How adeno-associated virus Rep78 protein arrests cells completely in S phase

Carole Berthet, Kenneth Raj*, Philippe Saudan†, and Peter Beard‡

Swiss Institute for Experimental Cancer Research (ISREC), 1066 Epalinges, Switzerland

Edited by Kenneth I. Berns, University of Florida College of Medicine, Gainesville, FL, and approved August 9, 2005 (received for review June 2, 2005)

Adeno-associated virus Rep78 protein has antiproliferative effects on cells. It inhibits cell cycle progression, and, in particular, Rep78 induces a complete arrest within S phase, a response rarely seen after cell DNA damage. We examined how Rep78 achieves such an efficient S phase block. Rep78 inhibits Cdc25A activity by a novel means in which binding between the two proteins stabilizes Cdc25A, thus increasing its abundance, while at the same time preventing access to its substrates cyclin-dependent kinase (Cdk) 2 and Cdk1. This effect alone does not induce a complete S phase block. In addition, Rep78, as well as Rep68, produces nicks in the cellular chromatin, inducing a DNA damage response mediated by ataxia telangiectasia mutated (ATM) leading to G_1 and G_2 blocks. Mutational analysis shows that the zinc finger domain and nuclease activity of Rep78 are both required for the S phase block. The results suggest that a true S phase block cannot be achieved through a single pathway, and that adeno-associated virus Rep78 protein arrests cells within S phase by interfering with two pathways that would normally lead to an S phase slow-down.

Cdc25A | cell cycle | DNA damage

A number of reports have highlighted the antineoplastic and antiproliferative properties of the helper-dependent parvovirus, adeno-associated virus (AAV) (1–5). The oncosuppressive effect of AAV has been suggested to be due to the inhibition of tumor-causing viruses such as adenovirus and human papillomavirus (HPV), a notion supported by some epidemiological studies showing that patients suffering from cervical cancer, which is caused largely by HPV, had lower titers of antibodies to AAV than healthy subjects (6). Subsequent work showed that, independently of other viruses, AAV DNA itself can induce a cell cycle block (7) or even cell death in p53-negative cells, by mimicking damaged DNA (8). Furthermore, the nonstructural proteins of AAV, the Rep proteins, inhibit replication of some tumor viruses and block their ability to transform cells (9–11). Rep78 has also been observed to exert antiproliferative effects on cells: it interferes with cell proliferation either by blocking the cell cycle in all of the phases (12) or by inducing apoptosis (13). How these effects are brought about by Rep78 is still not clear, although Rep78 has been reported to regulate several cellular promoters, either by binding to Sp1 or by interacting with proteins such as positive cofactor 4, TATA-binding protein, protein kinase A, protein kinase X, E2F, and p53 (14–19). One striking feature of the action of Rep78 on cells is total S phase arrest. Unlike conventional DNA damage-induced S phase inhibition, which merely slows down the rate of DNA synthesis (20), Rep78 causes the cell to stop DNA synthesis altogether within S phase (12). No other protein has been reported to do this. The mechanisms set in motion by Rep78 to bring about such a drastic effect on cellular DNA replication are not known, although it is clear that activation of the tumor suppressor protein, pRb, is essential (12). pRb acts in part by inhibiting expression of genes whose products are required either directly or indirectly for DNA synthesis. It is likely that this property of pRb, when induced by Rep78, causes an inhibition of S phase progression. Less clear, however, is how Rep78 could activate pRb to cause S phase arrest. We report here the finding that Rep78 causes damage to cellular DNA by virtue of its intrinsic endonuclease activity. Moreover, by binding to the cell cycle regulatory phosphatase Cdc25A, Rep78 prevents its access to substrates cyclin-dependent kinase (Cdk) 1 and Cdk2, resulting in the inactivation of cyclin-dependent kinases that are required for DNA replication to proceed. Although neither of these activities alone causes an S phase arrest, their combined action results in a potent arrest of DNA replication within S phase.

Materials and Methods

Cells, Transfection, Infection, and Reagents. HeLa and U-2-OS were maintained in high glucose DMEM supplemented with 10% FCS. For AT221EJ-T pEBS7 ($ATM^{-/-}$) and AT221EJ-T pEBS7-YZ5 ($ATM^{+/+}$) cells (a kind gift from Y. Shiloh, Tel Aviv University, Tel Aviv), 5% FCS and 100 μg/ml hygromycin were added to the usual medium. For expression of AAV2 Rep proteins, either transfection with Lipofectamine 2000 or retroviral infection was used. For infection, retroviruses pBP and pBP78 were constructed and used as described in ref. 12. Selection was done with 4.5 μg/ml puromycin and 100 μg/ml G418. Hydroxyurea was used at 2.5 mM for 24 h, H_2O_2 at 100 μM for 20 min at 4°C or 20 h at 37°C, and cycloheximide at 25 μg/ml for 0 or 30 min.

Plasmids. BP, BP68′, BP78, and $BP78^{CXXH}$ have been described (12). The N-terminal truncated mutant $Rep78^{\Delta 1-171}$ was a kind gift from J. Kleinschmidt (German Cancer Center, Heidelberg). It was subcloned into pBabepuro yielding $BP78^{\Delta 1-171}$. The P-loop mutant $Rep78^{K340H}$ was constructed by PCR-mediated mutagenesis. pRc/CMV Cdc25A was a kind gift of J. Bartek (Danish Cancer Society, Copenhagen).

Western Blot, GST-Pulldown, Coimmunoprecipitation, and Phosphatase Assays. Transfected cells were lysed in reporter lysis buffer (Promega) with protease inhibitor mixture set III (Calbiochem). Thirty micrograms of total cellular proteins were used for SDS/PAGE and immunoblotting, which were carried out with ECL reagent (Amersham Pharmacia). Loading was checked either with tubulin or by Ponceau (Serva) staining. γ-H_2AX, protein was extracted as described (21). For other phosphorylated proteins (ATM-P and Chk2-P), cells were lysed directly in 1× SDS/PAGE sample buffer and sonicated.

