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Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb

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Adeno-associated virus (AAV) has an antiproliferative action on cells. We investigated the effect of the AAV replication proteins (Rep) on the cell division cycle using retroviral vectors. Rep78 and Rep68 inhibited the growth of primary, immortalized and transformed cells, while Rep52 and Rep40 did not. Rep68 induced cell cycle arrest in phases G_1 and G_2, with elevated CDK inhibitor p21 and reduced cyclin E-, A- and B1-associated kinase activity. Rep78-expressing cells were also impaired in S-phase progression and accumulated almost exclusively with hypophosphorylated retinoblastoma protein (pRb). The differences between Rep78 and Rep68 were mapped to the C-terminal zinc finger domain of Rep78. Rep78-induced S-phase arrest could be bypassed by adenoviral E1A or papillomaviral E7 proteins but not by E1A or E7 mutants unable to bind pRb. $Rb^{-/-}$ primary mouse embryonic fibroblasts displayed a strongly reduced S-phase arrest when challenged with Rep78, compared with matched $Rb^{+/+}$ controls. These results suggest that physiological levels of active pRb can interfere with S-phase progression. We propose that the AAV Rep78 protein arrests cells within S-phase by a novel mechanism involving the ectopic accumulation of active pRb.

Keywords: AAV/cell cycle/pRb/Rep/S-phase arrest

Introduction

Adeno-associated virus type 2 (AAV) is a small DNA-containing parvovirus, which infects humans and depends on co-infection with a helper virus in order to produce progeny viruses (Berns and Linden, 1995). Low levels of AAV replication can also occur in cells exposed to genotoxic stress in the absence of helper virus (Yakinoglu *et al*., 1988). Under conditions that are not permissive for replication, AAV can establish a latent infection by integration of the viral DNA into the cellular genome, preferentially in a site-specific manner (Kotin *et al*., 1990). The AAV genome consists of two open reading frames, *rep* and *cap*, flanked by inverted terminal repeats that contain the replication origins (reviewed in Berns and Linden, 1995). The *cap* gene encodes three viral structural proteins. Four non-structural proteins are made from the *rep* gene by alternative promoter utilization and differential splicing. Rep78 and Rep68 are expressed from the p5 promoter and regulate many aspects of the viral life cycle, including DNA replication (Srivastava *et al*., 1983), transcription (Beaton *et al*., 1989; Pereira *et al*., 1997) and site-specific chromosomal insertion (Weitzman *et al*., 1994). They are multifunctional proteins with activities including specific DNA binding and site- and strand-specific endonuclease, helicase and ATPase activities (Im and Muzyczka, 1990; Wonderling *et al*., 1995). Rep52 and Rep40, which are expressed from the p19 promoter, do not bind DNA but have been shown to have ATP-dependent helicase activity (Smith and Kotin, 1998) and are involved in packaging single-stranded viral genomes into capsid structures (Chejanovsky and Carter, 1989). Rep68 lacks the 91 C-terminal amino acids of Rep78 and has an additional seven amino acids encoded by exon 2. Rep52 and Rep40 are N-terminally truncated versions of Rep78 and Rep68, respectively.

AAV is generally believed to have antiproliferative effects on cells and, since it interferes with tumour formation in animal models, has been proposed to have oncosuppressive activities (reviewed in Rommelaere and Cornelis, 1991; Schlehofer, 1994). However, very little is known about the molecular mechanisms involved. Depending on the cell type used, non-lytic AAV infection at high multiplicity can either induce differentiation (Klein-Bauernschmitt *et al*., 1992), inhibit cell growth or interfere with the cell cycle (Winocour *et al*., 1988; Bantel-Schaal, 1990; Hermanns *et al*., 1997; Kube *et al*., 1997). These effects do not require *de novo* Rep synthesis since they were also observed with recombinant or UV-inactivated AAV. Studies with cultured cells revealed that the Rep proteins inhibit transformation by viral and cellular oncogenes (Ostrove *et al*., 1981; Hermonat, 1989; Khleif *et al*., 1991), viral and cellular DNA synthesis (Hermonat, 1992; Yang *et al*., 1994, 1995) and transcription from a variety of promoters (Labow *et al*., 1987; Hermonat, 1991, 1994; Hörer *et al*., 1995). Since it is still not clear which components (capsid or the Rep proteins) are responsible for the proposed oncosuppressive activity of AAV, we decided to investigate whether the Rep proteins interfere with the cell division cycle.

The cell cycle is driven by a family of protein kinases called the cyclin-dependent kinases (CDKs). Their sequential activation controls the entry and passage through the different phases of the cell cycle: G_1, S, G_2 and M. In order to be activated, these CDKs must be associated with regulatory subunits, the cyclins, and have their inhibitory phosphates removed by the Cdc25 family of protein phosphatases (reviewed in Morgan, 1997). An additional level of regulation is achieved by two families of small inhibitory proteins the CDK-inhibitors (CKIs). CKIs play a key role in the response of cells to growth-

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inhibitory signals such as induction of differentiation, p53 activation in response to DNA damage, growth factor deprivation and senescence (reviewed in Sherr and Roberts, 1995; Harper and Elledge, 1996). The Cip/Kip family of CKIs includes $p21^{Cip}$, $p27^{Kip1}$ and $p57^{Kip2}$. These CKIs demonstrate a broad range of specificity and are able to inhibit G_1 cyclin–CDK complexes and, to a lower extent, cyclin B–Cdc2 complexes (Xiong *et al.*, 1993; Harper *et al.*, 1995). The second family includes the INK4 proteins $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, which are specific inhibitors of CDK4 and CDK6 (reviewed in Ruas and Peters, 1998).

The decision to complete a round of mitotic division takes place during the G_1-phase of the cell cycle at a stage called the restriction point, which precedes the onset of DNA synthesis in the S-phase (Pardee, 1989; Zetterberg *et al.*, 1995). The passage through this point is mainly controlled by the phosphorylation state of the retinoblastoma protein family consisting of pRb, p107 and p130, which are negative growth regulators (reviewed in Grana *et al.*, 1998). In their active hypophosphorylated form, Rb-family proteins associate with transcription factors of the E2F/DP family, repressing their target genes that include regulators of S-phase entry and genes required for DNA replication (for reviews see Weinberg, 1995; Helin, 1998). During the G_1–S transition, pRb is inactivated by phosphorylation through sequential activation of CDK4, CDK6 and CDK2. This hyperphosphorylated state is maintained until late mitosis when pRb is dephosphorylated by the PP1 family of phosphatases (reviewed in Mittnacht, 1998; Rubin *et al.*, 1998). Besides the well-documented importance of pRb in the G_1–S transition, recent reports based on over-expression of non-phosphorylatable pRb mutants suggest a novel function of active pRb in the inhibition of S-phase progression (Chew *et al.*, 1998; Knudsen *et al.*, 1998). The importance of pRb is further emphasized by the fact that DNA tumour viruses have evolved proteins (e.g. adenovirus E1A, human papillomavirus E7) that can bind and inactivate pRb, allowing progression into S-phase in the absence of mitogenic stimuli (reviewed in Moran, 1993).

