

Adeno-Associated Virus Replication Induces a DNA Damage Response Coordinated by DNA-Dependent Protein Kinase[∇]

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The parvovirus adeno-associated virus (AAV) contains a small single-stranded DNA genome with inverted terminal repeats that form hairpin structures. In order to propagate, AAV relies on the cellular replication machinery together with functions supplied by coinfecting helper viruses such as adenovirus (Ad). Here, we examined the host cell response to AAV replication in the context of Ad or Ad helper proteins. We show that AAV and Ad coinfection activates a DNA damage response (DDR) that is distinct from that seen during Ad or AAV infection alone. The DDR was also triggered when AAV replicated in the presence of minimal Ad helper proteins. We detected autophosphorylation of the kinases ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and signaling to downstream targets SMC1, Chk1, Chk2, H2AX, and XRCC4 and multiple sites on RPA32. The Mre11 complex was not required for activation of the DDR to AAV infection. Additionally, we found that DNA-PKcs was the primary mediator of damage signaling in response to AAV replication. Immunofluorescence revealed that some activated damage proteins were found in a pan-nuclear pattern (phosphorylated ATM, SMC1, and H2AX), while others such as DNA-PK components (DNA-PKcs, Ku70, and Ku86) and RPA32 accumulated at AAV replication centers. Although expression of the large viral Rep proteins contributed to some damage signaling, we observed that the full response required replication of the AAV genome. Our results demonstrate that AAV replication in the presence of Ad helper functions elicits a unique damage response controlled by DNA-PK.

Replication of viral genomes produces a large amount of extrachromosomal DNA that may be recognized by the cellular DNA damage machinery. This is often accompanied by activation of DNA damage response (DDR) signaling pathways and recruitment of cellular repair proteins to sites of viral replication. Viruses therefore provide good model systems to study the recognition and response to DNA damage (reviewed in reference 48). The Mre11/Rad50/Nbs1 (MRN) complex functions as a sensor of chromosomal DNA double-strand breaks (DSBs) and is involved in activation of damage signaling (reviewed in reference 41). The MRN complex also localizes to DNA DSBs and is found at viral replication compartments during infection with a number of DNA viruses (6, 40, 47, 70, 75, 77, 87, 93). The phosphatidylinositol 3-kinase-like kinases (PIKKs) ataxia telangiectasia mutated (ATM), ATM and Rad3-related kinase (ATR), and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) are involved in the signal transduction cascades activated by DNA damage (reviewed in references 43, 51, and 71). These kinases respond to distinct types of damage and regulate DSB repair during different phases of the cell cycle (5), either through nonhomologous end-joining (NHEJ) or homologous recombination pathways (reviewed in references 63, 81, and 86). The DNA-PK holoenzyme is composed of DNA-PKcs and two

regulatory subunits, the Ku70 and Ku86 heterodimer. DNA-PK functions with XRCC4/DNA ligase IV to repair breaks during NHEJ, and works with Artemis to process DNA hairpin structures during VDJ recombination and during a subset of DNA DSB events (46, 50, 86). While the kinase activity of DNA-PKcs leads to phosphorylation of a large number of substrates *in vitro* as well as autophosphorylation of specific residues (reviewed in references 16 and 85), it is currently unclear how DNA-PKcs contributes to signaling in cells upon different types of damage.

The adeno-associated virus (AAV) genome consists of a molecule of single-stranded DNA with inverted terminal repeats (ITRs) at both ends that form double-hairpin structures due to their palindromic sequences (reviewed in reference 52). The ITRs are important for replication and packaging of the viral genome and for integration into the host genome. Four viral Rep proteins (Rep78, Rep68, Rep52, and Rep40) are also required for replication and packaging of the AAV genome into virions assembled from the Cap proteins. Although the Rep and Cap genes are replaced in recombinant AAV vectors (rAAV) that retain only the ITRs flanking the gene of interest, these vectors can be replicated by providing Rep *in trans* (reviewed in reference 7). Productive AAV infection requires helper functions supplied by adenovirus (Ad) or other viruses such as herpes simplex virus (HSV) (reviewed in reference 27), together with components of the host cell DNA replication machinery (54, 55, 58). In the presence of helper viruses or minimal helper proteins from Ad or HSV, AAV replicates in the nucleus at centers where the viral DNA and Rep proteins accumulate (35, 76, 84, 89). Cellular and viral proteins involved in AAV replication, including replication protein A

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(RPA), Ad DNA-binding protein (DBP), and HSV ICP8, localize with Rep proteins at these viral centers (29, 33, 76).

A number of published reports suggest associations between AAV and the cellular DNA damage machinery. For example, transduction by rAAV vectors is increased by genotoxic agents and DNA damaging treatments (1, 62, 91) although the mechanisms involved remain unclear. Additionally, the ATM kinase negatively regulates rAAV transduction (64, 92), and we have shown that the MRN complex poses a barrier to both rAAV transduction and wild-type AAV replication (11, 67). UV-inactivated AAV particles also appear to activate a DDR involving ATM and ATR kinases that perturbs cell cycle progression (39, 60, 88). It has been suggested that this response is provoked by the AAV ITRs (60) and that UV-treated particles mimic stalled replication forks in infected cells (39). In addition to AAV genome components, the viral Rep proteins have been observed to exhibit cytotoxicity and induce S-phase arrest (3, 65).

The role of cellular repair proteins in AAV genome processing has also been explored by examining the molecular fate of rAAV vectors, which are converted into circular and concatemeric forms that persist episomally (18, 19, 66). Proteins shown to regulate circularization in cell culture include ATM and the MRN complex (14, 64), while *in vivo* experiments using mouse models have implicated ATM and DNA-PK in this process (14, 20, 72). Additionally, DNA-PKs and Artemis have recently been shown to cleave the ITR hairpins of rAAV vectors *in vivo* in a tissue-dependent manner (36). Despite these studies, it is not clear how damage response factors function together and how they impact AAV transduction and replication in human cells.