GST fusion proteins were expressed in bacteria as described (22). Proteins on glutathione beads were mixed with HeLa cell extracts (subconfluent HeLa cells lysed in reporter lysis buffer, Promega) in A20 buffer (20 mM Hepes-NaOH, pH 7.9/10% glycerol/1 mM EDTA/10 mM $MgCl_2$/4 mM DTT/20 mM KCl/protease inhibitors) for 1 h at 4°C. Beads were washed in A20 buffer, loaded on a 4–20% LongLife gel (Gradipore, Frenchs Forest, Australia), and

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AAV, adeno-associated virus; Cdk, cyclin-dependent kinase; siRNA, small interfering RNA; ATM, ataxia telangiectasia mutated.

*Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London, United Kingdom.

†Present address: Cytos Biotechnology AG, CH 8952 Schlieren, Switzerland.

‡To whom correspondence should be addressed at: Swiss Institute for Experimental Cancer Research, Chemin des Boveresses 155, 1066 Epalinges, Switzerland. E-mail: peter.beard@isrec.ch.

www.pnas.org/cgi/doi/10.1073/pnas.0504583102

immunoblotted as described above. Coimmunoprecipitation was performed as described (17) with minor modifications. The phosphatase assay was modified from Mailand *et al.* (23).

Immunofluorescence. Cells grown on glass coverslips were fixed in 5% formaldehyde for 10 min and permeabilized for 1 h in PBS plus 5% milk plus 0.5% Nonidet-P40. Antibodies were diluted in PBS plus 5% milk and incubated for 1 h. Cy™3-conjugated AffiniPure goat anti-rabbit, Cy™3-conjugated AffiniPure donkey anti-mouse and Alexa Fluor 488 goat anti-rabbit/mouse were used as secondary antibodies. Slides were mounted in DABCO glycerol solution (82 g/liter DABCO, Sigma plus 50% glycerol in PBS) and visualized on a Leica DMIRB DC200 microscope.

Nick Translation Assay. The *in situ* nick translation assay was performed as described (24) with an RNaseA treatment (0.1 mg/ml RNase A for 15 min at 37°C) before nucleotide incorporation .

Cell Cycle Analysis. Subconfluent cells were pulsed with 33 μM BrdUrd for 30 min, detached with trypsin, and fixed in 70% ethanol. Samples were prepared and analyzed by bivariate flow cytometry as described (12).

RNA Interference. To construct a small interfering RNA (siRNA) against Cdc25A with the sequence AAGGCGCUAUUUGGCGCUUCA (25), this oligonucleotide was cloned in pSUPERpuro plasmid yielding pSUPERpuroCdc25A. pSUPER plasmid was described in ref. 26. The puromycin resistance gene cloned in pSUPER plasmid was a kind gift from P. Reichenbach (Swiss Institute for Experimental Cancer Research). HeLa cells transfected with pSUPERpuro or pSUPERpuroCdc25A were analyzed 24 h posttransfection.

Antibodies. The following antibodies were used in this study: mouse anti-γ-H_2AX Ser 139 (clone JBW301 Upstate Cell Signaling Solutions, Charlottesville, VA), mouse anti-ATM-P Ser-1982 (Cell Signaling Technology, Beverly, MA), rabbit anti-Chk2-P T68 (Cell Signaling Technology), rabbit anti-Cdc25A (M-191, Santa Cruz Biotechnology), mouse anti-Cdc25A (Ab-3 DCS120 plus DCS121, NeoMarkers, Fremont, CA), mouse anti-tubulin (Abcam, Cambridge, MA), rabbit anti-Cdk1-P T14 plus Y15 (Calbiochem), rabbit anti-Cdk1 (Ab-1, Oncogene), rabbit anti-Cdk2 (Ab-1, Oncogene), mouse anti-cyclin B1 (GNS1, Santa Cruz Biotechnology), and mouse anti-Rep (a kind gift from J. Kleinschmidt).

Results

Rep78 Damages Cellular DNA. We have previously observed and reported that the Rep78 protein of AAV induces an S phase arrest of cells (12). Although protein-induced S phase arrest has not till now been described, DNA damage-induced S phase arrest has been extensively characterized. To understand how Rep78 arrests the S phase, we investigated whether DNA damage pathways are involved by testing whether ATM, H_2AX, and Chk2 were activated by phosphorylation. When U-2-OS and HeLa cells were transfected with the plasmid encoding Rep78 (BP78) or its empty version (BP), activation of both ATM and H_2AX was observed by immunofluorescence in cells expressing Rep78 (Fig. 1*A Left*). To ascertain whether Chk2, a substrate of ATM, is activated or not, puromycin-selected Rep78 transfectants were stained with phospho-Chk2 antibodies, which revealed that Chk2 was strongly activated in Rep78 transfectants but not in those with the empty vector. Therefore, it seems that Rep78 protein generates a DNA damage signaling cascade of which ATM and Chk2 are members. The activation of these proteins was also apparent by immunoblotting with the respective antibodies (Fig. 1*A Right*). Cells treated with hydroxyurea were used as positive control because it induces a DNA damage response (27). Therefore, we conclude that Rep78 induces a DNA damage response that is initiated by ATM and transduced by Chk2. We cannot, however, exclude a role of ATM-related (ATR) entirely although its substrate Chk1 is not activated by Rep78, and ATR activation was not detected (data not shown). To verify whether ATM is indeed the main DNA damage-signaling mediator involved, we expressed Rep78 in an ATM-null cell line and its wild-type isogenic partner, and tested to see whether a DNA damage signal, as indicated by γ-H_2AX staining, could be elicited. We observed that, whereas Rep78 expression causes cells with wild-type ATM to stain positively for γ-H_2AX, cells devoid of ATM failed to do so (Fig. 1*B*). Echoing this finding was the observation that, whereas Rep78 caused a decline in the number of cells incorporating BrdUrd (an indication of DNA replication), as an expected consequence of DNA damage signaling, this decline was absent in cells that lacked ATM (Fig.

