THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 8, pp. 5074–5082, February 21, 2014

Coupling of Human DNA Excision Repair and the DNA Damage Checkpoint in a Defined *in Vitro* System*

Received for publication, December 12, 2013, and in revised form, January 7, 2014 Published, JBC Papers in Press, January 8, 2014, DOI 10.1074/jbc.M113.542787

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Background: Nucleotide excision repair and the ATR-mediated DNA damage checkpoint responses are genetically coupled.
Results: We have analyzed the basic steps of ATR activation in a biochemically defined system.
Conclusion: ATR signaling requires enlargement of the DNA excision gap by EXO1.
Significance: The six excision repair factors, ATR-ATRIP, TopBP1, and EXO1 constitute the minimum essential set of proteins for ATR-activation upon UV-induced DNA damage.

DNA repair and DNA damage checkpoints work in concert to help maintain genomic integrity. *In vivo* data suggest that these two global responses to DNA damage are coupled. It has been proposed that the canonical 30 nucleotide single-stranded DNA gap generated by nucleotide excision repair is the signal that activates the ATR-mediated DNA damage checkpoint response and that the signal is enhanced by gap enlargement by EXO1 (exonuclease 1) 5′ to 3′ exonuclease activity. Here we have used purified core nucleotide excision repair factors (RPA, XPA, XPC, TFIIH, XPG, and XPF-ERCC1), core DNA damage checkpoint proteins (ATR-ATRIP, TopBP1, RPA), and DNA damaged by a UV-mimetic agent to analyze the basic steps of DNA damage checkpoint response in a biochemically defined system. We find that checkpoint signaling as measured by phosphorylation of target proteins by the ATR kinase requires enlargement of the excision gap generated by the excision repair system by the 5′ to 3′ exonuclease activity of EXO1. We conclude that, in addition to damaged DNA, RPA, XPA, XPC, TFIIH, XPG, XPF-ERCC1, ATR-ATRIP, TopBP1, and EXO1 constitute the minimum essential set of factors for ATR-mediated DNA damage checkpoint response.

DNA damage activates three major biochemical pathways in eukaryotic cells: DNA repair, DNA damage checkpoints, and apoptosis (1). The DNA damage checkpoint response delays or arrests cell cycle progression that helps prevent the mutagenic or lethal consequences of damage to the cell. In mammalian organisms two main DNA damage checkpoint pathways/networks have been defined based on the damage-sensing kinases that initiate the signal: the ataxia telangiectasia mutated (ATM)[4] pathway and the ATM and Rad3-related (ATR) pathway (2). Although there is some overlap and crosstalk between the two signaling pathways, in general, most studies support the view that ionizing radiation and other agents that produce double-strand breaks in DNA activate the ATM pathway, while ultraviolet (UV) light and other genotoxic agents that generate bulky base adducts activate the ATR-mediated checkpoint signaling pathway (3). The ATM kinase is activated by recruitment by the MRN complex to duplex termini generated by double-strand breaks and dimer to monomer transition or during oxidative stress by formation of disulfide crosslinks between the two subunits of the ATM homodimer (4). In the case of ATR, the signal could be the bulky adduct itself (5, 6), the stalled replication fork (7, 8), the transcription elongation complex stalled at the site of damage (9), the repair complex assembled at the site of damage (10), or the canonical 30 nucleotide gap generated by nucleotide excision repair (11–13). Although overwhelming evidence indicates that in S phase the stalled replication fork and the long stretches of ssDNA that result from replication fork stalling are the primary signals for the ATR-mediated checkpoint (14), the contributions of other factors to checkpoint activation in G_1 and G_2/M are not as well-defined (3). However, several studies have strongly supported a model whereby the canonical 30-nucleotide gap generated by nucleotide excision repair is enlarged by EXO1, and the enlarged single-stranded gap (presumably occupied by RPA protein) constitutes the major signal for the ATR-mediated checkpoint response outside of S phase (15–17).

While the *in vivo* data is compelling in support of the model, there are alternative explanations for some key observations upon which the model is based because transient knockdown of many gene products outside the core constituents of nucleotide excision repair have been reported to interfere with ATR-me-

* This work was supported by National Institutes of Health Grant GM32833 (to A. S.) and GM45190 (to P. M.).
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[4] The abbreviations used are: ATM, ataxia telangiectasia-mutated; UV, ultraviolet; RPA, replication protein A; ssDNA, single-stranded DNA; AAF, *N*-acetoxy-2-acetylaminofluorene; ATR, ataxia-telangiectasia-mutated and Rad3-related; AAD, ATR-activating domain.

diated checkpoint signaling (14). Thus, the basic model can be evaluated only *in vitro* in a system that contains components with precisely defined function which would eliminate the *in vivo* artifacts arising from mutations that affect the ATR pathway through secondary effects on cellular homeostasis. In this study, using highly purified minimal essential sets of both the human nucleotide excision repair system and the ATR checkpoint signaling pathway, we have reconstituted the ATR checkpoint system *in vitro*. We find that the nucleotide excision repair canonical 30 nucleotide gap enlarged by EXO1 is necessary and sufficient to activate ATR checkpoint signaling in the presence of the ATR co-activator TopBP1 protein. This is the first *in vitro* system that couples nucleotide excision repair and the ATR-mediated DNA damage checkpoint.