In this study we examined the cellular response to AAV replication in the context of Ad infection or helper proteins. We show that coinfection with AAV and Ad activates a DDR that is distinct from that seen during infection with Ad alone. The ATM and DNA-PKs damage kinases are activated and signal to downstream substrates, but the response does not require the MRN complex and is primarily mediated by DNA-PKs. Although expression of the large Rep proteins induced some DDR events, full signaling appeared to require AAV replication and was accompanied by accumulation of DNA-PK at viral replication compartments. Our results demonstrate that AAV replication induces a unique DNA damage signal transduction response and provides a model system for studying DNA-PK.

MATERIALS AND METHODS

Plasmids and transfections. The wild-type full-length AAV type 2 genome was supplied by the plasmid pNTC244 (12), and virus was produced by transfection in 293T cells. For production of rAAV vectors, 293T cells were transfected with three plasmids: pXX2, which supplied Rep and Cap proteins (90); pXX6, which contained the Ad helper functions (90); and the vector plasmid pAAV.GFP in which green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter is cloned between viral ITRs. The Ad helper proteins Ad-DBP and E4orf6 were expressed from the CMV promoter in expression vectors pRK5 or pcDNA3.1 (Clontech). The FLAG-Rep constructs were previously described (59) and were provided by M. Giacca. Rep78 was expressed under the control of the CMV promoter in the pcDNA3.1 plasmid (8). pGL2- and pGL3-based plasmids containing the AAV ITR, p5 promoter, or both elements have been previously described (9). Subconfluent monolayers of cells were transfected by calcium phosphate precipitation according to standard protocols or with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Cell lines and drug treatments. HeLa, U2OS, and 293 cells were purchased from the American Tissue Culture Collection. A stable cell line derived from HeLa cells that expresses wild-type E1b55K from a retrovirus vector has been described previously (6). A-TLD1 cells and complemented counterparts were previously described (6). NBS cells and complemented counterparts (10) were from P. Concannon. The HCT116-based cell lines (lacking DNA-PKs or expressing one copy of DNA-PKs) were provided by E. Hendrickson (61). A-T cells and their complemented counterparts were provided by Y. Shiloh. U2OS cells expressing inducible FLAG-tagged ATR that was kinase dead or wild-type were previously described (57) and were provided by S. Schreiber. All cells, except the MO59J fusion cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 or 20% fetal bovine serum (FBS) and penicillin/streptomycin, with appropriate selection at 37°C in a humidified atmosphere containing 5% CO₂. MO59J fusion cells, Fus9 (lacking DNA-PKs) and Fus1 (expressing DNA-PKs), were previously described (34) and were provided by T. Melendy. MO59J fusion cells were maintained in a 1:1 mixture of F10 medium and DMEM supplemented with 10% FBS and penicillin/streptomycin. Where indicated in the figure legends, cells were treated with the ATM inhibitor KU55933, the DNA-PK inhibitor NU7026 (both purchased from Calbiochem and used at a final concentration of 10 μM), or an equal volume of dimethyl sulfoxide (DMSO). Cells were pretreated for 1 h with inhibitors or DMSO before infection and for the duration of infection.

Viruses and infections. Ad serotype 5 (Ad5) was propagated on 293 cells, purified, and titrated as previously described (6). Wild-type and rAAV vector rAAV.GFP were produced in 293T cells and purified as previously described (30, 90). All AAV titers were determined by quantitative PCR using SYBR Green I double-stranded DNA binding dye and an ABI Prism 7700 Sequence detection system (PE Biosystems). All infections were performed on monolayers of cultured cells in DMEM supplemented with 2% FBS. After 2 h at 37°C, infection medium was replaced with DMEM with 10% or 20% FBS. Infections of Fus cells were performed in a 1:1 mixture of F10 medium and DMEM. Multiplicities of infection (MOIs) are detailed in the figure legends.

Antibodies, immunofluorescence, and immunoblotting. Commercially available antibodies used in this study were purchased from Abcam (anti-RPA32 phosphorylated at S33 [RPA32-P-S33] and RPA32-P-T21), American Research Products, Inc. (Rep clone 303.9), Bethyl (RPA32-P-S4/S8, and Chk1-P-S317), Cell Signaling (Chk2-P-T68 and Chk1-P-S345), Epitomics (ATM), Genetex (Mre11-12D7 and Rad50-13B3), NeoMarkers (DNA-PKs and Ku70), Novus (Nbs1), Research Diagnostics Inc. (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]), Rockland (ATM phosphorylated at S1981 [ATM-P-S1981] and SMC1 phosphorylated at S957 [SMC1-P-S957]), Santa Cruz (Ku86, ATR, and Chk2), Serotech (XRCC4), Sigma (FLAG-M2), and Upstate (γ-H2AX). The other primary antibodies used in this study were the following: Rep (rabbit polyclonal, a gift from J. Trempe), Rep (IF11, mouse monoclonal; a gift from J. Samulski), Ad-DBP (mouse monoclonal, a gift from A. Levine; and rabbit polyclonal, a gift from P. van der Vliet), E1b55K (mouse monoclonal B-6, a gift from A. Levine), RPA32 (mouse monoclonal, a gift from T. Melendy), and DNA-PK-P-S2056 (DNA-PK phosphorylated at S2056; rabbit polyclonal; a gift from B. Chen). Secondary antibodies for immunofluorescence were coupled to Alexa fluorophores (Invitrogen). Cells grown on coverslips in 24-well plates were processed for immunofluorescence as previously described (6, 75). Images were obtained on a Nikon microscope in conjunction with a charge-coupled-device camera (Cooke Sensicam) in double or triple excitation mode and processed using SlideBook and Adobe Photoshop. Immunoblotting was performed as previously described (6).

