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Infection of cells with replication deficient adenovirus induces cell cycle alterations and leads to downregulation of E2F-1

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Abstract

Gene products of recombinant replication-deficient adenovirus vectors of the first generation (Ad vector) can induce cell cycle dysregulation and apoptosis after infection in eukaryotic cells. The mechanisms underlying this complex process are largely unknown. Therefore, we investigated the regulation of the pRb/E2F-1 complex, which controls transition from G_0/G_1 to S phase of the cell cycle. As Ad vector infection results in a decrease in the number of cells in G_0/G_1 phase of the cell cycle, we observed a decline of the pRb protein level and, surprisingly, also a decrease of the E2F-1 protein and mRNA level in infected cell lines. Furthermore, in contrast to the reduction of cells in the G_0/G_1 phase we observed increased protein levels of p53 and p21 proteins. However, as experiments in p53 deficient cell lines indicated, the decrease of pRb and E2F-1 is independent of p53 and p21 expression. Moreover, results obtained with Rb deficient cell lines indicated that the reduced E2F-1 expression is independent of pRb. These results suggest that Ad vector-induced cell cycle dysregulation is associated with a specific downregulation of E2F-1 independent of Rb and p53 genomic status of cells.

Keywords: Adenovirus vector; pRb; E2F-1; p53; Cell cycle

1. Introduction

Recombinant replication-deficient adenovirus-based gene transfer vectors of the first generation (Ad vectors) are one of the most powerful and efficient means to transfer genetic material. In Ad vector, the E1 region of the adenovirus genome which is responsible for induction of viral replication has been deleted to prevent virus replication [1–3]. This deletion generates space for an expression cassette which allows transgene expression for an extended period [4,5]. However, the Ad vector still carries most of the adenoviral genes. Whereas they stimulate in general the host cell metabolism in favor of foreign gene expression, specific adenovirus gene products interfere with the cell cycle of the infected cell [2,6]. For instance, the E4orf6/7 protein binds to E2F proteins during the early phase of infection to stimulate E2

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promoter activity [7,8]. Moreover, the E4orf6 protein can bind to and inactivate p53 to control one of the major checkpoints of apoptosis and cell cycle regulation [9].

One of the most important checkpoints of the cell cycle in eukaryotic cells is the restriction point (R-point) which controls transition from G_0/G_1 phase to S phase [10]. Whereas the R-point is regulated by many factors, the ultimate substrate in this process is the Retinoblastoma protein (pRb) [11]. Its major activity is supposed to be sequestration or inactivation of the transcription factor E2F which is required for activation of S-phase genes [12]. The E2F family of transcription factors comprises a group of closely related proteins (E2F-1 through -5). One of the best characterized gene of the E2F family is E2F-1. During G_0/G_1 phase of the cell cycle hypophosphorylated pRb binds E2F-1 protein and this complex actively represses transcription from E2F sites in several promoters [13,14]. For transition from G_0/G_1 to S phase, phosphorylation of pRb by G_1 cyclin/cyclin-dependent kinase (cdk) complexes results in release of active E2F-1 protein [12,15]. This active protein directs due to its function as transcription activator the timely expression of cell cycle-controlling genes whose products are involved in DNA replication [16].

Several studies have shown that changes in the expression level of pRb and E2F-1 protein can lead to dysregulation of the cell cycle. Deficiency of pRb leads to inappropriate S phase entry, activation of E2F-response genes and apoptosis [17]. On the other hand, introduction of wild-type Rb gene into cells lacking functional pRb suppresses cell growth and apoptosis after DNA damage [18,19]. In contrast, overexpression of E2F-1 induces accumulation of cells in S and G_2/M phases and leads to apoptosis [20].

Because several studies have shown that Ad vector can induce cell cycle alterations [21,22], we analyzed the effect of Ad vector infection on expression of pRb and E2F-1 of the host cell. We found that Ad vector infection induces a decrease of pRb and also a decline of E2F-1 protein and mRNA expression. The downregulation of pRb and E2F-1 was independent of the p53 genomic status and the decline of E2F-1 was furthermore not dependent on the presence of pRb. Our results show that Ad vector-induced cell cycle dysregulation is associated with specific changes in the expression of cell cycle regulatory proteins.

