separated from fractions containing ATR-ATRIP during chromatography on CM Affi-gel blue and DEAE columns, respectively. The peak of ATR-ATRIP eluted at 220 mM KCl (fraction 8) from the Mono S column. L, load; E, eluate; FT, flow-through; 1-12, fraction numbers; asterisks, ATR and ATRIP. The line between the 100 and 150 molecular weight markers is the boundary between the 5 and 10% acrylamide regions of the gel. (C) Activation of purified ATR kinase activity by TopBP1. Kinase assays were performed with the Mono S column fractions to test for TopBP1-dependent ATR kinase activity. Mono S load (lane1 and 2), flow-through (lane 3 and 4), and eluted fractions (lane 5-12) were incubated under low ionic strength reaction conditions (45 mM total salt concentration) with His-Chk1-kd in the presence or the absence of GST-TopBP1-His. An equal volume of each fraction was pre-incubated for 15 min at 30°C with 5 nM GST-TopBP1-His. 20 nM Chk1-kd was then added into the reaction and incubated for 20 min at 30°C. Reactions were analyzed by immunoblotting for phosphorylated Chk1 (P-S345) (Top) and total Chk1 (Bottom).

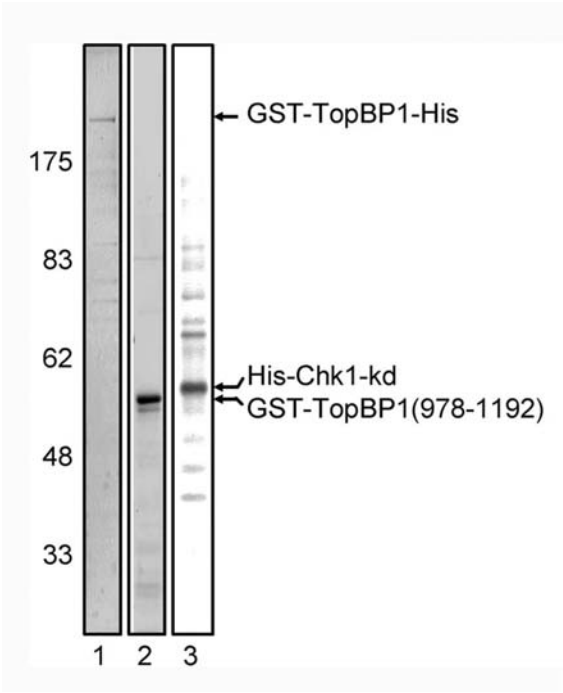

Figure 2.2. Purified human checkpoint proteins. The checkpoint proteins were analyzed by SDS-PAGE followed by Coomassie blue staining [GST-full-length TopBP1-His (lane 1) and GST-TopBP1 978-1192 fragment (lane2)] or silver staining [His-Chk1-kd (lane 3)]. 150 ng of GST-TopBP1-His, 1 μg of GST-TopBP1 978-1192 fragment, and 10 ng of His-Chk1-kd were resolved.

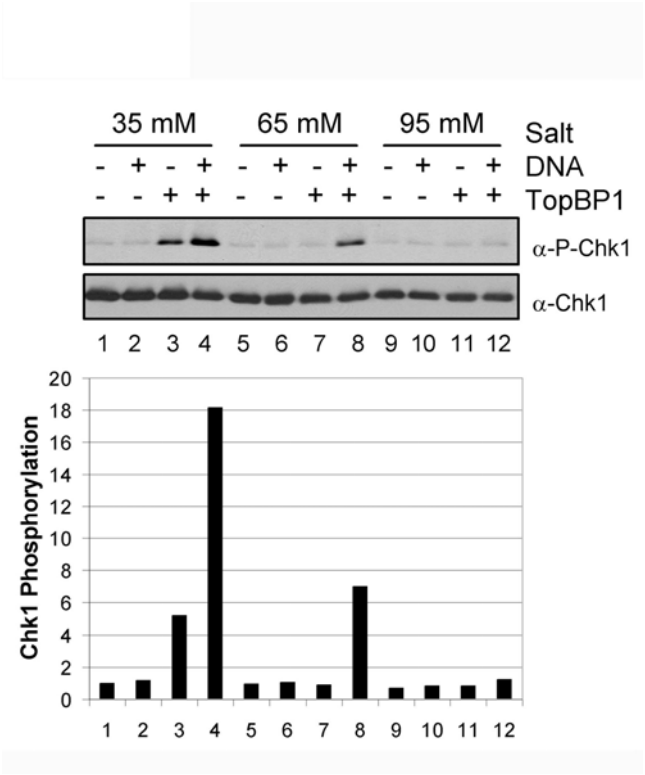

Figure 2.3. TopBP1-dependent stimulation of ATR kinase activity by DNA. Effects of ionic strength on TopBP1-dependent stimulation of ATR kinase activity. Kinase assays were carried out with ATR-ATRIP, His-Chk1-kd, GST-TopBP1-His, and double-stranded DNA under different ionic strength reaction conditions. 1 nM ATR-ATRIP was pre-incubated with or without 5 nM pUC19 plasmid in the presence and absence of 1 nM GST-TopBP1-His. 20 nM Chk1-kd was then added to the reaction and incubated for 20 min at 30°C. Levels of Chk1 phosphorylation were quantified and the results from three independent experiments were averaged and graphed at the bottom.

vitro reactions. As a model for both primer templates and excision repair gaps, deca-primed DNA has been reported to be optimal for stimulating *S. cerevisiae* $Mec1^{ATR}$ kinase (Majka et al., 2006). The addition of deca-primed DNA to our reactions results in up to a 5-fold stimulation of Chk1 phosphorylation by ATR in the presence of TopBP1 (Figure 2.4, lanes 6-8). However, the stimulatory effect was not specific to deca-primed DNA, as we observed essentially the same levels of stimulation of the TopBP1-dependent ATR kinase activity by both single-stranded (lanes 3-5) and double-stranded (lanes 9-11) plasmid DNAs. Because a number of studies have indicated that RPA-covered single-stranded DNA is a common, if not universal, signal for ATR-mediated checkpoint activation (Zou, 2007; Zou and Elledge, 2003), we tested the effect of RPA on the reaction using both deca-primed and double-stranded DNA. We only observed a slight stimulation upon the addition of RPA with both types of DNA (Figure 2.5). While these results do not eliminate RPA-covered single-stranded DNA or template/primer structures as checkpoint signals, they do show that under our experimental conditions of physiological ionic strength and limiting concentrations of TopBP1, all forms of DNA tested act similarly in activating ATR in a TopBP1-dependent manner.

TopBP1-Dependent Stimulation of ATR Kinase Activity by DNA Containing Bulky Base Lesions

Next, we tested DNA damaged by *N*-Aco-AAF and UV light. Both of these agents produce bulky base adducts, AAF-guanine and pyrimidine dimers, respectively. Moreover, evidence has been obtained indicating that checkpoint proteins bind directly to these lesions leading to checkpoint activation (Jiang and Sancar, 2006; Unsal-Kacmaz et al., 2002). Hence, we reasoned that DNA damaged by these agents may specifically activate ATR in the presence of TopBP1. In Figure 2.6*A*, we show the effect of various concentrations of *N*-Aco-AAF-damaged DNA on the kinase activity of ATR. As apparent from this figure, damaged DNA stimulates the ATR kinase 20-fold above

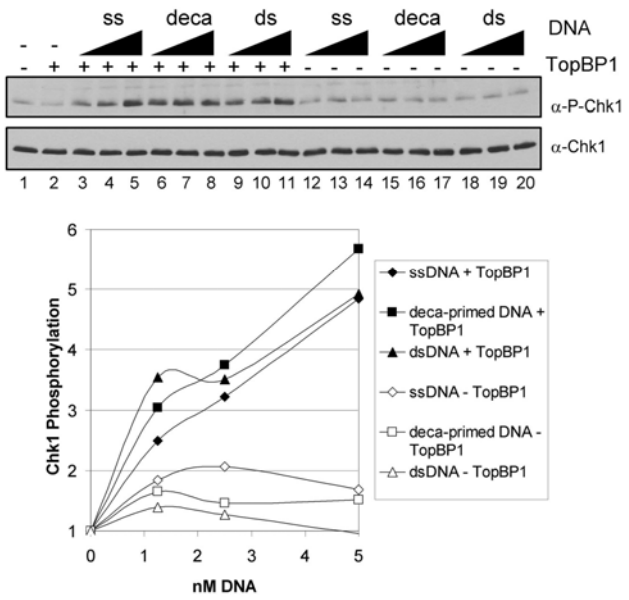

Figure 2.4. Stimulation of ATR kinase activity by various DNA substrates in the presence of TopBP1. Kinase assays were performed with ATR-ATRIP, His-Chk1-kd, GST-TopBP1-His, and different forms of DNA substrates under high ionic strength reaction conditions (85 mM total salt concentration) as described in Materials and Methods. 1 nM ATR-ATRIP was incubated with 1.25-5 nM single-stranded, deca-primed, or double-stranded pIBI25 plasmid in the presence and absence of 1 nM GST-TopBP1-His. The average levels of Chk1 phosphorylation from three independent experiments are quantitated at the bottom.

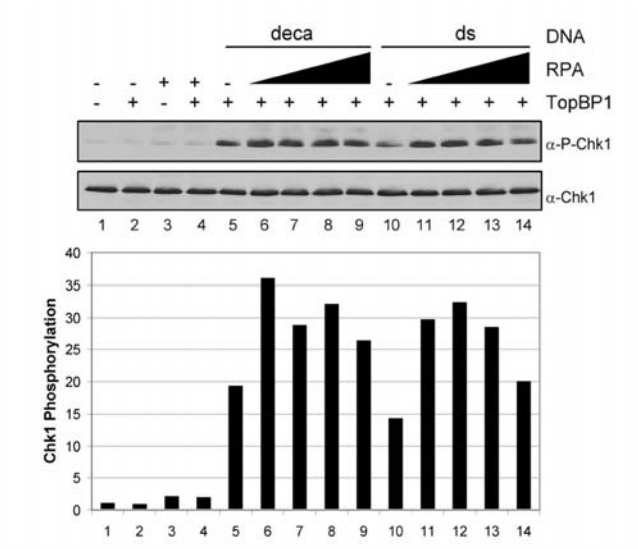

Figure 2.5. Effects of RPA on TopBP1-dependent activation of ATR kinase activity in the presence of DNA. Kinase assays were performed as in Figure 2.4, except that 5 nM deca-primed or double-stranded pIBI25 plasmid DNA was pre-incubated with 0.24-1.92 μM RPA for 10 min on ice. Reactions without DNA (lanes 3 and 4) contained 1.92 μM RPA.

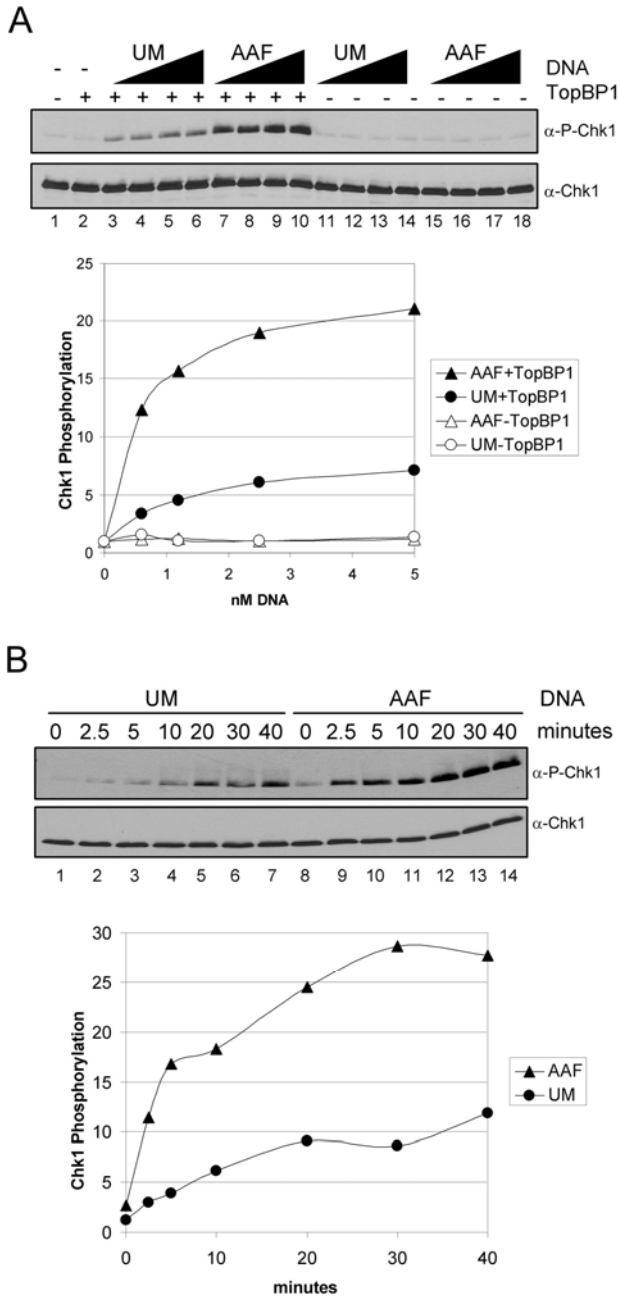

Figure 2.6. TopBP1-dependent stimulation of ATR kinase activity by N-Aco-AAF-damaged DNA. (A) Kinase assays were performed with 85 mM final salt concentration as in Figure 2.4, except with 0.62-5 nM unmodified (UM) or N-Aco-AAF-damaged (AAF) pUC19 plasmid DNA. The average levels of Chk1 phosphorylation from four independent experiments are quantitated at the bottom. (B) Time course of Chk1 phosphorylation. Kinase assays as in (A) were carried out with 0.62 nM DNA for the times indicated. The average levels of Chk1 phosphorylation from two independent experiments are quantitated at the bottom.

the level of TopBP1 alone, and more importantly, damaged DNA stimulates the TopBP1-dependent ATR kinase activity about 3-4-fold higher than undamaged DNA. We conducted kinetic experiments to analyze the reaction in more detail to determine the initial reaction rates (Figure 2.6*B*). From these experiments, we determined that the initial rate of the ATR kinase reaction was 5 times faster with *N*-Aco-AAF-damaged DNA than with undamaged DNA. Qualitatively similar but quantitatively less significant effects were observed with UV-damaged DNA as well (Figure 2.7). Once again, we observed increased specificity of the reaction under higher ionic strength conditions (Figure 2.8).