EXPERIMENTAL PROCEDURES

Protein Purification—The excision repair proteins His-XPA, XPC-HR23B, XPG, and XPF-ERCC1 were purified as recombinant proteins using the Sf21/baculovirus insect cell/vector system as previously described (18). The multi-subunit TFIIH complex was purified from HeLa Flp-In T-REx cells (19, 20) expressing tetracycline-inducible FLAG-p62 as described in the manufacturer's directions (Invitrogen), and purified with P11 chromatography and affinity chromatography with anti-FLAG-M2 agarose (Sigma) as previously described (21). The ATR-ATRIP complex was similarly purified from HeLa Flp-In T-REx cells containing a tetracycline-inducible Flag epitope-tagged ATRIP subunit by anti-FLAG-M2 affinity chromatography as previously described (22). The following proteins were purified as recombinant proteins expressed in *Escherichia coli* as previously described: GST-TopBP1-His (23), EXO1 (amino acids 1–450) (24), GST-p53 (Addgene plasmid 10852) (25), and RPA (26). The purified proteins were separated on 4–15% TGX-PAGE and analyzed by silver staining.

Cell Lines and Antibodies—Immortalized wild-type (WT) and *Exo1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured in DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. The *Exo1*$^{-/-}$ MEFs were obtained from Winfried Edelmann (Albert Einstein College of Medicine) (27). To drive cells into quiescence, cells were grown in DMEM containing 0.5% FBS for 3–4 days. Irradiation of cells with UV light involved the removal of the medium from the cells, exposure to a UV-C light source (254 nm), and replacement of the medium. Following a 1-h incubation, cells were washed with cold PBS, scraped from the plate into cold PBS, and then lysed in a buffer containing 25 mM HEPES-KOH pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 12.5% glycerol, 1 mM DTT, and 0.5% Nonidet P-40. Cell lysates were fractionated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting.

The following primary antibodies were obtained from the indicated companies and used at the indicated dilution: from Cell Signaling Technology, phospho-Chk1-Ser^{345} (catalogue no. 2348, 1:10,000), phospho-p53-Ser^{15} (catalogue no. 9284, 1:10,000); from Bethyl Laboratories, RPA1 (catalogue no. A300–241A, 1:2,000) and phospho-RPA2-Ser^{33} (catalogue no. A300–246A, 1:10,000); from Santa Cruz Biotechnology, Inc., Chk1 (catalogue no. sc-8408, 1:2,000), GST (catalogue no. sc-138, 1:1,000), and from Leica Biosystems, p53 (NCL-p53–505, 1:1,000).

Preparation of DNA Substrates—Gapped plasmid was generated by treating pBC-KS.nick (28) with Nt.BbvCI endonuclease which cuts only one strand of the plasmid 43 nucleotides apart. The excised oligomer was released by heat denaturation in the presence of excess complementary oligo. The ϕX174 ssDNA was purchased from New England Biolabs (N3023). *N*-Acetoxy-2-acetylaminofluorene (AAF) was obtained from the NCI Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). AAF-damaged plasmid DNA (pBC-KS.nick) was prepared as described previously (23). The concentration of AAF was empirically determined to generate ~3 adducts per plasmid.

Excision Repair Assay—The repair assay was performed as previously described (18). Unmodified or AAF-damaged plasmid DNA (100 ng) was incubated in a 12.5-μl reaction containing the core excision repair factors (XPA (86 ng), XPC-hR23B (17.5 ng), XPF-ERCC1 (7.5 ng), XPG (4 ng), TFIIH (100 ng), and 170 ng of RPA). The final reactions contained 23 mM Hepes-KOH (pH 7.9), 44 mM KCl, 2.5 mM $MgCl_2$, 2 mM ATP, 2.5% glycerol, 0.04 mM EDTA, and 0.2 mM DTT. After 90 min at 30 °C, 2.5 μl of the reaction was diluted 1:4 with TE buffer and reserved for kinase assays. To the remaining 10 μl, 2 μl of phenol and 12 μl of agarose gel-loading buffer containing TBE (0.1 M Tris, 0.1 M boric acid, 0.002 M EDTA), 1% SDS, 0.05% bromphenol blue, and 10% glycerol was added and then separated on an ethidium bromide-containing 1% agarose gel, which was then analyzed using a Bio-Rad Molecular Imager ChemiDoc XRS+ system.

Checkpoint Assay—The procedure was essentially as previously described (29). Briefly, kinase assay reactions contained 14 mM Hepes, pH 7.9, 30 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 2% glycerol, and 1 μM microcystin in a 12-μl final volume. Purified ATR-ATRIP (0.2 nM), TopBP1 (2.5 nM), RPA (100 nM), p53 (50 nM), and EXO1 (8 nM), where indicated, were incubated in reaction buffer for 20 min at 30 °C with DNA (2 ng) as indicated. The reactions were terminated by the addition of 3 μl of 5× SDS-PAGE loading buffer (100 mM Tris, pH 6.8, 10% (v/v) glycerol, 200 mM DTT, 2% (w/v) SDS, 0.01% (w/v) bromphenol blue) and then boiled and separated by 15% SDS-PAGE. Phosphorylation of p53 and RPA2 were detected by immunoblotting using the indicated phospho-specific antibodies, and the level of total protein was subsequently detected by immunoblotting the same membrane with the indicated antibodies. Chemiluminescent signals were visualized with Clarity Western blotting detection reagent (Bio-Rad) and analyzed with the Molecular Imager ChemiDoc XRS+ system (Bio-Rad). The highest phosphorylation signal on each blot was set to 100%, and the levels of phosphorylation of other samples were expressed relative to this value. Graphed values are the average and S.D. from at least two independent experiments.