RESULTS

AAV and Ad coinfection induces an MRN-independent DDR. We examined the cellular DDR to replicating AAV in the context of Ad helper virus. U2OS cells were infected with AAV and Ad, either alone or in combination (Fig. 1). Phosphorylation of damage response proteins was detected by immunoblotting (Fig. 1A) and visualized by immunofluorescence (Fig. 1B). Infections were confirmed by immunoblotting with antibodies to the DBP of Ad and Rep proteins of AAV. We have previously demonstrated that wild-type Ad5 infection does not induce significant signaling by the cellular damage machinery due to inactivation of the MRN complex, a sensor of DNA DSBs (6). Infection with Ad or AAV alone did not

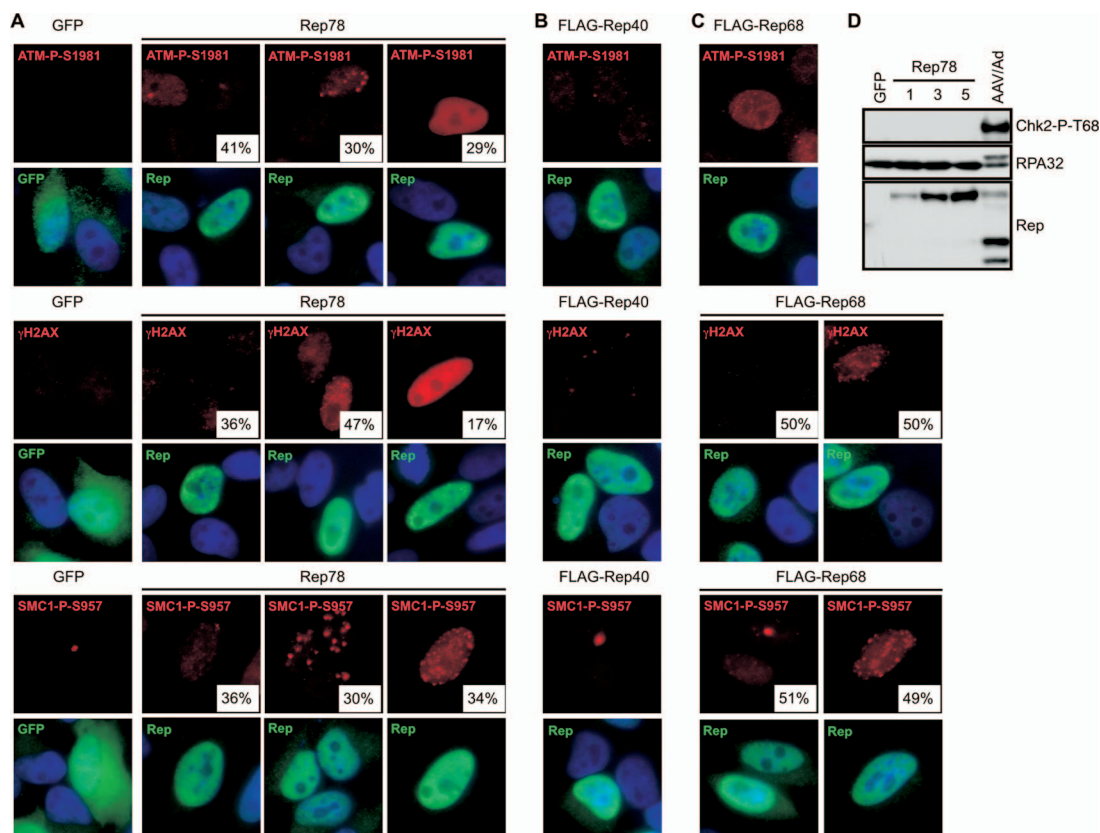


FIG. 2. The effects of Rep proteins on DNA damage signaling. HeLa cells were transfected with plasmids encoding GFP or Rep proteins. Cells were fixed for immunofluorescence approximately 36 h posttransfection and stained with the indicated antibodies to mark activated DDR proteins or Rep and with DAPI (4',6'-diamidino-2-phenylindole) to stain the nuclei. At least 100 cells were quantitated for the indicated percentages of each phenotype. (A) Rep78 activates DNA damage signaling. (B) FLAG-Rep40 does not activate damage signaling. (C) Damage signaling during FLAG-Rep68 expression. (D) Rep78 does not induce downstream signaling. HeLa cells were transfected with a plasmid encoding GFP or increasing amounts of a plasmid encoding Rep78. Total DNA transfected was the same between samples and was made up with pRK5. Cells were harvested 48 h posttransfection and processed for immunoblotting with the indicated antibodies. Coinfection with AAV and Ad served as a positive control.

ected to express either Rep78 or GFP as a control. DNA damage signaling was analyzed by immunoblotting of cell lysates (Fig. 3A). Phosphorylation of Chk2 and RPA32 was not detected in cells expressing Rep78 or in cells infected with rAAV alone in the presence or absence of Rep expression. Although Ad5 infection alone did not activate damage signaling, Rep78 expression in the presence of Ad5 induced a low level of phosphorylation on Chk2 and RPA32. This may reflect Rep interference with Ad replication (38, 84) or replication of human sequences mediated by Rep protein (79). However, robust DNA damage signaling was observed when rAAV was infected in the presence of Rep78 and Ad5, a condition sufficient to support rAAV replication. Phosphorylation of Chk2 and RPA32 was similar to that observed during wild-type AAV and Ad coinfection, suggesting that replication of the AAV genome may elicit the damage response.