Since several reports indicate that the ~30-nt gap produced by excision repair constitutes a strong signal for the ATR-mediated checkpoint response (Marini et al., 2006; Matsumoto et al., 2007; O'Driscoll et al., 2003), we considered the possibility that impurities in our ATR, Chk1, or TopBP1 preparations were at a sufficient level to excise the base damage and create excision gaps that would constitute a signal for checkpoint response. To test for this eventuality, we conducted an "excision assay" (Huang et al., 1992; Reardon and Sancar, 2006) with a mixture of the proteins used for checkpoint response. No excision was observed (Figure 2.9), suggesting that the base damage itself is the signal in our *in vitro* checkpoint system.

Dependence of DNA Binding Activity of TopBP1 for ATR Activation

The results presented so far are consistent with the idea that TopBP1 may recruit ATR to DNA damage sites and in so doing stimulate its kinase activity. To test this idea, we measured the DNA binding activity of TopBP1 for undamaged versus *N*-Aco-AAF-damaged DNA (Figure 2.10*A*). Full-length TopBP1 bound DNA with relatively high affinity and with ~5-fold preference for damaged DNA (lanes 5 and 6). Intrestingly, the ~5-fold TopBP1-dependent stimulation of ATR kinase activity by *N*-Aco-AAF-damaged DNA over undamaged DNA (Figure 2.6) correlates with the ~5-fold higher binding affinity of TopBP1 to damaged DNA. To determine whether DNA binding by TopBP1 was

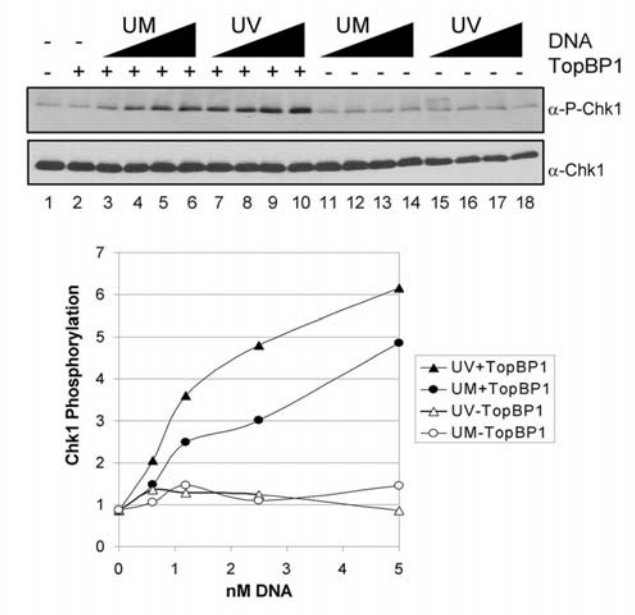

Figure 2.7. TopBP1-dependent stimulation of ATR kinase activity by UV-damaged DNA. Kinase assays were carried out as in Figure 2.6A, except with 0.62-5 nM unmodified or UV-damaged pUC19 plasmid DNA. Data from six independent experiments were quantitated, averaged, and graphed at the bottom.

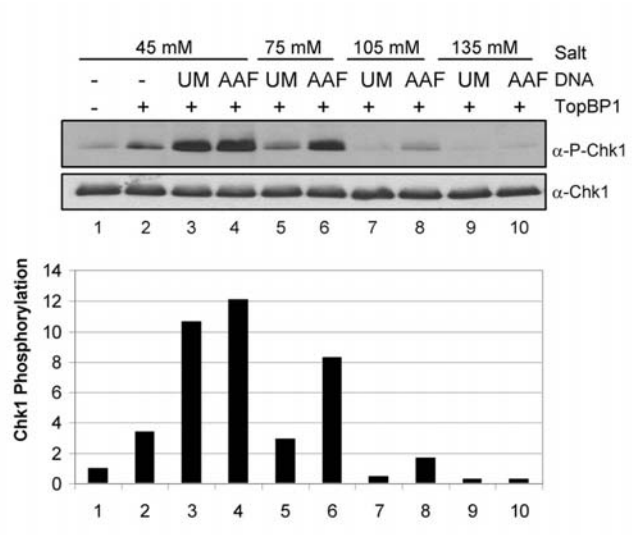

Figure 2.8. Requirement of high ionic strength reaction conditions for specificity of the TopBP1-dependent ATR-stimulation by N-Aco-AAF-damaged DNA. Kinase assays were performed as in Figure 2.3, except with unmodified DNA compared to N-Aco-AAF-damaged DNA and with different salt concentrations.

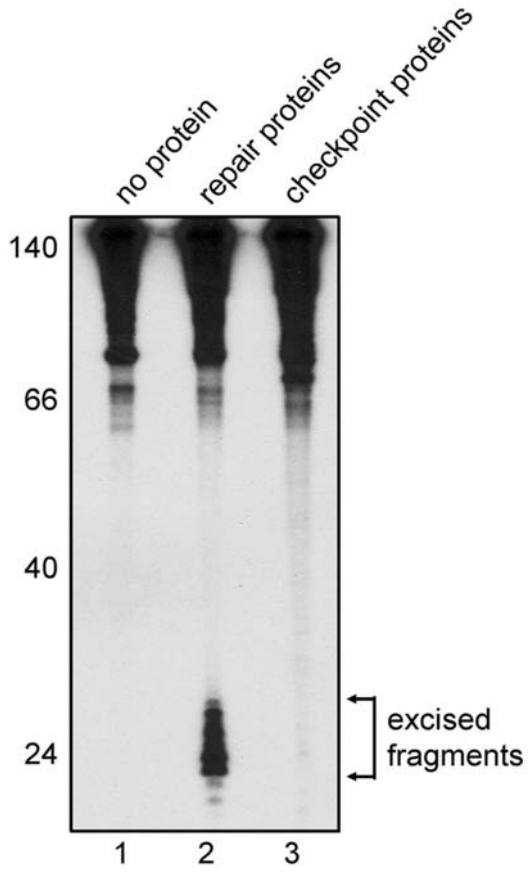

Figure 2.9. Excision assay with purified checkpoint proteins. Excision assays were performed with purified checkpoint proteins that were used for kinase assays, or purified repair factors that are sufficient for excision. 7.5 fmol N-Aco-AAF-damaged 148-bp DNA substrate was incubated at 30°C for 2 h with control buffer, purified repair proteins (27 nM XPA, 9 nM XPC-HR23B, 100 nM RPA, 300 ng partially purified TFIIH, 4 nM XPF-ERCC1, and 5 nM XPG) or with purified checkpoint proteins (1 nM ATR-ATRIP, 1 nM GST-TopBP1-His, and 20 nM His-Chk1-kd). Purified DNA was resolved in a 10% polyacrylamide gel and visualized by autoradiography.

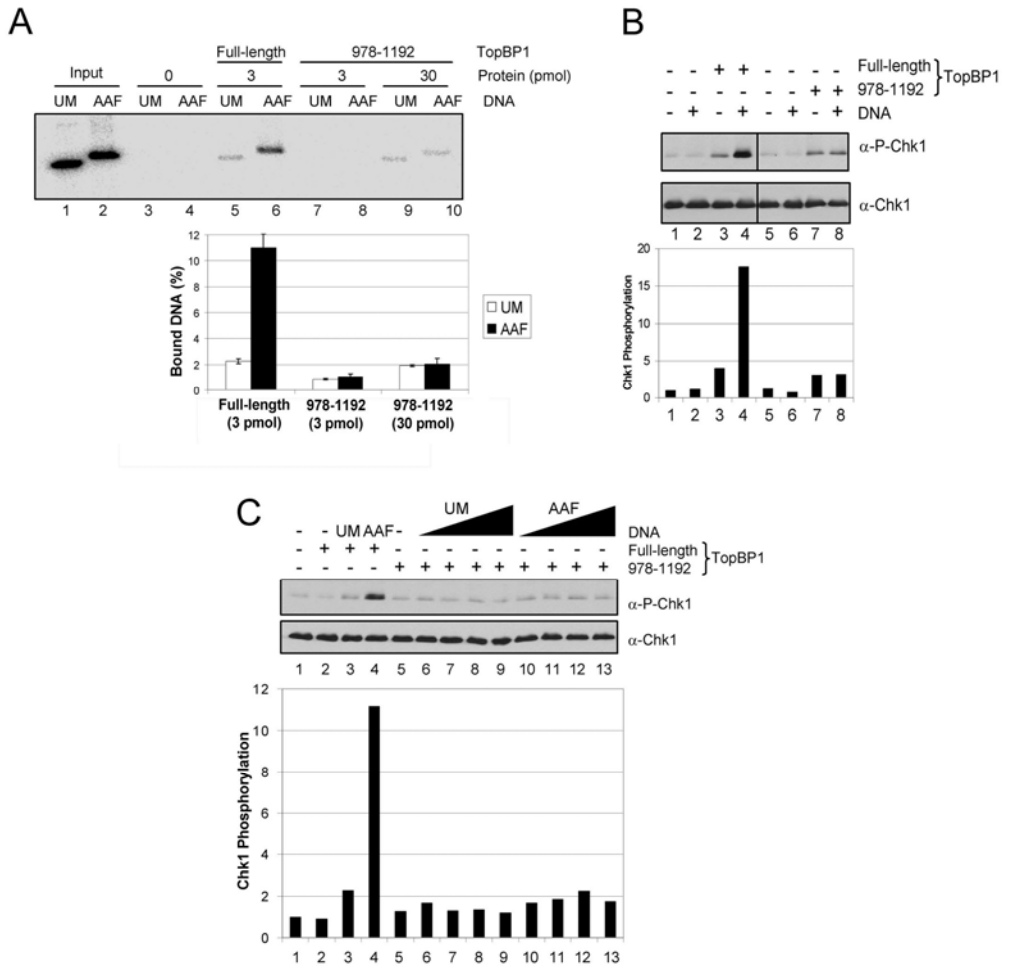

Figure 2.10. Dependence of DNA binding activity of TopBP1 for ATR activation. (A) Preferential binding of TopBP1 to N-Aco-AAF-damaged DNA. The indicated amounts of GST-TopBP1-His or GST-TopBP1 978-1192 fragment on glutathione-sepharose beads were incubated at 37°C for 10 min with 1 ng of unmodified or N-Aco-AAF-damaged DNA that had been 5′-end labeled. Bound DNA was eluted and analyzed by agarose gel electrophoresis. 50% of the DNA added into the reaction was loaded in the input lanes. Data from three independent experiments are presented as mean ± SD at the bottom. (B) Contribution of DNA binding activity of TopBP1 to stimulation of ATR kinase activity by double-stranded DNA. Kinase assays were performed as in Figure 2.3, except with TopBP1 978-1192 fragment compared to full-length TopBP1 under low ionic strength reaction conditions (35 mM total salt concentration). 15 nM of TopBP1 978-1192 fragment was used to obtain a comparable level of Chk1 phosphorylation to that observed with 1 nM full-length TopBP1 (compare lanes 3 and 7). (C) Requirement of DNA binding activity of TopBP1 for stimulation of ATR kinase activity by N-Aco-AAF-damaged DNA. Kinase assays were performed with 85 mM final salt concentration as in Fig. 2.6A, except with TopBP1 978-1192 fragment (15 nM) compared to full-length TopBP1 (1 nM). For the reactions containing full-length TopBP1, 5 nM unmodified or N-Aco-AAF-damaged DNA was added. The average levels of Chk1 phosphorylation from two independent experiments are quantitated at the bottom.

required for the DNA-dependent stimulation of ATR kinase activity, we used a C-terminal TopBP1 fragment encompassing amino acids 978-1192 which is known to activate ATR kinase (Kumagai et al., 2006) but lacks DNA binding activity (Yamane and Tsuruo, 1999). In fact, the 978-1192 fragment did not bind DNA at moderate enzyme concentrations (Figure 2.10*A*, lanes 7 and 8), and only bound weakly and non-specifically at 10-fold higher concentrations (lanes 9 and 10). We conducted ATR kinase assays to compare the ability of the full-length and 978-1192 fragment of TopBP1 to stimulate ATR in the presence or absence of DNA. In low ionic strength ATR kinase reactions, both the full-length and the 978-1192 fragment of TopBP1 had similar basal ATR-stimulatory activities (Figure 2.10*B* lanes 3 and 7); significantly however, only in the presence of the full-length TopBP1 was ATR kinase activity further stimulated 3-4-fold by DNA (lanes 4 versus 8). Next, we performed ATR kinase assays with the 978-1192 fragment of TopBP1 at higher ionic strength to compare the stimulatory effect of undamaged versus *N*-Aco-AAF-damaged DNA (Figure 2.11*C*). The results reveal that damaged DNA, in agreement with data in 2.6, stimulates full-length TopBP1-activated ATR kinase about 5-fold more than undamaged DNA (lanes 3 and 4). In contrast, no or only marginal stimulation by the 978-1192 fragment of TopBP1 is observed with damaged or undamaged DNA (lanes 6-13), which supports the model that TopBP1 binding to damaged DNA is essential for optimal stimulation of the ATR kinase activity on Chk1.