RESULTS

Purification of Nucleotide Excision Repair and ATR Checkpoint Signaling Proteins—In vitro assays with cell-free extract to test various models for ATR-mediated checkpoint signaling are hampered by the fact that, in humans, DNA-PK is the most

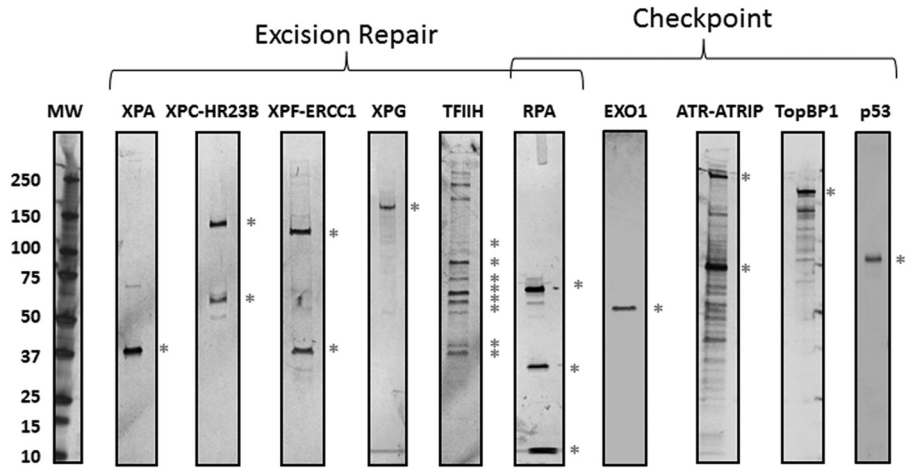

FIGURE 1. **Purified Repair and Checkpoint Factors.** Analysis of the 10 excision repair and checkpoint factors by silver staining. Approximately 5–20 ng of each factor was subjected to 4–15% TGX-PAGE analysis. The different subunits in the complexes are indicated by *asterisks*.

abundant member of the PIKK family kinases (ATM, ATR, DNA-PK) and has the most robust activity of the three kinases (30–33). As a consequence, it dominates the kinase activity in cell-free extracts with any putative ATM or ATR substrates, as there is considerable overlap among substrates of the PIKK family (28, 34). Use of kinase inhibitors only partially alleviates the problem (28, 34, 35). Perhaps most importantly, by using cell-free extracts, it is not possible to define the necessary and sufficient components of a biochemical pathway. For these reasons, we have not been able to test the various models for ATR checkpoint in cell-free extracts and found it necessary to purify the nucleotide excision repair and checkpoint proteins that are known to be essential for ATR-mediated checkpoint signaling.

Fig. 1 shows our highly purified nucleotide excision repair and DNA damage checkpoint proteins. The excision repair proteins XPA, XPC-HR23B, XPG, and XPF-ERCC1 were purified as recombinant proteins using the Sf21/baculovirus insect cell/vector system. The multisubunit TFIIH was purified from HeLa cells containing an inducible FLAG epitope-tagged p62 subunit through conventional chromatography steps and contained some minor high molecular weight contaminants. The identities of the main bands seen by silver staining as those corresponding to the known TFIIH subunits were confirmed by immunoblotting. The ATR-ATRIP complex was similarly purified from HeLa cells containing an inducible FLAG epitope-tagged ATRIP subunit by affinity chromatography, yielding a preparation in which the major protein bands on SDS-PAGE are ATR and ATRIP as confirmed by immunoblotting. The ATR co-activator, TopBP1, was purified as a recombinant protein expressed in *E. coli*, as were EXO1 nuclease, p53, and RPA.

A Model System for Excision Repair-Checkpoint Coupling—Two general models have been proposed for coupling of repair to the DNA damage checkpoint. In one, it is suggested that either the mismatch repair protein MutSα (36) or the nucleotide excision repair protein XPA (10) binds to a mismatch or to a bulky base adduct, respectively, and by some ill-defined mechanism recruits ATR to damage sites and stimulates its kinase activity. While these mechanisms may play some minor roles in ATR activation, attempts to demonstrate such effects in defined systems have not been successful. In the alternative mechanism, it is proposed that the canonical 30-nt-long excision gap generated by nucleotide excision repair either as such or after enlargement by exonucleases constitutes the structure/signal that couples nucleotide excision repair to ATR-initiated DNA damage checkpoint (11–13).

To test the model that the 30-nt-long nucleotide excision repair-gap either as is, or after processing by EXO1 exonuclease, constitutes the signal for ATR checkpoint, we first used a model DNA substrate. A plasmid DNA containing a 43-nucleotide gap was generated by treating the plasmid with Nt.BbvCI endonuclease, which cuts at two sites 43 nucleotides apart in only one strand of the plasmid. The gap generated by nicking with this enzyme followed by release of the excised oligomer by heat denaturation was used in our reconstituted ATR-ATRIP + TopBP1 kinase system with RPA2 (RPA32 subunit of RPA) as a substrate for ATR kinase, and the results are shown in Fig. 2*A*. The unprocessed gap was insufficient to activate ATR (*lane 5*). However, upon addition of human EXO1, which enlarges the gap by digesting DNA in the 5′ to 3′ direction, resulted in ATR activation as efficiently as ssDNA (compare *lanes 6* and *4*). Addition of EXO1 to reactions containing circular dsDNA had no significant effect (*lane 8*). Thus, we conclude that the canonical 30-nt excision gap would constitute a signal for ATR kinase after enlargement with EXO1.