AAV replication can be achieved by transfection of an infectious clone of the AAV genome together with the minimal helper genes from Ad (DBP, E1b55K, and E4orf6 genes) (67, 76). To examine the requirements for damage signaling in more detail, we used immunofluorescence to analyze AAV replication with this minimal helper system (Fig. 3B). A HeLa-

derived cell line expressing wild-type E1b55K (6) was transfected with plasmids expressing Ad helpers DBP and E4orf6 and a plasmid encoding the AAV genome, pNTC244 (12). Expression of the helper proteins alone in the E1b55K cell line did not activate a damage response (data not shown). However, cells that contained discrete AAV replication centers displayed phosphorylation of ATM, H2AX, SMC1, and RPA32 (Fig. 3B). Some cells with smaller Rep compartments had weaker staining for phosphorylated ATM. Phosphorylated RPA32 was found colocalized with AAV centers except at late stages, when RPA32-P-S33 staining was spread throughout the nucleoplasm to a greater degree. Together, our data indicate that the damage signaling observed during AAV and Ad coinfection is also activated with minimal helper-mediated AAV replication and demonstrate that replication of the AAV genome is sufficient to activate the cellular DDR.

DNA-PK mediates phosphorylation of damage response proteins during AAV and Ad coinfection. To determine the role of individual PIKKs in virus-induced damage signaling, we examined AAV and Ad coinfections in mutant cell lines and in the presence of chemical inhibitors. Infections were first carried out in ATM-deficient A-T cells and their complemented

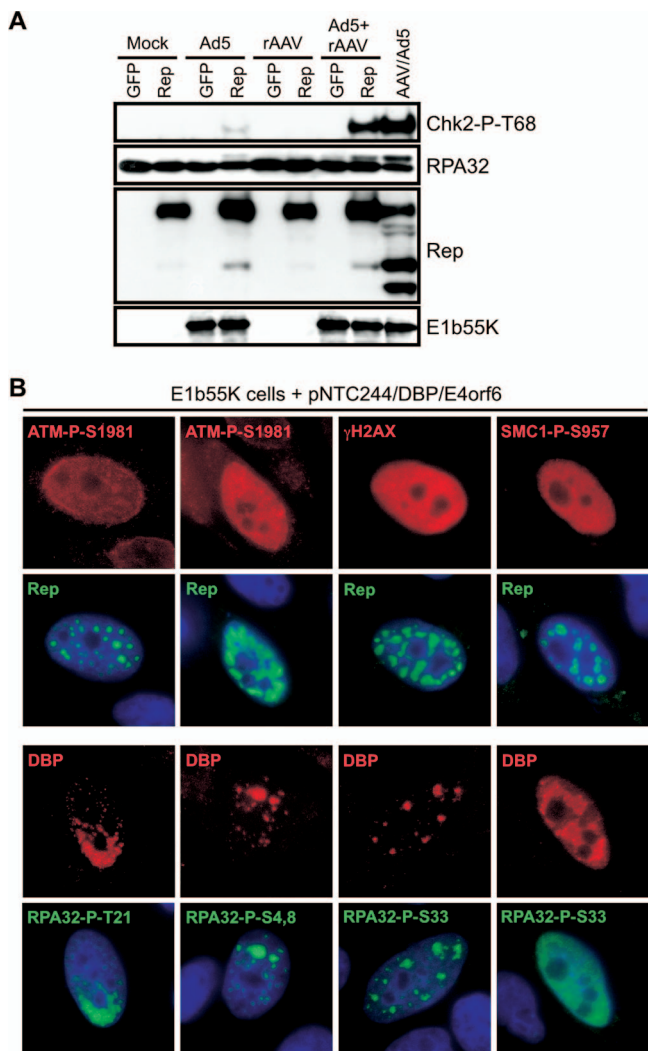


FIG. 3. AAV replication induces a DNA damage response. (A) HeLa cells were either uninfected (Mock) or infected with Ad5 (MOI of 50), rAAV-GFP (MOI of 1,000), or a combination of both viruses. At 6 h postinfection, cells were transfected with plasmids encoding GFP or Rep78 and harvested 30 h posttransfection. Lysates were processed for immunoblotting against the indicated proteins. Coinfection with wild-type AAV and Ad5 served as a positive control. (B) HeLa cells transduced to express E1b55K were transfected with pNTC244 and plasmids expressing DBP and E4orf6. At approximately 36 h posttransfection, cells were fixed for immunofluorescence and stained with the indicated antibodies. AAV replication centers are marked by DBP or Rep. DAPI (4',6'-diamidino-2-phenylindole) was used to mark cell nuclei.

counterparts (Fig. 4A). In A-T cells, we observed that coinfection induced phosphorylation of SMC1, XRCC4, Chk2, and RPA32, indicating that the signaling was not dependent upon ATM. This conclusion was supported by treatment of matched ATM-expressing cells with a small molecule inhibitor of ATM, which only minimally affected most signaling events (Fig. 4A and data not shown). Phosphorylation of most of these substrates was decreased in the presence of a DNA-PK inhibitor, implicating a role for DNA-PKcs in the signaling response to AAV replication. We next examined the role of ATR in cells expressing doxycycline-inducible wild-type or kinase-dead ATR