Discussion

Data from this study suggest that bulky DNA lesions are recognized by TopBP1, which recruits ATR to the damage site and potentiates its kinase activity on the checkpoint signal transduction kinase, Chk1 (Figure 2.11). We believe this is the first human *in vitro* checkpoint system that recapitulates a number of key features of the checkpoint response. However, the system in its present form has certain limitations. First, there are extensive data that primer/template structures, in which the single-stranded region is covered by RPA, generated by replication blocks or by nucleotide excision repair are strong signals for checkpoint activation (Zou, 2007). However, those data and our *in vitro* findings in this paper and *in vivo* data published previously (Jiang and Sancar, 2006) are not necessarily mutually exclusive, as it is quite likely that more than one type of DNA/protein structure may constitute a signal for checkpoint activation. Second, *in vivo* data indicate that in addition to TopBP1, several other proteins such as Rad17-RFC, the 9-1-1 complex, Claspin and Timeless are required for phosphorylation of Chk1 by ATR (Sancar et al., 2004; Zou, 2007). Indeed, a recent study indicates that the Rad9 subunit of the 9-1-1 complex plays a role in recruitment of TopBP1 to chromatin and thus facilitates ATR activation by this mechanism (Delacroix et al., 2007). Clearly, establishing an *in vitro* checkpoint response dependent on all known checkpoint components including damaged DNA and checkpoint proteins is undoubtedly a challenging problem. However, we believe that the *in vitro* system we describe here constitutes an important framework for incorporation of the other known checkpoint proteins, which may be required for amplification of the checkpoint signal observed under our reaction conditions or may be essential for checkpoint signaling under different and perhaps more stringent reaction conditions, resulting in reconstitution of a bona fide human DNA damage checkpoint signaling pathway in an entirely defined *in vitro* system.

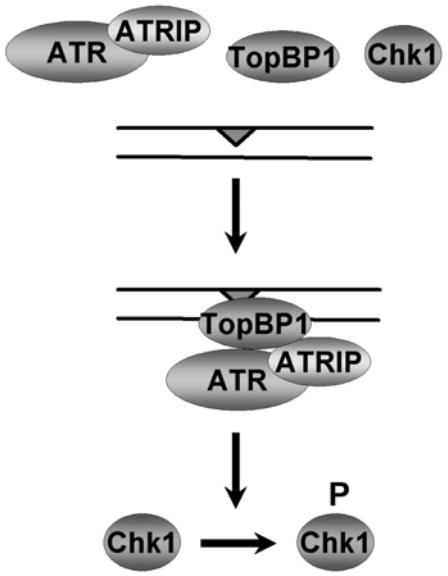

Figure 2.11. Model of TopBP1-dependent stimulation of ATR kinase activity by damaged DNA. Data from this study suggest that bulky DNA lesions are recognized by TopBP1, which recruits ATR to the damage site and potentiates its kinase activity on the checkpoint signal transduction kinase, Chk1.

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CHAPTER 3

COOPERATIVE ACTIVATION OF THE ATR CHECKPOINT KINASE BY TOPBP1 AND DAMAGED DNA

Abstract

TopBP1, acting in concert with DNA containing bulky base lesions, stimulates ATR kinase activity under physiologically relevant reaction conditions. Here we analyze the roles of the three components in ATR activation: DNA, base damage, and TopBP1. We show that base adducts caused by a potent carcinogen, benzo[a]pyrene diol epoxide (BPDE), constitute a strong signal for TopBP1-dependent ATR kinase activity on Chk1 and p53. We find that the C-terminus of TopBP1 binds preferentially to damaged DNA and is sufficient to mediate damaged DNA-dependent ATR activation in a manner similar to full-length TopBP1. Significantly, we find that stimulation of ATR by BPDE-damaged DNA exhibits strong dependence on the length of DNA, with essentially no stimulation with fragments of 0.2 kb and reaching maximum stimulation with 2 kb fragments. Moreover, TopBP1 shows preferential binding to longer DNA fragments, and in contrast to previous biochemical studies, TopBP1 binding is completely independent of DNA ends. We find that TopBP1 binds to circular and linear DNAs with comparable affinities and that these DNA forms result in the same level of TopBP1-dependent ATR activation. Taken together, these findings suggest a cooperative activation mechanism for the ATR checkpoint kinase by TopBP1 and damaged DNA.

Introduction

DNA damage checkpoints are signal transduction pathways that delay or arrest cell cycle progression in response to DNA damage or inhibition of replication. Checkpoints aid in maintaining genomic integrity and cell survival in unicellular organisms and are known or presumed to prevent genomic instability, cancer, and death in multicellular organisms. ATM and ATR are members of the phosphoinositide 3-kinase-related protein kinase (PIKK) family of protein kinases that function in the early stages of checkpoint signaling pathways. In general, the checkpoint response to double-strand breaks is initiated by ATM, whereas the checkpoint response to base adducts and inhibition of replication is induced by ATR (Abraham, 2001; Cimprich and Cortez, 2008; Sancar et al., 2004). It is commonly accepted that single-stranded DNA resulting from uncoupling of the replicative helicase and DNA polymerase because of base lesions or dNTP depletion during the S-phase (Byun et al., 2005), from processing of double strand breaks (Jazayeri et al., 2006; Myers and Cortez, 2006), or from damage removal in the form of approximately 30 nt-long oligomers in the G1 and G2 phases (Marini et al., 2006; Matsumoto et al., 2007; Stiff et al., 2008) constitutes the signal for the ATR-mediated DNA damage checkpoint response. While there is strong evidence that single-stranded DNA coated with replication protein A (RPA) is a signal for ATR activation (MacDougall et al., 2007; Zou and Elledge, 2003), there are also *in vivo* and *in vitro* data indicating that the base lesion itself acts as a signal for the ATR-mediated DNA damage checkpoint response (Choi et al., 2007; Jiang and Sancar, 2006; Unsal-Kacmaz et al., 2002).

The development of partially reconstituted checkpoint systems with purified proteins has been instrumental in obtaining mechanistic details about these important signal transduction pathways (Kumagai et al., 2006; Lee and Paull, 2004; Lee and Paull, 2005; Majka et al., 2006). Using a minimal *in vitro* system, Dunphy and coworkers made

the important discovery that the topoisomerase II binding protein 1 (TopBP1) is an essential co-activator of ATR (Kumagai et al., 2006). Subsequently, we demonstrated that under more physiologically relevant reaction conditions the TopBP1-dependent ATR kinase activity on the Chk1 signal transduction kinase was strongly stimulated by DNA, in particular DNA containing bulky base adducts induced by a model carcinogen *N*-acetoxy-2-acetylaminofluorene (*N*-Aco-AAF) (Choi et al., 2007). In the current study, we investigate whether other bulky base lesions act as checkpoint signals similarly to AAF-guanine adducts, and identify that the C-terminus of TopBP1 is sufficient for damaged DNA- and TopBP1-dependent stimulation of ATR kinase activity on Chk1 as well as p53 substrates. Finally, we present evidence that cooperative binding of TopBP1 to DNA may be essential for its function as the ATR co-activator. Collectively, our data support the view that TopBP1 is capable of recognizing unprocessed bulky DNA lesions, recruiting ATR to the damage site, and activating ATR via a cooperative mechanism.

Materials and Methods

Antibodies and purification of checkpoint proteins

Chk1 phospho-S345 (#2348) and p53 phospho-S15 antibodies (#9248) were purchased from Cell Signaling Technology (Danvers, MA) and Chk1 (sc-8408) and p53 (sc-6243) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Native ATR, which contains ATRIP but is free of other checkpoint proteins and PIKK family members, was purified from HeLa cell free extracts as previously described (Choi et al., 2007). GST-TopBP1-His, GST-TopBP1 fragments, GST-p53 and His-Chk1 kinase dead (Chk1-kd) were all purified by standard procedures as previously described (Choi et al., 2007).

Preparation of DNA substrates

For benzo[a]pyrene diol epoxide (BPDE)-damaged DNA, pUC19 plasmid (50 μg/ml) was treated with 50 μM BPDE (NCI Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas, MO) in 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA at 37ºC for 16 h in the dark. The reaction was followed by ether extraction and ethanol precipitation to remove the non-reacted excess BPDE. This treatment produces ~20 adducts/plasmid as previously determined (Gunz et al., 1996). To generate various sizes of DNA fragments, PCR was performed with pUC19 plasmid as a template.

DNA binding assays

Assays were carried out as described previously (Choi et al., 2007). Briefly, DNA substrates were 5′-end labeled with [γ-^{32}P]ATP and then mock-treated or treated with BPDE as described above. For circular DNA, labeled DNA was ligated and purified by agarose gel electrophoresis. Purified proteins (3 pmol) on glutathione beads were incubated at 37°C for 10 min with the DNA (0.6 fmol or 1 ng) in buffer B (10 mM Tris-Cl, pH 7.7, 1 mM EDTA, 0.5% NP 40) containing 0-300 mM NaCl. For DNA binding assays

in kinase reaction buffer, comparable amounts of TopBP1 and DNA to those used in kinase assays were used. After the incubation, the beads were washed three times with buffer B, and bound DNA was eluted by incubation with 0.1 μg/μl proteinase K at 37°C for 15 min. The DNA was resolved in a 0.8% agarose gel, dried, visualized by autoradiography, and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.2 software. The averages from independent experiments were graphed, and the error bars indicate the standard deviation of the mean.

Kinase assays

The procedure was essentially as previously described (Choi et al., 2007). Briefly, kinase assay reactions contained 14 mM Hepes, pH 7.9, 3 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 5% glycerol, 1% polyethylene glycol, 35 mM KCl, 50 mM NaCl, and 1 μM microcystin in a 10 μl final volume. For low-ionic strength conditions, the concentration of NaCl was reduced to 10 mM. 0.4 nM purified ATR was pre-incubated in the reaction buffer for 15 min at 30°C with the indicated amounts of recombinant full-length TopBP1 or TopBP1 fragments and with various DNA substrates where indicated. After the pre-incubation, 10 nM Chk1-kd, or p53 where indicated, was added into the reaction, incubated for 20 min at 30°C, terminated by the addition of SDS-PAGE loading buffer, and separated by SDS-PAGE. Chk1 or p53 phosphorylation was detected by immunobloting using the phospho-S345 or phospho-S15 antibody, respectively. Levels of phosphorylation were quantified using ImageQuant 5.2 software after scanning immunoblots. The highest level of Chk1 or p53 phosphorylation in each experiment was set equal to 100, and the levels phosphorylated protein in the other lanes were determined relative to this value. The averages from independent experiments were graphed, and the error bars indicate the standard deviation of the mean.

Results

DNA Binding Properties of TopBP1

We previously found that full-length TopBP1 bound with higher affinity to DNA containing AAF-guanine adducts than to undamaged DNA (Choi et al., 2007). Since it was reported that TopBP1 contains multiple DNA binding domains (Yamane and Tsuruo, 1999), we decided to determine which region of TopBP1 is responsible for the damaged-DNA binding activity. Towards this means, we generated several bacterial constructs to express full-length TopBP1 or fragments of the protein encompassing varying domains from the N- and C-termini (Figure 3.1A). TopBP1 contains 8 BRCA1 carboxyl-terminal (BRCT) motifs that are known to mediate protein-protein interactions (Garcia et al., 2005) and a region between the sixth and seventh BRCT domains that is sufficient for binding to and activation of ATR (Kumagai et al., 2006; Mordes et al., 2008a). Fragment A includes the two N-terminal BRCT domains; fragment B carries the ATR-activating domain but lacks a full BRCT motif; fragment C contains the ATR-activating domain as well as the two C-terminal BRCT motifs; fragment D has the N-terminal 6 BRCT motifs but lacks the ATR-interacting domain. All of these constructs were purified by GST affinity chromatography and were of high purity with the exception of the full-length protein and the large D fragment, both of which contain some degradation or premature termination products (Figure 3.1B).