Results

Rep78 and Rep68 have a strong antiproliferative activity

Recombinant retroviruses were generated that express the individual Rep proteins (Figure 1A) from the retroviral promoter, together with a puromycin selectable marker (Morgenstern and Land, 1990). Rep68′ and Rep40′ lack the seven amino acids encoded by exon 2 present in Rep68 and Rep40, and therefore correspond to C-terminal truncations of Rep78 and Rep52, respectively. The recombinant retroviruses were named BP78, BP68′, BP52 and BP40′.

NIH 3T3 cells were infected with these recombinant and control (BP, empty vector) retroviruses and seeded at serial dilutions under selective conditions. All uninfected cells had detached within 48 h of selection, at which time the more dense dishes were harvested for biochemical analysis. The lower dilutions were incubated further to assess colony outgrowth. These dishes were fixed and stained after 6 days in selective medium.

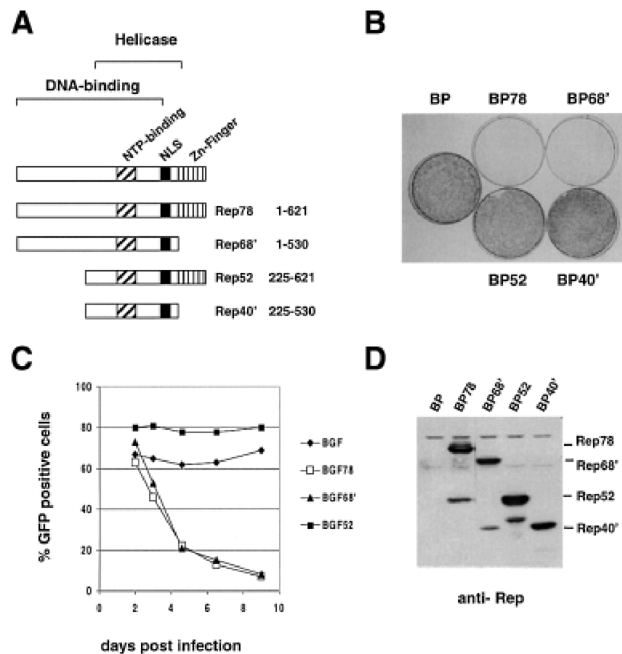

Fig. 1. Rep78 and Rep68′ have a strong antiproliferative activity whereas Rep52 and Rep40′ do not affect cell growth. (**A**) Schematic representation of the different Rep proteins used in this work. Rep78 is shown at the top with the different functional domains known [modified from Smith *et al*. (1997)]. The numbers shown on the right refer to amino acid positions in Rep78. (**B**) NIH 3T3 cells were infected with the indicated recombinant retroviruses, split and seeded in selective medium. Colonies were fixed and stained on dishes 6 days after infection. (**C**) Outgrowth curves of BGF, BGF78, BGF68′ and BGF52-transduced NIH 3T3 cells. The percentage of GFP-positive cells was monitored at the indicated time point by flow cytometry. (**D**) Immunoblot analysis of retrovirally expressed Rep proteins, 3 days after infection.

Rep52 and Rep40′ had no effect on cell growth whereas Rep78 and Rep68′ strongly suppressed colony outgrowth (Figure 1B). Microscopic analysis showed that dishes seeded with BP78- or BP68′-infected cells contained numerous single cells that were enlarged, suggesting that these Rep proteins induce cell cycle arrest as opposed to cell death. Moreover, these results show that the absence of colonies is not due to the down-regulation of the resistance gene by the Rep proteins. Similar results were obtained with primary human lung fibroblasts (HLFs) and the human osteosarcoma cell line U2-OS transduced with pBP78 and 68′ (data not shown). Western blot analysis of infected cells showed that the different Rep proteins are expressed at similar levels (Figure 1D). In BP78 and BP68′, the AAV p19 promoter from which Rep52 and Rep40 are expressed is present and accounts for the low levels of these proteins present in BP78- and BP68′-transduced cells, respectively.

To rule out possible effects of the two large Rep proteins on viral titres and expression of the resistance gene, we created a new retroviral vector named BabeGFP (BGF), which instead of the drug resistance, co-expresses the enhanced green fluorescence protein (EGFP). The recombinant versions expressing Rep78, Rep68′ and Rep52 were generated (BGF78, BGF68′ and BGF52) and used to infect NIH 3T3 cells. Infected cells were passed every 2–3 days, and aliquots were fixed and analyzed by flow cytometry for the percentage of infected cells (Figure 1C). Although

similar infection rates were observed initially with the four different viruses, the GFP-positive population was rapidly outgrown in BGF78- and BGF68′-infected cells, with kinetics corresponding to a growth arrest, whereas this population remained constant for BGF- and BGF52-infected cells. Living cells were analyzed by fluorescence microscopy. Dishes seeded with BGF78- or BGF68′-transduced cells revealed green cells that were enlarged, with a morphology typical of arrested cells (data not shown) in accordance with the cell cycle block induced by Rep78 and Rep68′.

Differential cell cycle arrest mediated by Rep78 and Rep68

Having demonstrated the growth-suppressive activity of Rep78 and Rep68′ in this system, we wanted to know whether these cells are blocked in specific phases of the cell cycle. Asynchronously growing NIH 3T3 cells were infected with BP, BP78 and BP68′, and 4 days after infection subconfluent cells were pulse-labelled with bromodeoxyuridine (BrdU) for 45 min and fixed for flow cytometry. Nuclei were prepared, stained with propidium iodide and fluorescein isothiocyanate (FITC)-coupled anti-BrdU antibody, and analyzed by bivariate flow cytometry.

We found that Rep78 and Rep68′ have different effects on cell cycle distribution (Figure 2). Rep68′ led to a strong reduction of S-phase accompanied by accumulation of cells at the G_2–M boundary, while the percentage of cells in G_1 was not significantly changed. This pattern shows that the cells were blocked both in G_1 and G_2. In contrast, cells expressing Rep78 not only arrested in G_1 and G_2, but also in S-phase, as documented by the appearance of cells with S-phase DNA content that did not incorporate BrdU. Similar results were obtained with primary mouse embryonic fibroblasts (MEFs) and primary human lung fibroblasts (HLFs) (Figure 2) suggesting that the arrest observed here reflects a general action of the two Rep proteins.