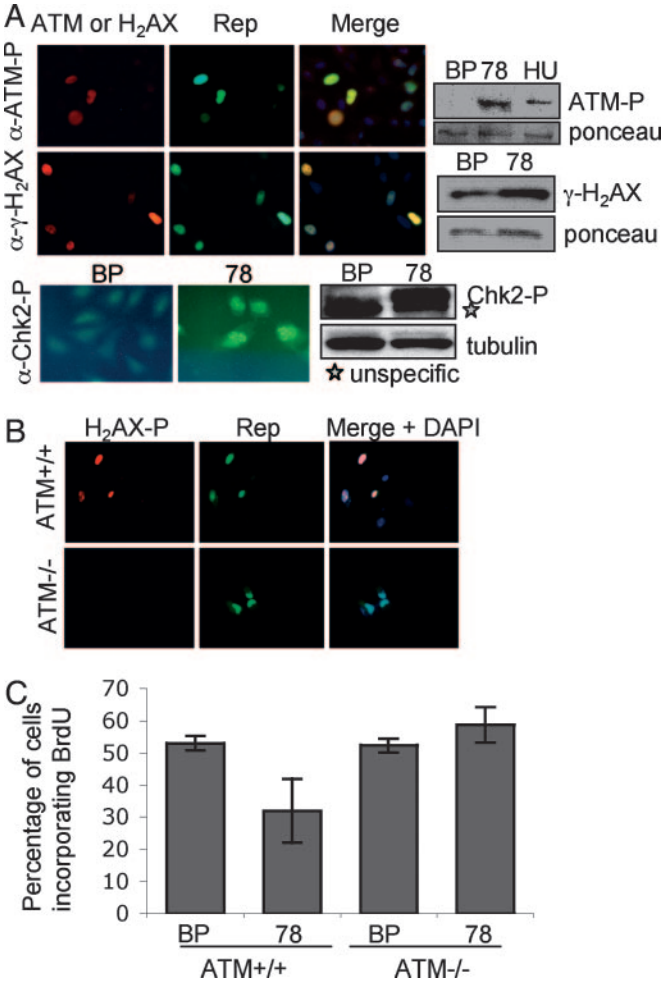

Fig. 1. Rep78 induces a DNA damage response. (*A*) Cells were transfected with the Rep78-expressing plasmid BP78 (78) or its empty version BP. As positive control for DNA damage response, cells were treated with hydroxyurea (HU). DNA damage protein activation was monitored in HeLa and U-2-OS cells by immunofluorescence staining and Western blot with phosphospecific antibodies. ATM and H_2AX activation were monitored 2 days posttransfection without selection, and Chk2 activation at 3 days posttransfection after complete selection. Ponceau staining is used as loading control. (*B*) Analysis of H_2AX phosphorylation in $ATM^{-/-}$ Rep78 transfectants that were not selected. Rep is in green and γ-H_2AX in red. (*C*) Percentages of $ATM^{+/+}$ and $ATM^{-/-}$ cells incorporating BrdUrd in the presence or in the absence of Rep78. The results are based on four independent bivariate flow cytometry analyses of cells without selection.

1*C*). All these results point to ATM being the mediator of Rep78-induced DNA damage signaling.

Having established that Rep78 activates a DNA damage response, we set out to understand how this is elicited. The site-specific endonuclease activity of Rep78 is well documented and stands out as a plausible cause of the DNA damage response seen when this protein is expressed in cells. We therefore tested whether Rep78 could cause a substantial number of nicks in the cellular chromatin. HeLa cells transfected with BP or BP78, or treated with 100 μM H_2O_2 as a positive control (28, 29), were fixed 2 days posttransfection and subjected to an *in situ* nick translation assay (24) (Fig. 2*A*). Rhodamine-labeled dUTP incorporated at the sites of nicked chromatin enables visualization with a fluorescence microscope. Although a low background staining was seen in the negative control (BP) cells (contributed presumably by spontaneous DNA damage or incomplete DNA replication), a strong and uniform staining was readily observed in cells treated with H_2O_2 and in those transfected with the Rep78-expressing vector. This observation implies that the Rep78 protein is capable of causing substantial amounts of damage to the cellular DNA and, as such, could well be the trigger of the DNA damage response that we characterized above. If this finding is so, then mutant Rep proteins that cannot nick DNA would not be predicted to induce a DNA damage response in cells. We tested Rep68′, which is a shorter form of Rep68 that lacks the 7 aa of the second exon, as well as $Rep78^{CXXH}$, which is a Rep78 protein with a mutated zinc finger domain (12), mutant Δ1-171, an N-terminal truncation of 171 aa of Rep78 that cannot bind and nick DNA and the $Rep78^{K340H}$ mutant, which has lost the ATPase and therefore the endonuclease activity, but still binds DNA (30). The results bore out this prediction: whereas Rep78, Rep68′ and $Rep78^{CXXH}$ proteins, all of which can nick DNA, triggered a DNA damage response, the $Rep78^{\Delta 1-171}$ and the $Rep78^{K340H}$ proteins, which are unable to nick DNA, failed to do so, as indicated by the lack of H_2AX activation (Fig. 2*B*).

Rep78-Induced DNA Damage Is Necessary, but Not Sufficient, to Induce a Complete S Phase Block. Having established that Rep78 protein, by damaging cellular DNA, activates a DNA damage response, we asked whether this is the way by which Rep78 induces the cell cycle arrest that we previously observed (12). Briefly, in that work we saw that Rep78 arrested cells in the G_1, G_2, and S phases. The Rep78-induced S phase arrest abolished cellular DNA replication completely, and it was this unusual total S phase block by Rep78 that we were interested in addressing. We measured cell cycle distributions of HeLa and 3T3 cells expressing Rep78 and the various mutants by BrdUrd incorporation and DNA-content flow cytometry (Fig. 2 *C* and *D*). In this assay, S phase arrest is documented by the appearance of cells with S phase DNA content that did not incorporate BrdUrd. The proportion of such cells was ≈2% in control BP-transfected cells but rose to >20% in BP78 transfectants, as previously observed. The results showed that nicking of the cellular DNA could not itself be responsible for the complete S phase arrest because Rep68′, which nicks DNA equally well, was unable to do this (Fig. 2*C*). Instead, cells accumulated in the G_2 and, to a lesser extent, G_1 phases.