2. Materials and methods

2.1. Cell lines and cell culture

All cell lines used were purchased from ATCC. The human colon carcinoma cell lines LoVo, LS174T, SW620, human breast cancer cell line BT549, cervix cancer cell line C33A, lung cell (NSCLC) cancer line H1299 and 293 cells (human embryonic kidney cells) were maintained in DMEM or RPMI 1640 (LS174T, SW620, H1299), supplemented with 10% FCS (fetal calf serum) and 2 mM glutamine at 37°C, 5% CO_2 in a humidified atmosphere. For experiments, cells were seeded, treated and analyzed as indicated.

2.2. Recombinant adenovirus vectors

The recombinant Ad vector AdRSV-hAAT.2 carrying the cDNA of the human α_1-antitrypsin driven by the Rous-Sarcoma-Virus promoter was a gift from M. Kay (Department of Genetics, Stanford University, Palo Alto, CA, USA). The recombinant Ad vector AdCLX.Null without expression cassette and AdRSV.β-Gal, carrying the cDNA of β-galactosidase, were a gift from R. Crystal (Cornell University Medical College, New York, NY). Vectors were propagated on 293 cells and purified by cesium chloride density centrifugation, titered by plaque assay and stored at −80°C [5]. Multiplicity of infection (MOI) resulting in equal levels of infection in the different cell lines were determined by infecting the cells with AdRSV.β-Gal carrying the reporter gene β-galactosidase. Infection of cells was performed for 1.5 h at 37°C in serum free medium. After 1 day of infection, cells were fixed and stained for β-galactosidase (β-Gal) activity.

2.3. Western blotting

Cell extracts were lysed on ice (50 mM HEPES

(pH 7.5), 200 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml leupeptin). Protein concentrations were determined by DC Protein Assay (Bio-Rad). For Western blotting, 10 μg protein per lane were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes (Hybond-N, Amersham) by standard procedures. Primary monoclonal antibodies against Rb (G3-245), E2F-1 (KH95/E2F), p53 (DO-1) and p21 (SX118) were purchased from Pharmingen and the antibody against β-actin (N 350) from Amersham. The biotinylated secondary antibody was derived from Vector Laboratories. In all cases detection was performed with streptavidin coupled peroxidase and ECL (Amersham).

2.4. Assessment of cell cycle distribution by FACS analysis

For FACS analysis 8×10^5 cells were seeded as triplicates in 10-cm dishes, allowed to adhere overnight and infected with Ad vectors at the indicated multiplicity of infection (MOI). The cells were harvested at the times indicated, were stained with FITC-conjugated Annexin V and propidium iodide (PI) using the Annexin V kit (Immunotech) and analyzed by flow cytometry (FACScan, Becton Dickinson) for the presence of viable (Annexin V- and PI-negative) and apoptotic (Annexin V-positive) cells which include both, primary (PI-negative) and secondary (PI-positive), apoptotic subpopulations.

To study cell cycle distributions, samples (5×10^5 cells) were fixed and permeabilized by addition of 2 ml of ice-cold 70% ethanol for 1 h at 4°C. After washing, the cells were resuspended in 0.5 ml PBS containing 50 mg/ml PI, pH 7.5. Following treatment with 10 μl of 10 mg/ml RNase (type I-A; Roche Diagnostics) for 30 min at room temperature in the dark, the cells were stored at 4°C until analysis by flow cytometry. Cell cycle analysis was performed using either CellQuest software (Becton Dickinson) or ModFit LT (Verity, Topsham, ME, USA). Cell aggregates were excluded from the analysis by using the Doublet Discriminating Module (DDM, Becton Dickinson).