We and others have previously reported that Ser^{33} of RPA2 is phosphorylated by ATR in a manner dependent on ssDNA in *in vitro* kinase assays (37, 38). This residue of RPA2 is known to be phosphorylated by ATR in cells treated with UV light (39). However, it has not been reported whether RPA phosphorylation at this site occurs in a manner dependent on excision repair and EXO1. Therefore, we examined phosphorylation of RPA2 after UV in WT and $Exo1^{-/-}$ mouse embryonic fibroblast (MEF) cells, and the results are shown in Fig. 2*B*. We find that in quiescent cells, where UV-induced ATR activation is known to be dependent on nucleotide excision repair (11–13, 40), that indeed, RPA2 is phosphorylated on Ser^{33}, and the phosphorylation is dependent on the presence of EXO1 (compare *lanes 2* and *4*). As was previously shown (15), p53 phosphorylation on Ser^{18} (equivalent to Ser^{15} in human p53) is also dependent on EXO1 under these conditions. Also as previously reported (13),

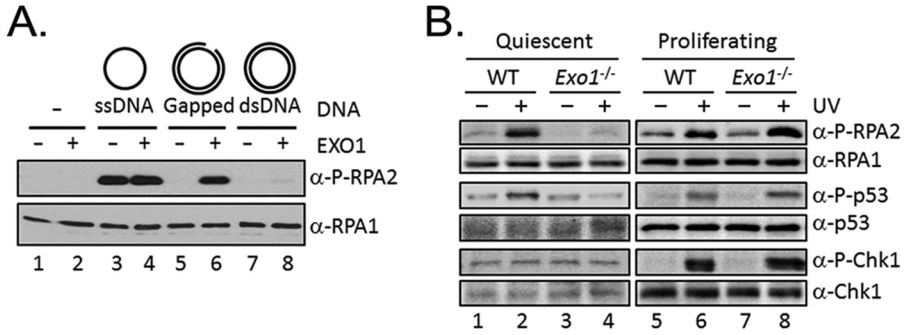

FIGURE 2. **EXO1-dependent ATR Activation.** *A*, a model system for excision repair-checkpoint coupling. ATR kinase reactions were carried out with ATR-ATRIP, TopBP1, RPA, and EXO1 as indicated. 0.6 ng (27 pM) single-stranded ϕX174 DNA (*ssDNA*), plasmid DNA (*dsDNA*), or gapped DNA was added to the reaction as indicated and incubated 20 min at 30 °C. Reactions were analyzed by immunoblotting for phospho-RPA2 (Ser^{33}) and RPA1. *B*, EXO1-dependent phosphorylation of RPA2 (Ser^{33}) and p53 (Ser^{18}) in quiescent cells after UV damage. Serum-starved (quiescent) or asynchronously growing (proliferating) wild-type (*WT*) and *Exo1*$^{-/-}$ MEFs were exposed to 20 J/m^2 of UVC light and harvested 1 h later. Cell lysates were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies.

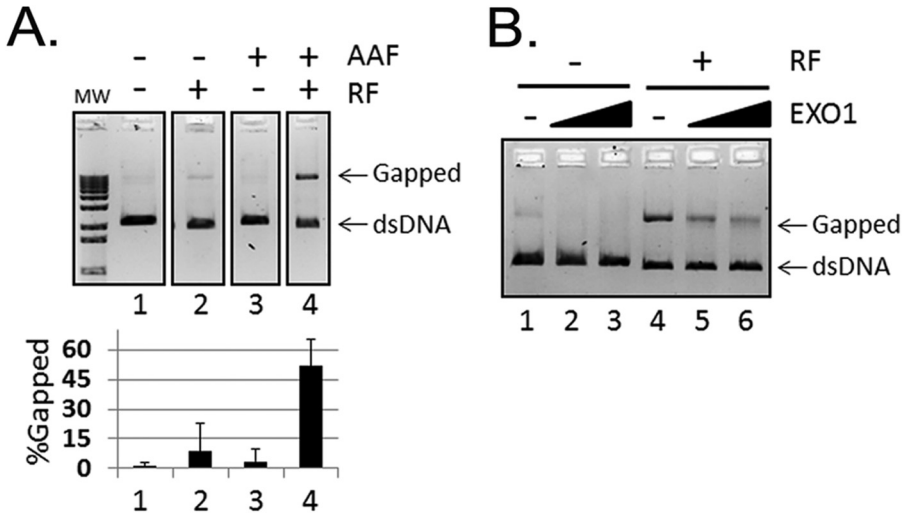

FIGURE 3. **Repair factor- and damage-dependent gap generation and resection by EXO1.** *A*, repair factor (*RF*)-dependent generation of gapped-DNA. Excision reactions were performed with unmodified DNA (*lanes 1* and *2*) or AAF-damaged DNA (*lanes 3* and *4*) in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of the 6 core excision repair factors (*RF*). The percentage of gapped DNA was quantified from identical repeats of the experiment and presented as mean ± S.D. ($n \geq 3$). *B*, EXO1 specifically digests the gapped DNA generated by repair factors. After 90 min, excision reactions with AAF-damaged DNA without (*lanes 1–3*) or with repair factors (*lanes 4–6*), EXO1 was added at the indicated concentrations for an additional 10 min before analysis by agarose gel electrophoresis (0: *lanes 1* and *4*, 4 nM: *lanes 2* and *5*, 8 nM: *lanes 3* and *6*).

