The adenovirus E4 ORF6 and E1b 55 kDa proteins cooperate in a p53-independent manner to enhance transduction by recombinant adeno-associated virus vectors

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The observation that exposure of target cells to genotoxic stress or adenovirus infection enhances recombinant adeno-associated virus (rAAV) transduction is an important lead towards defining the rAAV transduction mechanism, and has significant implications for the exploitation of rAAV in gene therapy applications. The adenovirus-mediated enhancement of rAAV transduction has been mapped to the E4 ORF6 gene, and expression of E4 ORF6 alone has been considered necessary and sufficient to mediate this effect. Since p53 subserves an important function in the cellular response to genotoxic stress, and interacts with the E4 ORF6 gene product during adenovirus infection, we hypothesized that p53 function might be essential to the rAAV enhancement resulting from these cellular insults. In the current study, using the p53-null cell lines H1299 and Saos-2, we find that p53 is not essential to either genotoxic stress or adenovirus-mediated enhancement of rAAV transduction. We further demonstrate using HeLa, H1299 and Saos-2 cells that E4 ORF6 expression alone is not sufficient to enhance rAAV transduction and that coexpression of the adenovirus E1b 55 kDa protein is necessary. Together, these observations indicate that the mechanism by which adenovirus infection enhances rAAV transduction involves cooperative and interdependent functions of the E4 ORF6 and E1b 55 kDa proteins that are p53-independent.

Introduction

Recombinant adeno-associated virus (rAAV) vectors show significant promise for gene therapy applications, and are currently the subject of intense investigation (Flotte & Carter, 1995; Xiao et al., 1997; Russell & Kay, 1999). AAV is a non-pathogenic helper-dependent human parvovirus that under physiological conditions requires coinfection with adenovirus (Atchison et al., 1965) or herpesvirus (Buller et al., 1981) for productive infection. In the absence of helper virus coinfection the AAV genome, encapsidated as a single-stranded DNA molecule of either sense (Borns & Rose, 1970), is capable of conversion to a double-stranded molecule that can undergo integration into host cell DNA to establish latent infection (Laughlin et al., 1986). The genome conversion and integration events do not require de novo viral gene expression and the essential elements required in cis for virus replication, packaging and integration are encoded in the 145 nucleotide terminal repeat (TR) sequences that flank the genome. These biological properties have been exploited in the development of rAAV vectors that retain only the viral TR sequences and can accommodate heterologous gene expression cassettes up to approximately 4–8 kb in length (McLaughlin et al., 1988; Samulski et al., 1989; Muzyczka, 1992).

While rAAV stocks can be generated that have extremely high virus titre, the proportion of single-stranded input genomes successfully converting to transcriptionally active double-stranded molecules varies dramatically with different target cell types (Halbert et al., 1995; Xiao et al., 1996; Russell & Kay, 1999) and as a function of the cell cycle (Russell et al., 1994). This critical dependence of rAAV transduction efficiency on the state of the intracellular milieu remains poorly understood, and currently limits the potential utility of rAAV in many gene therapy applications. The demonstration, however, that rAAV transduction can be dramatically enhanced by exposing target cells to genotoxic stress (Alexander...
et al., 1994; Russell et al., 1995; Ferrari et al., 1996) or adenovirus infection (Fisher et al., 1996; Ferrari et al., 1996) offers the prospect of overcoming this apparent biological limitation and optimizing the potential of this gene delivery system.

Adenovirus-mediated enhancement of rAAV transduction has previously been mapped to the E4 ORF6 gene product, the function of which has been described as necessary and sufficient to mediate this effect (Fisher et al., 1996; Ferrari et al., 1996). An additional contribution from the E1 region has also been recognized, but considered to be cooperative and dependent on E4 ORF6 (Fisher et al., 1996). Even less is known about the mechanism by which genotoxic stress enhances rAAV transduction, although induction of DNA repair activities has been proposed (Alexander et al., 1994). We are interested in identifying the cellular proteins and activities mediating the enhancement of rAAV transduction efficiency, and initially hypothesized that p53 may subserve a crucial function in this process because p53 is upregulated in response to genotoxic stress (Levine, 1997; Steele et al., 1998), and conversely is targeted for degradation by interaction with the E4 ORF6 protein during an adenovirus infection (Querido et al., 1997; Steegenga et al., 1998). We therefore chose to establish whether or not these cellular insults that result in enhanced rAAV transduction are dependent on the p53 pathway.