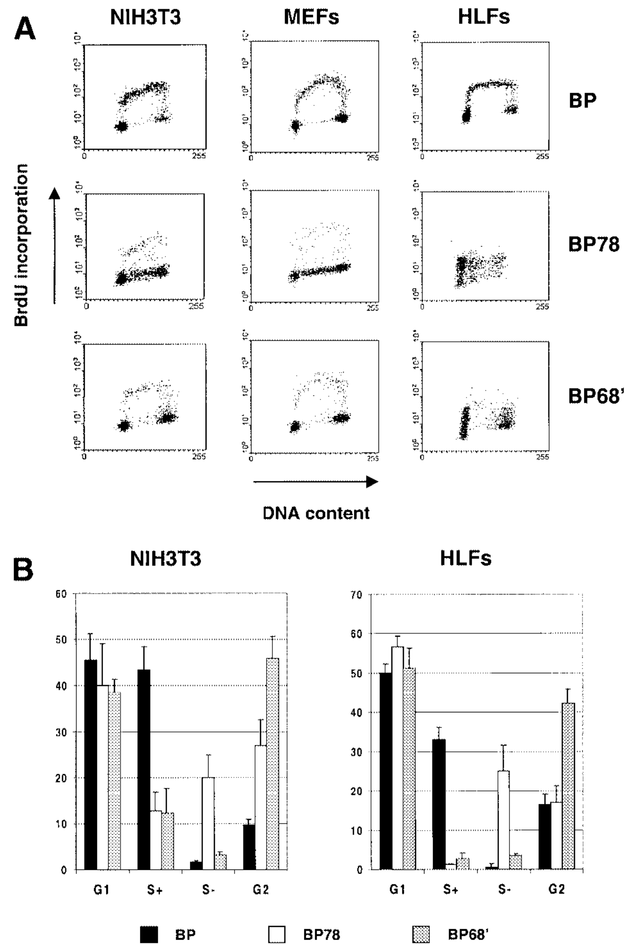

Fig. 2. Rep78 and Rep68′ arrest the cells at different phases of the cell division cycle. (**A**) Cell cycle distribution of Rep78- and Rep68′-expressing cells compared with cells transduced with empty vector as measured by BrdU incorporation and DNA-content flow cytometry on nuclei. BrdU incorporation is monitored with an FITC-anti-BrdU antibody (vertical axis) and DNA content by propidium iodide staining (horizontal axis). The different cell types are indicated at the top, and the different retroviruses at the right of the panels. (**B**) Quantitation of the different phases of the cell cycle in transduced NIH 3T3 cells and HLFs. The S-phase is differentiated into cells that synthesize DNA (S+) as measured by BrdU incorporation, and cells with S-phase DNA content that do not synthesize DNA (S–). BP-infected cells are shown as black, BP78 as white and BP68′ as grey bars. Error bars represent the standard deviations of four independent experiments.

Analysis of cell cycle proteins

To characterize the nature of the cell cycle arrest caused by the Rep proteins, we examined the expression of several cell cycle regulatory proteins by immunoblot analysis. NIH 3T3 cells were transduced with empty vector or viruses expressing either Rep78 or Rep68′, and the cells were collected for analysis 3–4 days after infection (Figure 3A). Both Rep proteins were expressed at similar levels in the transduced cell population. Neither Rep78 nor Rep68′ had a significant effect on the protein levels of CDK4, 6 and 2, the G_1 cyclins D1 and E, and the CKI p27. In contrast, expression of Rep78 and Rep68′ led to the induction of the CKI p21.

Expression of Rep78 and Rep68′ had opposite effects on the levels of the S-phase cyclin (A) and the G_2 cyclin (B1). Both cyclins were strongly down-regulated in BP78-transduced cells but present at higher levels in BP68′-transduced cells. The two Rep proteins also differentially affected Rb-family proteins. The phosphorylation status of these proteins can be monitored by SDS–PAGE, in which the hyperphosphorylated forms have slower migration. Rep68′-expressing cells accumulated with a mixture of hyper- and hypo-phosphorylated pRb and slightly increased levels of p130, whilst the levels and phosphorylation status of p107 remained unchanged. However, BP78-transduced cells arrested with increased levels of p130, decreased levels of p107 and essentially only the hypophosphorylated form of pRb.

To gain further insight into the nature of the cell cycle arrest induced by the Rep proteins, we measured kinase activities associated with the cyclins E, A and B1 in a histone H1 phosphorylation assay (Figure 3B). Rep78- and Rep68′-expressing cells had a 10-fold reduced cyclin B1-associated kinase activity whereas cyclin E-associated kinase activity was reduced by a factor of two. Cyclin A-associated kinase activity was strongly reduced in BP78, and reduced ~2-fold in BP68′-transduced cells. In summary, Rep68-expressing cells are arrested in G_1 and G_2–M. The increase in the fraction of hypophosphorylated pRb and the slight decrease in cyclin E activity appears

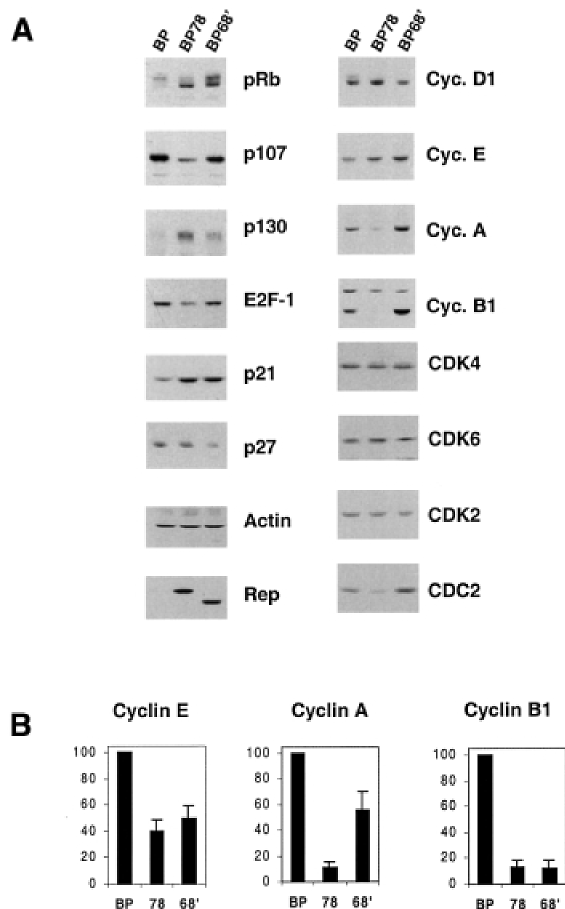

Fig. 3. (**A**) Immunoblot analysis of cellular proteins in transduced NIH 3T3 cells. Cells were infected with BP, BP78 and BP68′ as indicated at the top. At the time of analysis (3–4 days post-infection) all cells were subconfluent. The cellular proteins visualized in each panel are indicated to the right. (**B**) Cyclin E, cyclin A or cyclin B1 as indicated were immunoprecipitated from NIH 3T3 cell lysates. Histone H1 kinase activities were measured in the immunoprecipitates and normalized to the activities in control cells (BP, 100%). Error bars represent the standard deviation of at least three independent experiments.

consistent with the G_1 arrest. We presume that the elevated levels of the cyclins A and B1 can be attributed to the increased population in G_2, since we observed similar effect on these cyclins in NIH 3T3 cells chemically blocked in G_2 with etoposide (data not shown). In addition, Rep78 interferes with S-phase progression. Although Rep78-expressing cells are arrested throughout the cell cycle, they accumulate almost exclusively with the hypophosphorylated form of pRb. The reduced activity of cyclin A- and B1-associated kinases can be attributed to the markedly reduced levels of the corresponding cyclins.