We performed DNA pull-down assays to investigate the effect of DNA damage on binding by full-length TopBP1 and the fragments. As seen in Figure 3.1C, full-length TopBP1 (lanes 3 and 4), the C-terminal fragment carrying the ATR-activating domain and the last two BRCT motifs (fragment C) (lanes 9 and 10), and the N-terminal fragment of the protein (fragment D) containing the first 6 BRCT motifs (lanes 11 and 12) bind to damaged DNA preferentially, with the full-length protein exhibiting the highest affinity. In contrast, the N-terminal fragment carrying the first two BRCT motifs (fragment

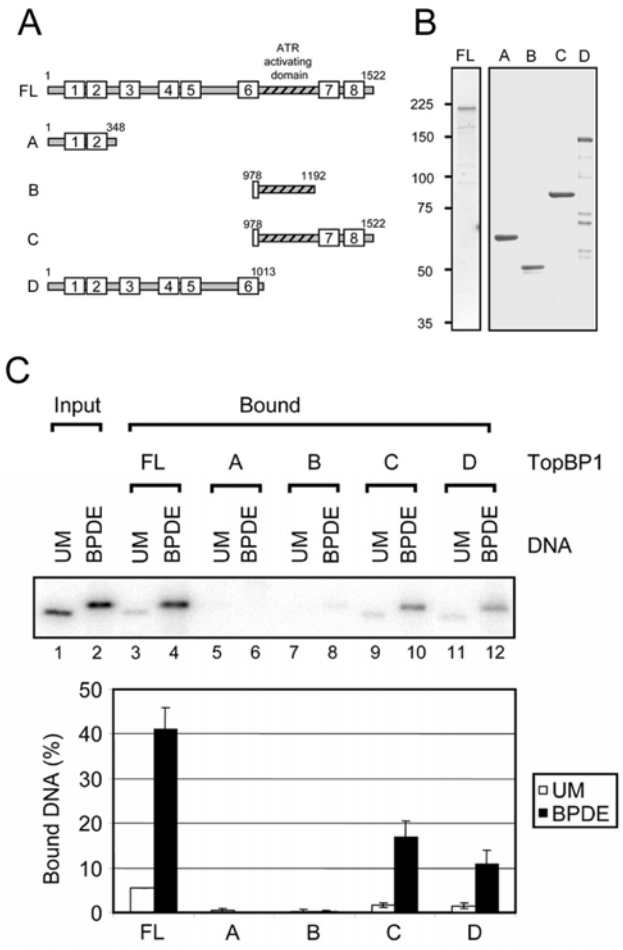

Figure 3.1. TopBP1 fragments used in this study and their DNA binding properties. (A) Schematic of human TopBP1 and its fragments that were purified for structure-function experiments. The amino acid positions are indicated, and the 8 boxes indicate the BRCT motifs. The ATR activating domain between BRCT domains 6 and 7 is indicated. (B) The GST-fusion proteins visualized by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining. (C) Preferential binding of TopBP1 and TopBP1 fragments to BPDE-damaged DNA. (Top panel) Unmodified (UM) or BPDE treated pUC19 plasmid DNAs (1 ng) which had been labeled with [γ-^{32}P]ATP were incubated with 3 pmol of full-length TopBP1 (FL) bound beads or beads carrying 3 pmol of the TopBP1-A, -B, -C, or -D fragments in buffer containing 200 mM NaCl. The bound DNA was eluted by proteinase K, analyzed by agarose gel electrophoresis, and visualized by autoradiography. The input lanes contain fifty percent of DNA added to the reaction. The results from two experiments were quantified and plotted.

A), which was previously shown to have DNA binding activity (Yamane and Tsuruo, 1999), fails to exhibit measurable binding to either undamaged or damaged DNA under our experimental conditions (lanes 5 and 6). Fragment B, containing the activation domain but no BRCT motifs, also fails to bind undamaged or BPDE-damaged DNA (lanes 7 and 8), which is in agreement with our previous results with *N*-Aco-AAF-damaged DNA (Choi et al., 2007). Thus, we conclude that the two DNA-binding domains located in the N- and C-terminal halves of TopBP1 contribute to the preferential binding of this protein to DNA containing bulky base lesions such as BPDE-guanine adducts. We then proceeded to analyze the effect of BPDE damage on ATR activation mediated by full-length TopBP1 and the TopBP1 fragments.

Stimulation of the ATR Kinase by TopBP1 Fragments

It is known that in buffers of low ionic strength, both full-length TopBP1 and the ATR-activating domain of TopBP1 can activate the ATR kinase in the absence of DNA (Choi et al., 2007; Kumagai et al., 2006). We directly compared the ATR-stimulatory activities of the TopBP1 fragments under low ionic strength reaction conditions, and the results are shown in Figure 3.2A. We find that full-length TopBP1 is the most efficient in stimulating the ATR kinase (lanes 2-5), and that fragments B and C (lanes 6-9 and lanes 10-13, respectively) which carry the ATR-activating domain are also capable of stimulating ATR, albeit at less than 10% of the efficiency of the full-length protein. The D fragment, which does not contain the ATR-activating domain, fails to stimulate ATR (lanes 14-17).

We previously reported that under conditions of high ionic strength, TopBP1 failed to stimulate ATR, and that some stimulation was observed only when DNA was included in the reaction mixture and that the strongest stimulation was seen when DNA was damaged by *N*-Aco-AAF (Choi et al., 2007). To gain even deeper insight into the role of damaged DNA binding by TopBP1 in ATR activation, we tested the fragments of

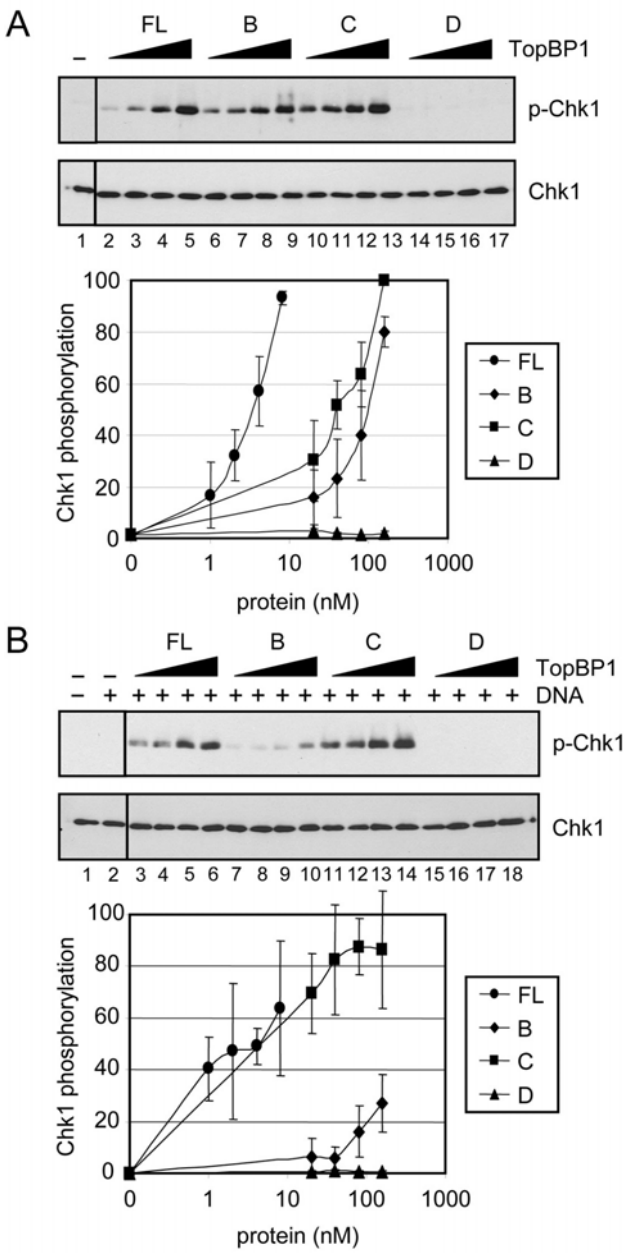

Figure 3.2. Stimulation of the ATR kinase by TopBP1 and its fragments. (A) TopBP1 fragments containing the ATR activating domain stimulate ATR in the absence of DNA. ATR (0.4 nM) was incubated with Chk1 (10 nM) in the presence of full-length TopBP1 (1–8 nM) or the indicated TopBP1 fragments (20–160nM) under low ionic strength conditions (45 mM total salt concentration). ATR kinase activity was determined by immunoblotting for phospho-Chk1 (S345) and Chk1 as indicated. The graph shows quantitative analysis of the data from three independent experiments. (B) DNA stimulates the kinase activity of ATR in the presence of full-length TopBP1 or the C-terminal fragment. Kinase assays were performed as described in Figure 3.2A, except with 5 ng of BPDE-damaged DNA under high ionic strength conditions (85 mM total salt concentration). The average levels of Chk1 phosphorylation from three independent experiments were quantitated and graphed.

TopBP1 for their abilities to stimulate ATR in the presence of BPDE-damaged DNA. As shown in Figure 3.2B, in addition to full-length TopBP1 (lanes 3-6), the C fragment (lanes 11-14), which has DNA binding activity as well as the activation domain, also efficiently stimulates ATR in the presence of BPDE-damaged DNA. DNA slightly stimulates the ATR kinase in the presence of high concentrations of the B fragment (lanes 7-10), which carries the activation domain but lacks DNA binding activity. DNA has no effect on the ATR kinase in the presence of the D fragment (lanes 15-18), which binds DNA but lacks the activation domain. These data support our previous model that the formation of a damaged DNA-TopBP1-ATR ternary complex is essential for ATR stimulation by damaged DNA *in vitro* (Choi et al., 2007).

Since the results in Figure 3.2B demonstrate that the C-terminal one-third of TopBP1 is sufficient for mediating damaged DNA-dependent stimulation of the ATR kinase, we wished to determine whether the observed stimulation by the C fragment of TopBP1 is indeed damaged-DNA specific, as was previously reported with full-length TopBP1 (Choi et al., 2007). For this purpose, we tested increasing amounts of either unmodified or BPDE-damaged DNA in ATR kinase reactions containing full-length TopBP1 or the C fragment (Figure 3.3). In the presence of full-length TopBP1, BPDE-damaged DNA stimulates the ATR kinase ~5 fold more than unmodified DNA (lanes 7-10 vs lanes 3-6), which is very consistent with what we previously reported for DNA damaged with *N*-Aco-AAF (Choi et al., 2007). Importantly, in the presence of the C fragment of TopBP1, BPDE-damaged DNA stimulates the ATR kinase ~9 fold more than unmodified DNA (lanes 17-20 vs lanes 13-16). Together, we conclude that like full-length TopBP1, the C fragment preferentially binds to DNA containing bulky base lesions and specifically stimulates ATR kinase activity. Therefore, the C-terminal one-third of TopBP1 is sufficient for damaged DNA-dependent ATR activation.

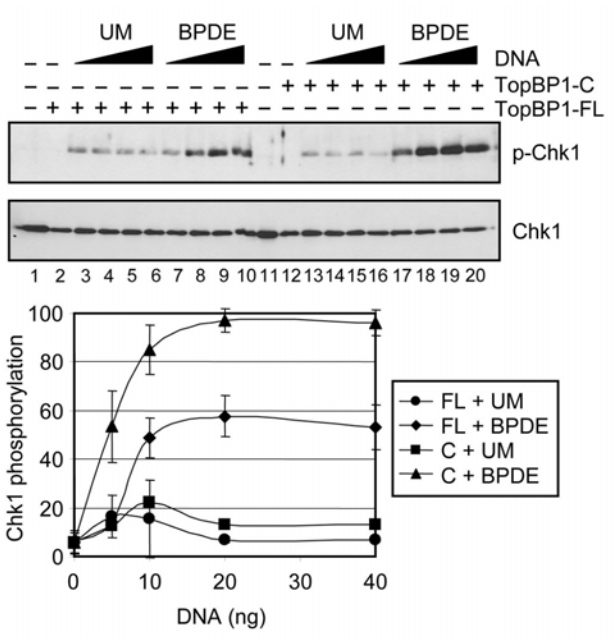

Figure 3.3. TopBP1 C-terminal fragment stimulates the ATR kinase in a manner dependent on the presence of damaged DNA. Kinase assays were carried out with ATR (0.4 nM), Chk1 (10 nM), and unmodified (UM) or BPDE-damaged DNA (5-40 ng) in the presence of full-length TopBP1 (0.5 nM) or TopBP1 C-fragment (10 nM) under high ionic strength conditions. The average levels of Chk1 phosphorylation from three independent experiments were quantitated and graphed.

We next examined whether the TopBP1- and DNA-dependent activation of ATR is specific to the Chk1 substrate or whether it is a general mechanism applicable to other known ATR substrates as well. Therefore, we tested another key checkpoint protein and ATR substrate, p53, in our assay, and the results are shown in Figure 3.4A. We observe ~5-fold more phosphorylation of serine 15 of p53 in the presence of DNA (lane 4) than in the absence (lane 3), which is comparable to the level of DNA-dependent stimulation of Chk1 phosphorylation (lanes 1 and 2). As with the Chk1 substrate, the DNA-dependent p53 phosphorylation by ATR occurs in the presence of either full-length or the C-fragment of TopBP1 (Figure 3.4A and B). More importantly, BPDE-damaged DNA specifically stimulates TopBP1-dependent ATR kinase activity ~4 fold more than undamaged DNA (Figure 3.4B lanes 2 vs 3). Therefore, the TopBP1- and damaged DNA-dependent stimulation of ATR is not specific to the Chk1 substrate.