2.5. Semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was isolated from 1×10^6 cells infected with Ad vector or mock infected using RNeasy Mini kit (Qiagen). The extracted RNA was quantified by 260 nm and 1 μg was subjected to DNase I digestion (Gibco-BRL) at room temperature for 15 min. DNase I was heat-inactivated by addition of EDTA (2,5mM) and incubation for 15 min at 65°C. After DNase digestion RNA was subjected to reverse transcription using oligo(dT) primer following the protocol described in a Reverse Transcription System kit (Promega). Samples were amplified by PCR for Rb and E2F-1. GAPDH was used as control. The primer used for amplification were as follows: Rb forward 5′-CCGGAGGACC-TGCC-TCTCGT-3′, Rb reverse 5′-AACGACATCTCATC-TAGGTC-3′, E2F forward 5′-ACCTTCGTAGC-ATTG-CAGACC-3′, E2F reverse 5′-TTCTTGC-TCCAGGCTGAGTAG-3′, GAPDH forward 5′-GCAGGGGGG-AGCCAAAAGGG-3′, GAPDH reverse 5′-TGC-CAGCCCCAGCGTCAAAG-3′. Each amplification mixture (20 μl) containing 1 μl cDNA, 2 μl Reaction-Buffer (Promega), 1 U *Taq* polymerase (Promega), 200 μM dNTP each, and 10 pg forward and reverse primers each. The optimized PCR conditions were one cycle denaturation at 94°C for 2 min, followed by gene specific cycles (Rb and E2F-1: 30 cycles; GAPDH: 22 cycles) of denaturation at 94°C for 30 s, gene-specific annealing (Rb: 55°C; E2F-1: 58°C; GAPDH: 62°C) for 30 s, and extension at 72°C for 30 s. The amplification cycles were followed by an additional extension at 72°C for 7 min. The PCR products were run on 2% agarose gel with ethidium bromide staining. The intensity for each band was determined using the Gel Doc System (Bio-Rad).

2.6. UV inactivation of Ad vector

Virus (AdRSV.β-Gal) was diluted in 2 ml serum-free medium in a six-well plate. The plate was placed on a UV table and irradiated for 30 min (302 nm). One ml of serum-free medium containing UV inactivated Ad vector was used to infect cells as described above. As control we used virus, which was placed in the six-well plate for 30 min without UV irradiation.

Table 1
Cell cycle distribution of LoVo cells infected with Ad vectors

	Mock	AdRSV.hAAT	AdCLX.Null
G_0/G_1	61.3 ± 5.2%	35.8 ± 1.7%*	43.7 ± 4.8%*
S	28.2 ± 6.7%	33.6 ± 1.7%	36.5 ± 3.2%
G_2/M	10.6 ± 1.5%	30.6 ± 2.0%*	19.8 ± 5.3%*

LoVo cells were mock infected, or infected with 50 MOI of AdRSV-hAAT.2, or AdCLX.Null. Cells were harvested after 72 h of infection and the DNA content was determined by FACS analysis. Percentages of cells in G_0/G_1, S and G_2/M phases are derived from DNA histograms. Data are mean ± S.D. ($n = 3$). *Significantly different to mock ($P < 0.05$) using Student's *t*-test.

After 1 day of infection, cells were fixed and stained for β-galactosidase activity.

3. Results

3.1. Effect of Ad vector on cell cycle regulation and induction of apoptosis

Gene products of the Ad vector can alter the cell cycle regulation of the host cell after infection [23–25]. To further examine the underlying molecular mechanisms, LoVo cells were infected with an Ad vector carrying the human α_1-antitrypsin gene (AdRSV-hAAT.2) as reporter gene or with an Ad vector without an expression cassette (AdCLX.Null). Seventy-two hours after infection, cells were counted and subjected to analysis of cell cycle distribution. Consistent with earlier reports cell cycle analysis showed a substantial decrease in the relative number of cells in the G_0/G_1 phase and an increase in the relative number of cells in the G_2/M phase of the cell cycle (Table 1). The differences in G_0/G_1 and G_2/M phases between mock-infected cells and cells infected with the Ad vectors were statistically significant. Parallel to the changes in cell cycle distribution, we observed a significant increase ($P < 0.05$) in the number of apoptotic cells (mock: 4.7 ± 0.6%; AdRSV-hAAT.2: 11.0 ± 1.0%; AdCLX.Null: 7.8 ± 1.2%). Similar results were obtained with the Ad vector carrying the cDNA for β-galactosidase under the control of the RSV promoter (Table 2). These data indicate that Ad vector infection induces dysregulation of the cell cycle, and that this is independent of the cDNA contained in the expression cassette of Ad vector.