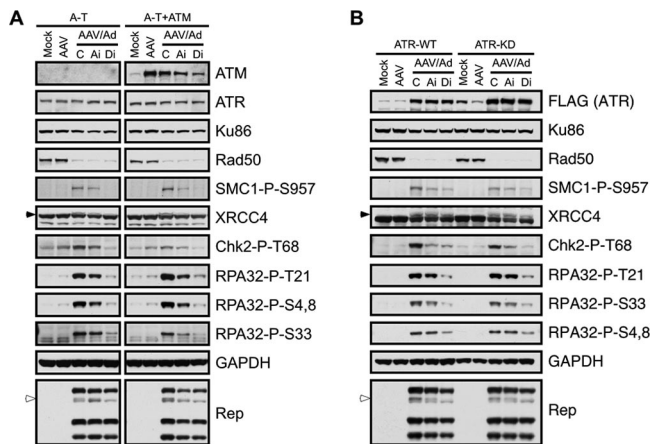


FIG. 4. ATM and ATR are not the kinases predominantly responsible for signaling in response to AAV and Ad coinfection. Immunoblotting was used to analyze AAV-induced damage signaling. Cells with individual PIKKs inactivated were either uninfected (Mock) or infected with AAV (MOI of 2,000) or AAV and Ad (MOI of 25). Infections were performed in the presence of DMSO as a control (C) or inhibitors to ATM (Ai) and DNA-PKcs (Di). Mock- and AAV-infected cells were also treated with DMSO. Cells were harvested 24 h postinfection and processed for immunoblotting with the indicated antibodies. (A) Infections in A-T cells or a matched line complemented with ATM. Right- and left-hand panels were from the same gels, and lanes not relevant to these results were removed from the figure. (B) Infections in cells expressing inducible ATR that is wild-type (ATR-WT) or kinase-dead (ATR-KD). Cells were induced to express ATR by doxycycline treatment for 48 h before infections, which then proceeded for a further 24 h. The inducible ATR protein is tagged with a FLAG epitope. GAPDH served as a loading control. The open arrowheads indicate a slower-migrating band for Rep68, and the filled arrowheads highlight specific phosphorylated proteins.

that acts as a dominant-negative mutant (57). Phosphorylation of SMC1, XRCC4, Chk2, and RPA32 was observed after AAV and Ad coinfection even in the presence of kinase-dead ATR (Fig. 4B), suggesting that ATR is not absolutely required. In further support of this conclusion, we found that caffeine treatment of A-T cells at a concentration that inhibits both ATM and ATR (2.5 mM) had a minimal effect on the DDR during AAV and Ad coinfection (data not shown). Although treatment of kinase-dead ATR cells with the ATM inhibitor slightly diminished some phosphorylation events, they were more drastically affected by the DNA-PK inhibitor. In these experiments we obtained inconsistent results for Chk1 phosphorylation and were therefore unable to conclude which PIKK activated Chk1. In addition to the phosphorylation of DNA damage substrates, we observed slower-migrating forms of the larger Rep proteins. This was most discernible with Rep68, which appeared as a doublet (Fig. 4A and B). In the presence of the DNA-PK inhibitor, this form of Rep68 was lost, with no major effect on the total Rep levels. Together, these data suggest that DNA-PK may be the predominant PIKK responsible for the DDR to AAV replication and that Rep may also be a phosphorylation substrate.

To investigate further the dependence on DNA-PK, we infected two different DNA-PKcs-deficient cell lines with both viruses and analyzed AAV-induced damage signaling by immunoblotting. We first compared signaling between two MO59J-derived cell lines that lack or express DNA-PKcs (34)

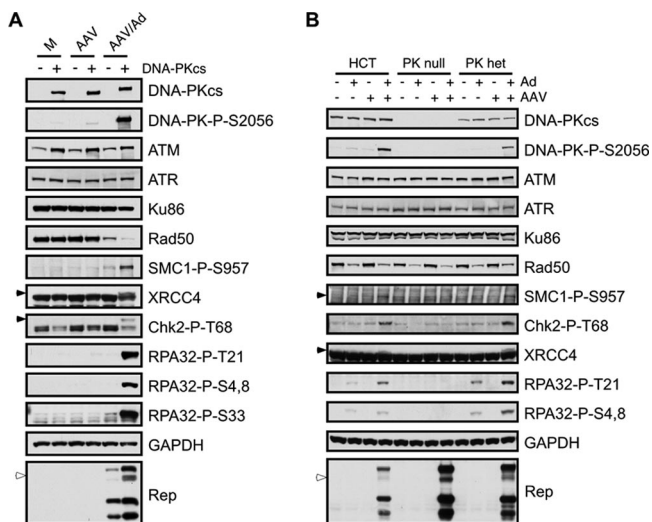


FIG. 5. DNA-PKcs is required for signaling in response to AAV and Ad coinfection. Immunoblotting was used to analyze damage signaling to AAV and Ad coinfection in cells that lack DNA-PKcs. Cells were either uninfected or infected with AAV (MOI of 1,000 to 2,000), Ad (MOI of 25), or both viruses and harvested 24 h postinfection. Lysates were processed for immunoblotting with the indicated antibodies. The open arrowheads indicate a slower-migrating band for Rep68, and the filled arrowheads highlight specific phosphorylated proteins. (A) Infections in M059J-derived cells lines that lack DNA-PKcs (Fus9) or express the kinase (Fus1). (B) Infections in HCT-derived cells that are heterozygous or null for DNA-PKcs.

and found that most phosphorylation events during AAV and Ad coinfection were dependent on this kinase (Fig. 5A). Minimal signaling was detected in the absence of DNA-PKcs and during infections with AAV or Ad alone. Additionally, we noted the activation of DNA-PKcs, as measured by a phospho-specific antibody against S2056 (13). Since levels of ATM were slightly lower in the cells that lacked DNA-PKcs (34) (Fig. 5A), we also tested damage signaling in another set of matched DNA-PKcs-deficient cells. We analyzed infections from HCT116-derived cells in which DNA-PKcs was knocked out using homologous recombination to generate cells heterozygous or null for the kinase (61). Immunoblotting of lysates from these cells and the parental HCT116 cells confirmed that AAV and Ad coinfection induced damage signaling mediated by DNA-PKcs (Fig. 5B). Interestingly, one copy of DNA-PKcs was sufficient for this response (Fig. 5B, PK het lanes). We also observed that the doublet of Rep68 was lost in both sets of DNA-PKcs-deficient cells (Fig. 5), further suggesting that Rep is a likely target of this kinase. These data confirm that DNA-PKcs is activated and induces many of the signaling events during the response to AAV and Ad coinfection.