Cooperativity in ATR Activation

It was recently shown that the MRN (Mre11-Rad50-Nbs1)-mediated cooperative binding of ATM to DNA greatly stimulated the ATM kinase activity (You et al., 2007). To determine whether ATR exhibited cooperativity similarly to ATM, BPDE-damaged DNAs ranging in size from 0.2 to 2.6 kb were tested at identical DNA mass (and therefore adduct) concentrations for activation ATR in the presence of TopBP1. The results are shown in Figure 3.5A. Fragments of 0.2 kb had no effect on TopBP1-dependent stimulation of the ATR kinase (lanes 18-20), whereas, at longer DNA sizes, the stimulation increased essentially uniformly, approaching maximum at the 2-2.6 kb range (lanes 3-11). It is important to note that the number of DNA ends in the reaction does not contribute to the DNA length-dependent stimulation of ATR, as there is no difference in the ATR kinase stimulation induced by linear and circular DNAs (lanes 3-8 and Figure 3.6B), and the length effect is also observed with equal molar quantities of DNA (data not shown). We have also observed similar DNA length-dependent stimulation of ATR

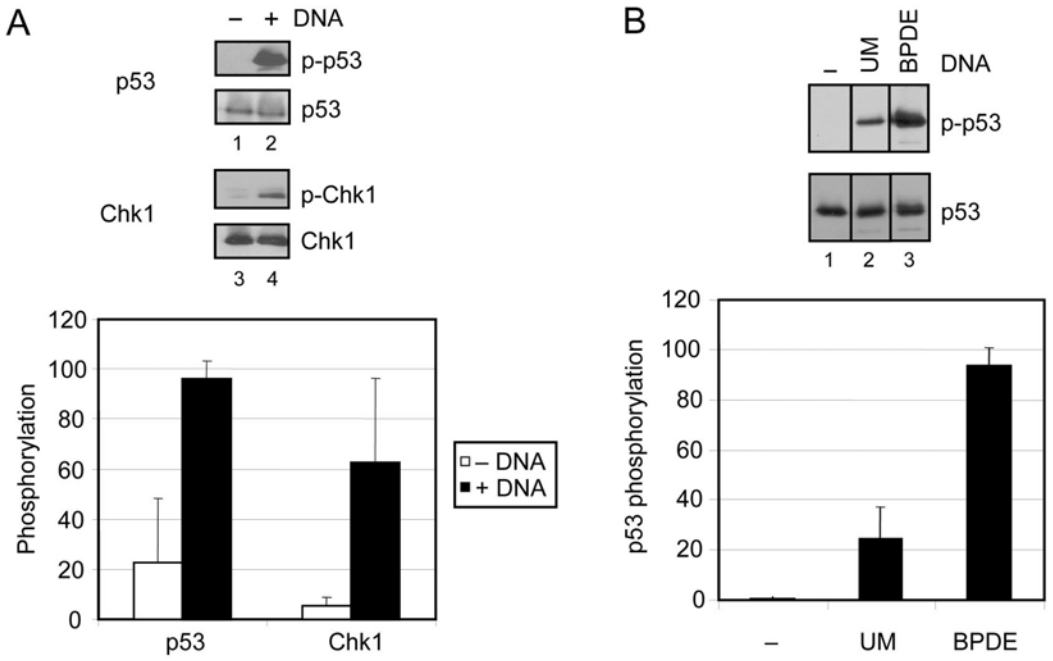

Figure 3.4. Damaged DNA –dependent stimulation of ATR is independent of the substrate. (A) Addition of DNA stimulates the ATR kinase activity toward another key downstream target, p53 in the presence of TopBP1. Kinase assays were carried out with ATR and TopBP1-C fragment in the absence or presence of 5 ng of unmodified circular DNA in reactions containing 5 nM Chk1 (lane 1 and 2) or 5 nM p53 (lane 3 and 4) as described in Figure 3.3. The average levels of Chk1 and p53 phosphorylation from three independent experiments were quantitated and graphed. (B) Phosphorylation of p53 is strongly stimulated by BPDE-damaged DNA. Kinase assays were performed with ATR, p53, and 5ng of unmodified (UM) (lane 2) or BPDE-damaged DNA (BPDE) (lane 3) in the presence of full-length TopBP1 as described in Figure 3.3. The average levels of p53 phosphorylation from two independent experiments were quantitated and graphed.

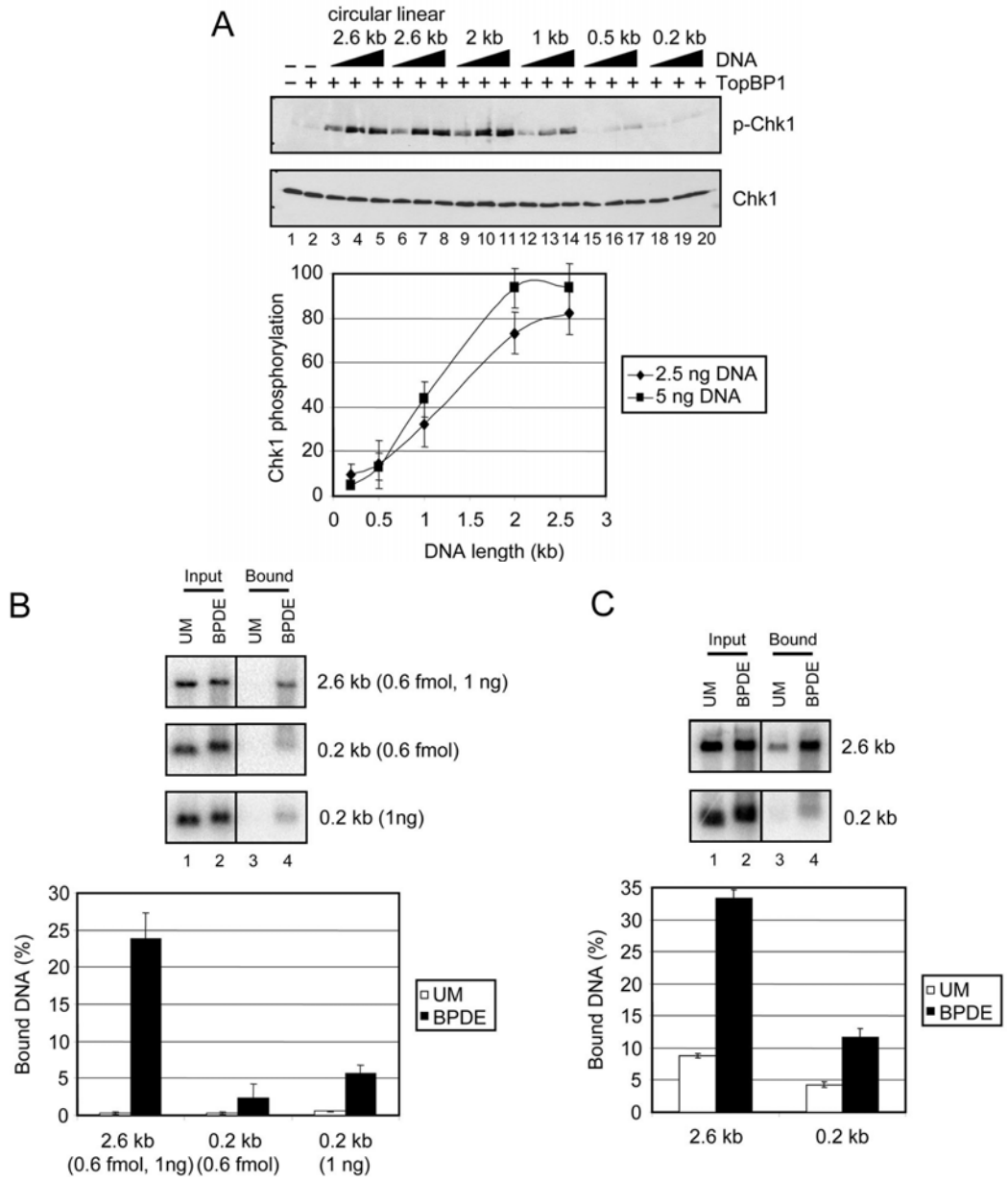

Figure 3.5. Efficient ATR activation and DNA binding by TopBP1 depend on the length of DNA. (A) TopBP1-dependent ATR stimulation increases with DNA length. Kinase assays were performed as described in Figure 3.3, except with different length of DNAs ranging from 0.2 to 2.6 kb. The average levels of Chk1 phosphorylation from three independent experiments were quantitated and graphed. (B) TopBP1 preferentially binds to longer DNA. DNA binding assays were carried out as described in Figure 3.1C, except with equal molar or mass amounts of 0.2 or 2.6 kb DNA fragments in buffer containing 300 mM NaCl. The results from two experiments were quantified and plotted. (C) TopBP1 preferentially binds longer DNAs under kinase reaction conditions. DNA binding assays were performed with 0.15 pmol of TopBP1 and 3.5 fmol of 0.2 or 2.6 kb DNA fragments under conditions used for kinase reactions. The results from two experiments were quantitated and graphed.

in the presence of TopBP1-C fragment and with undamaged DNA (data not shown). Thus, it appears that, as in the case of ATM, cooperative binding of ATR to DNA is required for activation of the kinase activity of this PIKK member.

To address whether TopBP1 exhibits cooperative DNA binding properties, we performed DNA pull-down assays with different size fragments of DNA. The results are shown in Figure 3.5B and C. We observe preferential binding of TopBP1 to longer BPDE-modified DNA fragments under conditions with either equal DNA mass or molar quantities of the two DNA fragments (Figure 3.5B, lane 4). Under these experimental conditions, we observe negligible binding to unmodified DNAs of either length (lane 3). Because these DNA binding experiments were carried out under conditions different from the kinase reaction conditions, we then repeated the DNA binding experiment under kinase reaction buffer conditions, and we obtained similar results (Figure 3.5C). There was significantly more binding of TopBP1 to the longer DNA fragments whether the DNAs were unmodified (lane 3) or BPDE-modified (lane 4). Therefore, we conclude that TopBP1 preferentially binds to longer DNAs which results in cooperative recruitment and activation of ATR.

Independence of DNA Ends on the DNA Binding and ATR Stimulatory Activities of TopBP1

It was previously reported that TopBP1 binds preferentially to DNA duplex ends and nicks *in vitro* indicating the TopBP1 may have an important role in recognition of DNA breaks (Yamane and Tsuruo, 1999). However, we observed no preferential binding to DNA ends under our experimental conditions (Figure 3.5B). Since our results seem to be contradictory to the previous report, we wished to directly address whether TopBP1 has different binding affinities for circular versus linear DNA. Therefore, we performed DNA pull-down assays according to the previously described procedures with the A and C fragments which are equivalent to the TopBP1 fragments used in the report

(Yamane and Tsuruo, 1999). The results are shown in Figure 3.6A. The C-terminal fragment (lanes 7 and 8) binds about 10-fold more efficiently to DNA than the N-terminal fragment (lanes 5 and 6); and importantly, there are no significant differences between the affinities of either fragment to linear, nicked or circular DNA, leading us to conclude that TopBP1 has no preference for DNA ends. It is unclear why our results differ from the previous report showing similar DNA binding affinities for the two equivalent TopBP1 fragments as well as an absolute dependence on DNA ends for binding (Yamane and Tsuruo, 1999). However, we have also conducted independent experiments using electromobility shift assays which also confirm our results (data not shown).

Since we did not observe a noticeable difference in the binding affinity of TopBP1 to circular or linear DNA in the DNA pull-down assays, we used the kinase assay to probe for a difference in the ability of these two forms of DNA to stimulate TopBP1-dependent ATR kinase activity. Figure 3.6B shows that circular (lanes 3-8) and linear (lanes 9-14) DNAs, which were either mock- or BPDE-treated, were nearly identical in their ability to stimulate ATR kinase activity in the presence of TopBP1. The presence of unmodified DNA (lanes 3-5 and lanes 9-11) in the kinase reaction resulted in more than 10-fold stimulation of ATR activity relative to no DNA (lane 2), and the addition of BPDE-modified DNA (lanes 6-8 and lanes 12-14) resulted in ~8-fold further stimulation over unmodified DNA, independent of whether the DNAs were linear or circular. We conclude from these results that in contrast to the MRN-mediated cooperative activation of ATM which depends on free DNA ends, DNA termini are not required for TopBP1 DNA binding or DNA-dependent cooperative activation of ATR.

Discussion

In this study we demonstrate that TopBP1 has two DNA binding sites and binds DNA with no preference for DNA termini. Moreover, we find that both sites aid in preferential binding of TopBP1 to DNA damaged by BPDE. In addition, we demonstrate

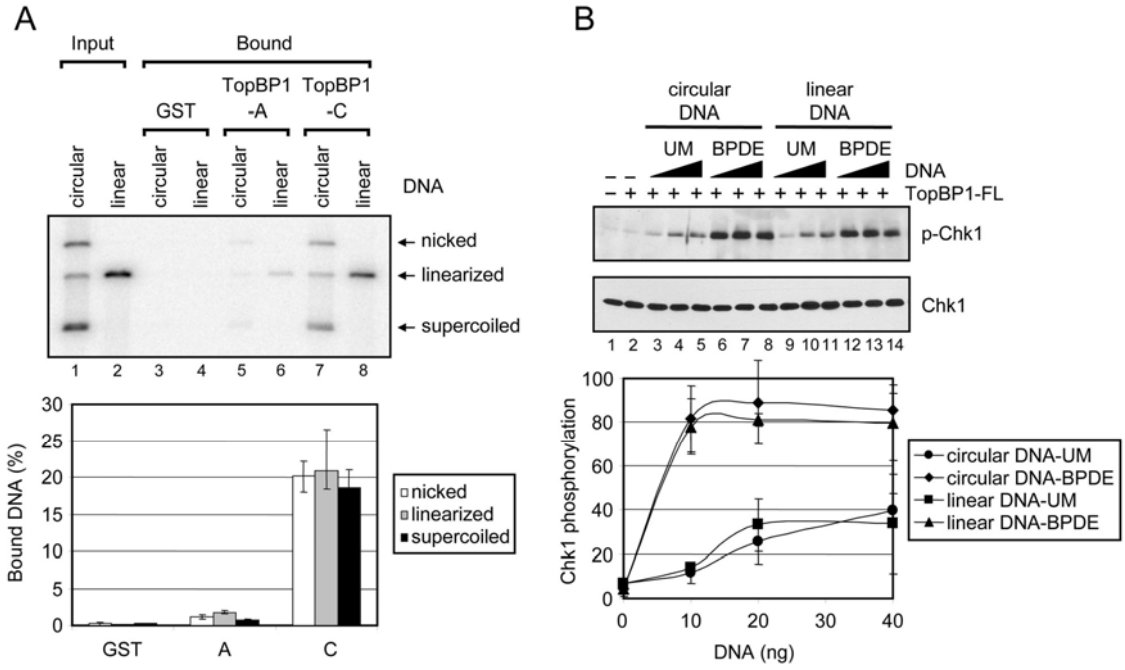

Figure 3.6. DNA binding and ATR stimulatory activities of TopBP1 are independent of DNA ends. (A) TopBP1 has no preferential binding to DNA ends. Covalently closed circular and linear DNA that had been labeled with [γ-^{32}P]ATP were incubated with GST alone, the TopBP1-A, or TopBP1-C fragment immobilized on glutathione beads. After extensive washing, the bound DNA was eluted and analyzed on an agarose gel containing ethidium bromide. The gel was dried and visualized by autoradiography. Note that the 'covalently closed DNA' sample contains covalently closed, nicked, and linear DNAs and hence gives a fair representation of the relative affinities of these three forms to the TopBP1 fragments. The input lanes contain fifty percent of DNA added to the reaction. The results from five experiments were quantified and plotted. (B) DNA double-strand breaks has no effect on TopBP1-dependent ATR activation. Kinase assays were carried out with ATR, Chk1, and TopBP1 in the presence of unmodified (UM) or BPDE-damaged circular or linear DNA as described in Figure 3.3. The average levels of Chk1 phosphorylation from three independent experiments were quantitated and graphed.