In the current study, using HeLa cells and two p53-null cell lines, H1299 and Saos-2, we show that cellular p53 is not essential for the enhancement of rAAV transduction efficiency following exposure of target cells to genotoxic stress or adenovirus infection, confirming the recent report of Griffon et al. (1999). We further demonstrate that, in the cell lines examined, the E1b 55 kDa protein subserves a function in adenovirus-mediated enhancement of rAAV transduction equally as important as that already attributed to the E4 ORF6 gene product. Indeed, the rAAV enhancement effect of these adenovirus proteins is best characterized as cooperative and interdependent and this paper reports the first empirically derived data supporting this hypothesis. Ongoing analysis of the mechanism by which adenovirus infection changes the intracellular milieu in favour of rAAV transduction should therefore be focused on the cooperative effects of the E4 ORF6 and E1b 55 kDa proteins that do not involve interaction with p53. This information should ultimately lead to an improved understanding of the cellular proteins and activities mediating rAAV transduction.

Methods

Plasmids and plasmid construction. The E4 ORF6 open reading frame was amplified by PCR from adenovirus dl309 (Jones & Shenk, 1979) DNA using primers 5′ ccaatgccacagcaatcggcggt 3′ (forward) and 5′ ccaatggcagcagcctactacaggg 3′ (reverse) and cloned into pGEM-T (Promega). Both primers contain an EcoRI half-site (underlined). The E4 open reading frame in pGEM-T was verified by sequencing and then subcloned into the EcoRI site of pCI-neo (Promega), placing it under the transcriptional control of a CMV immediate-early promoter, to create plasmid pCI40Rf6. The rAAV vector plasmid, pCMVnLacZ, was constructed, using standard molecular techniques (Sambrook et al., 1989), by inserting the CMV promoter from pCI-neo and the nuclear localizing LacZ open reading frame and polyadenylation signal from pAB11 (Goodman et al., 1994) into the XhoI site of pusb201 (Samulski et al., 1987). Plasmids pCMVE1b55 (Goodrum et al., 1996), pFG140 (Graham, 1984), pAAV/Ad (Samulski et al., 1989), pXX6 and pXX2 (Xiao et al., 1998) have been described previously.

Cell culture and DNA transfection. HeLa cells, 293 cells and the p53-null cell lines H1299 and Saos-2 have been described elsewhere (Gey et al., 1995; Ferrari et al., 1996). An additional contribution from the E1 region has also been recognized, but considered to be cooperative and dependent on E4 ORF6 (Fisher et al., 1996). Even less is known about the mechanism by which genotoxic stress enhances rAAV transduction, although induction of DNA repair activities has been proposed (Alexander et al., 1994). We are interested in identifying the cellular proteins and activities mediating the enhancement of rAAV transduction efficiency, and initially hypothesized that p53 may subserve a crucial function in this process because p53 is upregulated in response to genotoxic stress (Levine, 1997; Steele et al., 1998), and conversely is targeted for degradation by interaction with the E4 ORF6 protein during an adenovirus infection (Querido et al., 1997; Steegenga et al., 1998). We therefore chose to establish whether or not these cellular insults that result in enhanced rAAV transduction are dependent on the p53 pathway.

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Propagation and analysis of adenovirus mutants. Adenovirus stocks were propagated on 293 cells. The adenovirus E3 mutant dl309, and the E1b 55 kDa mutants dl338 and dl1520, have been described (Pilder et al., 1986; Barker & Berk, 1987). The dl338 mutant has a 524 bp in-frame deletion between nucleotides 2805 and 3329 and does not produce E1b 55 kDa (Pilder et al., 1986). The dl1520 mutant has an 827 bp deletion combined with a point mutation at nucleotide 2222 that introduces a premature stop codon (Barker & Berk, 1987). Stocks of dl309 were purified by equilibrium density-gradient centrifugation in CsCl and stocks of the E1b 55 kDa mutants were made from clarified crude cell lysates as described (Snyder et al., 1996). Virus titre was assigned for each preparation by plaque assay on 293 cells (Snyder et al., 1996). To test the ability of adenovirus mutants to enhance rAAV transduction, cells were seeded into 12-well plates at a density of 1.5 to 3 x 10^5 cells per well in 0.5 ml of medium. Six hours after replating cells were simultaneously exposed to rAAV vector and the adenovirus mutant under investigation. Each adenovirus mutant was used at an m.o.i. of approximately 5 p.f.u. per cell.