in quiescent cells Chk1 protein levels are low and there is no detectable phosphorylation at Ser^{345}. In contrast, as expected, in asynchronous cells where ATR activation after UV is largely the result of replication fork stalling, phosphorylation of RPA2, p53, and Chk1 is not dependent on EXO1 (*lane 8*). Thus, we conclude that RPA2 phosphorylation on Ser^{33} and p53 phosphorylation on Ser^{15} are the physiologically relevant readouts for ATR activation dependent on excision gap enlargement by EXO1 after UV-induced DNA damage in quiescent cells, and we set out to test this model in our defined system *in vitro*.

Human Nucleotide Excision Repair in Vitro and Excision Gap Enlargement with EXO1—*N*-Acetoxy-2-acetylaminofluorene (*N*-Aco-AAF)-damaged DNA is one of the best substrates for nucleotide excision repair (41), and is considered to be a UV-mimetic (42). Therefore, for our checkpoint assay we used AAF-damaged plasmid as a substrate for the reconstituted human excision nuclease system to generate the excision gaps that have been proposed to initiate checkpoint signaling. First, we tested the specificity of our reconstituted excision nuclease system by using undamaged and AAF-modified plasmids with the 6 core repair factors in a nicking assay for excision repair. As seen in Fig. 3*A*, the purified repair factors are virtually free of nonspecific endonucleases as evidenced by the lack of nicking activity on undamaged DNA under conditions where on average one gap per plasmid is produced in damaged DNA (Fig. 3*A*, *lane 2 versus lane 4*). Because it has been proposed that the enlargement of the excision gap significantly amplifies the checkpoint signal we tested the effect of EXO1 on the gapped plasmid. As seen in Fig. 3*B*, with increasing concentration of EXO1, the gapped plasmid band becomes more diffuse in the agarose gel, while the band corresponding to covalently closed DNA remains unchanged, consistent with the prediction that EXO1 enlarges the excision repair gap. Because the excision gap is enlarged in individual gapped molecules to varying degrees, the "open circular" plasmid band on the agarose gel has a diffuse appearance.

Coupling of Excision Repair with ATR Checkpoint—To test the model of the checkpoint response to UV and UV-mimetic agents, we treated undamaged and AAF-damaged plasmid DNAs with various combinations of repair and checkpoint fac-

tors in the absence and presence of EXO1 and tested for ATR signaling using RPA2 phosphorylation as a readout. The results are shown in Fig. 4. As is clear from the figure, even though a low level signal is seen with undamaged DNA, in agreement with earlier data (23), only the combination of AAF-DNA + Repair Factors + ATR-ATRIP + TopBP1 resulted in strong checkpoint signaling well above all other combinations including signal with undamaged DNA (*lane 7 versus lane 1*, $p < 0.01$), with damaged DNA in the absence of repair factors (*lane 7 versus lane 9*, $p < 0.01$), or with both damaged DNA and repair factors but in the absence of EXO1 (*lane 7 versus lane 12*, $p < 0.01$). The low level of ATR kinase activity observed in the presence of TopBP1 and undamaged DNA (*lanes 1* and *3*) and with damaged DNA in the absence of repair factors (*lane 9*) is consistent with previous observations that under certain experimental conditions DNA + TopBP1 are sufficient to cause moderate checkpoint activation both *in vitro* (23) and *in vivo* (43).

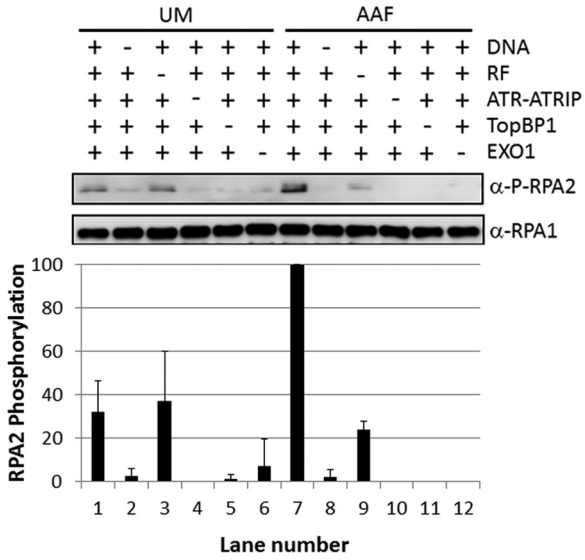

FIGURE 4. **Repair-checkpoint coupling as measured by RPA phosphorylation by ATR.** Kinase reactions containing 100 nM RPA as a substrate were incubated 20 min at 30 °C. 0.2 nM ATR-ATRIP, 2.5 nM TopBP1, and 8 nM EXO1 were added, as indicated, to kinase reactions containing 4 ng unmodified (*UM*) or AAF DNA from excision reactions with or without repair factors (*RF*) as indicated. All six repair factors were required, as determined by omission studies (data not shown). Reactions were analyzed by immunoblotting for phospho-RPA2 (Ser[33]). The blots were also analyzed for RPA1 to control for loading. The relative levels of phosphorylated RPA2 from identical repeats of the experiment were quantified and presented as mean ± S.D. ($n \geq 3$).

