Adeno-associated virus infection of murine fibroblasts with help provided by mouse adenovirus

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A B S T R A C T
Adeno-associated virus (AAV-2) replicates to high titers when host cells are coinfected with a helper virus. Here we analyzed the coinfection of AAV-2 and mouse adenovirus (MAV-1) in murine fibroblasts. We observed that AAV-2/MAV-1 coinfected NIH 3T3 cells produced approximately 10–40-fold less AAV-2 DNAase resistant particles than Hela cells. Levels of AAV-2 DNA replication were approximately 30-fold less in 3T3 cells as compared to Hela cells coinfected with human adenovirus (Ad-5). A study of these lower levels of infection in 3T3 cells compared to Hela cells revealed that receptor binding and internalization of AAV-2 in 3T3 and Hela cells was comparable. However, AAV-2 did not enter into the nucleus of mouse cells as efficiently as it does in human cells. Furthermore, viral DNA replication levels of AAV-2 DNA were found to be lower in mouse cells than human cells, indicating limitations in the murine nucleus for viral replication.

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Introduction

Adeno-Associated Virus (AAV) is a non-pathogenic, single-stranded DNA parvovirus. It has been classified in the genus Dependovirus because it needs a helper virus for productive replication (Atchison et al., 1965). The most common helper virus for AAV-2 is adenovirus (Ad) (Hoggan et al., 1966) however human cytomegalovirus (CMV) (McPherson et al., 1985), Herpes Simplex Virus (HSV) (Buller et al., 1981) and Human Papilloma Virus (HPV) (Walz et al., 1997) also provide helper functions. In the absence of helper virus, AAV-2 establishes a latent infection (Cheung et al., 1980) by preferential integration in the long arm of chromosome 19 (Kotin et al., 1989; Grifman et al., 1999; Samulski and Shenk, 1988). AAV-2 DNA replication is limited to the host cell alone; AAV-2 also inhibits replication of its helper viruses. During coinfection AAV-2 decreases adenovirus DNA replication 20–200-fold (Jing et al., 2001; Timpe et al., 2006). AAV-2 also decreases adenovirus cytotoxicity and production in cell culture. In animal studies of AAV infection, AAV-1 was detected in kidneys and lungs of fetuses and newborns, when pregnant mice were injected subcutaneously with AAV-1 and murine adenovirus (Lips and Mayor, 1980). However, mice carrying AAV-1 acquired via placental infection were protected against lethal infection by MAV (Lips and Mayor, 1982).

Productive AAV infections occur in a wide range of cells and tissues from a variety of species. However, a large number of non-permissive cell types have also been identified (Bartlett et al., 1999; Girod et al., 2007). AAV-2 is considered to be non-pathogenic it inhibits proliferation of transformed cells (Batchu et al., 1999), and represses transformation of mouse fibroblasts by heterologous oncogenes (Khleif et al., 1991). AAV-2 infection inhibits cell-cycle progression (Berthet et al., 2005), causes cell death in p53 negative cells (Raj et al., 2001), promotes differentiation and alters expression of several cell-cycle regulated (Winocour et al., 1988) genes. Effects of AAV-2 replication are not limited to the host cell alone: AAV-2 also inhibits replication of its helper viruses. During coinfection AAV-2 decreases adenovirus DNA replication 20–200-fold (Jing et al., 2001; Timpe et al., 2006). AAV-2 also decreases adenovirus cytotoxicity and production in cell culture. In animal studies of AAV infection, AAV-1 was detected in kidneys and lungs of fetuses and newborns, when pregnant mice were injected subcutaneously with AAV-1 and murine adenovirus (Lips and Mayor, 1980). However, mice carrying AAV-1 acquired via the placental route were protected against lethal infection by MAV (Lips and Mayor, 1982).

The AAV-2 genome has two translational open reading frames (ORF). The right ORF encodes three structural capsid proteins as a result of alternative splicing (VP1, VP2, and VP3) (Tratschin et al., 1984; Trempe and Carter, 1988) and the left ORF encodes four non-structural proteins (Rep78, Rep68, Rep52, and Rep40) (Mendelson et al., 1986). The Rep mRNAs are under the control of transcriptional promoters at map unit 5 and 19 (p5 and p19) whereas Cap mRNAs are under the control of a transcriptional promoter at map unit 40 (p40) (Hermonat et al., 1984). Rep proteins have pleiotropic effects in the life cycle of AAV-2 regulating DNA replication as well as RNA transcription (Labow et al., 1986; Tratschin et al., 1986) both positively and negatively (Beaton et al., 1989).