Although nicking of cellular DNA by itself is not sufficient, this activity is nevertheless required for an S phase arrest because mutant Rep proteins that are unable to nick DNA ($Rep78^{K340H}$ and $Rep78^{\Delta 1-171}$) were defective in arresting cells in S phase. It is noteworthy that Rep52, which possesses ATPase activity but not nicking activity, does not induce S phase arrest (12), ruling out the likelihood that depletion of cellular ATP by the ATPase activity of Rep was responsible for the S phase arrest. The results, which were similar for 3T3 and HeLa cells (Fig. 2 *C* and *D*), suggested that nicking of cellular DNA is coupled with another activity of Rep78 together to elicit the S phase arrest. It is clear that this other activity resides in the zinc finger domain of the Rep78 protein, because Rep68′ and $Rep78^{CXXH}$, which both lack this domain, while still being proficient in nicking DNA, failed to arrest cells in S phase. Thus, the combined effects of nicking of cellular DNA and an activity found in the zinc finger domain of Rep78 are required for a total S phase arrest. Moreover, two mutants defective for either one of these activities ($Rep78^{CXXH}$ and $Rep78^{\Delta 1-171}$) were able to complement each other to arrest cells in S phase (Fig. 2*D*). Therefore, we characterized the activity of the zinc finger domain of Rep78.

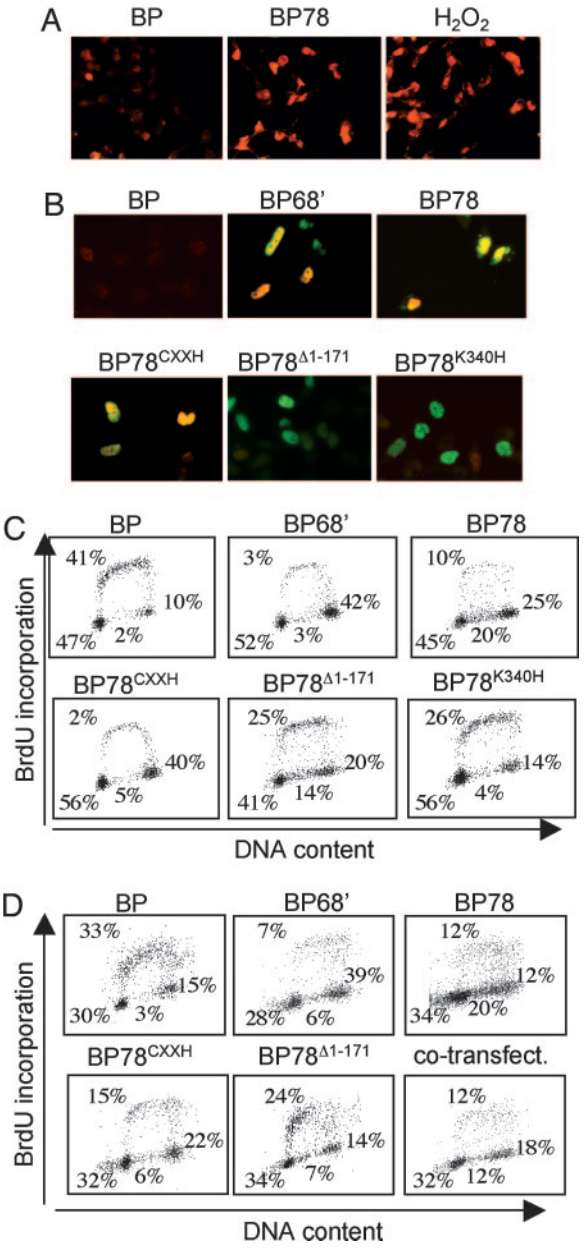

Fig. 2. Nicking of cellular DNA by Rep78 is not sufficient to completely block cells in S-phase. (*A*) Nick translation assay in HeLa cells transfected with Rep78. H_2O_2 treatment was used as a positive control. (*B*) H_2AX activation (red) in cells transfected with different forms of Rep (green). (*C*) Cell cycle analysis by bivariate flow cytometry of 3T3 cells infected with different forms and mutants of Rep, 5 days postinfection with selection. (*D*) Cell cycle analysis of HeLa cells transfected with different forms and mutants of Rep, 2 days posttransfection, with selection. Co-transfect., $BP78^{CXXH}$ plus $BP78^{\Delta 1-171}$.

Rep78 Increases the Level of Cdc25A by Binding to It and Stabilizing It. To determine what activities Rep78 possesses that could be elicited by the zinc finger domain, we focused on cell cycle regu-

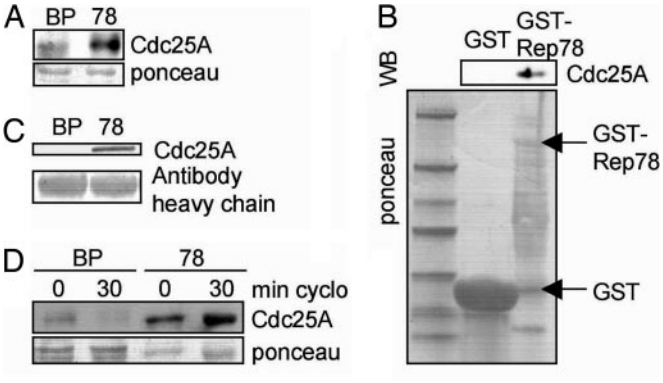

Fig. 3. Rep78 increases Cdc25A levels by binding and stabilizing it. In *A*, *C*, and *D*, cells were transfected with BP78 or BP. (*A*) Cdc25A levels were detected in U-2-OS cells by Western blot after 2 days selection. (*B*) GST and GST-Rep78 were produced in bacteria and used in pull-down assays with total HeLa cell extract. (*C*) Coimmunoprecipitation of Cdc25A with an anti-Rep78 antibody from HeLa cells. (*D*) Rep78 stabilizes Cdc25A protein. Shown is Western blot analysis of Cdc25A in U-2-OS cells after 0 or 30 min of cycloheximide treatment (min cyclo). Ponceau staining is used as loading control.