DNA Concentration Effect and Kinetics of ATR Checkpoint Signaling—Because DNA is a key component of the ATR checkpoint pathway, we next determined the effect of DNA concentration on excision repair + EXO1 enlargement-dependent ATR checkpoint signaling. We find that under our reaction conditions ~4 ng DNA per reaction yields the best signal-to-noise ratio (Fig. 5*A*). Next, using this amount of DNA we carried out a time course experiment. As apparent in Fig. 5*B*, at all time points the complete reaction is significantly more effective in promoting RPA2 phosphorylation by ATR than partial reactions in which the repair factors or damaged DNA were omitted. Taken together, these data indicate that our *in vitro* system is a faithful representation of the ATR signaling system defined genetically in yeast (3) and mammalian cells or with partial reactions in *Xenopus* extracts (43).

Repair-Checkpoint Coupling as Measured by p53 Phosphorylation—Although RPA2 phosphorylation is a commonly used readout for ATR checkpoint signaling *in vivo* and in some studies with cell-free extracts (34, 35, 38), its significance in delaying or arresting cell cycle progression (checkpoint response) in G_1/quiescent cells is not known. In contrast, the phosphorylation of p53 by ATR is known to be an important step in ATR signaling during G_1 (44). Therefore, we wished to ascertain that our *in vitro* repair checkpoint coupling system was operative on p53 as well. Data shown in Fig. 6 indicate that phosphorylation of p53 at Ser[15] by ATR is dependent on damaged DNA + repair factors (*lane 2 versus lane 4*, $p < 0.05$). This indicates that our *in vitro* system is a true representative of the ATR checkpoint signaling pathway.

DISCUSSION

ATM and ATR Checkpoints—In humans, the two main DNA damage checkpoint pathways are the ATM- and ATR-medi-

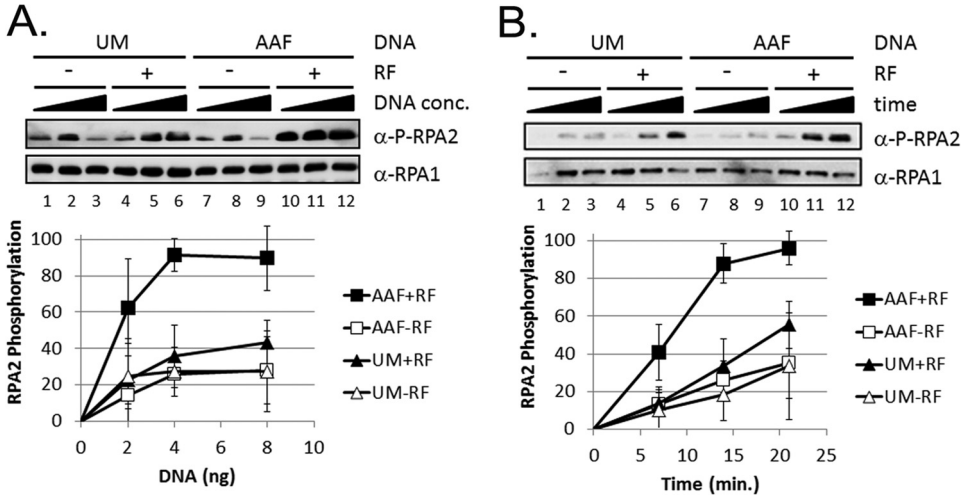

FIGURE 5. **DNA concentration effect and kinetics of ATR checkpoint signaling.** *A*, titration of unmodified (*UM*) or AAF DNA (2, 4, or 8 ng) from excision reactions with or without repair factors (*RF*) into kinase reactions containing ATR-ATRIP, TopBP1, RPA, and EXO1 as in Fig. 4. *B*, time course analysis of RPA2 phosphorylation in kinase reactions with 4 ng of DNA as in *panel A*. The graphs below show the relative levels of phosphorylated RPA2 from identical repeats of the experiments quantified and presented as mean ± S.D. ($n \geq 3$).

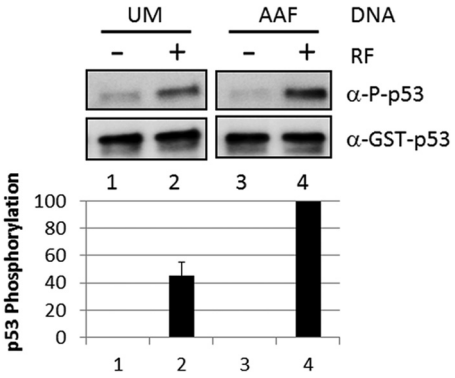

FIGURE 6. **Repair-checkpoint coupling as measured by p53 phosphorylation.** Kinase reactions containing p53, ATR-ATRIP, TopBP1, RPA, EXO1, and the indicated DNA were performed as in Fig. 4. Reactions were analyzed by immunoblotting for phospho-p53 (Ser^{15}). The blots were also analyzed for GST-p53 to control for loading. The relative levels of phosphorylated p53 from identical repeats of the experiment were quantified and presented as mean ± S.D. ($n \geq 2$).