Transcription of AAV-2 DNA begins at a site known as AAVS1. A 34 bp sequence (Giraud et al., 1994; Linden et al., 1996) located in the first exon of the myosin binding subunit 85 of protein phosphatase 1 (Grifman et al., 1999; Samulski and Shenk, 1988). AAV serotype 2 (AAV-2) has a single-stranded DNA genome which is 4780 nucleotide long (Srivastava et al., 1983). Both ends of the AAV-2 genome have a 145 nucleotide long inverted terminal repeat (ITR) (Lusby et al., 1980) encoding the viral origin of replication (Senapathy et al., 1984) and genome packaging signal. The AAV-2 genome has two
whereas 3T3 cells were harvested 48, 72 and 96 h post-infection. Hela cells were harvested 48 h post-infection. Hela cells were coinfected with 500 MOI of AAV-2 and 5 MOI of MAV-1 or Ad-5, respectively. We chose NIH-3T3 cells as a model murine cell line and compared the amount of DRP produced from these cells to that of AAV-2 in murine cells with helper functions provided by mouse adenovirus. We determined if AAV-2 DNAse resistant particle (DRP) production from NIH 3T3 cells versus Hela cells was due to the inefficient transport of AAV-2 from the plasma membrane to the nucleus because AAV-2 particles were observed in the perinuclear space and AAV-2 DNA was detected in cytosolic fraction after infection.

Results

**AAV-2 DNAse I resistant particle production from NIH 3T3 cells versus Hela cells**

There have been few studies to analyze replication of wild type AAV-2 in murine cells with helper functions provided by mouse adenovirus. We determined if AAV-2 DNAse resistant particle (DRP) were produced after coinfection of murine fibroblasts with AAV-2 and MAV-1. We chose NIH-3T3 cells as a model murine cell line and compared the amount of DRP produced from these cells to that produced by Hela cells. To this end, equivalent numbers of 3T3 and Hela cells were coinfected with 500 MOI of AAV-2 and 5 MOI of MAV-1 or Ad-5, respectively. Hela cells were harvested 48 h post-infection whereas 3T3 cells were harvested 48, 72 and 96 h post-infection.

As demonstrated in Fig. 1 at 48 h post-infection there was a negligible number of AAV-2 DRPs produced from 3T3 cells. After 72 h the number of AAV-2 DRPs from 3T3 cells was approximately 40-fold less than the amount produced from Hela cells after 48 h. After 96 h, the number of particles produced from 3T3 cells was approximately 10-fold less than those produced by Hela cells at 48 h. Hela cells demonstrated extensive cytopathic effects at MOI 5 of Ad-5 after 48 h, hence, the infections were stopped after 48 h to determine the number of AAV-2 DRP. After 96 h the cytopathic effect in 3T3 cells due to MAV-1 at MOI 5 was comparable to Ad-5 induced cytopathic effects in Hela cells 48 h post-infection. Since the temporal progression of MAV-1 infection appeared to be more prolonged than Ad-5 infection we performed time course infections in 3T3 and Hela cells and measured cytotxicity using MTT assays. This experiment verified that Ad-5 infections in Hela cells progress more rapidly than MAV-1 infections in 3T3 cells. The level of Ad-5 induced cytotxicity in Hela cells at 24 h was comparable to that observed in MAV-1 infected 3T3 cells at 48 h (data not shown). Therefore these conditions were used for subsequent experiments unless indicated otherwise.