A zinc finger motif in the C-terminus of Rep78 is responsible for the differential response of cells to Rep78 and Rep68′

We wanted to explore further the difference between Rep78 and Rep68′. As mentioned earlier, Rep68′ represents a C-terminal truncation of Rep78. A closer look at the C-terminus absent in Rep68′ reveals a zinc finger motif composed of three CXXC and three CXXH repeats (Figure 4C). This motif was shown to be capable of binding zinc *in vitro*. Individual mutation of either of these two repeated motifs, $(CXXC)_3$ to $(RXXR)_3$, and $(CXXH)_3$ to $(SXXH)_3$, abolished zinc binding suggesting that both repeats are necessary to form a functional zinc finger (Hörer *et al.*, 1995). To test whether this motif is responsible for the functional differences observed here between Rep78 and Rep68′, recombinant retroviruses were generated that express the $(RXXR)_3$ mutant version of Rep78 ($BP78^{CXXC}$), and analyzed as before in NIH 3T3 cells. Cells transduced with 78^{CXXC} behaved like cells transduced with Rep68′ (Figure 4A and B). They arrested in G_1 and G_2, accumulated with increased levels of p21 and cyclins A and B1, and displayed hypo- and hyper-phosphorylated pRb. Similar results were obtained with the $(SXXH)_3$ mutant (data not shown). In conclusion, a functional zinc finger motif is required for the S-phase arrest observed with Rep78 and for the quantitative accumulation of pRb in its hypophosphorylated form, as well as for the down-regulation of cyclins A and B1.

Rapid appearance of hypophosphorylated pRb upon Rep78 expression

Given the profound effect of Rep78 on the pRb phosphorylation pattern, we determined the kinetics of pRb accumulation in its hypophosphorylated form. NIH 3T3 cells were transduced with BGF or BGF78, and the phosphorylation status of pRb was examined in cells harvested at different time points between 24 and 48 h later (Figure 5). The percentage of infected cells was 80–90% as determined by flow cytometry (data not shown). Maximal Rep78 protein levels were observed 28 h after infection and remained constant thereafter. At this time point, the pRb phosphorylation pattern was still unchanged as compared with BGF-infected cells. However, 4 h later already half of the Rb protein was found in its hypophosphorylated form, and after an additional 4 h most of the Rb protein was hypophosphorylated. In contrast, cyclin A started to decline only 8 h after the onset of Rep expression. These results are in accordance with a central role played by pRb in response to Rep78, and suggest that the disappearance of cyclin A is subsequent to, rather than a cause of, pRb dephosphorylation.

Bypass of the Rep78-mediated arrest by the adenovirus E1A protein

We wanted to elucidate further the possible role of pRb in the S-phase arrest mediated by Rep78. The fact that in contrast to Rep68′, Rep78 arrests cells throughout the cell cycle with almost exclusively hypophosphorylated pRb prompted us to hypothesize that pRb could be responsible for the observed S-phase arrest. Two recent reports show that over-expression of active pRb can interfere with S-phase progression (Chew *et al.*, 1998; Knudsen *et al.*, 1998). This effect could be attenuated by over-expression of the adenovirus E1A protein, which functionally inactivates pRb by direct binding. Thus, we reasoned that if the Rep78-mediated S-phase arrest is due to active pRb, the adenovirus E1A protein should overcome this block. To test this hypothesis we used serial retroviral infections as described previously (Vlach *et al.*, 1996). NIH 3T3 cells were first infected (INF.1) with pBH (control vector) or pBH–E1A, expanded under hygro-

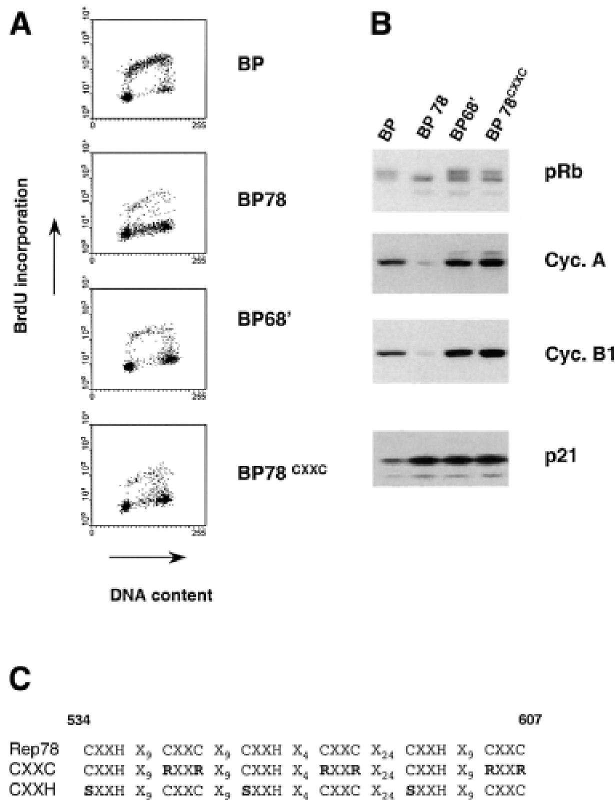

Fig. 4. The zinc finger motif in the C-terminus of Rep78 is responsible for the S-phase arrest. NIH 3T3 cells were transduced with BP, BP78, BP68′ and BP78^{CXXC}. (**A**) Cell cycle distribution of the different samples, analyzed as described in Figure 2. (**B**) Immunoblot analysis of cellular proteins as described in Figure 3. (**C**) Sequence of the zinc finger motif in the C-terminus of Rep78 in comparison with the mutant constructs 78^{CXXC} and 78^{CXXH} (Hörer *et al*., 1995).

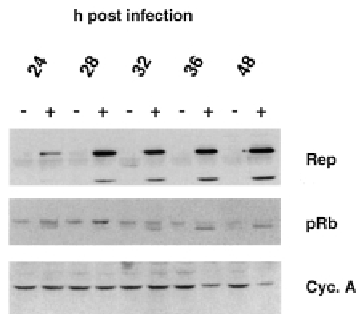

Fig. 5. Rapid appearance of hypophosphorylated pRb upon Rep78 expression. NIH 3T3 cells were infected with pBGF (–) and pBGF78 (+) for 12 h, split and seeded at different dilutions. Whole-cell lysates of samples harvested at the indicated time points were analyzed by immunoblotting for Rep and cyclin A protein levels and pRb phosphorylation status.

mycin selection, superinfected (INF.2) with BP or BP78, and seeded in puromycin selective medium. Cells were harvested 4 days after infection, and analyzed by bivariate flow cytometry (Figure 6A). E1A partially rescued Rep78-mediated cell cycle arrest. In comparison with control cells arrested with BP78, the number of cells expressing both E1A and Rep78 arrested in S and G_2 was reduced concomitant with an increased population of cells actively synthesizing DNA.