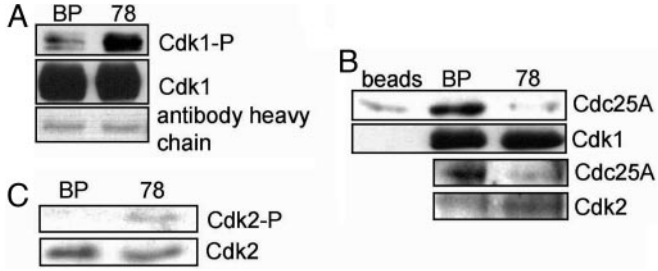

Fig. 4. Rep78 binding to Cdc25A inhibits its phosphatase activity. Cells were transfected with BP78 or BP. (*A*) *In vitro* phosphatase assay was performed by mixing cyclinB-Cdk1 immunoprecipitated from HeLa cells treated with doxorubicin, with Cdc25A immunoprecipitated from HeLa cells either expressing Rep78 or not. (*B*) Coimmunoprecipitation of Cdc25A from HeLa cells with anti-Cdk2 or anti-Cdk1 antibodies. (*C*) Western blot analysis of Cdk2 and its phosphorylated form in U-2-OS cells.

latory proteins that might physically interact with Rep78. We looked for changes in the amounts of these proteins in cells expressing Rep78 that could be a consequence of such an interaction. We observed that Rep78 induced an increase in the level of the Cdc25A protein (Fig. 3*A*). This result was unexpected because Cdc25A actually facilitates cell cycle progression (31). It was not immediately clear how, by increasing the amount of Cdc25A in the cell, Rep78 could cause an S phase arrest. However, it was thought possible that Cdc25A might be stabilized by Rep78 in the same way as p53 is by SV40T (32). To test this hypothesis, we examined whether Rep78 can bind to Cdc25A in a GST pull-down assay (Fig. 3*B*). The results revealed that Cdc25A does indeed associate with GST-Rep78. To see whether this interaction also occurs in the cell, we performed coimmunoprecipitation experiments on protein extracts of HeLa cells that were transfected with either BP or BP78. Rep78-binding proteins immunoprecipitated from these extracts were subjected to immunoblotting with antibodies against Cdc25A. The results in Fig. 3*C* show that Cdc25A was brought down along with Rep78, confirming that Rep78 and Cdc25A interact physically in cells. This binding was specific: Rep78 did not interact with other proteins tested, including cyclin B, MCM3, and DNA polymerase ε (data not shown).

The half-life of Cdc25A of $\approx$30 min is reduced even more in the presence of a DNA damage response (23, 33). One way by which Rep78 could increase the Cdc25A protein level is by protecting it from degradation. To test this possibility, U-2-OS cells transfected with BP or BP78 and puromycin-selected were treated with cycloheximide to inhibit protein synthesis, and Cdc25A levels were monitored (Fig. 3*D*). In cells transfected with the empty vector, Cdc25A was almost completely absent after 30 min of cycloheximide treatment. In contrast, in cells expressing Rep78, the Cdc25A level did not decrease after this time, demonstrating that Cdc25A was indeed stabilized by Rep78.

Rep78 Inhibits the Activity of Cdc25A. Knowing that Rep78 binds and stabilizes Cdc25A explains the increased level of Cdc25A but does not explain how this increase translates to S phase arrest by Rep78. As mentioned above, Cdc25A facilitates the cell cycle. Therefore, it would have been intuitively acceptable had Rep78 decreased Cdc25A levels. Because we observed the opposite, we asked whether the Rep78-Cdc25A interaction is inhibitory to Cdc25A function. To test this possibility, we performed *in vitro* phosphatase assays for Cdc25A. To obtain a Cdc25A substrate, HeLa cells were treated with doxorubicin to induce phosphorylation of Cyclin B1-Cdk1 on Thr-14 and Tyr-15. The CyclinB1-Cdk1-P complexes were then immunoprecipitated from protein extracts of these cells. In parallel, other plates of HeLa cells were transfected with BP or BP78 and harvested 24 h later. Cdc25A was immunoprecipitated from these cell extracts, and equal amounts of the immunoprecipitated cyclinB1-Cdk1-P and Cdc25A were mixed in a phosphatase buffer for 30 min. The phosphorylated form of Cdk1 was then analyzed by immunoblotting. A decreased level of Cdk1 phosphorylation was clearly apparent for the mix with an extract from normal cells, compared with one from cells expressing Rep78 (Fig. 4*A*), indicating that, although Cdc25A protein was stabilized by Rep78, its activity was severely diminished.

We then asked how Rep78 inhibits Cdc25A activity. We tested the hypothesis that Rep78 prevents Cdc25A from interacting with its target proteins. By precipitating Cdk1 or Cdk2 with their respective antibodies and measuring the amount of coprecipitated Cdc25A, we observed a clear decrease of Cdc25A associating with these proteins in cells expressing Rep78, compared with controls (Fig. 4*B*). This result indicates that Rep78 prevents Cdc25A from binding to its substrates, Cdk1 and Cdk2. To ascertain whether the inhibitory effect of Rep78 on Cdc25A also occurs *in vivo*, phosphospecific antibodies to Cdk2 were used to reveal the extent to which Cdk2 was inactivated in cells that express Rep78, compared with control cells. We found that there was indeed a higher proportion of phosphorylated (inactive) Cdk2 in cells expressing Rep78 (Fig. 4*C*). Together, these results demonstrate that the inhibitory effect of Rep78 on Cdc25A is brought about by the prevention of Cdc25A from binding its targets, such as Cdk2 and Cdk1. This finding could explain, at least in part, the cell cycle block observed in cells expressing Rep78.

Cdc25A Inhibition Is Necessary but Not Sufficient for Blocking Cells in S Phase. To return to the reasoning behind these experiments, we asked whether the binding and inactivation of Cdc25A are conferred by the zinc finger domain of the Rep78 protein, a region that is required for a full S phase arrest by Rep78. To test this possibility, we compared the ability of Rep78 and Rep78^{CXXH}, which lacks the zinc finger domain, to bind Cdc25A. Coimmunoprecipitations were performed as above and quantified, and the results are presented in Fig. 5. From these results, it is clear that the ability of Rep78 to bind Cdc25A was drastically compromised by the absence of the zing finger domain (Fig. 5*A*). This result is consistent with the fact that this Rep mutant was also defective in preventing Cdc25A from binding to Cdk1 *in vivo*, whereas Rep78 and Rep78$^{\Delta 1-171}$, both of which possess the zinc finger domain, efficiently inhibited this binding (Fig. 5*B*).