3.2. Effect of Ad vector on expression of G_1 regulatory proteins

A variety of DNA viruses like the adenovirus use the transcription machinery of the host cell to replicate their own genome by activating cell cycle progression due to overriding the G_1 checkpoint of cell cycle regulation [2,6]. In this context G_1 regulatory proteins like p53 and pRb are one of the main targets for adenovirus gene products leading to inactivation of the G_1 checkpoint. Therefore, we analyzed the expression of G_1 regulatory proteins after infection with AdRSV-hAAT.2 and AdCLX.Null. Western blot analysis in Ad vector-infected LoVo cells demonstrated that the expression of endogenous p53 was increased when compared with mock-infected cells (Fig. 1A). Consequently, we also observed an increase of p21 which can be transcriptionally activated by p53 [26]. One of the important functions of p53-induced p21 expression is the arrest of cells in G_1 mediated by pRb [27]. However, as

Table 2
Cell cycle distribution of LoVo cells and number of β-galactosidase-positive cells infected with AdRSV.β-Gal after UV inactivation

	Mock	AdRSV.β-Gal	AdRSV.β-Gal (UV[a])
G_0/G_1	71.9 ± 2.4	50.4 ± 6.8*	69.5 ± 3.7
S	18.8 ± 3.6	31.2 ± 7.2*	21.1 ± 2.9
G_2/M	9.3 ± 2.1	18.4 ± 7.1*	9.4 ± 4.1
β-Gal-positive cells	0%	> 95%	< 1%

LoVo cells were mock infected, or infected with 50 MOI of native AdRSV.β-Gal and after UV irradiation. Cells were harvested after 72 h of infection and DNA content was determined by FACS analysis. Percentages of cells in G_0/G_1, S and G_2/M phases are derived from DNA histograms. Data are mean ± S.D. ($n = 3$). *Significantly different to mock ($P < 0.05$) using Student's *t*-test.
[a]UV inactivated.