Two functional domains of E1A are required to bypass Rep78 mediated arrest

E1A is a multifunctional protein. To identify which functional domains are required to bypass Rep78-mediated cell cycle arrest, we analyzed a panel of different E1A mutants. These were E1A$^{124/135A}$, which is unable to bind and inactivate pRb; E1A^{R2G}, which has lost the ability to bind to p300/CBP but can still functionally inactivate pRb (reviewed Bayley and Mymryk, 1994); and an N-terminal deletion (E1AΔ$^{26–35}$), which still functionally inactivates pRb but has lost the ability to bind p400, a cellular protein with as yet unknown functions (Barbeau *et al*., 1994). Cell populations expressing wild-type (wt) E1A, E1A$^{124/135A}$, E1A^{R2G}, E1AΔ$^{26–35}$ and control cells (BH) were generated by retroviral transduction as described above, and superinfected with BP and BP78. Four days after infection, cells were harvested and analyzed by flow cytometry (Figure 6B). The mutant that cannot bind pRb (E1A$^{124/135A}$) failed to rescue the Rep78-mediated arrest. Similar results were obtained with HPV16 E7 protein. Wild-type E7 partially restored a normal cell cycle distribution, whereas the pRb non-binding mutant E7^{C24G} had no effect on the Rep78-imposed cell cycle arrest (data not shown). E1A^{R2G}, which has lost the ability to bind p300/CBP, showed a similar rescue to the one observed with wt E1A. Interestingly, E1AΔ$^{26-35}$ rescued the Rep78-mediated S and G_2 phase arrest, as documented by the reduced populations blocked in S and G_2 compared with control cells. However, this mutant did not rescue the Rep78-mediated G_1 arrest, since these cells accumulated in G_1. In conclusion, the pocket-binding domain and an N-terminal domain are required to bypass Rep78-mediated cell cycle arrest. Whereas the N-terminal domain residues 26–35 are essential to rescue the G_1 arrest, this domain is dispensable for the bypass of the S and G_2 arrest. These results provide further evidence for the implication of pRb in the S-phase arrest, and in addition reveal that Rep78 also targets a pRb-independent function required for the G_1–S transition.

We have shown above that Rep78 leads to the down-regulation of several cell cycle regulatory proteins including the E2F target genes, cyclin A and Cdc2, as well as the mitotic cyclin B1. We wondered whether the reduction of these protein levels was a consequence of the activation of pRb or whether this reflected a pRb-independent activity of Rep78. In order to test this, we analyzed the levels of these proteins in control cells (BH) and cells expressing E1A or E1A$^{124/135A}$ superinfected with BP and BP78 (Figure 6C). Cells expressing the E1A mutant deficient in pRb binding (E1A$^{124/135A}$) displayed similar patterns to control cells when challenged with Rep78. In contrast, the levels of cyclin A and Cdc2 were restored in cells expressing both E1A and Rep78. Interestingly, the levels of cyclin B1, which is not an E2F target gene, were also restored in cells expressing E1A. However, recently J.Lukas *et al*. (1999) provided a link between pRb phosphorylation status in S-phase and cyclin B1 stability (see Discussion). In conclusion, these results suggest that the reduced levels of cyclins A and B1,

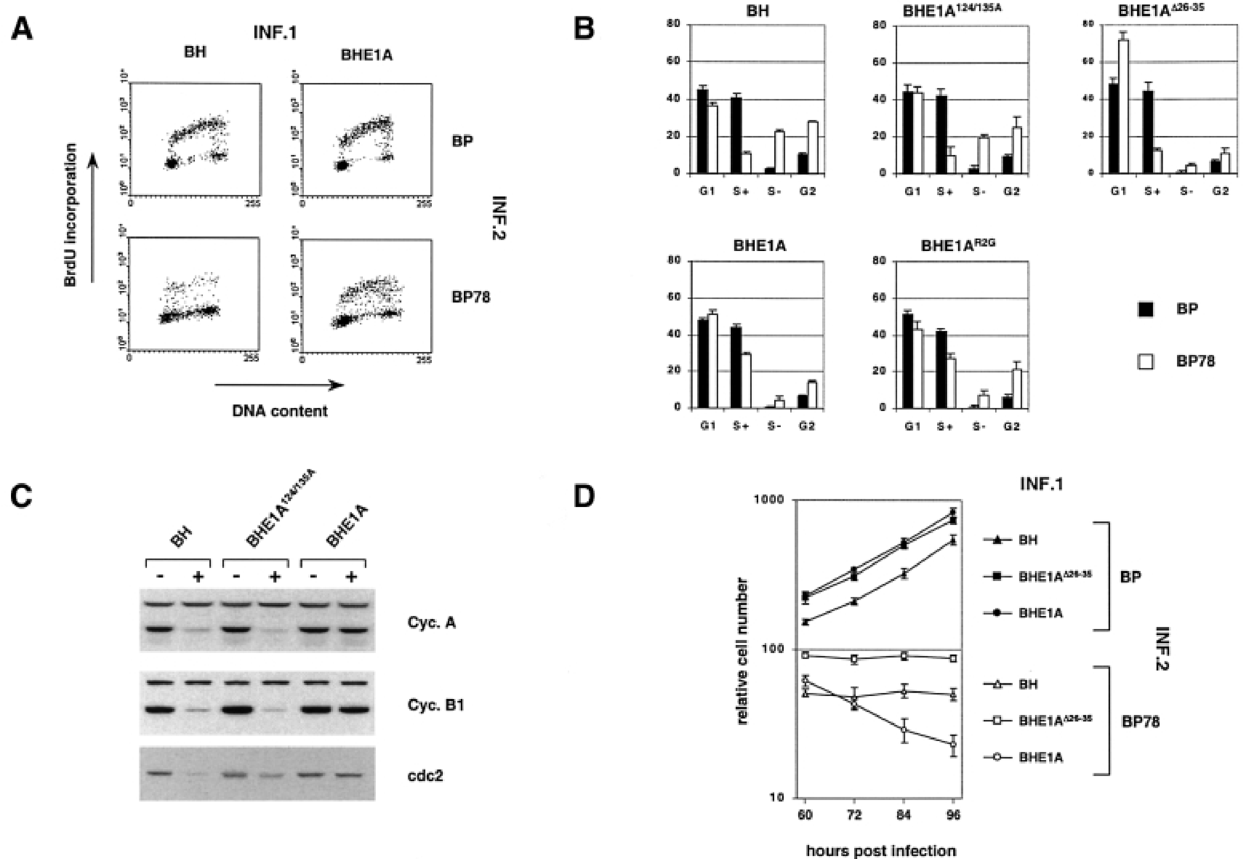

Fig. 6. Bypass of the Rep78-mediated cell cycle arrest by the Ad-E1A protein. (**A**) Cell cycle distribution of infected cells prepared and analyzed by flow cytometry as described in Figure 2. NIH 3T3 cells were infected first (INF.1) with a retrovirus expressing E1A or with the empty control vector (pBH), followed by BP retroviruses with or without Rep78 (INF.2). (**B**) pRb binding but not p300 binding of E1A is required to rescue Rep78 mediated S-phase arrest. Quantitation of the different phases of the cell cycle serially-infected NIH 3T3 cells. NIH 3T3 cells were infected first with BH retroviruses expressing E1A, $E1A^{R2G}$, $E1A^{124/135A}$ and $E1A\Delta^{26-35}$ or with the empty control vector (BH) followed by a second infection with BP or BP78. The different cell populations infected are indicated above the graphs. BP-infected cells are shown as black bars and BP78 as white bars. The S-phase is differentiated into cells that synthesize DNA (S+) as measured by BrdU incorporation and cells with S-phase DNA content that do not synthesize DNA (S–). Error bars represent the standard deviations of three independent experiments. (**C**) pRb inactivation by E1A is required to restore cyclin A, B1 and cdc2 protein levels in cells expressing Rep78. Cell populations expressing E1A, $E1A^{124/135A}$ or infected with control virus (BH) were infected with BP (–) or BP78 (+) as indicated at the top. Immunoblot analysis was performed as described in Figure 3. The antibodies used are indicated to the right of the individual panels. (**D**) Cells expressing both E1A and Rep78 die. Cells infected with BH, BHE1A and $BHE1A\Delta^{26-35}$ retroviruses were superinfected with BP and BP78 viruses as described above. After complete selection (48 h post-splitting) resistant cells were counted at 12 h intervals. Averages and the standard deviation of triplicates are shown.

and of Cdc2 are a consequence of pRb activation rather than a pRb-independent function of Rep78.