Although Rep78, via its zinc-finger domain, clearly inactivates Cdc25A, we asked whether this is the required factor (in concert

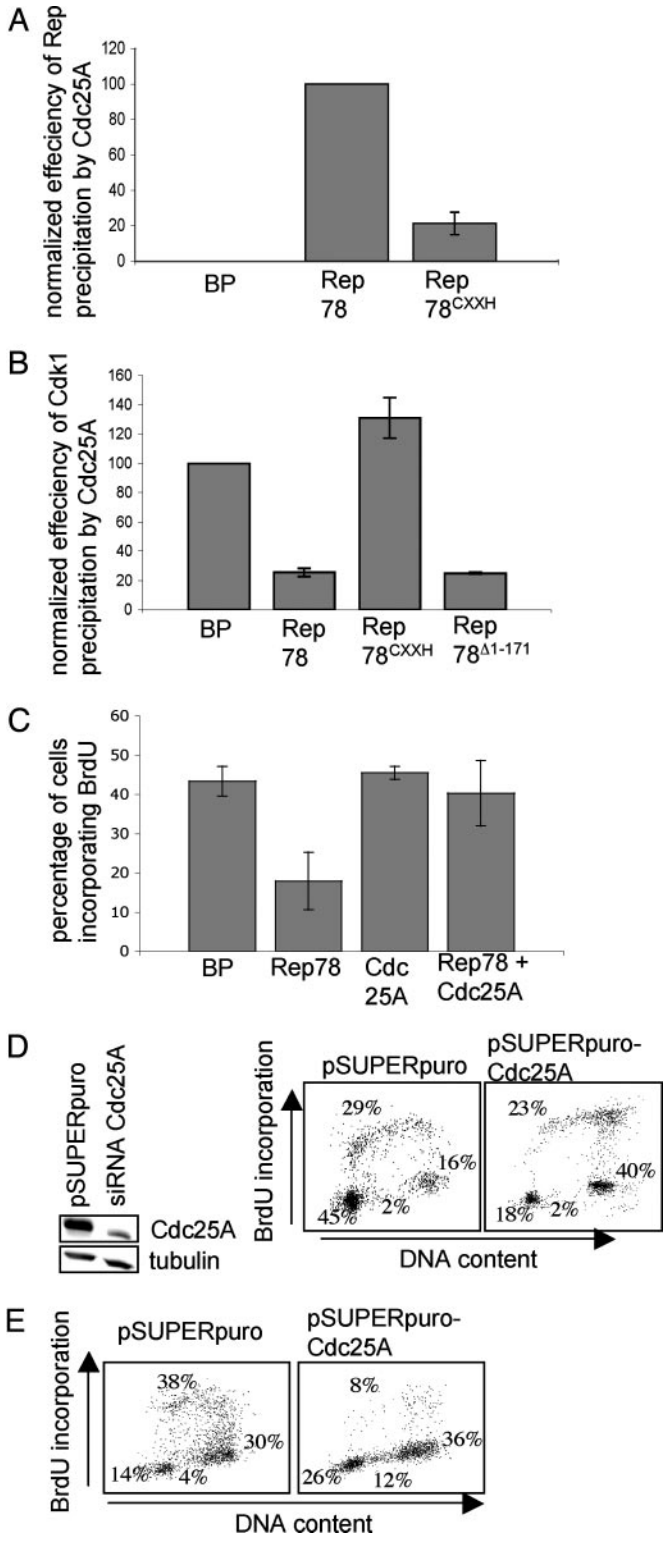

Fig. 5. Rep78 lacking the zinc-finger domain is defective in binding and inhibition of Cdc25A activity. (*A*) Measurements of Rep78 or Rep78^{CXXH} protein that coimmunoprecipitated with Cdc25A. (*B*) Measurement of Cdk1 that coimmunoprecipitated with Cdc25A either in the absence or presence of Rep78, Rep78^{CXXH}, or Rep78$^{\Delta 1-171}$. Error bars represent standard deviations of three experiments. (*C*) Percentages of HeLa cells incorporating BrdUrd in the presence of Rep78 and Cdc25A overexpression. Cells transfected with Rep78 were not antibiotic-selected before analysis. The error bars represent the standard deviations from four independent experiments. (*D Left*) Western blot analysis of Cdc25A levels in HeLa cells transfected with pSuperpuro or pSuperpuroCdc25A 2 days after transfection and antibiotic selection. (*Right*) Bivariate flow cytometry analysis of these cells after a 30-min BrdUrd pulse, 2 days after transfection and antibiotic selection. (*E*) Rep78-induced cell cycle block can be mimicked by H_2O_2 treatment of cells expressing siRNA against Cdc25A. Both cell populations were H_2O_2–treated and either expressed siRNA against Cdc25A or not.

with Rep nicking of DNA) for the S phase block. If it is, then over-expressing Cdc25A in cells would be expected to rescue them from the arrest elicited by Rep78. To test this, HeLa cells transfected with pRc/CMVCdc25A to overexpress Cdc25A were first generated. These cells and control cells were subsequently transfected with BP or BP78 and pulsed with BrdUrd 2 days later before analyses by bivariate flow cytometry (Fig. 5*C*). As shown before, there was a clear decline in BrdUrd incorporation by Rep78 transfectants. This decrease was absent in cells overexpressing Cdc25A, supporting the conclusion that Cdc25A inhibition is necessary for the S phase block induced by Rep78.

Because Cdc25A prevented Rep78 from arresting cells, we asked whether depletion of Cdc25A is sufficient to induce an S phase arrest. We expressed siRNA against Cdc25A in HeLa cells to lower the level of this protein (Fig. 5*D*). As a consequence, the level of Cdc25A was reduced to 16% of that of the control cells. Bivariate flow cytometry analyses of these cells revealed that they were blocked primarily in G_2, with some cells being blocked in G_1, in agreement with the findings of Mailand *et al.* (34). However, depletion of Cdc25A did not arrest cells within the S phase, as Rep78 does. From this, we conclude that, whereas inactivation of Cdc25A is clearly needed for Rep78 to arrest cells in the S phase, this function by itself is not sufficient.