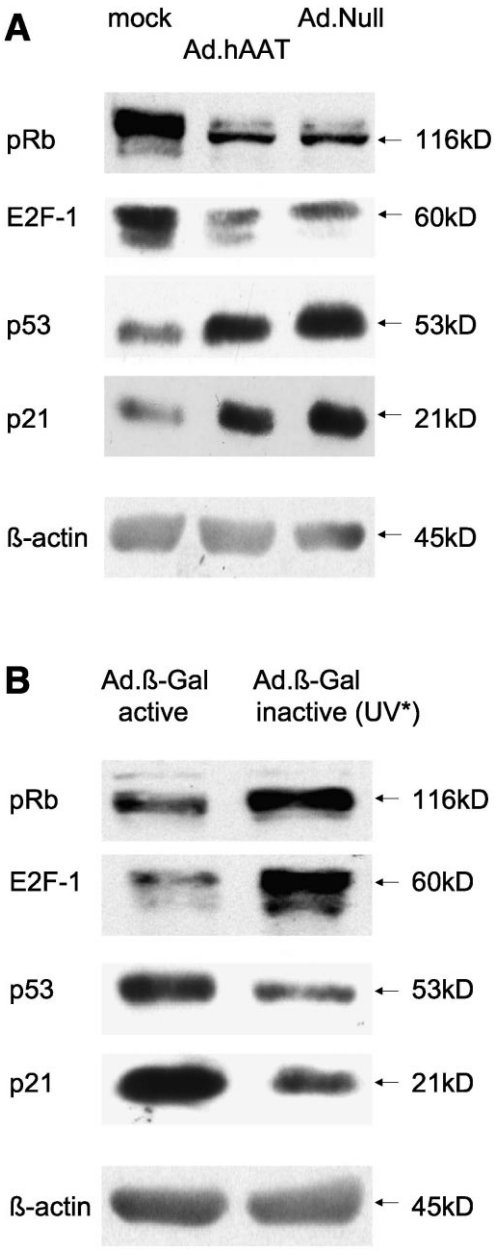

Fig. 1. Expression of G_1 regulatory proteins in LoVo cells infected with Ad vectors. LoVo cells were (A) mock infected or infected with 50 MOI of AdRSV-hAAT.2, or AdCLX.Null, and (B) infected with 50 MOI of AdRSV.β-Gal or of AdRSV.β-Gal after UV irradiation. After 72 h of infection floating and adherent cells were harvested, combined and subjected to Western blot analysis for protein levels of pRb, E2F-1, p53 and p21. Equal amounts (10 μg) of cellular protein from each sample were separated by SDS–PAGE (8% for pRb, p53 and E2F-1, 15% for p21). After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membrane was probed with monoclonal anti-human pRb, E2F-1, p53 and p21 antibodies and protein bands were visualized. Multiple bands of pRb and E2F-1 represent different phosphorylation states. β-Actin was used to control protein loading. *UV inactivated. ←

described, Ad vector induces a decrease in the fraction of cells in G_1 phase. Therefore we also analyzed the protein level of pRb, which should be downregulated if cells are lost in G_1 [11]. Infection of LoVo cells with Ad vectors induced a reduction in the level of pRb (Fig. 1A). Since the loss and/or inactivation of pRb can lead to overexpression of E2F-1 [28,29], we next investigated the protein expression of E2F-1, which is required for activation of S-phase genes [12]. Interestingly, in contrast to the well known upregulation of E2F-1 during progression of transition of G_1 to S phase, we observed a dramatic decline of E2F-1 protein expression after Ad vector infection (Fig. 1A). The observed effect of decreased pRb and E2F-1 protein levels after Ad vector infection was dependent on the dose (Fig. 2) and on the duration of infection (data not shown). These results indicate that infection of cells with the Ad vector is associated with downregulation of the protein levels of pRb and E2F-1. Further, these changes in the expression of cell cycle regulatory factors were also independent of the cDNA contained in the expression cassettes of the Ad vector.

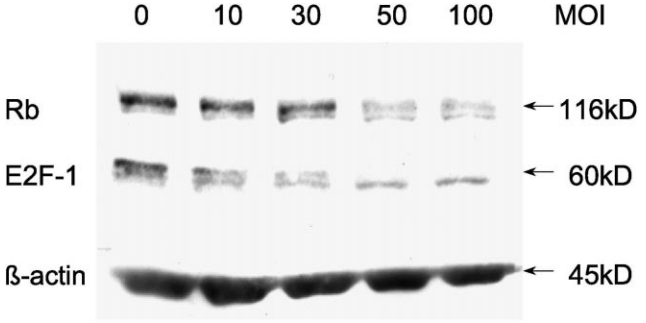

Fig. 2. Expression of pRb and E2F-1 in LoVo cells infected with various doses of Ad vector. LoVo cells were infected with increasing doses (0, 1, 10, 30, 50 and 100 MOI) of AdRSV-hAAT.2. After 72 h incubation, cells were harvested and protein expression of pRb and E2F-1 was examined by Western blot analysis as described in Fig. 1.

3.3. UV-inactivated Ad vector has no effects on cell cycle progression and its regulation

In order to examine whether the uptake of Ad vector particles itself may have the potential to induce cell cycle changes, we inactivated the viral DNA by exposition to UV light. Infection of LoVo cells with an Ad vector containing the cDNA of β-galactosidase showed that enzyme activity was lost after UV inactivation of Ad vector. Whereas about 95% of cells infected with the control vector were stained, less than 1% of cells infected with UV-inactivated vector showed β-galactosidase activity (Table 2). This means that the transcriptional activity of Ad vector is abrogated and any observed effects are independent of viral gene expression.

Analysis of cell cycle after infection of cells with UV inactivated Ad vector showed no difference in the cell cycle distribution compared with mock-infected cells, whereas the untreated vector again showed a significant decrease in G_0/G_1 phase and an arrest in G_2 (Table 2). Further, the Ad vector-induced changes in the expression of cell cycle regulating proteins such p53, p21, pRb, and E2F-1 were abrogated by UV inactivation (Fig. 1B). These results indicate that adenoviral gene expression is necessary for the induction of cell cycle changes.