that while under conditions of low ionic strength any TopBP1 fragment that carries the ATR binding domain (Kumagai et al., 2006; Mordes et al., 2008a) is sufficient for ATR activation, only the C-terminal fragment that contains the ATR-activating domain together with a DNA binding domain can stimulate the ATR kinase in a manner dependent on the presence of BPDE-damaged DNA under physiologically relevant ionic strength. Interestingly, whereas the N-terminal half of TopBP1 is conserved through evolution from yeast to humans and plays an essential role in replication initiation, the C-terminal half of TopBP1, containing the ATR-activation domain, is only conserved in metazoans and is essential for checkpoint activation (Hashimoto et al., 2006; Yan et al., 2006). In fact, the C-terminal half of TopBP1 is sufficient for Chk1 phosphorylation induced by oligonucleotides in *Xenopus* egg extracts (Hashimoto et al., 2006). The budding yeast TopBP1 homolog, Dpb11, while lacking sequence homology to the ATR-activation domain, is still able to activate the $Mec1^{ATR}$ kinase in an *in vitro* system (Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008a). However, the yeast $Dpb11^{TopBP1}$ also lacks the C-terminal region that we have identified to be important in the human protein for mediating DNA-dependent ATR activation, and does not support DNA-dependent $Mec1^{ATR}$ activation (Navadgi-Patil and Burgers, 2008a). While results from our reconstituted checkpoint system indicate that the C-terminus of TopBP1 is sufficient for direct binding to damaged DNA and activation of ATR kinase activity, there are recent reports that the N-terminus of TopBP1 is required for its recruitment and resulting activation of ATR via an interaction with Rad9 in mammalian cell lines (Delacroix et al., 2007) and *Xenopus* egg extracts (Lee et al., 2007). Work is underway to develop an *in vitro* system that depends on Rad9 and the other factors identified genetically for optimal activation of the ATR-mediated checkpoint response.

An unexpected finding of this study has been the DNA length-dependence of TopBP1 stimulatory activity. Although a similar length dependence was reported for

ATM autophosphorylation, in that case there was an absolute requirement for DNA ends for cooperative binding and autophosphorylation of ATM (You et al., 2007). In contrast, in the case of ATR there is no requirement for DNA termini. It also must be noted that the length effect on ATM activation was ascribed to chromatinization of the DNA added to the egg extract, the minimum requirement for DNA of 0.2 kb coinciding with the DNA length required for efficient formation of nucleosomes, which are presumed to be required for recruitment of ATM to DNA flanking double-strand breaks and subsequent activation (You et al., 2007). Clearly, this is not the case for ATR in our system in which purified proteins are used in the checkpoint reconstitution. However, studies on ATM activation using purified proteins also demonstrated a very similar DNA length dependence for ATM activation (Lee and Paull, 2005). In that report, fragments of 0.384 kb had a minimal effect on MRN-dependent stimulation of ATM kinase, and the stimulation increased essentially uniformly with longer DNA sizes, with the maximum at 2.3 kb. Therefore, it appears that both ATM and ATR share similar DNA length-dependence of MRN and TopBP1 stimulatory activity, respectively. It should also be noted in the case of ATM, that by increasing the DNA concentration the ATM-stimulatory activity of a 0.2 kb fragment approached that achieved by a 2 kb fragment (You et al., 2007), whereas in the case of ATR, increasing the amount of the 0.2 kb fragment did not affect the outcome. This is in line with the argument that the length-dependent cooperative effects of DNA with double-strand breaks, in the case of ATM, and DNA with base damage, in the case of ATR, result from different mechanisms.

We use the word cooperativity as an operational definition, not in the strict mechanistic sense, because at present, we do not have a mechanistic model for the DNA-induced cooperativity of the ATR kinase. Although ATM and ATR have distinctly different modes of damage sensing and activation, our *in vitro* findings regarding the cooperative activation of ATR cooperativity of the ATR kinase are in line with the recent

report that the lac operator/lac repressor-mediated binding of budding yeast Ddc2^{ATRIP} (and therefore Mec1^{ATR}) to DNA exhibits a similar type of cooperativity for Mec1^{ATR} kinase activation *in vivo* (Bonilla et al., 2008). It was found that at least 40 repressor/operator complexes with the repressor-fused Ddc2^{ATRIP} were required for significant Mec1^{ATR} activation as measured by Rad53$^{Chk1/2}$ phosphorylation. There is also evidence for cooperative activation of ATR *in vitro* in *Xenopus* egg extracts where Chk1 phosphorylation is dependent on the size of the single stranded DNA gap (MacDougall et al., 2007).

While in wild-type human and yeast cells, in addition to ATR (Mec1), the 9-1-1 complex and TopBP1 (Dpb11) are required to act coordinately to initiate the checkpoint response after DNA damage, the requirement for the 9-1-1 complex, TopBP1, or DNA damage can be circumvented under special reaction conditions *in vitro* (Choi et al., 2007; Kumagai et al., 2006; Majka et al., 2006; Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008a), by overexpressing the TopBP1 ATR activating domain *in vivo* (Kumagai et al., 2006), or by artificially tethering these checkpoint proteins to the DNA *in vivo* (Bonilla et al., 2008; Delacroix et al., 2007). Hence, we believe that our *in vitro* system in which damaged DNA-bound TopBP1 recruits ATR and activates its kinase function is a reasonable approximation to ATR activation *in vivo,* and provides a useful platform for mechanistic studies of the ATR-mediated DNA damage checkpoint.

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CHAPTER 4

THE EFFECT OF RPA-COATED SINGLE-STRANDED DNA ON TOPBP1-DEPENDENT ATR ACTIVATION *IN VITRO*

Abstract

DNA damage caused by endogenous and exogenous assaults threatens the maintenance of genomic integrity. In order to prevent the catastrophic consequences of DNA damage, eukaryotic cells activate the DNA damage checkpoint response which delays cell cycle progression until damage is repaired. Although extensive data indicate that RPA-coated ssDNA is an important component in the ATR-mediated checkpoint response, so far there is no human *in vitro* system where the effect of RPA-coated ssDNA can be observed. Here we show that RPA significantly stimulates TopBP1-dependent ATR activation in our *in vitro* checkpoint system. The observed stimulation depends on the presence of ssDNA, and the stimulatory effect of RPA is specific for ssDNA. Interestingly, we find that alternative RPA, which does not support DNA replication *in vitro*, functions in a manner very similar to canonical RPA for TopBP1-dependent ATR activation. However, other ssDNA-binding proteins, such as human SSB1 and *E. coli* SSB, have only marginal effects on ATR activity under identical reaction conditions. Taken together, the data suggest that the stimulatory effect of RPA-coated ssDNA on TopBP1- dependent ATR activation can be observed in a well-defined *in vitro* system.

Introduction

Upon DNA damage, cells activate the DNA damage checkpoint response which delays cell cycle progression (Sancar et al., 2004). This signal transduction pathway is essential to preventing genomic instability which results in cancer development. ATR is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs) and a major ragulator of the checkpoint response to incompletely replicated DNA and various forms of damaged DNA, including UV-induced DNA damage (Abraham, 2001). A central question in the ATR-mediated checkpoint response is how ATR is recruited to sites of DNA damage.

RPA (Replication Protein A) is a trimeric complex composed of 70 kDa, 32 kDa, and 14 kDa subunits. RPA is highly conserved in all eukaryotic cells and is essential for many aspects of DNA metabolism, including DNA replication, repair, recombination, and DNA damage checkopoints (Wold, 1997). RPA interacts with ssDNA in the cell and interacts with numerous proteins involved in these processes (Iftode et al., 1999; Wold, 1997). It has been reported that RPA is sufficient to recruit ATR to ssDNA, and RPA-coated ssDNA can be a signal for ATR activation (You et al., 2002). Therefore, it was proposed that RPA-coated ssDNA interacts with ATRIP and recruits the ATR-ATRIP complex (Zou and Elledge, 2003). However, ATR phosphorylates Chk1 without ATRIP binding to RPA, suggesting that RPA-coated ssDNA is not essential for ATR activation (Ball et al., 2005; Kumagai et al., 2006). It seems likely that additional components might be also required to induce robust ATR activation (Byun et al., 2005; MacDougall et al., 2007; Michael et al., 2000; Stokes et al., 2002; Unsal-Kacmaz et al., 2002). More importantly, recent observations from our lab have shown that primary DNA lesions induced by *N*-Aco-AAF and UV light can be a signal for checkpoint activation *in vivo* and *in vitro*, and checkpoint proteins bind to these lesions directly (Choi et al., 2007; Jiang and Sancar, 2006; Unsal-Kacmaz et al., 2002).

Although there are still some conflicting data, it is clear that RPA-coated ssDNA is an important component of the checkpoint signal (Zou, 2007). In this study we investigate whether RPA stimulates TopBP1-dependent ATR activation in the presence of ssDNA in our reconstituted checkpoint system.

Materials and Methods

Antibodies and preparation of checkpoint components

Chk1 phospho-S345 and p53 phospho-S15 antibodies were purchased from Cell Signaling Technology (Danvers, MA) and Chk1 and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Native ATR-ATRIP, GST-TopBP1-His, GST-TopBP1 fragments, and His-Chk1 kinase dead (Chk1-kd) were all purified as previously described (Choi et al., 2007). RPA was purified as described (Henricksen et al., 1994), and alternative RPA was obtained from M. S. Wold (University of Iowa, Iowa City, IA). Human SSB1 was purified as described (Richard et al., 2008), and *E. coli* SSB1 was obtained from J. D. Griffith (University of North Carolina, Chapel Hill, NC). Single- and double-stranded forms of PhiX174 DNA were purchased from New England Biolabs (Ipswich, MA).

Kinase assays

The procedure was slightly modified from the previously described assay (Choi et al., 2007; Choi et al., 2009). Briefly, kinase assay reactions contain 15 mM Hepes, pH 7.9, 35 mM KCl, 10 mM NaCl, 3 mM $MgCl_2$, 1 μM microcystin, 1 mM ATP, 0.5 mM DTT, 5% glycerol, 1% polyethylene glycol (6000) in 10 μl final volumes. For binding of RPA, the indicated amount of DNA substrates were pre-incubated with RPA (4-100 nM) for 10 min at 30°C. After preincubation, 0.2 nM ATR-ATRIP, 5 nM GST-TopBP1-His, and 10 nM His-Chk1-kd were added into the reactions and incubated for 20 min at 30°C. The reactions were then terminated by the addition of SDS-PAGE loading buffer, and separated by SDS-PAGE. Chk1 phosphorylation was detected by immunobloting using phospho-S345 antibodies. Levels of Chk1 phosphorylation were quantified using ImageQuant 5.2 software after scanning immunoblots. The highest level of Chk1 in each experiment was set equal to 100, and the levels of phosphorylated protein in the other lanes were expressed relative to this value.

Results

Effect of RPA on TopBP1-dependent ATR activation in the presence of ssDNA

We previously observed slight stimulation by RPA in the presence of primed ssDNA in our checkpoint system containing the ATR-ATRIP kinase, the TopBP1 activator, and the Chk1 substrate (Choi et al., 2007). We reasoned that this marginal stimulation might be enhanced under more optimized reactions. Thus, we have improved our kinase assay to enhance the previously observed RPA effects on ATR activation, optimizing reaction conditions (e.g. concentrations of proteins and DNA, and ionic strength of reaction buffer). We have now established conditions where we clearly observe significant stimulation of TopBP1-dependent ATR activation in the presence of ssDNA upon addition of RPA (Figure 4.1). ssDNA stimulates ATR kinase in the presence of TopBP1 to some extent as expected from our previous work, and this stimulation was greatly enhanced by the addition of RPA. RPA-coated ssDNA stimulates the ATR kinase 10-fold more than the level of DNA alone. The observed stimulation was dependent on the presence of DNA since no or only marginal effect was seen in the absence of DNA. Next, we asked whether the observed effect of RPA is specific to ssDNA or not. To address this question, we carried out ATR kinase assays with three different forms of DNA, including ssDNA, nicked dsDNA, and supercoiled dsDNA. Interestingly, while RPA greatly stimulates ATR kinase activity in the reactions with ssDNA, there was only slight stimulation in the presence of the two forms of dsDNA, which even in the absence of RPA strongly stimulate TopBP1-dependent ATR kinase activity (Figure 4.2).