ated checkpoints (2). To a first approximation, the ATM checkpoint response is activated by DNA double strand breaks and the ATR checkpoint response is activated by inhibition of replication in S-phase and by UV and UV-mimetic agents that introduce bulky base adducts in G_1 and G_2/M phases (1). Substantial progress has been made in mechanistic understanding of the ATM signaling pathway. It appears that ATM is activated by two mechanisms (4). In one, activation is initiated by double strand breaks: The MRN complex (Mre11/Rad50/Nbs1) binds to duplex DNA ends and unwinds the duplex by the Rad50 helicase activity to generate long stretches of single-stranded DNA (~2,000 nucleotides is optimal for activity) to which ATM binds and undergoes dimer-to-monomer transition concomitant with unmasking of the ATM kinase activity on MRN and signal transducing- and effector proteins such as the Chk2 kinase and the p53 transcription factor. In the second mode of activation, it was reported that oxidative stress, independent of its genotoxic effect, causes disulfide bond formation between the ATM monomers, producing a stable dimer and in the process induces a conformational change that activates the ATM kinase (4).

In the case of ATR, early on it was realized that inhibition of replication by genotoxic agents or by depletion of the dNTP pools, both of which uncouple the activities of the replication helicases and polymerases and result in the formation of long stretches of ssDNA, is a potent signal for ATR activation and therefore it was concluded that ssDNA-RPA filaments constituted the primary structure for ATR signaling (45, 46). However, other studies indicated that the ATR-mediated checkpoint can be activated in G_1 and G_2/M phases in cells by UV damage or by base pair mismatches, and models were proposed for mechanisms of ATR activation in the absence of DNA replication (12, 13, 36).

Experiments in yeast, *Xenopus* egg extracts, and human cell lines and cell-free systems have led to three general models for checkpoint activation by ATR outside of S-phase: 1) Direct Recruitment by DNA Damage. Evidence has been presented that ATR and the 9-1-1 checkpoint clamp assemble at the site of bulky base damage or DNA mismatches and that this assembly of ATR-ATRIP/Rad17-RFC/9-1-1 complex on DNA activate the ATR kinase (6). 2) Recruitment by Repair Proteins. It has been reported that the nucleotide excision repair protein XPA and the mismatch repair protein MSH2 bind to the respective damage/mismatch sites and recruit ATR (Mec1 in budding yeast) to chromatin, leading to its activation (36, 47, 48). 3) Recruitment by the Repair Gap. Nucleotide excision repair generates a canonical 30 nucleotide gap which acts as a signal for ATR checkpoint. There are several variants of this model. In one, the 9-1-1 (Ddc1-Rad17-Mec3 in budding yeast) checkpoint clamp is loaded onto the 5′ terminus of the gap occupied by RPA, and ATR-ATRIP (Mec1-Ddc2) is recruited to the gap occupied by RPA through RPA-ATRIP interaction, placing ATR in proximity of 9-1-1 and RPA-coated DNA, resulting in ATR kinase activation (49). Presumably, TopBP1 is not required for this mode of activation. In the second model, the MRN complex binds to the 5′ terminus of the RPA-coated excision gap; and, independent of MRN, the 9-1-1 complex is also loaded at the 5′-end of the gap; ATR-ATRIP is recruited to the gap through ATRIP-RPA interaction (50). MRN recruits TopBP1 to the 5′-end through direct protein-protein interaction. Then, TopBP1 binds to the tail of Rad9 in the 9-1-1 complex causing a conformational change in TopBP1, exposing its AAD (ATR-activating domain) which then interacts with ATR and activates its kinase function (51, 52). In a third model, it is proposed that enlargement of the canonical 30-nt gap by the 5′ to 3′ exonuclease, EXO1, both in yeast and in humans, is necessary for optimal activation of ATR/Mec1 checkpoint (15–17). In support of this model it was reported that the ATR/Mec1 checkpoint signaling was severely attenuated in EXO1 mutant yeast or EXO1 knockdown in human cell lines. In further support of this model, it was found that in *Xenopus* egg extract, a 35-nt gap was only marginally capable of activating the ATR checkpoint and that larger gaps in the range of 2000–5000-nt were optimal for activation (53). In support of the notion that gaps of relatively large size are required for ATR/Mec1 checkpoint activation, it has been reported that DNA damage by agents that produce non-bulky base lesions that are mainly repaired by base excision repair do not activate ATR/Mec1 checkpoint in G_1 phase; but, when cells are defective in base excision repair the damage is primarily repaired by nucleotide excision repair which generates larger gaps (possibly after processing by EXO1) activate Mec1/ATR checkpoint (54).

Minimal Essential Set of Factors for ATR Activation in the Absence of DNA Replication—In addition to the DNA and protein components discussed here, numerous other genes have been implicated in the ATR checkpoint response. It is beyond the scope of this discussion to critique the data on which these conclusions were based and what might be direct and indirect effects of mutations affecting DNA dynamics and metabolism on ATR activation. *In vitro* reconstitution experiments are necessary to differentiate direct from indirect effects and to define the ATR checkpoint at a mechanistic level.