Cells expressing both E1A and Rep78 die

Although E1A partially rescued a Rep78-mediated cell cycle arrest, E1A-expressing cells transduced with BP78 fail to form colonies in a colony outgrowth assay (data not shown). Microscopic analysis revealed that there were very few cells recovered expressing both E1A and Rep78, suggesting that these cells were dying. To test this further we compared the growth properties of these cells with control and $E1A\Delta^{26-35}$-expressing cells that are both arrested by Rep78. The different cell populations were transduced with BP or BP78, selected in puromycin and, 48 h post splitting, were counted at 12 h intervals (Figure 6D). The number of control and $E1A\Delta^{26-35}$-expressing cells transduced with BP78 remained constant over this time period, reflecting cell cycle arrest. However, cells expressing the E1A protein decreased in number, suggesting cell death. The cell populations infected with BP showed exponential growth. Taken together with the observations from the flow cytometry analysis above, these results show that E1A-expressing cells that fail to arrest upon Rep78 expression, die.

Reduced S-phase arrest in $Rb^{-/-}$ MEFs

Thus far, several lines of evidence suggest that the S-phase arrest observed in Rep78-expressing cells is mediated by pRb. If this arrest depends on the appearance of hypophosphorylated pRb in S-phase, cells that lack pRb should not be blocked within S-phase if challenged with Rep78. Therefore, we analyzed MEFs derived from $Rb^{-/-}$ embryos and matched controls ($Rb^{+/+}$) for their response to BP or BP78 infection. The $Rb^{-/-}$ and $Rb^{+/+}$ MEFs were infected with BP and BP78, harvested 4 days after infection and analyzed by flow cytometry as described above (Figure 7).

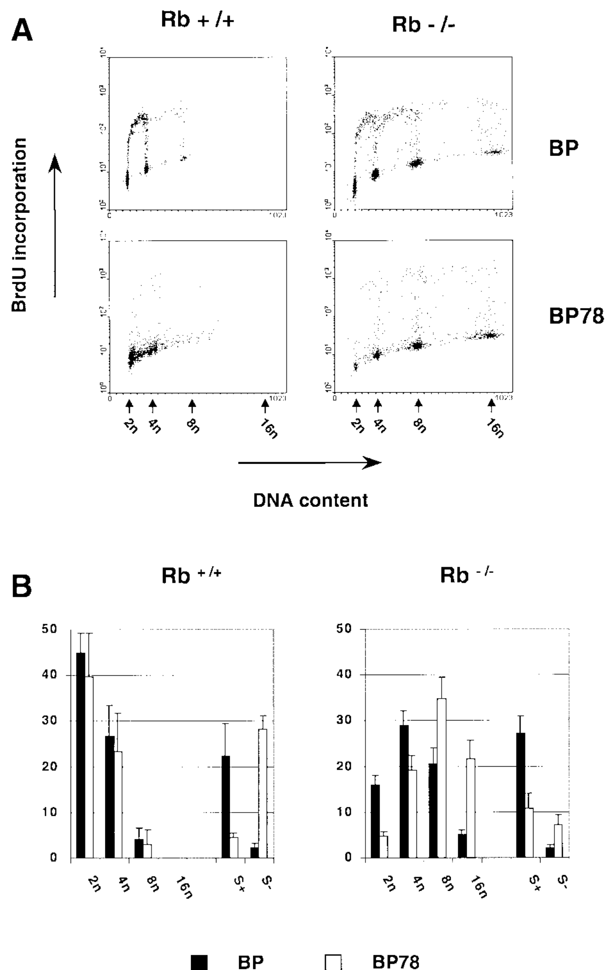

Fig. 7. Strongly reduced S-phase arrest in $Rb^{-/-}$ MEFs transduced with BP78. (**A**) Cell cycle distribution of $Rb^{-/-}$ and $Rb^{+/+}$ MEFs (indicated at the top) infected with BP or BP78 (indicated to the right) analyzed as described in Figure 2. The 2n, 4n, 8n and 16n populations are indicated at the bottom of the panels by arrows. (**B**) Quantitation of the different phases of the cell cycle in transduced $Rb^{-/-}$ and $Rb^{+/+}$ MEFs. The percentage of cells with 2n, 4n, 8n and 16n DNA content, the percentage of cells incorporating BrdU (S+) and the percentage of cells with intermediate DNA content that do not incorporate BrdU (S–) are shown. BP-infected cells are shown as black, BP78 as white bars. Error bars represent the standard deviations of three independent experiments.

Whereas wild-type MEFs ($Rb^{+/+}$) displayed very low levels of re-replication, $Rb^{-/-}$ cells showed a high propensity to undergo multiple rounds of DNA replication without cell division when infected with control virus (BP). A clear difference between $Rb^{+/+}$ and $Rb^{-/-}$ cells was observed when the cells were infected with BP78. $Rb^{+/+}$ MEFs showed a tight arrest in all phases of the cell cycle, as observed above in different cell types. In contrast, $Rb^{-/-}$ MEFs displayed a strongly reduced proportion of cells arrested within S-phase (S–) when challenged with Rep78. These cells undergo even more rounds of re-replication as documented by the increased populations with 8n and 16n DNA content, respectively. They did not proliferate (data not shown) and had a reduced population actively synthesizing DNA (S+). In conclusion, these results show that pRb plays the major role in mediating the Rep78-imposed S-phase arrest.

Discussion

We have studied the role of the Rep proteins in the proposed antiproliferative activity of AAV using a retroviral system. We show that Rep78 and Rep68′ have a strong antiproliferative activity in primary, immortalized and transformed cells of different origins. In contrast, Rep52 and Rep40′, which lack the DNA binding domain, did not interfere with cell proliferation. Our results reveal a clear biological difference between the two large Rep proteins as documented by a distinct cell cycle arrest and differential modulation of cell cycle regulatory proteins.