UV irradiation. A Stratalinker 1800 (Stratagene) was used to expose cell monolayers to 15 J/m^2 UV irradiation (254 nm). Tissue culture medium was aspirated from cell monolayers which were then exposed to UV irradiation immediately prior to rAAV transduction.

Immunohistochemistry. The mouse monoclonal antibody MAb3, which recognizes an epitope within the N terminus of E4 ORF6, was obtained from Tom Shenk, Princeton University (Marton et al., 1990). The mouse monoclonal antibody 2A6, specific for the E1b 55 kDa protein, was obtained from Arnold Levine, Princeton University (Sarnow et al., 1982). Both antibodies were provided as hybridoma tissue culture supernatant and were diluted in TBS pH 7.4–2% BSA. The 2A6 antibody was detected with an indirect immunoperoxidase system according to the manufacturer’s instructions (Dako). Peroxidase activity was visualized with DAB (Sigma). MAb3 binding was detected with an FITC-labelled goat α-mouse secondary antibody (Becton Dickson) and visualized under epifluorescent illumination on a Leica DMLB upright microscope.

Results

Adenovirus infection and genotoxic stress enhance rAAV transduction in p53-null cell lines

The p53-null cell lines H1299 and Saos-2 were used to test the hypothesis that p53 function is required for enhanced rAAV transduction in target cells after (i) adenovirus infection or (ii) exposure to genotoxic stress. Exponentially growing H1299 and Saos-2 cells were exposed to rAAV in the absence or presence of pre-treatment with either (i) adenovirus dl309 or (ii) UV irradiation. In both cell lines the transduction efficiency of rAAV was markedly increased by pre-treatment with either adenovirus infection or UV irradiation (Fig. 1).

E4 ORF6 expression enhances rAAV transduction in 293 cells but not in H1299 and Saos-2 cells

An E4 ORF6 expression construct, designated pCIE4ORF6, was generated by subcloning the PCR amplified gene into the mammalian expression vector, pCI-neo. This E4 ORF6 construct was functional based on (i) expression studies using MAb3 monoclonal antibody (Fig. 2A, B), (ii) the nuclear relocalization of E1b 55 kDa (Fig. 2C, D) and (iii) the enhancement of rAAV transduction in 293 cells after transient transfection (Fig. 2E).

In contrast to results obtained in 293 cells, there was no enhancement of rAAV transduction after transient transfection of H1299 or Saos-2 cells with the E4 ORF6 construct (Fig. 3A). To preclude the possibility that this negative result might reflect low transient transfection efficiencies in H1299 and Saos-2 cells, stable E4 ORF6-expressing lines were generated by G418-selection as described (Methods), and designated Saos–2–E4 ORF6 and H1299–E4 ORF6, respectively. Consistent with data obtained after transient transfection with pCIE4ORF6, there was no increase in rAAV transduction efficiency in either Saos–2–E4 ORF6 or H1299–E4 ORF6 cells when compared to the parental cell lines (Fig. 3B).

Interestingly, an attempt to generate a stable E4 ORF6-expressing 293 cell line was unsuccessful. The number of G418-resistant colonies obtained after transfection with pCIE4ORF6 was approximately 5-fold lower than the number obtained after transfection with pCI-neo, and only the occasional cell (fewer than 5%) surviving G418-selection expressed E4 ORF6 as determined by immunohistochemical staining using MAb3. In contrast, the number of G418-resistant colonies obtained after transfection of H1299 and Saos-2 cells was equivalent for each plasmid, and more than 90% of cells expressed E4 ORF6 (data not shown).

Transient transfection of a plasmid encoding the dl309 genome enhances rAAV transduction in H1299 and HeLa cells

The effect of transient transfection of plasmids containing the entire adenovirus dl309 genome (pFG140) or the E4 ORF6 gene alone (pCIE4ORF6) on the efficiency of rAAV transduction was compared in H1299 cells (p53-null) and HeLa cells.
Fig. 2. Functional validation of the E4 ORF6 expression construct pCIE4ORF6. Analyses were performed 48 h after transient transfection of 293 cells with either pCI-neo (control) or pCIE4ORF6. Immunohistochemical analysis of E4 ORF6 expression using the MAb3 monoclonal antibody in 293 cells transfected with either pCI-neo (A) or pCIE4ORF6 (B). Immunohistochemical localization of E1b 55 kDa using the 2A6 monoclonal antibody in 293 cells transfected with either pCI-neo (C) or pCIE4ORF6 (D). Relative transduction efficiency of rAAV in 293 cells after transfection with pCI-neo or pCIE4ORF6 (E). Error bars represent standard error of the mean (n = 3).