**Replication of AAV-2 DNA in NIH-3T3 and Hela cells**

The dramatic difference between the AAV-2 particle numbers produced from 3T3 cells compared to that of AAV-2 particles produced from Hela cells at comparable points of cytopathic effect prompted us to determine if AAV-2 DNA replication in 3T3 cells with help provided by MAV-1 is equivalent to that AAV-2 DNA replication in Hela cells with help provided by Ad-5. Comparable numbers of 3T3 or Hela cells were coinfected with increasing MOI of AAV-2 (0.1, 1, 10, 100, and 500) and 5 MOI of MAV-1 or Ad-5, respectively. 3T3 or Hela cells were harvested 48 or 24 h later, respectively, low molecular weight AAV-2 DNA was extracted and analyzed by Southern hybridization. This experiment revealed that MAV-1 was able to provide helper functions for AAV-2 DNA replication in 3T3 cells, because replicative forms of AAV-2 DNA were observed at AAV MOI of 10, 100 and 500 (Fig. 2a). However, replicative form DNA was detected in Hela cells even at the MOI of 0.1 (Fig. 2b). The replication signal was approximately 200-fold lower in 3T3 cells compared to Hela cells as determined by phosphor-imager analysis (data not shown) depending on the MOI of AAV-2.

To obtain a more quantitative assessment of the differences in replication of AAV-2 between 3T3 cells and Hela cells we determined AAV-2 genome copy numbers per cell line after cooinfection with AAV-2 and MAV-1 or Ad-5. To this end, we coinfected 3T3 or Hela cells with increasing MOI of AAV-2 and 5 MOI of MAV-1 or Ad-5. 3T3 and Hela cells were harvested 48 h or 24 h later, respectively. Low molecular weight AAV-2 DNA was extracted and real time PCR was performed to amplify AAV-2 DNA. As shown in Figs. 2c and d the number of AAV-2 genome copies per cell at the highest MOI in 3T3 cells was approximately 30-fold less than the AAV genome copies in Hela cells. This difference in DNA replication and genome copy number corresponds to the differences noted in AAV-2 DRPs produced from 3T3 and Hela cells.

We also observed that L-929 murine fibroblast cells supported AAV-2 replication when coinfected with MAV-1 however the kinetics of replication were even slower than replication of AAV-2 in 3T3 cells (data not shown). These data demonstrate that AAV-2 DNA replicated in murine cells with MAV-1 provided helper functions but the level of replication was lower than that observed in Hela cells which are highly permissive for AAV-2 infection.

**Adenovirus replication in 3T3 and Hela cells**

Previous observations prompted us to question if MAV-1 replicates efficiently in 3T3 cells compared to the replication of Ad-5 in Hela cells. To determine the replication levels of MAV-1 in 3T3 cells and the replication of Ad-5 in Hela cells, equal numbers of 3T3 or Hela cells were infected with 5 MOI of MAV-1 or Ad-5 respectively with or
without coinfection of AAV-2. 3T3 and Hela cells were harvested 48 and 24 h later respectively. Low molecular weight adenovirus DNA was extracted and analyzed by Southern hybridization. As shown in Fig. 3a we observed a robust replication of MAV-1 in 3T3 cells in the absence of AAV-2. At the highest MOI (500) of AAV in coinfected 3T3 cells the levels of MAV-1 DNA replication seems to decrease by a modest 2-fold as quantified by phosphor imager analysis (data not shown). As expected Ad-5 DNA replication in Hela cells was robust. In agreement with previously published studies (Timpe et al., 2006) Ad-5 DNA replication decreases with increasing MOI of AAV-2 (Fig. 3b). Quantification by phosphor imager analysis (data not shown) revealed that Ad-5 DNA decreased by approximately 15-fold at the highest MOI of AAV.

To directly compare the replication of MAV-1 in 3T3 and Ad-5 in Hela cells and to determine the effect of AAV-2 on adenovirus DNA replication we determined the genome copy number of adenovirus DNA by real time PCR. 3T3 or Hela cells were infected with 5 MOI of MAV-1 or Ad-5, respectively, and increasing MOI of AAV-2. Cells were harvested 48 or 24 h later respectively, low molecular weight DNA was extracted and real time PCR was performed. As shown in Fig. 3d there was only a slight difference in genome copy number of MAV-1 and Ad-5 per cell in the absence of AAV-2. Further, with increasing MOI of AAV-2, the Ad-5 genome copy number decreased approximately 16-fold at the highest MOI of AAV. However, the effect of AAV-2 on MAV-1 replication was not as pronounced and showed a decrease of about 5-fold at the highest MOI of AAV. These results show that MAV-1 replication in 3T3 cells is less affected by AAV-2 than Ad-5.