3.4. Role of p53/p21 pathway on downregulation of pRb and E2F-1 by Ad vector infection

We found an increase in the level of p53 protein expression in p53 wild-type (wt) LoVo cells after Ad vector infection. Because p53 can indirectly inhibit the E2F-1 transcriptional activity by maintaining pRb in its hypophosphorylated form [30], we were interested to see if the Ad vector-induced decrease of pRb and E2F-1 protein expression might be related to the genetic status and/or the presence of p53. To this end, we infected the p53-negative cell line H1299 with the Ad vector. As observed in p53-wt LoVo cells, we noted a decrease of pRb and E2F-1 protein levels after infection with an equivalent dose of Ad vector (Fig. 3). The same results were obtained when SW 620, a cell line which overexpresses mutated p53 was used (data not shown).

The protein expression of p21 which can be transcriptionally activated by p53 is increased in LoVo

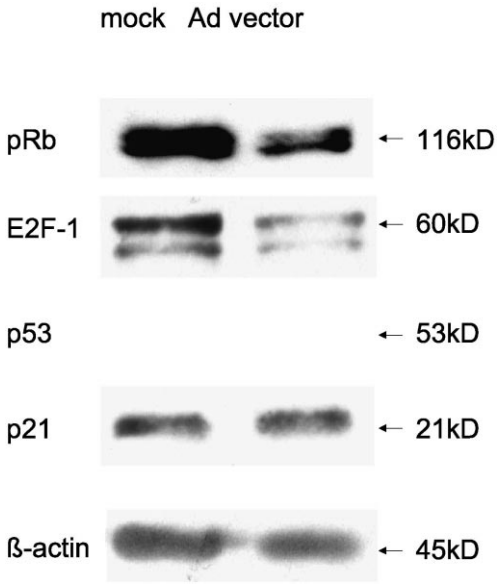

Fig. 3. pRb and E2F-1 expression in p53-negative cells infected with Ad vector. The p53-negative cell line H1299 was mock infected or infected with 25 MOI AdRSV-hAAT.2. After 96 h incubation, cells were harvested and expression of pRb, E2F-1, p53 and p21 was examined by Western blot analysis as described in Fig. 1.

cells infected with Ad vector. Because transcription of the p21 gene can also be activated by p53-independent mechanisms [31], we also analyzed the protein level of p21 in p53-deficient cells after Ad vector infection. In contrast to wt p53 LoVo cells we found no difference in the protein level of p21 between Ad vector and mock-infected H1299 cells (Fig. 3). In mutant-type (mt) p53 cells such as BT549, C33A or SW620 which either do not express p21 or only at low levels, we also observed no difference in the pattern of expression of p21 between Ad vector and mock-infected cells (data not shown). This demonstrates that the decrease of pRb and E2F-1 protein levels after Ad vector infection is not dependent on a functional p53/p21 pathway.

3.5. Effect of Ad vector on mRNA levels of Rb and E2F-1

To investigate whether the changes observed in the Rb and E2F-1 protein levels are paralleled by similar changes in the level of mRNA, we determined mRNA expression of Rb and E2F-1 in infected LoVo cells by semi-quantitative RT–PCR. We de-

tected that the mRNA level of E2F-1 was decreased in Ad vector-infected LoVo cells to about 50% of the level in uninfected control cells, whereas the mRNA level of Rb remained unchanged (Fig. 4). Investigations with the p53-negative cell line H1299 showed the same results (data not shown). This demonstrates that E2F-1 mRNA is downregulated in Ad vector-infected cells whereas pRb appears to be affected only at the level of protein.

3.6. *Influence of pRb expression on E2F-1 downregulation*

The mRNA expression of pRb and E2F-1 indicated that both genes are differently transcriptionally regulated. Because pRb can regulate the E2F-1 expression [11,12], we next examined whether downregulation of E2F-1 protein depends on expression of pRb. To this end, we investigated the influence of Ad vector infection on the level of E2F-1 protein expression in two pRb defective cell lines; BT549, which is Rb deleted and C33A, which expresses a smaller and inactive form of pRb at a very low level. We observed in these cells the same cell cycle alterations as in pRb-positive cells (data not shown). Further, similar to the results in cell lines expressing intact pRb, infection of cells carrying deleted or inactive pRb with Ad vector also leads to a decline of E2F-1 protein expression (Fig. 5). These observations suggest that the downregulation of E2F-1 after infection with Ad vector occurs independently of pRb.