Effects of various ssDNA-binding proteins on ATR activation

An alternative form of RPA (aRPA), in which the RPA 32 subunit is replaced by the product of the RPA4 gene, was identified in some human tissues (Keshav et al., 1995). Recent work has shown that alternative RPA and canonical RPA seem to have

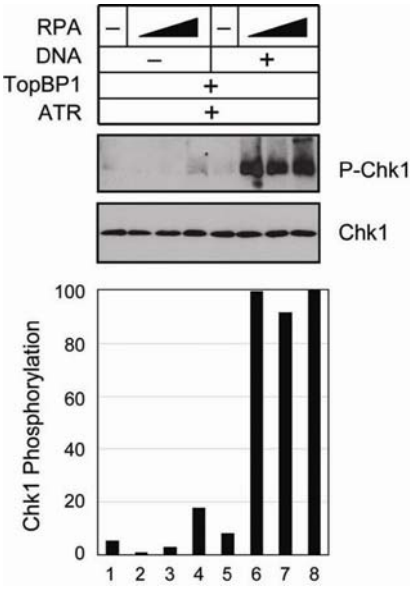

Figure 4.1. Effects of RPA on TopBP1-dependent activation of ATR kinase activity in the presence of ssDNA. Kinase assays were performed with 0.2 nM ATR-ATRIP, 5 nM GST-TopBP1-His, 10 nM His-Chk1-kd, 20-100 nM RPA, and 0.07 nM single stranded phiX174 DNA. RPA was pre-incubated with ssDNA for 10 min on ice. ATR-ATRIP, GST-TopBP1-His, and His-Chk1-kd were then added to the reaction and incubated for 20 min at 30°C. Reactions were analyzed by immunoblotting for phospho-Chk1 (S345) and Chk1 (top panel). The levels of Chk1 phosphorylation were quantified and graphed (bottom panel).

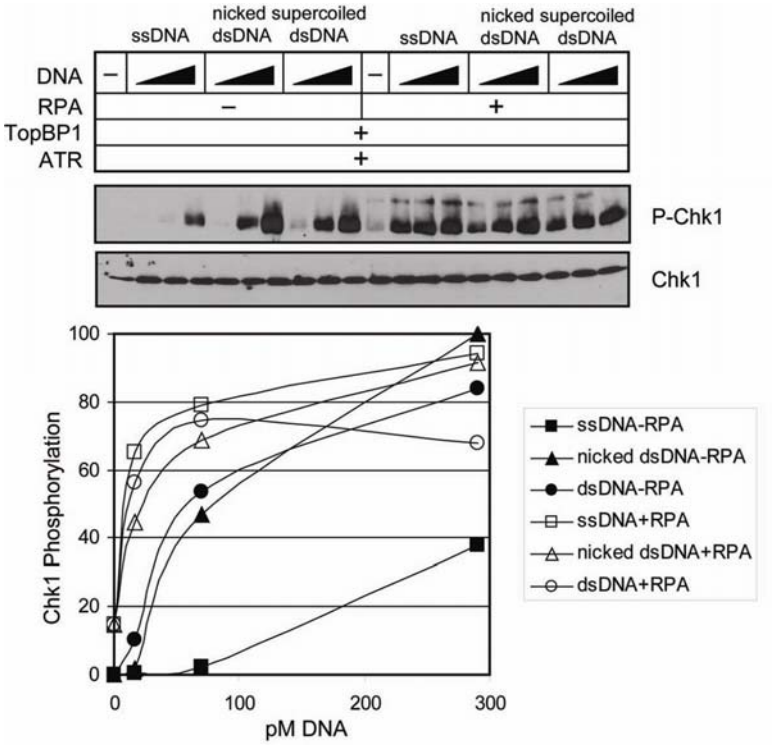

Figure 4.2. Effects of RPA on stimulation of ATR kinase activity by various DNA substrates in the presence of TopBP1. Kinase assays were performed as described in Figure 4.1, except 11 nM RPA and 17-290 pM plasmid DNA were used. The graph shows quantitative analysis of the data.

similar biochemical properties, but different functions in DNA replication (Mason et al., 2009). Although aRPA binds ssDNA with high affinity similar to canonical RPA, it is unable to support DNA replication *in vitro* and moreover, when overproduced, inhibits DNA replication and cell proliferation *in vivo*. (Mason et al., 2009). Therefore, we wished to determine whether aRPA has a function in the DNA damage checkpoint response as shown with canonical RPA. To this end, we tested the effect of aRPA in the ATR kinase reactions containing ssDNA. As shown in Figure 4.3, aRPA also significantly stimulates TopBP1-dependent ATR activation in the presence of ssDNA. Interestingly, the stimulation levels of aRPA are closely comparable to those of canonical RPA, suggesting aRPA substitutes the role of canonical RPA in our checkpoint system.

The results in Figure 4.3 demonstrate that both RPA complexes, which preferentially bind to ssDNA, are capable of stimulating ssDNA-mediated ATR activation *in vitro*. To ascertain that the observed effect was specific to canonical and alternative RPAs and not the result of a non-specific effect of any DNA binding protein, we tested other ssDNA-binding proteins, including human SSB1 and *E. coli* SSB, under identical reaction conditions since both proteins are known to specifically bind ssDNA (Meyer and Laine, 1990; Richard et al., 2008). As shown in Figure 4.4, while we consistently observed significant stimulation of ATR kinase by either RPA or aRPA, there was only a marginal effect of either human SSB1 or *E. coli* SSB on TopBP1-dependent ATR activation. Therefore, we conclude that the observed stimulatory effects of RPA and aRPA are specific features of these proteins, indicating that alternative RPA, in contrast to its inability to substitute for RPA in DNA replication, can substitute for canonical RPA in the DNA damage checkpoint response.

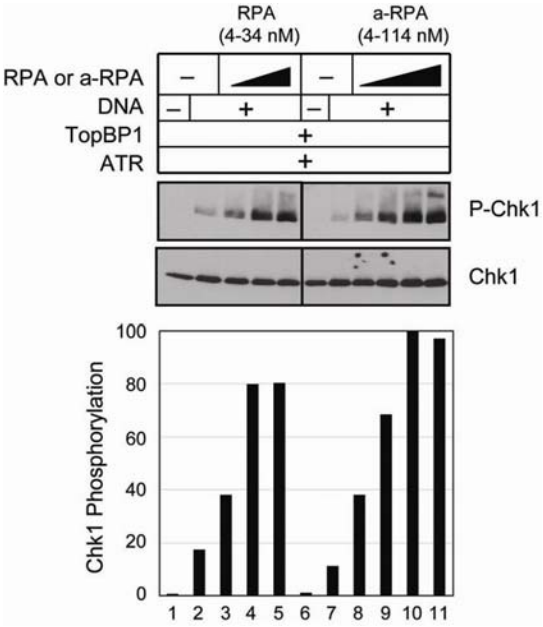

Figure 4.3. Effects of alternative RPA on TopBP1-dependent ATR activation in the presence of ssDNA. Kinase assays were performed as described in Figure 4.1. The concentrations of RPA and aRPA were 4, 11, 34 nM for RPA and 4, 11, 34, 114 nM for aRPA as indicated. The levels of Chk1 phosphorylation were quantified and graphed (bottom panel).

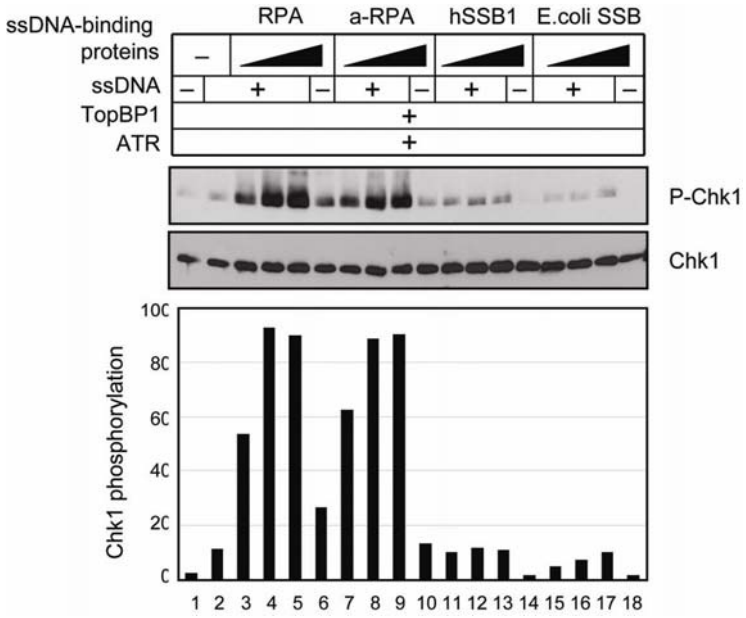

Figure 4.4. Effects of various ssDNA-binding proteins on ATR activation by TopBP1. Kinase assays were performed as described in Figure 4.1, except that RPA and aRPA were compared with human SSB1 and E. coli SSB at the same concentrations (4, 11, 34 nM). The levels of Chk1 phosphorylation from two independent experiments were averaged and graphed.

Discussion

Although extensive data indicate that RPA-coated ssDNA is an important component in the ATR-mediated checkpoint response, so far there is no human *in vitro* system where the effect of RPA-coated ssDNA can be observed. Recently, our group has developed a human ATR-mediated checkpoint system with purified proteins (Choi et al., 2007), and observed TopBP1-dependent activation of ATR kinase activity as shown in *Xenopus* (Kumagai et al., 2006). Previously, Cortez and colleagues reported that addition of RPA or RPA-ssDNA to ATR kinase reactions containing TopBP1 failed to stimulate ATR kinase activity toward its substrate (Ball et al., 2007). In our *in vitro* system, however, upon addition of RPA we observed a slight stimulation of ATR in the presence of DNA (Choi et al., 2007). Although these observations seem to be inconsistent, it should be noted that we used full-length TopBP1 instead of the ATR-activating domain lacking DNA binding activity used in the other study. It is not clear whether the different observations are due to TopBP1-DNA interaction or not. Although it is not known whether TopBP1 interacts with RPA and how this interaction, if present, enhances ATR kinase activity, TopBP1-RPA interaction might be another possible reason for the difference.

Recently, Burgers and colleagues have developed an *in vitro* system with yeast purified proteins and shown that Dpb11 (human TopBP1 ortholog) also directly activates the Mec1-Ddc2 kinase (human ATR-ATRIP ortholog) to phosphorylate its substrate, Rad53 (human Chk1/2 ortholog) (Navadgi-Patil and Burgers, 2008b). Interestingly, this yeast *in vitro* system failed to exhibit a stimulatory effect of DNA and only a marginal effect of RPA-coated ssDNA. In the current study, we show that under more optimized reaction conditions, RPA significantly stimulates TopBP1-dependent activation of the ATR kinase in the presence of ssDNA *in vitro*. The stimulation strongly depends on the

presence of DNA, and the stimulatory effect of RPA seems to be specific for ssDNA. In the presence of dsDNA, RPA has no marked stimulatory effect.

Previous studies indicate that ATRIP is required for ATR function and binds RPA-coated ssDNA that accumulates at sites of DNA damage (Ball et al., 2005; Falck et al., 2005; Namiki and Zou, 2006; Zou and Elledge, 2003). Recent biochemical studies show that the N-terminal OB (oligonucleotide-oligosaccharide)-fold domain of the large subunit of RPA, RPA70 is responsible for interaction with ATRIP (Ball et al., 2007). Moreover, an RPA70 mutant with double mutations in the OB-fold domain no longer interacts with ATRIP, resulting in some defects in the ATR signaling pathway (Xu et al., 2008). This is in line with our observation that aRPA, which contains the RPA70 subunit and binds ssDNA, also stimulates ATR kinase activity in the presence of ssDNA. aRPA is found in placenta and colon tissues and some immortalized cell lines but is absent in most cell lines. In these tissues aRPA might substitute for RPA in some aspects of DNA metabolism, including the ATR-mediated response to DNA damage. However, further investigation is required to determine the role of aRPA in checkpoint pathways.

It will be interesting to test the RPA70 mutant, which was used in previous work, in our *in vitro* system. However, it should be noted that similar mutations in RPA70 reduce ssDNA-binding affinities by one-third (Haring et al., 2008), and thus may contribute to ATR activation defects. The importance of the N-terminal domain of RPA70 might be the reason why the other ssDNA-binding proteins, such as human SSB1 and *E. coli* SSB, have no significant influence. Recent work has shown that upon ionizing radiation, human SSB1 is phosphorylated by ATM and is required for efficient ATM activation, suggesting it has an important role in ATM signaling in response to double-strand breaks (Richard et al., 2008). Although human SSB1 binds specifically to ssDNA and is recruited to sites of DNA damage, the protein is not colocalized with RPA in

response to IR, suggesting that human SSB1 might have completely different functions from RPA (Richard et al., 2008).

The imporatance of RPA-coated ssDNA has emerged from extensive previous studies. We believe that the well-defined *in vitro* system we describe here will be a useful tool to study the detailed biochemical mechanism of RPA-coated ssDNA-mediated checkpoint responses. Since RPA interacts with multiple proteins, this system will allow us to test for the contribution of many checkpoint proteins identified genetically to be in the ATR-mediated checkpoint response.

Acknowledgements

I thank M. S. Wold (University of Iowa, Iowa City, IA) for supplying alternative RPA and L. A. Lindsey-Boltz for purifying RPA. I am also grateful to Dr. J. D. Griffith for providing *E. coli* SSB and to S. Bereketoglu for purifying human SSB1.