To determine if the number of 3T3 cells supporting MAV-1 replication is similar to the number of Hela cells supporting Ad-5 replication. 3T3 and Hela cells were plated in an 8 well chamber slide. These cells were infected with 5 MOI of MAV-1 or Ad-5 respectively. Two hours post-infection cells were treated with 5 mM of bromodeoxyuridine (BrDU). Hela and 3T3 cells were fixed 24 and 48 h later respectively and immuno-stained for BrDU-substituted DNA. DNA needs to be denatured (Fox et al., 1991). However because adenovirus DNA replicates by single-strand displacement mechanism (Lechner and Kelly, 1977) thereby generating single-stranded DNA hence denaturation of DNA is not required (Bosher et al., 1992). This allows us to specifically detect adenoviral DNA replication by staining for BrDU. As shown in Figs. 4a and c, we did not see any signal from uninfected cells however however BrDU stained cells were observed in Ad-5 or MAV infected Hela or 3T3 cells, respectively (Figs. 4b and d). The number of cells showing BrDU staining and therefore supporting replication of MAV-1 or Ad-5, was comparable at approximately 8%.

To determine if the number of receptors available for binding of AAV-2 in 3T3 cells and Hela cells are comparable, we infected equal numbers of cells with an MOI of 105 of AAV-2. A high MOI was used to saturate the receptors available on the cell surface with AAV-2.

Available of receptors, receptor avidity and internalization of AAV-2 in 3T3 cells and Hela cells

Our results suggest that lower levels of AAV-2 replication in 3T3 cells as compared to Hela cells may be due to inefficient AAV-2 infection of 3T3 cells. This inefficiency of infection can be attributed to (a) availability and binding of AAV to its receptor on the cell surface (b) internalization of AAV (c) transport of AAV from the cell membrane to the nucleus (d) AAV protein expression or (e) DNA replication. We analyzed each of these steps during the infection of AAV in 3T3 and Hela cell lines.

To determine if the number of receptors available for binding of AAV-2 in 3T3 cells and Hela cells are comparable, we infected equal numbers of cells with an MOI of 105 of AAV-2. A high MOI was used to saturate the receptors available on the cell surface with AAV-2.
Infections were performed at 4 °C for 1 h to prevent virus internalization. One hour later, cells were washed extensively with cold PBS on ice to remove any free virus. Cells were harvested and viral DNA was extracted. Real time PCR of AAV-2 DNA was performed to determine the number of AAV-2 particles bound per cell. As shown in Fig. 5a the number of AAV-2 particles bound per cell was comparable in both 3T3 and Hela cells suggesting that initial virus interaction with receptors was similar in each cell type.

Next, we sought to determine if receptor binding of AAV-2 in 3T3 cells is similar to that of Hela cells. 3T3 and Hela cells were coinfected with increasing MOI of AAV-2 and 5 MOI of MAV-1 or Ad-5, respectively, in serum free medium. In this experiment, lower MOIs for AAV-2 infection were used to mimic the conditions used for analyzing AAV-2 DNA replication. Infections were conducted for 1 h at 4 °C to prevent internalization of virus. Viral DNA was extracted and real time PCR of AAV DNA was performed to determine the number of AAV-2 particles bound per cell. As shown in Fig. 5b, the number of AAV-2 particles bound per cell was comparable for both 3T3 and Hela cells. This indicated that binding of AAV-2 for its receptor in 3T3 and Hela cells was comparable.

Next we analyzed if the internalization of virus was different in the cell lines. To this end 3T3 and Hela cells were coinfected with increasing MOI of AAV-2 and 5 MOI of MAV-1 and Ad-5, respectively. Cells were incubated at 37 °C for 2 h following which cells were washed with PBS three times and trypsinized to remove any virus bound to the receptors on cell surface. Viral DNA was extracted and real time PCR was performed to detect the number of AAV-2 particles internalized. Fig. 5c shows comparable number of particles internalized per cell for both 3T3 and Hela cells. These results (Figs. 5a, b, and c) show that the number of available receptors, receptor binding and the internalization of AAV-2 is comparable in both 3T3 and Hela cells. Thus AAV-2 gains entry into both the cell lines with comparable efficiency.