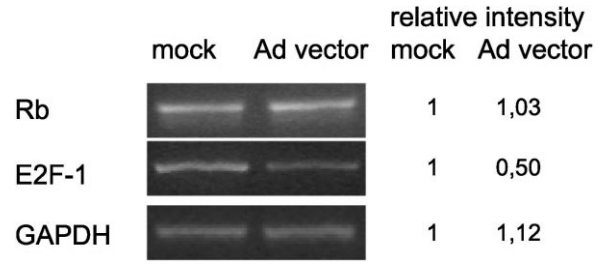

Fig. 4. Rb and E2F-1 mRNA expression in LoVo cells infected with Ad vector. LoVo cells were mock infected or infected with AdRSV-hAAT.2 as described in Fig. 1. After 72 h cells were harvested and total RNA was isolated. Steady-state level of Rb and E2F-1 mRNA was determined by semi-quantitative RT–PCR. GAPDH was used as control. The data presented are representative of three samples.

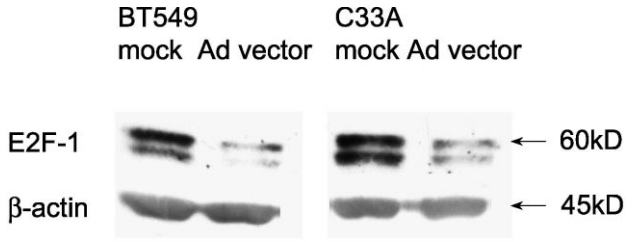

Fig. 5. Expression of E2F-1 protein in pRb-negative cells infected with Ad vector. The Rb-negative cell lines C33A and BT549 were mock infected or infected with AdRSV-hAAT.2(125 MOI/C33a, 80 MOI/BT549). Cells were harvested 96 h after infection and subjected to Western blot analysis for the protein level of E2F-1 as described in Fig. 1.

4. Discussion

In our study we investigated the influence of Ad vector of the first generation on the protein complex of pRb and E2F-1, known to be the ultimate substrate in regulation of transition from G_0 to G_1 phase of the cell cycle. Consistent with previous studies [21,22], we observed in our study a decrease in the number of cells in the G_0/G_1 phase and simultaneously an arrest of cells in G_2/M after infection with Ad vector, suggesting that cell cycle regulation may be altered during infection. We found that these cell cycle changes were accompanied by a decrease of pRb and E2F-1 protein levels. Further, we observed an upregulation of p53 and p21 in p53-wt cells.

The decrease of pRb protein expression was expected, because downregulation of pRb by viral products is not unusual in order to promote cellular transcription and viral replication. For example, the SV40 large T antigen [32], HPV16 E7 oncoprotein [33], and the adenovirus E1A protein [34], bind to pRb and inhibit its tumor suppressor function. The Epstein–Barr virus (EBV) immediate-early protein BRLF1 downregulates pRb and induces transition of G_1 to S phase [29]. As we used E1-deficient adenovirus, our results strongly indicate that adenoviral proteins other than E1A can also cause, directly or indirectly, the loss of pRb and, consequently, the function of pRb in the G_1–S phase transition. Moreover, pRb has been found to be important in the regulation of G_2/M transition [35,36]. However, we

found the observed cell cycle changes also in pRb-negative cells. This fact shows that Ad vector-induced cell cycle dysregulation is not dependent on pRb expression.

As the decreased level of pRb was paralleled by an increase in protein levels of p53 and p21 in cells expressing wild-type p53, its loss during Ad vector infection was suggested not to be caused by general protein degradation. Moreover, this reduced pRb level may explain why after Ad vector infection the activated p53/p21 pathway does not result in G_1 arrest of the cell cycle [37]. P21 induces G_1 arrest through inactivation of the cyclin-dependent kinase 2 (cdk2), which is therefore blocked to phosphorylate pRb. The resulting hypophosphorylated pRb prevents cell cycle transition from G_1 to S phase [30]. Because Ad vector infection induces a loss of the ultimate substrate, i.e., the pRb protein, an upregulated p53/p21 pathway is functionally insufficient to mediate G_1 arrest.