Having identified DNA nicking and Cdc25A inactivation as properties of Rep78 that are required for S phase arrest, we asked whether these two effects, if induced independently of Rep78, will also block cells in S phase. HeLa cells were both transfected with pSUPERpuroCdc25A and treated with H_2O_2 for 20 h, then analyzed by bivariate flow cytometry. The results (Fig. 5 *D* and *E*) revealed that, whereas the siRNA against Cdc25A and H_2O_2 treatment separately failed to arrest cells in S phase, together they were capable of doing this, suggesting that these two activities are necessary and sufficient for Rep78-induced S phase arrest.

Discussion

In this study, we have identified two actions of the AAV2 Rep78 protein on the cell cycle that, in concert, induce an arrest of the S phase that is unlike any seen with conventional DNA-damaging agents or proteins. The first is the nicking of cellular DNA by Rep78. The fact that Rep78 produces nicks in the cellular chromatin is not unexpected for a number of reasons. The site-specific endonuclease activity of Rep78/68 is known (35) and is a function required for AAV DNA replication and integration in the human genome. Moreover, expression of Rep78/68 in the absence of AAV is sufficient to rearrange and amplify chromosomal Rep-binding sites, estimated at 2×10^5 sites in the human genome (36). Therefore, it is conceivable that Rep78 binds to these sites and causes DNA damage. Furthermore, it was suggested that Rep78/68 initiates replication at the AAV integration site in the host genome, which may provoke repeated reinitiation (37). The resulting multiple 5′ flaps could be recognized as damage. Whatever the mechanism, it is clear that Rep78, by damaging cellular chromatin, causes the cell to trigger a DNA damage response that is mediated by ATM and Chk2. This result at first sight seems surprising because ATM activation is commonly associated with double-strand breaks, not DNA nicks. Although it would be tempting to claim that the action of Rep78 suggests otherwise, single-strand breaks or nicks can be converted to double-stand breaks in cells, and ATM can be activated by as few as two double-strand breaks. Furthermore, mere

alteration of the chromatin structure can be sufficient to activate ATM (38), as can treatment of cells with thymidine, which is not known to cause double-strand breaks (39). What is clear however is that, by damaging cellular DNA, Rep78 causes activation of ATM. The results of ATM activation, which include triggering of G_1/S, intra-S, and G_2/M checkpoints and apoptosis (40, 41), are evident in cells expressing Rep78, as we have previously reported. In this study, we see further evidence of ATM activation in Chk2 activation and arrest within S phase. The primacy of ATM in mediating the DNA damage signal is demonstrated by the lack of such signaling when Rep78 was expressed in ATM-null cells. It was clear, however, from the outset that the damaging of DNA by Rep itself would not be sufficient to induce a total S phase arrest. Thus, studies with Rep mutants showed that some mutants able to nick DNA were nevertheless defective in arresting the S phase. From these studies, it was equally clear that the zinc-finger domain of the Rep78 protein was needed for this activity. We demonstrated a direct binding of Cdc25A by the zinc-finger domain of Rep78. This binding stabilizes Cdc25A, which is normally very short-lived. The consequence of Rep78 binding is the inhibition of Cdc25A from accessing its substrates, Cdk1 and Cdk2. Therefore, although Rep78 induces an increase in the Cdc25A level, its final effect is a decrease in Cdc25A activity. It is interesting to note that this mechanism of regulation of Cdc25A, by direct binding, has not been described before. However, this second activity of Rep78 is also not by itself sufficient to account for the complete S phase block, consistent with the reported observation that inhibition of Cdc25A in itself does not induce an S phase block (34, 42, 43). Thus, taken alone, no single one of the activities of Rep78 described above can arrest cells in S phase. However, either cotransfection of two mutants each defective for one of these activities, or H_2O_2 treatment of cells partially depleted for Cdc25A by siRNA, is able to induce a total S phase arrest, indicating that the DNA-nicking and Cdc25A-inactivation functions of Rep78 are uncoupled and sufficient to induce an S phase arrest.

How do these two events lead to total S phase arrest? In our previous work, we demonstrated that Rep78 causes a strong and irreversible activation of the pRb protein, and that this activation is absolutely required for the total S phase arrest. Inactivation of pRb by viral proteins such as adenovirus E1a and human papillomavirus E7, or a complete lack of pRb, as is the case with pRb-null cells, results in the inability of Rep78 to induce the total S phase block. Thus, it is reasonable to postulate that the DNA damage induced by the nicking activity of Rep78 together with Rep78-induced inactivation of Cdc25A are required to attain a level of pRb activation that is extremely strong, if not total. The failure of most conventional DNA damage regimes to elicit a total S phase arrest as seen with Rep78 may be due to incomplete or transient activation of pRb. This notion is consistent with the fact that activated pRb arrests DNA replication by at least two pathways. The first is by attenuation of the Cdk2 activity, and the second by depletion of nucleotides, achieved by repressing dihydrofolate reductase, ribonucleotide reductase, and thymidylate synthase production (44). It would be of interest to determine the relative efficiency with which active pRb represses the two pathways. pRb may need to target them both to bring a total halt to DNA replication. Although Rep78 succeeds in this inhibition, DNA-damaging treatments may achieve only partial inhibition, which is manifested as a slow-down of DNA replication. Answers to these questions should reveal the difference between the action of Rep78 and pure DNA damage. In conclusion, the results presented here, together with those we reported previously, delineate the way by which the AAV Rep78 protein elicits a total S phase arrest when expressed in cells. They are relevant from the virology viewpoint. Thus, in a latent infection, Rep78 might lead to a transient S phase arrest, which would give the virus a greater chance to integrate into the cell genome. They are also pertinent for understanding the checkpoints that govern S phase progression.