AAV-2 protein expression in 3T3 and Hela cells

Observing a comparable number of AAV-2 particles bound to the receptors and internalized in both 3T3 and Hela cells we wanted to confirm if AAV Rep and Cap proteins were expressed in 3T3 cells to support AAV-2 DNA replication and packaging. 3T3 cells were coinfected with 10 or 100 MOI of AAV-2 and 5 MOI of MAV-1. Cells were harvested 48 h post-infection and nuclear extracts were prepared, separated by SDS-PAGE electrophoresis and proteins were transferred to a nitrocellulose membrane and probed for AAV-2 Rep and Cap proteins. As shown in Fig. 6a, all of the Rep and Cap proteins of AAV-2 were expressed in coinfected 3T3 cells. We also observed that the abundance of Rep and Cap proteins from 3T3 cells was lower than
Hela cells. It could be because of the lower levels of AAV-2 DNA replication in 3T3 cells.

To determine if there was a difference in the number of cells supporting AAV protein expression between 3T3 and Hela cells, we plated equal number of cells in an 8 well chamber slide. Cells were then coinfected with increasing MOI of AAV-2 and 5 MOI of MAV-1 or Ad-5, respectively. 3T3 and Hela cells were fixed 48 or 24 h later, respectively, and stained for Cap proteins. As shown in Figs. 6b and c there was robust expression of Cap proteins in Hela cells however the number of 3T3 cells showing Cap expression was much lower (data shown only for 500 MOI of AAV-2). The average of Cap-positive cells from three independent experiments indicated that at 500 MOI of AAV-2 and 5 MOI of Ad-5 or MAV-1, the percentage of Cap-positive Hela cells was approximately 12-fold higher than that of Cap-positive 3T3 cells.

Localization of Cap proteins in 3T3 and Hela cells

Having observed comparable numbers of AAV-2 particle bound to the receptor and internalized in 3T3 and Hela cells we next determined if a difference in trafficking of AAV-2 in either cell line accounted for different levels of DNA replication and protein expression of AAV-2 in 3T3 and Hela cells. 3T3 or Hela cells were plated in 8 well chamber slides. Cells were coinfected with AAV-2 MOI of 10, 100 and 500 and 5 MOI of MAV-1 or Ad-5. Cells were fixed 24 or 48 h post-infection and stained for Cap proteins in both cell lines. As seen in Fig. 7a, we observed foci of Cap proteins in the nuclei of Hela cells; a hallmark of Cap expression. However, in 3T3 cells we observed the signal for Cap proteins in the peri-nuclear space Fig. 7b (results shown only at the 500 MOI of AAV-2). The presence of AAV-2 Cap protein in the peri-nuclear space suggests that it is input virus rather than newly synthesized virus for two reasons; we see this signal only with high MOI and that newly synthesized Cap expression is localized exclusively in the nucleus. It has been demonstrated that phosphorylation of the AAV-2 vectors at tyrosine residues directs them for ubiquitination followed by proteosome mediated degradation (Zhong et al., 2008). The absence of Cap signal in 3T3 cell at lower MOIs can be because of rapid ubiquitination followed by degradation of input viral particles in 3T3 cells as compared to Hela cells.

To further confirm the localization of AAV-2 DNA we infected 3T3 and Hela cells with 10 MOI of AAV-2 with or without adenovirus. Cells were incubated with virus for 2 h at 37 °C. Cells were harvested and nuclear and cytoplasmic fractions were separated. AAV-2 DNA was amplified by PCR from both the fractions and separated by agarose gel electrophoresis. As shown in Fig. 8 it is clear that AAV-2 DNA in Hela cells was present exclusively in the nucleus both with and without Ad-5. However, in 3T3 cells the majority of AAV-2 DNA was present in the cytoplasmic fraction. This observation is in agreement with Hansen et al. (2000) wherein the majority of AAV-2 DNA was detected in the cytosolic fraction in 3T3 cells as compared to Hela cells. In the presence of MAV-1, AAV-2 DNA was detected in the cytoplasm even though it is known that human adenovirus aids in the trafficking of AAV-2 from cell membrane to the nucleus (Xiao et al., 2002). As mentioned earlier the lower levels of AAV-2 DNA in 3T3 cells as compared to Hela cells may be due to rapid degradation of AAV-2 particles in them. These experiments suggest that there is a trafficking defect in 3T3 cells as compared to Hela cells for AAV-2. This defect keeps AAV-2 in the cytosol in 3T3 cells thereby causing a decreased replication of AAV-2 in 3T3 cells as compared to Hela cells, even in the presence of adenovirus.
AAV-2 protein expression after plasmid transfection