In yeast, experiments with purified proteins led to reconstitution of an *in vitro* system consisting of primed-DNA + RPA + Mec1/Ddc2 + Rad24-RFC/Ddc1-Rad17-Mec3 combination with RPA and Rad53 (Chk1/Chk2 ortholog) as substrates (49). This system closely recapitulated the Mec1 signaling pathway,

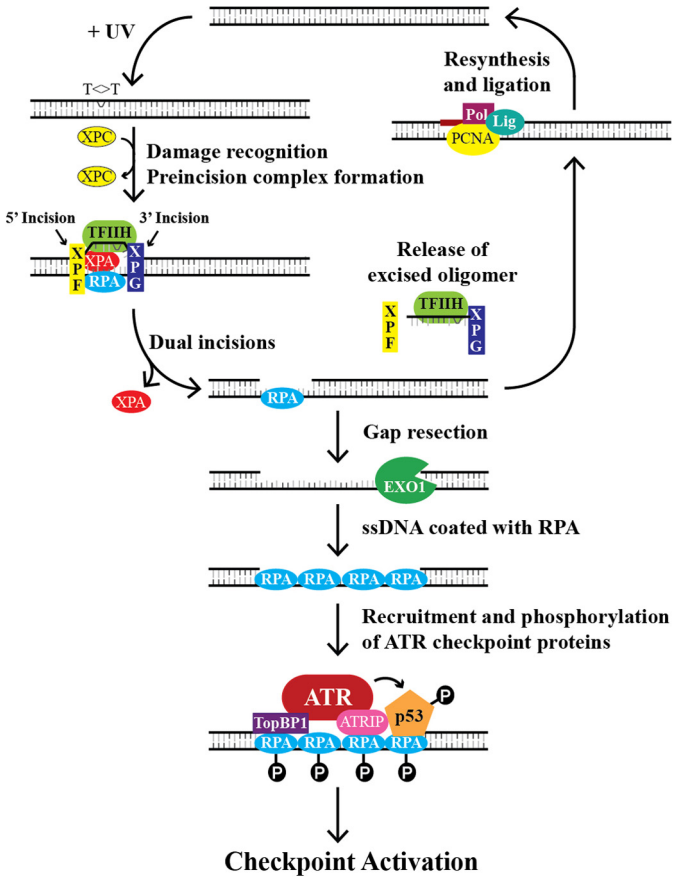

FIGURE 7. **Model of activation of the ATR-signaling pathway during G_1/G_0 or G_2-M phases of the cell cycle.** When DNA is damaged by UV or a UV-mimetic agent, the core excision repair factors (RPA, XPA, XPC, TFIIH, XPG, XPF-ERCC1) excise a ~30 nt oligomer containing the damage. (Note that XPC, after playing an essential role in damage recognition, dissociates from the repair complex prior to the dual incisions (1) (18), and hence it is not shown in the incision complex.) The resulting gap is either filled in by polymerases or the gap is enlarged by EXO1. The enlarged ssDNA gap is coated with RPA, which recruits ATR-ATRIP, TopBP1, and substrates including p53. The close proximity of the ATR kinase with its activator, TopBP1, and substrate, p53, results in phosphorylation and checkpoint activation.

but was independent of Dpb11 (TopBP1 ortholog). Dpb11 does activate Mec1/Ddc2 in yeast (55, 56), but does not appear to play the essential role that human TopBP1 has in ATR activation (43, 57) as it is functionally redundant with Ddc1 and Dna2 (58–60).

In humans, partial reconstitution reactions have been reported with ATR-ATRIP + DNA (5); ATR-ATRIP + ssDNA + RPA (22); and ATR-ATRIP + TopBP1 + ssDNA + RPA ± Claspin with substrates that included RPA, Chk1, and p53 (23, 29, 37, 61–64). In addition to these systems with purified proteins, a number of other *in vitro* systems with cell-free extracts have been reported (28, 34–36, 65). However, because of the limitations of cell-free extracts to unambiguously assign functions to specific proteins, their utility in defining the ATR checkpoint is also limited and therefore those systems will not be taken into consideration in formulating a mechanistic model for ATR checkpoint. In this report we have described a system encompassing purified nucleotide excision repair factors, purified checkpoint proteins, an exonuclease (EXO1) that couples the two pathways along with DNA damaged by a UV-mimetic agent and appropriate substrates for checkpoint signaling. We have demonstrated that the activation of the signaling pathway is dependent on all of these components. Note, that while other nucleases, such as *E. coli* EXOIII, can substitute for EXO1 (data not shown) to generate the ssDNA to activate ATR *in vitro* as in Fig. 2*A*, the *in vivo* data in Fig. 2*B* indicate that EXO1 is the most physiologically relevant nuclease for gap enlargement in the cell. Therefore, we propose that the following constitutes the minimal essential set of factors for ATR checkpoint signaling (Fig. 7): Signal: DNA damaged by UV or a UV-mimetic agent; Core Excision Repair Factors: RPA, XPA, XPC, TFIIH, XPG, XPF-ERCC1; Core ATR Checkpoint Factors: ATR-ATRIP, TopBP1, RPA; Excision Repair-Checkpoint Coupling Factor: EXO1. This minimal set of factors is sufficient to enable ATR to phosphorylate RPA and p53 without the need for additional proteins.

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