We thank J. Bartek, J. Kleinschmidt, and Y. Shiloh for gifts of plasmids, antibodies, and cells, and our colleagues N. Paduwat, P. Reichenbach, and J. Thevenat for their help. This work was supported by the Swiss National Science Foundation, the National Center of Competence in Research (NCCR) on Molecular Oncology, and Cancer Research Switzerland.

1. Rommelaere, J. & Cornelis, J. J. (1991) *J. Virol. Methods* **33,** 233–251.
2. Schlehofer, J. R. (1994) *Mutat. Res.* **305,** 303–313.
3. Berns, K. & Linden, R. (1995) *Bioessays* **17,** 237–245.
4. Winocour, E., Callaham, M. F. & Huberman, E. (1988) *Virology* **167,** 393–399.
5. Hermanns, J., Schulze, A., Jansen-Db1urr, P., Kleinschmidt, J. A., Schmidt, R. & zur Hausen, H. (1997) *J. Virol.* **71,** 6020–6027.
6. Georg-Fries, B., Biederlack, S., Wolf, J. & zur Hausen, H. (1984) *Virology* **134,** 125–137.
7. Raj, K., Ogston, P. & Beard, P. (2001) *Nature* **412,** 914–917.
8. Jurvansuu, J., Raj, K., Stasiak, A. & Beard, P. (2005) *J. Virol.* **79,** 569–580.
9. Antoni, B. A., Rabson, A. B., Miller, I. L., Trempe, J. P., Chejanovsky, N. & Carter, B. J. (1991) *J. Virol.* **65,** 396–404.
10. Yang, Q., Chen, F., Ross, J. & Trempe, J. (1995) *Virology* **207,** 246–250.
11. Heilbronn, R., Burkle, A., Stephan, S. & zur Hausen, H. (1990) *J. Virol.* **65,** 3012–3018.
12. Saudan, P., Vlach, J. & Beard, P. (2000) *EMBO J.* **19,** 4351–4361.
13. Schmidt, M., Afione, S. & Kotin, R. (2000) *J. Virol.* **74,** 9441–9450.
14. Weger, S., Wendland, M., Klenschmidt, J. & Heilbronn, R. (1999) *J. Virol.* **73,** 260–269.
15. Hermonat, P., Santin, A., Batchu, R. & Zhan, D. (1998) *Virology* **245,** 120–127.
16. Batchu, R. B., Shammas, M. A., Wang, J. Y. & Munshi, N. C. (2001) *J. Biol. Chem.* **276,** 24315–24322.
17. Batchu, R., Shammas, M., Wang, J. & Munshi, N. (1999) *Cancer Res.* **59,** 3592–3595.
18. Chiorini, J., Zimmermann, B., Yang, L., Smith, R., Ahearn, A., Herberg, F. & Kotin, R. (1998) *Mol. Cell. Biol.* **18,** 5921–5929.
19. Di Pasquale, G. & Stacey, S. (1998) *J. Virol.* **72,** 7916–7925.
20. Bartek, J., Lukas, C. & Lukas, J. (2004) *Nat. Rev. Mol. Cell. Biol.* **5,** 792–804.
21. Ward, I. M. & Chen, J. (2001) *J. Biol. Chem.* **276,** 47759–47762.
22. Costello, E., Saudan, P., Winocour, E., Pizer, L. & Beard, P. (1997) *EMBO J.* **16,** 5943–5954.
23. Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J. & Lukas, J. (2000) *Science* **288,** 1425–1429.
24. Op De Beeck, A. & Caillet-Fauquet, P. (1997) *J. Virol.* **71,** 5323–5329.
25. Turowski, P., Franckhauser, C., Morris, M. C., Vaglio, P., Fernandez, A. & Lamb, N. J. C. (2003) *Mol. Biol. Cell.* **14,** 2984–2998.
26. Brummelkamp, T. R., Bernards, R. & Agami, R. (2002) *Science* **296,** 550–553.
27. Yabro, J. (1992) *Semin. Oncol.* **19,** 1–10.
28. Thannickal, V. J. & Fanburg, B. L. (2000) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279,** L1005–1028.
29. Storz, G. & Imlay, J. (1999) *Curr. Opin. Microbiol.* **2,** 188–194.
30. Wonderling, R. S., Kyöstiö, S. R. & Owens, R. A. (1995) *J. Virol.* **69,** 3542–3548.
31. Nilsson, I. & Hoffmann, I. (2000) *Prog. Cell Cycle Res.* **4,** 107–114.
32. Tiemann, F. & Deppert, W. (1994) *J. Virol.* **68,** 2869–2878.
33. Falck, J., Mailand, N., Syljuasen, R., Bartek, J. & Lukas, J. (2001) *Nature* **410,** 842–847.
34. Mailand, N., Podtelejnikov, A., Groth, A., Mann, M., Bartek, J. & Lukas, J. (2002) *EMBO J.* **21,** 5911–5920.
35. Im, D. & Muzyczka, N. (1990) *Cell* **61,** 447–457.
36. Young, S. M., Jr., McCarty, D. M., Degtyareva, N. & Samulski, R. J. (2000) *J. Virol.* **74,** 3953–3966.
37. Young, S. M., Jr., & Samulski, R. J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 13525–13530.
38. Bakkenist, C. J. & Kastan, M. B. (2003) *Nature* **421,** 499–506.
39. Bolderson, E., Scorah, J., Helleday, T., Smythe, C. & Meuth, M. (2004) *Hum. Mol. Genet.* **13,** 2937–2945.
40. Kurz, E. U. & Lees-Miller, S. P. (2004) *DNA Repair* **3,** 889–900.
41. Kastan, M., Onyekwere, O., Sidransky, D., Vogelstein, B. & Craig, R. (1991) *Cancer Res.* **51,** 6304–6311.
42. Hoffmann, I., Draetta, G. & Karsenti, E. (1994) *EMBO J.* **13,** 4302–4310.
43. Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H. & Okayama, H. (1994) *EMBO J.* **13,** 1549–1556.
44. Angus, S. P., Wheeler, L. J., Ranmal, S. A., Zhang, X., Markey, M. P., Mathews, C. K. & Knudsen, E. S. (2002) *J. Biol. Chem.* **277,** 44376–44384.