The two outstanding features of Rep78-expressing cells are the observed S-phase arrest and the accumulation of hypophosphorylated pRb. We speculated that these two events might be linked, and that pRb plays a key role in the cellular response to Rep78. This notion is supported by the fact that pRb rapidly appears in its hypophosphorylated active form upon Rep78 expression. Furthermore, the reduced levels of cyclin A, p107 and Cdc2, which are all E2F target genes (Helin, 1998), can be attributed to the accumulation of active pRb. Surprisingly, levels of cyclin E, although an E2F target gene (Ohtani *et al.*, 1995), were unchanged in Rep78-expressing cells. However, C.Lukas *et al.* (1999) similarly observed unchanged cyclin E protein levels in cells arrested either by p16 or by constitutively active pRb, and proposed that physiological levels of cyclin E can be reached independently of E2F activity. Interestingly, J.Lukas *et al.* (1999) could show that repression of E2F activity in S-phase using constitutively active pRb leads to the degradation of cyclin B1 through unscheduled activation of APC. These observations link pRb status during S-phase to the levels of cyclin B1 and provide an explanation for the strongly reduced cyclin B1 levels observed in Rep78-expressing cells in our experiments. We therefore postulate that the modulation of different cell cycle regulatory proteins (cyclins A, B1, Cdc2 and p107) is a consequence of the activation of pRb we observed, rather than a pRb-independent activity of Rep78. This is supported by the observation that cyclin A levels declined subsequent to pRb activation, and by the fact that E1A restored cyclins A and B1, and Cdc2 levels, whereas an E1A mutant unable to inactivate pRb did not.

Two recent reports based on the over-expression of non-phosphorylatable and thus constitutively active pRb mutants revealed that pRb has the potential to interfere with S-phase progression (Chew *et al.*, 1998; Knudsen *et al.*, 1998). However, these effects were observed with unphysiological levels of active pRb. Nevertheless, in both studies the pRb-mediated S-phase arrest could be attenuated by the co-expression of the adenovirus E1A protein, which can functionally inactivate pRb. To address the role of pRb in the observed S-phase arrest we tested several different E1A mutants for their ability to bypass Rep78-mediated cell cycle arrest. The adeno-E1A protein partially restored a normal cell cycle profile in cells expressing Rep78, although these cells did not proliferate and ultimately died. In contrast, a mutant defective in pRb-binding ($E1A^{124/135A}$) had no effect on Rep78-mediated cell cycle arrest. Similar results were obtained with wt and mutant HPV16 E7 proteins (data not shown). Furthermore, rescue of Rep78-mediated cell cycle arrest did not depend

on E1A's interaction with p300/CBP since E1A^{C2R} displayed a similar pattern to the one observed with E1A. Interestingly, cells expressing E1AΔ$^{26–35}$ and Rep78 were arrested in G_1. This mutant has been shown to be able to bypass a p16-mediated, but not a p27-mediated cell cycle arrest (Alevizopoulos *et al*., 1998). These observations imply that E1AΔ$^{26–35}$ is fully functional in pRb inactivation, and that besides its role in pRb phosphorylation CDK2 activity is required for another event essential for G_1–S progression. The fact that E1AΔ$^{26–35}$ bypasses the S and G_2 arrest but not the G_1 arrest of Rep78 further confirms the essential role of pRb in the Rep78-mediated S-phase arrest, and also shows that Rep78 targets a pRb-independent function required for G_1–S transition.

Altogether, these results provide good evidence for the central role played by pRb in the observed S-phase arrest, and suggest that Rep78-mediated S-phase arrest should not occur in pRb-negative cells. Indeed, a strong reduction of the S-phase arrest was observed in $Rb^{-/-}$ MEFs compared with matched control MEFs, which are $Rb^{+/+}$, when challenged with Rep78. As expected from the E1A results, and from the fact that the C-terminal truncation of Rep78 (Rep68′) arrests cells in G_1 and G_2 without drastically affecting pRb, $Rb^{-/-}$ MEFs still failed to proliferate upon Rep78 expression. The interpretation of this result is complicated by the fact that $pRb^{-/-}$ MEFs also underwent several rounds of re-replication when infected with control viruses. However, when challenged with Rep78 a shift was observed towards the 8n and 16n population, respectively, showing that under these circumstances they displayed an even higher propensity to re-replicate. This may be due to the increased levels of p21 in Rep78-expressing cells since it was previously shown that p21 can induce re-replication in cells negative for pRb (Niculescu *et al*., 1998). The residual low levels of cells arrested within S-phase may be due to the effects of other pocket proteins. The fact that $Rb^{-/-}$ cells, in contrast to E1A- or E1AΔ$^{26–35}$-expressing cells, fail to undergo mitosis when challenged with Rep78, suggests that the Ad-E1A protein also targets a pRb-independent event required for the G_2–M transition. In conclusion, our findings show that pRb is the major player in the Rep78-mediated S-phase arrest and in addition show that physiological levels of pRb can interfere with S-phase progression. Furthermore, they reveal that conditions exist that result in the appearance of hypophosphorylated pRb beyond the restriction point late in G_1.

Interestingly, Rep78's ability to activate pRb and to induce S-phase arrest depended on a functional zinc finger motif within the C-terminus. Since this region is dispensable for Rep's site-specific DNA binding activity, the phenotypes observed with Rep78 may be mediated by protein–protein interactions through this motif. Recently, Rep78 was shown to bind to and inhibit two protein kinases (PKA and PrKX), and this interaction was mapped to the C-terminus of Rep78 (Chiorini *et al*., 1998; Di Pasquale and Stacey, 1998). However, Rep52 also inhibits these kinases (Chiorini *et al*., 1998) yet fails to arrest cells, arguing against a role for these interactions in the observed cell cycle arrest. Nevertheless, it will be interesting to explore the C-terminus and especially the zinc finger motif for interacting proteins since this may help to understand the mechanism by which pRb is activated upon Rep78 expression.

Different mechanisms can be envisaged for the activation of pRb; either pRb kinases could be inhibited or a pRb phosphatase could be induced. The observation that pRb dephosphorylation precedes the decline of cyclin A, taken together with the fact that cyclin E/CDK2 was only reduced 2-fold, raises the possibility that Rep78-mediated appearance of hypophosphorylated pRb could be achieved by the induction of a pRb phosphatase. Emerging data suggest that induction of phosphatases is implicated in the activation of pocket proteins. Hypoxia was shown to lead to the activation of the pRb-phosphatase PP1 (Krtolica *et al*., 1998) Similarly, PP2A was shown to be implicated in the rapid dephosphorylation of p107 upon UV irradiation (Voorhoeve *et al*., 1999). However, little is known about how these phosphatases are induced following the different stimuli.

Besides the novel function of Rep78 leading to the activation of pRb and subsequent S-phase arrest, the Rep proteins contain additional activities able to arrest cells. The C-terminal truncation of Rep78 (Rep68′), which does not drastically affect pRb, has lost the ability to arrest cells within S-phase but still blocks cells in G_1 and G_2. Since it is unlikely that the 2-fold reduction in cyclin E CDK2 activity can account for the arrest in G_1, Rep68′ (like Rep78) may also inhibit pRb-independent functions required for the G_1–S transition. In contrast, the strongly reduced cyclin B1-associated kinase activity provides the molecular mechanism for the inability of these cells to enter mitosis, since this kinase has been shown to be crucial for the G_2–M transition (Draetta *et al*., 1989). We did not find increased p21 levels associated with this complex (data not shown) and neither cyclin B1 nor Cdc2 protein levels were reduced. Therefore, we presume that the kinase is inhibited via inhibitory phosphorylation of Cdc2 as proposed for the G_2 arrest upon DNA damage (Sanchez *et al*., 1997). Considering that Rep78 and Rep68 are multifunctional proteins with activities including specific DNA binding, site-specific endonuclease and helicase, it is plausible that they may interfere with cellular DNA and trigger a DNA damage response. This hypothesis is supported by the fact that both cell cycle arrest in G_1 and G_2, and the induction of p21, have been linked to DNA damage response (Kastan *et al*., 1991; Dulic *et al*., 1994). Furthermore, the replication protein NS1 of the autonomous parvovirus minute virus of mice was shown to block cell cycle progression in certain cell types (Op De Beeck and Caillet-Fauquet, 1997); the authors proposed that this was due to NS1-induced nicks in cellular chromatin.