(p53-positive). Transfection with the E4 ORF6-encoding plasmid failed to significantly enhance rAAV transduction in either cell line, whereas transfection with the plasmid encoding the entire adenoviral genome significantly enhanced rAAV transduction in both cell lines (Fig. 4).

**E1b 55 kDa mutant adenoviruses exhibit a reduced capacity to enhance rAAV transduction in H1299 cells**

To address the contribution of E1b 55 kDa, the ability of the E1b 55 kDa mutant adenoviruses (dl338 and dl1520) to enhance rAAV transduction was examined in H1299 and 293 cells. Both adenoviral E1b 55 kDa mutants exhibited a significantly reduced capacity to enhance rAAV transduction compared to dl309 in H1299 cells, but were equally effective in 293 cells (Fig. 5).

**E4 ORF6 requires E1b 55 kDa coexpression to enhance rAAV transduction in H1299, Saos-2 and HeLa cells**

To confirm the importance of E4 ORF6 and E1b 55 kDa coexpression in the absence of other adenoviral gene products, H1299, Saos-2 and HeLa cells were transfected with E4 ORF6
E4 ORF6 and E1b 55kDa enhance rAAV transduction

Fig. 3. Expression of E4 ORF6 does not enhance rAAV transduction in H1299 and Saos-2 cells. H1299 and Saos-2 cells were transiently (A) or stably (B) transfected with pCI-neo (control) or pCIE4ORF6, and then exposed to rAAV at low multiplicity as described (Methods). Relative transduction efficiency was calculated by dividing the number of cell foci expressing β-galactosidase in treated cultures by the number present in control cultures. Error bars represent standard error of the mean (n = 6).

Fig. 4. Transfection of H1299 or HeLa cells with plasmids encoding the entire dl309 adenoviral genome (pFG140) but not E4 ORF6 alone (pCIE4ORF6) enhances rAAV transduction. H1299 and HeLa cells were transiently transfected with pCI-neo (control), pCIE4ORF6 or pFG140 and then exposed to rAAV at low multiplicity as described (Methods). Relative transduction efficiency was calculated by dividing the number of cell foci expressing β-galactosidase in treated cultures by the number present in control cultures. Error bars represent standard error of the mean (n = 6).

Fig. 5. E1b 55 kDa mutant adenoviruses exhibit a reduced capacity to enhance rAAV transduction in H1299 cells but not in 293 cells. H1299 or 293 cells were infected with dl309, dl338 or dl1520 (m.o.i. ≈ 5) and exposed to rAAV at low multiplicity as described (Methods). Relative transduction efficiency was calculated by dividing the number of cell foci expressing β-galactosidase in treated cultures by the number present in control cultures. Error bars represent standard error of the mean (n = 6).

Fig. 6. E4 ORF6 requires E1b 55 kDa coexpression to enhance rAAV transduction in H1299, Saos-2 and HeLa cells. H1299, Saos-2 and HeLa cells were transiently transfected with pCMVE1b55 and pCIE4ORF6 alone or in combination and then exposed to rAAV at low multiplicity as described (Methods). Relative transduction efficiency was calculated by dividing the number of cell foci expressing β-galactosidase in treated cultures by the number present in control cultures. Error bars represent standard error of the mean (n = 6).