CHAPTER 5

FINAL CONCLUSIONS

The DNA damage checkpoint is a signal transduction pathway that coordinates the cell cycle with other cellular responses and thus prevents the segregation of damaged chromosomes. In response to DNA damage, cells activate the DNA damage checkpoint response which delays cell cycle progression to allow time for DNA repair before entering S phase or mitosis (Abraham, 2001; Sancar et al., 2004; Zhou and Elledge, 2000). This cellular mechanism operates throughout the cell cycle and induces G1/S, intra-S, and G2/M checkpoints in the presence of DNA lesions. Defects in DNA damage checkpoints can result in pathological conditions, such as Ataxia telangiectasia, which is characterized by cancer predisposition or the autosomal recessive disorder Seckel syndrome, which is characterized by retarded development or Li-Fraumeni syndrome, which is characterized by predisposition to cancer (O'Driscoll et al., 2003; Savitsky et al., 1995; Shiloh, 2003). Moreover, mutations in several DNA damage checkpoint genes have been identified in sporadic cancers, breast cancers, and ovarian cancers in humans (Bargonetti and Manfredi, 2002; Hartwell and Kastan, 1994; Kastan and Bartek, 2004; Miki et al., 1994). The importance of DNA damage checkpoints in human pathology has led to significant progress in characterizing the molecular components of the pathways. Among the checkpoint components, members of the phosphoinositide kinase-related family of protein kinases (PIKKs), such as ATM and ATR, are central to this signal-transduction pathway (Abraham, 2001). While ATM responds primarily to double-strand breaks, ATR plays an important role in the response to a much broader range of DNA damage, including UV-induced DNA lesions.

In the studies described here, I have focused on the human ATR-mediated DNA damage checkpoint response with the goal of elucidating the molecular mechanism of the pathway. To understand the molecular mechanism of this checkpoint response in detail, it is necessary to develop an *in vitro* checkpoint system reconstituted from purified proteins. Such a reconstituted system will provide a powerful tool for assessing the

potential effect of each checkpoint component. Moreover, the examination of the combined effects of two or more components in a well defined system will provide important information on how the checkpoint components are incorporated into the complex, mulistep process. Until very recently, numerous *in vitro* studies for the ATR-mediated DNA damage checkpoint response have used cell-free systems derived from *Xenopus* eggs, which has made significant contributions but has certain limitations, such as the complexity due to the number of potential checkpoint proteins in the pathway as well as their other cellular functions, in developing a mechanistic model for ATR checkpoint pathway (Guo et al., 2000; Hashimoto et al., 2006; Kumagai et al., 1998; MacDougall et al., 2007; Michael et al., 2000; Yan et al., 2006). Therefore, the objective of my work has been to reconstitute a human ATR-mediated DNA damage checkpoint *in vitro* with purified proteins and to use this system for studying checkpoint activation induced by DNA damage. The recent finding that TopBP1 is a strong co-activator of ATR kinase has led us to develop an *in vitro* system that incorporates many of the components of the genetically defined human ATR-mediated checkpoint signaling.

In chapter 2, I successfully purified three human proteins which are key components of the ATR-mediated checkpoint response. Most importantly, using the purified proteins, I developed a human *in vitro* system in which TopBP1 stimulates the kinase activity of ATR only in the presence of DNA. These results provide the first experimental *in vitro* evidence of DNA- and TopBP1-dependent stimulation of ATR kinase activity. Previous studies have indicated that common DNA structures, such as RPA-coated ssDNA or primer/template DNA structure generated by processing of damage sites are initial signals to activate checkpoint responses and have led to the widely held notion that RPA-coated ssDNA is the universal signal for ATR signaling pathway (Zou, 2007). However, our laboratory has demonstrated that primary DNA lesions induced by *N*-Aco-AAF and UV light can also be a signal for checkpoint

activation *in vivo* and checkpoint proteins bind to these lesions directly (Jiang and Sancar, 2006; Unsal-Kacmaz et al., 2002). To address these conflicting claims, I tested the effects of various DNA substrates on TopBP1-dependent stimulation of ATR kinase activity using the *in vitro* system. I found that under optimized conditions DNA damaged by *N*-Aco-AAF, BPDE and UV light specifically stimulates TopBP1-dependent activation of ATR *in vitro*. Some studies reported that excision gaps generated by processing DNA damage can initiate ATR-activation *in vivo* (Marini et al., 2006; Matsumoto et al., 2007). To exclude the possibility that contaminating excision repair proteins in our ATR preparation may have generated such a gap, we tested for nucleotide excision repair in our ATR checkpoint reactions and found that no excision of damaged DNA occurs in our reactions, indicating that unprocessed DNA damage can be a signal at least under some conditions. More recently, we have observed that like *N*-Aco-AAF-damaged DNA, BPDE-damaged DNA also significantly stimulates TopBP1-dependent ATR activation in our system in chapter 3. Interestingly, UV-damaged DNA, which contains the two major types of DNA photoproducts, cyclobutane pyrimidine dimmers (CPDs) and (6-4) photoproducts (6-4 PP), shows less significant effects. It should be noted that although (6-4) photoproducts are better substrates to be recognized by repair proteins, they are much less abundant than CPDs (Reardon and Sancar, 2003). It is likely that the UV-damaged DNA substrate contains only a few (6-4) photoproducts which are efficiently recognized by checkpoint proteins, and thus results in less significant checkpoint responses under our experimental conditions. Therefore, it will be interesting to compare the effect of DNA containing a single (6-4) photoproduct with that of DNA carrying a CPD in our *in vitro* checkpoint system. Surprisingly, I observed that TopBP1 has higher binding affinity to DNA containing bulky base lesions than to undamaged DNA. The data provide strong evidence of the role of TopBP1 in sensing damaged DNA under certain circumstances, in addition to activating ATR kinase activity. Moreover, TopBP1 binding

to damaged DNA is essential for efficient stimulation of ATR kinase activity in the checkpoint system. Altogether, the data from this study suggests that TopBP1 recognizes unprocessed bulky DNA lesions, recruits ATR-ATRIP to the sites of DNA damage, and consequently activates ATR kinase activity to phosphorylate its downstream target, Chk1.

I further characterized the DNA binding and ATR stimulatory activities of TopBP1 using the *in vitro* checkpoint system in chapter 3. The data demonstrate that TopBP1 has two DNA-binding domains located in the N- and C-terminus, and both domains contribute to preferential binding to damaged DNA containing bulky base adducts. Interestingly, I found that the C-terminal one third of TopBP1 is sufficient for damaged DNA-dependent activation of the ATR kinase. Consistent with this observation, previous studies in *Xenopus* systems suggest that the C-terminal half is responsible for the checkpoint response, while the N-terminal half is involved in replication initiation (Hashimoto et al., 2006; Yan et al., 2006). It is interesting that the budding yeast TopBP1 homolog, Dpb11 directly activates the ATR ortholog, Mec1 kinase although Dpb11 has no sequence homology to the C-terminal region of TopBP1, which contains ATR activating domain (Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008b). However, it turns out that Dpb11 contains the Mec1 activation domain in its C-terminal region, suggesting the functional mechanism of Dpb11/TopBP1 for Mec1/ATR activation is conserved from yeast to humans (Mordes et al., 2008b). Recently, it has been reported that the N-terminus of TopBP1 interacts with Rad9 and that this interaction is required for efficient activation of the ATR kinase (Delacroix et al., 2007; Lee et al., 2007). However, our results along with the original report on TopBP1-ATR interaction reveal that the Rad9 requirement can be bypassed in our *in vitro* checkpoint system or there might be some pathways that are independent of Rad9 interaction due to a variety of forms of DNA damage. Interestingly, I observed that TopBP1-dependent ATR activation

is significantly stimulated by longer DNA substrates, indicating that the observed ATR activation is dependent on the length of DNA. This DNA length dependence of ATR activation seems to be correlated with preferential binding of TopBP1 to longer DNA fragments. Therefore, the data suggest that TopBP1 preferentially binds to longer DNAs which results in cooperative recruitment and activation of ATR. Consistent with this idea, larger ssDNA gaps induce higher levels of Chk1 phosphorylation in *Xenopus* egg extracts, suggesting cooperative activation of ATR (MacDougall et al., 2007). In contrast to previous biochemical studies showing specific binding of TopBP1 to DNA ends, I observed that TopBP1 binding is independent of DNA ends (Yamane and Tsuruo, 1999). Moreover, TopBP1-dependent ATR activation in our checkpoint system is also independent of the presence of DNA termini, indicating that unlike ATM activation, DNA ends are not required for ATR activation by TopBP1. Therefore, the data from this study suggest a cooperative activation mechanism for TopBP1- and damaged DNA-dependent ATR activation. Together with the data presented in Chapter 2, I believe that in addition to the current model of ATR activation, unprocessed bulky DNA lesions can be recognized by the checkpoint proteins, resulting in cooperative activation of the ATR kinase (Figure 5.1).

Recent studies from our lab and my work reported in this dissertation indicate that primary DNA lesions induced by *N*-Aco-AAF, BPDE, and UV light can be a signal for checkpoint activation *in vivo* and *in vitro*, and checkpoint proteins bind to these lesions directly (Choi et al., 2007; Choi et al., 2009; Jiang and Sancar, 2006; Unsal-Kacmaz et al., 2002). In addition, a number of previous studies suggest that RPA-coated ssDNA is an important component in the ATR-mediated checkpoint response (Michael et al., 2000; You et al., 2002; Zou, 2007). Upon DNA damge, ssDNA can be generated by nucleotide excision repair or by uncoupling of MCM helicase and DNA polymerase activities at stalled replication forks (Cimprich and Cortez, 2008; You et al., 2002). RPA then

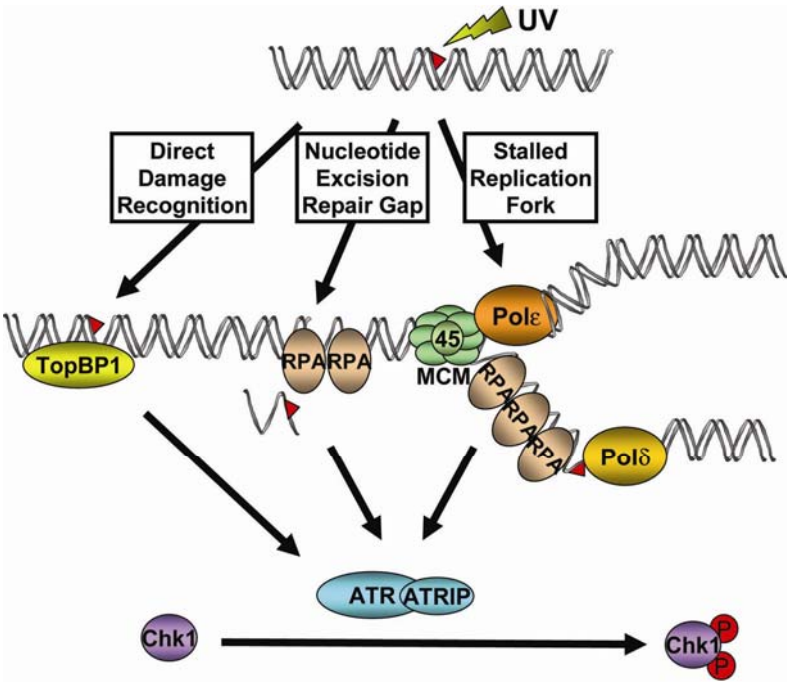

Figure 5.1. Potential DNA damage detection mechanisms for initiating checkpoint responses. The primary damage it self may be recognized by damage-specific proteins that may activate the checkpoint directly. RPA-coated single-stranded DNA gaps generated by nucleotide excision repair and the extensive RPA-coated single-stranded DNA generated by replication forks stalled at a damage site are known to be strong signals for checkpoint activation. Adapted from (Lindsey-Boltz and Sancar, 2007).

accumulates on the ssDNA regions and interacts with checkpoint proteins including ATR-ATRIP at sites of DNA damage (Iftode et al., 1999; Wold, 1997; Zou and Elledge, 2003). Therefore, I investigated whether RPA stimulates TopBP1-dependent ATR activation in the presence of ssDNA in our *in vitro* checkpoint system in chapter 4. Using *in vitro* systems with purified proteins from yeast or human, previous studies failed to observe any significant effect of RPA in the absence or presence of ssDNA, although they confirmed TopPB1/Dpb11-dependent activation of ATR/Mec1 *in vitro* (Ball et al., 2007; Navadgi-Patil and Burgers, 2008b). In this study, I found more optimized reaction conditions to detect the effect of RPA *in vitro* and observed that RPA significantly stimulates TopBP1-dependent activation of the ATR kinase in the presence of ssDNA. I also tested the effect of alternative RPA, which contains an alternative form of RPA 32 subunit, in the ATR kinase reactions. Although alternative RPA has similar biochemical properties to RPA, it is unable to support DNA replication *in vitro* (Mason et al., 2009). Surprisingly, I found that aRPA also significantly stimulates TopBP1-dependent ATR activation in the presence of ssDNA, and moreover the stimulation levels are comparable to those of RPA. Under identical reaction conditions, there were only marginal effects of the other ssDNA-binding proteins, such as human SSB1 and *E. coli* SSB, indicating the specific function of the RPA complexes in our *in vitro* checkpoint system. Collectively, the data provide the first *in vitro* evidence indicating the stimulatory effect of RPA-coated ssDNA on TopBP1- dependent ATR activation.

In summary, I have established a human *in vitro* checkpoint system which recapitulates key elements of the ATR-mediated DNA damage checkpoint response. Using this system I was able to study the effects of various types of DNA on ATR activation and the requirements for the checkpoint response. This system will ultimately be a useful framework for reconstituting the complete checkpoint response *in vitro*, using all identified checkpoint components *in vivo*. Importantly, a well-defined system for

studying the DNA damage checkpoint response will provide a valuable tool for designing new therapeutic approaches that target the DNA damage checkpoint pathway.

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