We next attempted to determine how DNA-PK activation affects AAV using these two sets of DNA-PKcs-deficient cells. However, we obtained inconsistent results between the mutant cell lines when we measured both rAAV transduction and wild-type AAV replication (data not shown). Additionally, we found that treatment of cells with the small-molecule DNA-PK inhibitor did not affect levels of Rep proteins (Fig. 4) or viral replication (data not shown). While it is unclear from these data how DNA-PK affects AAV transduction and replication,

it is evident that DNA-PKcs is activated and induces most of the signaling events during AAV and Ad coinfection.

DNA-PK localizes to AAV replication centers. Given the involvement of DNA-PK in the damage response to AAV, we next examined the intracellular localization of holoenzyme components (DNA-PKcs, Ku70, and Ku86) during AAV replication. Immunofluorescence of mock-treated cells showed that all three proteins were located diffusely throughout the nucleus, and this pattern was unaffected by infections with Ad or AAV alone (Fig. 6A). In contrast, during AAV and Ad coinfection, the DNA-PKcs, Ku70, and Ku86 proteins all became concentrated at Rep-staining compartments (Fig. 6A). The dramatic redistribution of DNA-PK components to AAV replication centers was also observed under minimal replication conditions induced by transfection of the AAV genome and Ad helper proteins (DBP and E4orf6) into wild-type E1b55K protein-expressing cells (Fig. 6B). Additionally, we noted that autophosphorylated DNA-PKcs localized with DBP at the AAV replication compartments. These data suggest that AAV replication centers serve as a signal for DNA-PK recognition and activation during the DDR.

Since AAV replication promoted DNA-PK holoenzyme relocalization, we further examined the requirements for recruitment of these proteins. Previously, it was shown that plasmids containing an AAV ITR or p5 element can establish replication centers in the presence of Rep and HSV proteins (28). To test whether these AAV elements are sufficient to induce DNA-PK localization, we transfected 293 cells with luciferase-based plasmids containing an ITR, p5 promoter, or both components (9) in the presence of Rep and Ad helpers. DNA-PK component accumulation at replication centers was examined by immunofluorescence. When 293 cells were transfected to express Rep, DBP, and E4orf6 alone, there was no effect on DNA-PK distribution (data not shown). However, when replication centers were established using plasmids containing the ITR origin, we found that DNA-PKcs, Ku70, and Ku86 all localized to these compartments (Fig. 7 and data not shown). Although we found the p5 promoter to be a much less efficient origin for Rep-mediated replication, any replication centers that were formed with this plasmid also showed costaining for DNA-PK components. These data demonstrate that replication centers established with AAV *cis* sequences are sufficient to recruit the DNA-PK holoenzyme.

DISCUSSION

In this study we examined the cellular response to replicating AAV and found that a robust cellular DDR was induced. Immunoblotting and immunofluorescence demonstrated that a number of DNA damage substrates, including ATM, DNA-PK, Chk1, Chk2, SMC1, and H2AX, were strongly phosphorylated during AAV and Ad coinfection and during AAV replication with minimal Ad helper proteins. Under these conditions, we found that the majority of signaling events were dependent on DNA-PK rather than ATM and ATR. Additionally, the damage response was independent of the MRN complex. This latter observation was surprising, given the requirement for MRN in signaling elicited by mutant Ad infection (6), HSV-1 infection (47), and chromosomal DSBs (6, 41, 80). In the situations where MRN is required, ATM orchestrates dam-