Discussion

Treatment of target cells with genotoxic stress (Alexander et al., 1994; Russell et al., 1995; Ferrari et al., 1996) or expression of the adenovirus E4 ORF6 gene product have been shown to dramatically enhance the transduction efficiency of rAAV vectors, and this effect has been correlated with an increased proportion of input single-stranded DNA genomes (pCIE4ORF6) and E1b 55 kDa (pCMVE1b55) expression plasmids individually or together. In all three cell lines, coexpression of E4 ORF6 and E1b 55 kDa led to a marked enhancement of rAAV transduction efficiency. In contrast, expression of either protein alone failed to enhance rAAV transduction efficiency, with the exception of E1b 55 kDa in HeLa cells where a moderate level of enhancement was observed (Fig. 6).
undergoing second-strand synthesis (Fisher et al., 1996; Ferrari et al., 1996; Sanlioglu et al., 1999). There is significant potential to exploit this biological property of rAAV in gene therapy applications, such as cancer immunotherapy, where rAAV-mediated gene delivery might be coordinated with conventional anti-cancer treatment modalities that have been demonstrated to enhance rAAV transduction efficiency in vitro (Alexander et al., 1994; Russell et al., 1995; Maass et al., 1998). Pursuit of this strategy however, necessitates an improved understanding of the intracellular events mediating the enhancement of rAAV transduction. We initially focused on the possible involvement of p53 in this process because of the crucial function this protein subserves in mediating cellular responses to genotoxic stress (Levine, 1997; Steele et al., 1998), and the known interactions between p53 and E4 ORF6 (Dobner et al., 1996; Moore et al., 1996; Nevels et al., 1997; Querido et al., 1997; Steegenga et al., 1998). The observation, however, that UV irradiation or adenovirus dl309 infection enhance rAAV transduction in the p53-null cell lines H1299 and Saos-2 provides unequivocal evidence that these cellular insults are capable of enhancing rAAV transduction in a p53-independent manner. These results support the conclusion of Grimfam et al. (1999), who also reported that overexpression of p53 in 293 cells does not inhibit rAAV transduction.

Two separate groups, using mutant adenoviruses, have previously mapped the enhancement effect of adenovirus infection on rAAV transduction to E4 ORF6, and concluded that the E4 ORF6 gene product is necessary and sufficient to enhance rAAV transduction (Fisher et al., 1996; Ferrari et al., 1996). Interestingly, one of these groups also noted, but did not further dissect, an additional contribution by the E1 region to enhanced rAAV transduction that was described as cooperative and dependent on expression of E4 ORF6 (Fisher et al., 1996). We therefore anticipated that expression of E4 ORF6 alone would, at least partially, recapitulate the enhancement effect of adenovirus dl309 infection on rAAV transduction observed in H1299 and Saos-2 cells. Intriguingly, while transient expression of E4 ORF6 in 293 cells enhanced rAAV transduction as previously described (Fisher et al., 1996; Ferrari et al., 1996), neither transient nor stable expression of E4 ORF6 enhanced rAAV transduction in H1299 and Saos-2 cells. Important differences between 293 cells and H1299 cells or Saos-2 cells that may account for this observation include the presence of p53 and adenovirus E1 gene products in 293 cells. The ability of adenovirus infection to enhance rAAV transduction in a p53-null environment and the observation that p53 overexpression does not inhibit rAAV transduction (Grimfam et al., 1999) support the hypothesis that the relevant difference is the expression of adenoviral E1 proteins in 293 cells. This possibility is further supported by the observation that transient transfection of H1299 cells or HeLa cells with a plasmid encoding only E4 ORF6 failed to enhance rAAV transduction in either cell line. In contrast, transfection with a plasmid encoding the entire adenovirus dl309 genome, including the E1 and E4 regions, enhanced rAAV transduction in both cell lines. Collectively, these results demonstrate that in the cell lines examined E4 ORF6 expression alone is not sufficient to enhance rAAV transduction, and that a contribution from the E1 region is necessary.

Among the proteins encoded by the E1 region we chose to investigate the contribution of the E1b 55 kDa gene product to adenovirus-mediated enhancement of rAAV transduction. This protein is known to form a functional complex with the E4 ORF6 gene product (Sarnow et al., 1984; Cuit et al., 1987) and both proteins have been identified as providing helper function in the rescue and replication of wild-type AAV (Richardson & Westphal, 1984; Samulski & Shenk, 1988). Infection of H1299 cells with the E1b 55 kDa mutant adenoviruses dl338 and dl1520 resulted in a markedly diminished enhancement of rAAV transduction compared to infection with dl309. In contrast, all three mutant viruses enhanced rAAV transduction to a similar extent in 293 cells. In transient transfection experiments, free of the complicating influence of adenovirus infection, expression of the E1b 55 kDa gene product was required for the E4 ORF6-mediated effect on rAAV enhancement in HeLa, H1299 and Saos-2 cell lines. Unexpectedly, expression of the E1b 55 kDa gene product alone enhanced rAAV transduction in HeLa cells, although optimal levels of rAAV enhancement required coexpression of E4 ORF6. The explanation for this observation in HeLa cells remains unclear, and is confounded by the expression of papillomavirus type 18 E6 and E7 oncoproteins (Schneider-Gadicke & Schwarz, 1986).