What benefit could the strong cytostatic activity of the Rep proteins have for AAV? Some clues to a function of the observed S-phase arrest for the AAV life cycle are provided by recent reports analyzing transduction efficiency of AAV vectors that do not contain the Rep gene. It has been shown that the adenovirus E4orf6 protein strongly enhances transduction mediated by AAV vectors (Ferrari *et al*., 1996). Grifman *et al*. (1999) further analyzed this issue and could show that upon E4orf6 expression, the cells were arrested in S-phase with strongly reduced cyclin A levels. Furthermore, the E4orf6-mediated increase in transduction efficiency was abolished by transient transfection of cyclin A. Hence, in a wild-type AAV infection, Rep78, which we have shown to affect the

cells similarly, could promote the establishment of a latent infection. This could be achieved by the Rep proteins shown to be associated with the AAV capsids (Kube *et al.*, 1997) and may not require *de novo* Rep synthesis. During latent infection, Rep is undetectable due to Rep-mediated repression of its own promoters (Beaton *et al.*, 1989) thereby preventing interference with the host cell. We have shown that the adeno-E1A protein can rescue a Rep78-mediated cell cycle arrest. Thus, during a productive infection in the presence of adenovirus, where Rep levels are high, Rep no longer interferes with DNA synthesis allowing efficient replication of the AAV genome.

It was previously reported that infection of primary human fibroblasts with AAV leads to a cell cycle arrest (Hermanns *et al.*, 1997). These arrested cells displayed reduced levels of the cyclins A and B1, hypophosphorylated pRb and p107, and increased amounts of the CKI p21. The fact that the effects of Rep78 we have found are strikingly similar to those observed with AAV virions suggests that the major component of AAV's antiproliferative activity is provided by the Rep proteins. Cell cycle interference by Rep proteins could therefore be at the basis of AAV's reported oncosuppressive properties.

Materials and methods

Retroviral expression vectors

The retroviral expression vectors pBabe-Puro (pBP) and pBabe-Hygro2 (pBH) have been described previously (Morgenstern and Land, 1990). pBabe-GFP (pBGF) was constructed by replacing the SV40-puro resistance cassette in pBP with the *EGFP* gene under the control of a CMV promoter from pEGFP-C1 (Clontech). The AAV2 *Rep78* gene (nucleotides 321–2252), *Rep52* gene (nucleotides 993–2252), exon 1 of *Rep68* (nucleotides 321–1906) and exon 1 of *Rep40* (nucleotides 993–1906) were amplified by PCR from pAV2 (Laughlin *et al.*, 1983), and cloned into pBP or pBGF, and the resulting recombinant retroviral vectors were named pBP or pBGF78, pBGF52, pBGF68′ and pBGF40′, respectively. cDNAs encoding the zinc finger mutants were a kind gift of J.Kleinschmidt (Hörer *et al.*, 1995) and were subcloned into pBP, generating $pBP78^{CXXC}$ and $pBP78^{CXXH}$. The recombinant retroviral vectors expressing the different E1A mutants, pBH–E1A, $pBH–E1A\Delta^{26–35}$, $pBH–E1A^{C2R}$ and $pBH–E1A^{124/135A}$ were a kind gift of K.Alevizopoulos and have been described previously (Alevizopoulos *et al.*, 1998).

Cell culture, retroviral infections and cell cycle analysis

Cells were maintained in high glucose Dulbecco's-modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 10% donor calf serum for NIH 3T3 cells and derivatives thereof, or 10% fetal calf serum (FCS) for all other cells.

High titre retroviral supernatants were generated by transient transfection of phoenix E or A cells and used to infect NIH 3T3 cells and derivatives thereof (phoenix E), or various human cells (phoenix A) as described (Pear *et al.*, 1996). Infected cells were selected with the appropriate drug: hygromycin (Calbiochem, 150 µg/ml) or puromycin (Sigma, 2.5 µg/ml). For cell cycle analysis, subconfluent cells were pulsed with 33 µM BrdU for 30–45 min, detached with trypsin and fixed in 70% ethanol. Samples were prepared as described previously (Tlysty *et al.*, 1995) with modifications as follows (centrifugation at 2000 r.p.m. in an MSE Super Minor centrifuge between each step): pepsin digestion (0.08% pepsin in 0.1 N HCl) for 20 min at 37°C; 2 N HCl for 20 min at 37°C; wash in 0.1 M sodium borate pH 8.0; wash in IFA (10 mM HEPES pH 7.4, 150 mM NaCl, 4% FCS, 0.1% sodium azide, 0.5% Tween 20); incubation in FITC-conjugated anti-BrdU (Beckton Dickinson) diluted 1/3 in IFA, without Tween 20 for 45′, at 4°C; wash in IFA; incubation in IFA containing 0.1 mg/ml RNase A at 37°C for 30 min. Nuclei were then stained with 10 µg/ml propidium iodide and analyzed by two-dimensional flow cytometry.

Biochemical analysis of infected cells

Biochemical analysis of infected cells was done as described previously (Vlach *et al.*, 1996). Briefly, infected cells were lysed in mild lysis buffer (MLB) containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitiol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin, 20 mM NaF, and 0.3 mM ortho-sodium-vanadate. Between 20 and 40 µg of total cellular protein (determined by Bradford assay, Bio-Rad) were used for SDS–PAGE and immunoblotting. After incubation with primary antibodies, reactive proteins were detected with peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and enhanced chemiluminescence (ECL, Amersham). For kinase assays, cells were lysed in MLB and 60–200 µg of total protein were immunoprecipitated with 2 µl of the respective antibody for 2 h at 4°C, in the presence of protein A–Sepharose. Beads were washed four times in MLB and once in kinase reaction buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). Beads were then resuspended in 40 µl of kinase reaction buffer supplemented with 0.1 mg/ml bovine serum albumin, 50 µM ATP, 1 µg of histone H1, 7 µCi of $[\gamma$-$^{32}P]$ATP and incubated for 20 min at 30°C. The reaction was stopped by the addition of 3× SDS loading buffer, radiolabelled histone H1 was resolved by SDS–PAGE and quantified by PhosphorImager.

Antibodies

Antibodies against cyclin A (sc-751), cyclin B1 (sc-245), cyclin D1(sc-450), cyclin E (sc-481), CDK2 (163), CDK4 (sc-749), CDK6 (sc-177), Cdc2 (sc-54), p107 (sc-318), p130 (sc-317), p21(sc-397) and E2F-1 (sc-193) were from Santa Cruz, and anti-pRb (14001A) was from Pharmingen. The polyclonal antibody against Rep was generated against a GST–Rep78 fusion protein.

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