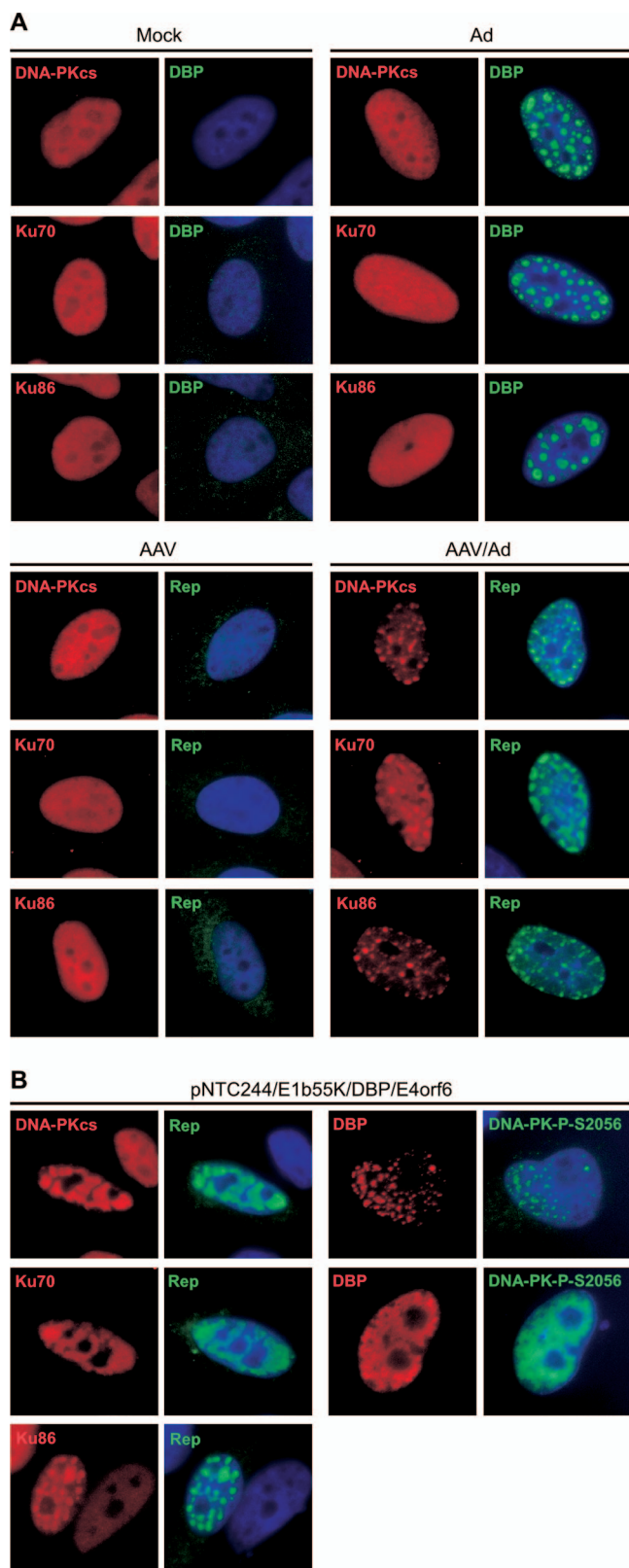


FIG. 6. DNA-PK components localize to AAV replication centers. (A) DNA-PK components localize to viral replication centers during AAV and Ad coinfection. HeLa cells were uninfected (Mock) or infected with Ad5 (MOI of 25), AAV (MOI of 1,000), or both viruses for 24 h before fixing for immunofluorescence. Cells were stained with

age signaling, whereas we found that in our experiments DNA-PK was the primary kinase mediating the DDR during AAV replication. Interestingly, H2AX is also phosphorylated by DNA-PKcs after DSBs, but only when MRN and ATM are absent (22, 74). MRN and/or ATM may therefore potentially influence DNA-PK signaling to certain substrates. This may be particularly relevant to AAV since ATM is known to have a negative effect on rAAV (64, 92), and MRN negatively impacts AAV transduction and replication (11, 67). Under Ad helper conditions where MRN is present at AAV centers (11, 67), we observed differences in the accumulation of repair factors at AAV centers (R. A. Schwartz and M. D. Weitzman, unpublished data), suggesting that MRN may impact the organization of viral replication compartments. Taken together, these results suggest that the cellular response to AAV replication may be affected by several DNA damage response regulators, including MRN, ATM, and DNA-PK.

In our experiments we also detected the DNA-PK-dependent phosphorylation of RPA32 on multiple sites and localization of these activated forms at AAV centers. RPA, replication factor C (RFC), PCNA, minichromosome maintenance proteins, and DNA polymerase δ have been previously shown to be required for AAV replication in vitro and in cells (54, 55, 58). Interestingly, RPA phosphorylation excludes it from cellular sites of replication (25, 82) and may alter its DNA affinity in order to promote repair (23). We have also previously shown that Rep can bind RPA (76) although we do not know how this interaction may be affected by phosphorylation. In addition to RPA32, it is possible that other cellular replication proteins are modified during the AAV-induced DDR. Cellular minichromosome maintenance proteins are phosphorylated during DNA damage (17, 37, 68) while RFC2/RFC4 and PCNA can become ubiquitinated (44, 78). It will be interesting to determine how AAV-induced signaling impacts these replication factors and their function during productive AAV replication.

We found that activated DNA-PKcs, Ku70, and Ku86 all localized to AAV centers during infection and to compartments formed during Rep-mediated replication of AAV origins (p5 and the ITR), further supporting the role of DNA-PK as a key responder to AAV replication. These data suggest that AAV *cis* sequences may provide a signal for recognition by DNA-PK, perhaps through the formation of DNA hairpin structures, which are known to activate DNA-PK (50, 85). Protein-protein interactions could also contribute to the recruitment or maintenance of DNA-PK components at viral centers. Indeed, recent proteomic studies identified the Ku70/Ku86 complex and DNA-PKcs as Rep-interacting proteins (56, 59). Additionally, RPA and other proteins that localize to AAV centers may help to stabilize DNA-PK at AAV compartments. This observation is consistent with DNA-PK accumu-

the indicated antibodies. Viral replication centers are detected with antibodies to DBP and Rep. DAPI (4',6'-diamidino-2-phenylindole) marks the cell nuclei. (B) HeLa cells expressing E1b55K were transfected with pNTC244, DBP, and E4orf6 expression plasmids for approximately 36 h before cells were fixed for immunofluorescence. Viral Rep centers and DAPI staining are as described in panel A.

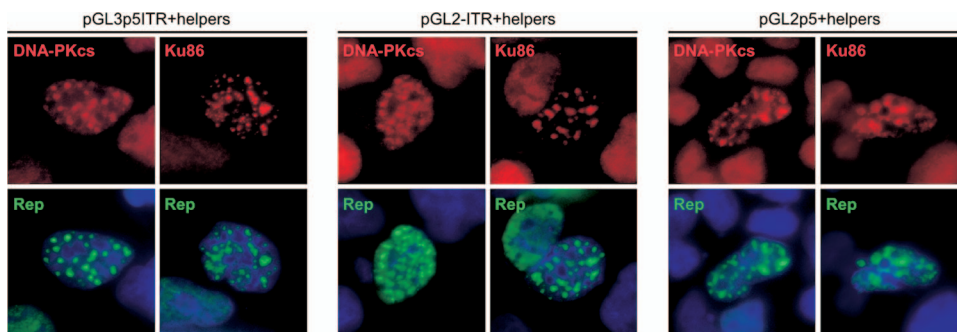


FIG. 7. DNA-PK is recruited to Rep-mediated replication compartments. 293 cells were transfected with pGL-based plasmids containing the 5' ITR (pGL2-ITR), the p5 promoter (pGL3-p5), or both elements (pGL3-p5ITR) in the presence and absence (not shown) of E4orf6, DBP, and Rep78 expression plasmids. Cells were fixed approximately 36 h posttransfection and processed for immunofluorescence with the indicated antibodies. Rep marks replication centers induced by all pGL-based plasmids, and DAPI (4',6'-diamidino-2-phenylindole) stains the cell nuclei.