The major activity of pRb during cell cycle regulation is to control the R-point by sequestration or inactivation of the transcription factor E2F-1, which is required for S-phase entry [11,12]. In the G_0/G_1 phase E2F-1 is inactivated by binding to hypophosphorylated pRb. Inactivation of pRb by phosphorylation and/or destruction results in liberation of free active E2F-1 which in turn activates expression of genes involved in the G_1 to S phase transition. This suggests that the phenomenon of reduced fraction of cells in G_0/G_1 may be easily explained by elevated levels of liberated E2F-1 after loss of pRb. However, we unexpectedly found a dramatic decrease of E2F-1 expression in the infected cells. An explanation for this observation of a reduced G_0/G_1 population in spite of downregulated E2F-1 could be that other members of the E2F protein family might substitute for E2F-1 [38] or other mediators can promote the transition from G_0/G_1 into S phase. In fact, whereas E2F-1 is one of the most prominent key regulators for S-phase entry, recent studies demonstrated that ectopic expression of cyclin/cdk complexes can induce transition from G_0/G_1 to S phase in the absence of free E2F-1 [39]. Since Wersto et al. found that cyclin proteins such as cyclins A, B1, and D are elevated in Ad vector-infected cells [21], it is conceivable that rather than E2F-1, these cyclins with their associated cdks are responsible for the cell cycle progression leading to a reduced fraction of cells in G_0/G_1.

The increase of the proportion of cells in the G_2/M phase is also a prominent effect of Ad vector infection. Normally, during S phase cyclin A binds to E2F-1 which results in inhibition of E2F-1 DNA-binding activity [40]. This inhibition of E2F-1 is important for correct cell cycle progression [41]. The unscheduled presence of active E2F-1 or the induction of E2F-1 expression in cells induces a decline in the G_1 population with concomitant increase in the S and G_2/M population followed by apoptosis [42,43]. However, we observed a decrease of E2F-1. Therefore, we suppose that the G_2/M arrest of cells is not directly caused by the decline of E2F-1.

The mechanism causing the decrease of E2F-1 and its physiological role after Ad vector infection are at present unclear. Alterations of cell cycle in response to DNA damage are often associated with altered expression of p53/p21 [44]. Therefore, we analyzed whether E2F-1 downregulation in Ad vector-infected cells is associated with activation of p53/p21. However, we also observed a decrease of E2F-1 expression in Ad vector-infected p53-deficient cells. Therefore, we conclude that the downregulation of E2F-1 in Ad vector-infected cells is not dependent on p53 and p21 protein expression.

Overexpression of E2F-1 has been shown to lead to cell cycle dysregulation and to induction of apoptosis [20]. All adenoviral interference with the host cell cycle described so far has pointed to the induction of E2F-1 to promote viral replication [7,8]. However, we observed a decrease of E2F-1 mRNA and protein which is associated with alterations of cell cycle distribution and induction of apoptosis. Therefore, it is possible that the downregulation of E2F-1 is a reaction of the cell in order to prevent inappropriate cell cycle progression. This process is counteracted by gene products of the E1 region in wild-type adenovirus and is detectable only in E1-deleted recombinant vectors. This mechanism might be an equivalent to upregulation of p53 and p21 after DNA damage which both serve to arrest the cell cycle at several important checkpoints [37,45]. Observations in UV-irradiated BUdR-sensitized cells showed an upregulation of p53 and p21 and also a decrease of E2F-1 protein expression [46].

Nevertheless, the fact is that in infected cells the

decrease of E2F-1 cannot maintain the cells in G_0/G_1 phase and does not protect against apoptosis. It is likely that the Ad vector is still able to override the protective mechanisms of the cell. Thus, further investigations of E2F-1 regulation after Ad vector infection will give more information about the nature of cell cycle control and may help to understand cytotoxic side effects of first-generation Ad vector, and may aid the design of improved Ad vectors.

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