To our knowledge, the only published data directly supporting the conclusion that E4 ORF6 expression is both necessary and sufficient to enhance rAAV transduction were also generated in HeLa cells (Fisher et al., 1996). In these experiments a HeLa cell line stably expressing E4 ORF6 under the transcriptional control of a zinc-inducible sheep metallothionein promoter was used. Exposure of these cells to zinc sulphate at concentrations up to 250 µM correlated with enhanced rAAV transduction. Why these results differ from our own HeLa data remains a matter of conjecture. Possible explanations include (i) differences in the biological properties of the HeLa cell lines employed, (ii) differences in the experimental conditions used (such as the input m.o.i. of rAAV) and (iii) possibly the ability of zinc to act directly as a cellular stress. In support of this last possibility we have found that zinc treatment of unmodified HeLa cells enhances rAAV transduction (5-fold enhancement at 200 µM) (unpublished observation). This might also explain why there was little correlation between enhanced transduction following zinc treatment and the detection of duplex replicative form monomers in HeLa cells, which was observed in 293 cells (Fisher et al., 1996). Recently published data provide evidence that, in contrast to adenovirus-mediated enhancement of rAAV transduction, genotoxic stress-mediated enhancement does.

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not appreciably increase the appearance of replicative form rAAV genome intermediates (Sanioğlu et al., 1999). In summary, while we concede that E4 ORF6 expression alone may enhance rAAV transduction in some cellular contexts, our data support the conclusion that E1b 55 kDa suberves an equally crucial, and largely overlooked function in this process.

The demonstrated interdependence of the E4 ORF6 and E1b 55 kDa proteins for enhancement of rAAV transduction in H1299 and Saos-2 cells directs analysis of the underlying mechanism to the cooperative functions performed by these proteins which are independent of cellular p53. The E4 ORF6 and E1b 55 kDa proteins have been shown to form a functional complex (Cutt et al., 1987), one effect of which is to promote the cytoplasmic accumulation of viral mRNAs (Samulski & Shenk, 1988). Indeed, this effect of the E4 ORF6–E1b 55 kDa protein complex has already been postulated to account for the previously observed contribution of the E1 region to adenovirus-mediated enhancement of rAAV transduction (Fisher et al., 1996). While our data provide further support for this mechanism, it alone is insufficient to fully account for our results. Post-transcriptional effects mediated by the E4 ORF6–E1b 55 kDa protein complex might well increase the amount of rAAV-encoded reporter protein expressed per transductant, but are unlikely to be sufficient to account for the E1b 55 kDa protein-dependent increase in the absolute number of individual transduction events observed. We therefore conclude that it is likely that the E1b 55 kDa protein also functions to enhance critical events earlier in the rAAV transduction pathway. Clearly, a key possibility is the conversion of input single-stranded rAAV genomes to transcriptionally active double-stranded molecules, a process that as already stated has been linked to the E4 ORF6 protein (Fisher et al., 1996; Ferrari et al., 1996). Candidate activities of the E4 ORF6 and E1b 55 kDa proteins, identified in the context of adenovirus infection, that might facilitate this genome conversion process include the redistribution and recruitment of cellular proteins to specific intranuclear sites for viral DNA replication (Doucas et al., 1996). Adenovirus infection has also been shown to recruit input single-stranded rAAV genomes to intranuclear virus replication centres (Weitzman et al., 1996). This rAAV mobilization phenomenon, however, can be achieved with either E4 or E1b mutant adenoviruses, thereby precluding a requirement for E4 ORF6 or E1b 55 kDa in this process.

In conclusion, our results provide further insight into the mechanisms subserving adenovirus-mediated enhancement of rAAV transduction efficiency. Importantly, we have shown that cellular p53 is not essential for this effect and that the E1b 55 kDa protein plays a more critical role than previously appreciated. Further dissection of the interactions of E4 ORF6–E1b 55 kDa with the host cell will undoubtedly contribute to the elucidation of the cellular proteins and activities mediating rAAV transduction. Such knowledge will be of great value in fully exploiting the biology of rAAV for research and gene therapy applications.

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