lation at HSV-1 centers via either the viral ICP8 protein or RPA (77, 87). We did not observe DNA-PK localization at Ad replication centers for wild-type or mutant virus (Schwartz and Weitzman, unpublished data), which was surprising given the involvement of DNA-PK in concatemerization of mutant Ad genomes (4, 75). It is possible that association with Ad centers is masked under our immunofluorescence conditions or is too transient to detect in these experiments. Based on our data, recruitment of DNA-PK appears to be specific to AAV replication centers and may be due to a combination of AAV structures and protein interactions.

Multiple viral factors are likely responsible for activation of the DDR during AAV replication. Consistent with a previous report (3), we found that expression of the larger Rep proteins resulted in some damage signaling, perhaps due to their DNA nicking capabilities. However, we also saw that damage signaling was stronger during AAV replication, implying that Rep expression cannot account for all of the DDR events. AAV ITRs have single-strand/double-strand DNA junctions that could resemble stalled replication forks, and it has been suggested that AAV induces a DDR that mimics these structures (39). Although AAV ITRs have been proposed to be sufficient to activate a DDR (60), subsequent work demonstrated that rAAV vectors do not provoke this response (24). Instead, wild-type AAV, UV-inactivated particles, or rAAV genomes with a p5 element were required to induce damage signaling (24). These previous reports have mainly involved very high MOIs (MOIs of 10,000 to 20,000) in the absence of helper proteins (24, 39, 60). In accordance with these studies, we observed that wild-type AAV infection alone resulted in some H2AX phosphorylation even at a much lower MOI. Since damage signaling was far more robust during viral replication, the full DDR is most likely activated in response to a combination of Rep functions, ITRs, and replication intermediates. We detected the damage phosphorylation events with minimal helper proteins in the absence of the E1a gene, and therefore this Ad helper protein is not directly required for the response to replicating AAV. Since E1a activates the p5 promoter to induce Rep expression and thus increase replication (52), the response is likely to be quantitatively increased in the presence of all the Ad helper functions. During natural infection, the MOI of AAV may be lower than that used in our experiments, and only the minimal response will be activated. In the absence

of helper functions, the virus will integrate in to the host genome (52), and it is likely that the cellular DNA repair machinery will play an important role in integration.

During the course of our analysis of the AAV-induced DDR, we noticed altered migration of the large Rep proteins. Our results are consistent with other reports showing that all Rep proteins are phosphorylated during AAV and Ad coinfection (15). Although the kinases involved and the sites they modify on Rep are largely unknown, Rep phosphorylation has been correlated with decreased DNA binding and AAV replication (15, 53). Our data suggest that DNA-PKcs may mediate modification of the large Rep proteins. This result draws an interesting parallel to the simian virus 40 (SV40) large tumor antigen, which has similar functions to Rep78/68 during SV40 replication and is phosphorylated by ATM during infection (69). Phosphorylation by DNA-PK also inactivates large tumor antigen during *in vitro* SV40 DNA replication (83). The preferred target sites for phosphorylation by PIKKs are SQ and TQ motifs. The large Rep proteins share five of these sites while Rep78 contains one additional C-terminal SQ motif. It will be interesting to determine whether these sites are modified by DNA-PKcs during AAV replication and what impact this has on Rep functions.

In addition to AAV, DNA-PK has been previously implicated in the cellular response to infection by a number of other viruses (48). Some viral proteins subvert DNA-PK repair functions, such as the Tax oncoprotein of human T-cell leukemia virus type 1 (21) and the ICP0 protein of HSV-1 which degrades DNA-PKcs (45). The Ad E4orf6 and E4orf3 have also been reported to bind DNA-PK and to inhibit NHEJ (4, 32, 75). E4orf6 may indirectly affect DNA-PK and its autophosphorylation by inhibiting protein phosphatase 2A activity (31, 32). While our studies have established AAV replication as a model system to study DNA-PK, the role of this kinase beyond signaling is not completely clear. One previous report suggested that DNA-PKcs inhibits AAV integration (73), which may benefit replication of the episomal genome. Other *in vivo* studies have implicated DNA-PKcs in rAAV vector processing in certain murine tissues (14, 20, 36, 64, 72). However, DNA-PKcs did not affect transduction unless gene expression required vector circularization (14). Despite many attempts, we have obtained conflicting AAV transduction and replication data when we compared infections between the two sets of

DNA-PKcs-deficient cells employed in the experiment shown in Fig. 5 (data not shown). The impact of DNA-PK signaling on AAV replication and transduction may depend on the cellular context and the viral genome structure. In this report we have uncovered a unique role for DNA-PK in AAV-induced damage signaling, expanding our knowledge of the links between viral replication and cellular DDR pathways.

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ADDENDUM IN PROOF

After this paper was accepted, an independent study also observed a cellular DNA damage response activated by AAV and Ad coinfection with DNA-PD as the most prominent kinase mediating these effects (R. F. Collaco, J. M. Bevington, V. Bhargava, V. Kalman-Maltese, and J. P. Trempe, *Virology*, in press).

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