After observing a defect in AAV-2 trafficking in 3T3 cells compared to Hela cells we decided to analyze if the protein expression and DNA replication of AAV were comparable in 3T3 and Hela cells when transfected with a plasmid carrying the AAV-2 genome. This approach bypasses the intracellular trafficking of AAV-2 and allows us to directly compare levels of AAV-2 replication. 3T3 or Hela cells were plated in an 8 well chamber slide and transfected with pNTC244 and infected with MAV-1 or Ad-5. 3T3 and Hela cells were fixed and stained for Cap proteins. As expected we observed Cap protein expression in transfected cells, however the number of Cap-expressing 3T3 cells, as shown in Figs. 9a and b, was much lower than Cap-expressing Hela cells. This result shows that even without a need for virus transport to the nucleus the levels of AAV-2 proteins remain lower in 3T3 cells compared to Hela cells.

AAV-2 DNA replication after plasmid transfection

To analyze if there was a difference in DNA replication between 3T3 and Hela cells, pNTC244 was transfected in the presence of adenovirus and AAV-2 replication was determined by Southern hybridization. To verify that equivalent transfection efficiency was obtained for 3T3 and Hela cells, each cell line was transfected with a GFP expressing plasmid. Twenty four hours post-transfection; both cell lines showed a comparable number of green cells (approximately 30% data not shown). To further compare the transfection efficiency, equal numbers of 3T3 and Hela cells were transfected with pNTC244. 6 h post-transfection, cells were harvested and low molecular weight plasmid DNA was extracted. Extracted DNA was subjected to DpnI digestion to remove any free plasmid DNA, separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then probed for replicative forms of AAV-2 DNA using a radio-labeled AAV-2 DNA fragment. As seen in Figs. 10a and b we observed AAV-2 DNA replication in both Hela and 3T3 cell lines respectively. However, replication of AAV-2 DNA in 3T3 cells was approximately 5-fold less than the replication of AAV-2 in Hela cells. This result indicates that along with having a defect in trafficking there was also a defect in replication of AAV-2 DNA in 3T3 cells. These experiments indicate that AAV-2 is also defective for replication in 3T3 nuclei as compared to the Hela nuclei.

Discussion

Given the nature of the effects of AAV-2 on host cells and helper virus, and due to the interest in the use of AAV-2 as a vector for gene
therapy, it is essential that the effects of AAV-2 on the host and helper virus in animal models be studied in detail. As a prelude to studying the coinfection of AAV-2 and adenovirus in mice, we studied coinfection of AAV-2 and MAV-1 in murine fibroblasts and compared it to AAV-2 replication in Hela cells with Ad-5 provided helper functions. We observed that even after 96 h the number of AAV particles produced from 3T3 cells was approximately 10-fold less than the number of particles produced from Hela cells. Further, AAV DNA replication in 3T3 cells was approximately 30-fold less than AAV DNA replication in Hela cells even though the levels of MAV-1 and Ad-5 replication in 3T3 and Hela cells were comparable as detected by Southern blotting. Real time PCR and BrDu incorporation.

It is well established that the transduction of rAAV vectors in different cell types can be limited at multiple steps (Ding et al., 2005; Sanlioglu et al., 2001). These include availability of the receptors or co-receptors on the cell surface thereby allowing binding and internalization of virus, trafficking from the cell membrane to the nucleus (Bartlett et al., 2000; Duan et al., 2000) and conversion of single-stranded AAV DNA to a transcriptionally active double-stranded conformation (Ferrari et al., 1996; Fisher et al., 1996). These systematically analyzed if infection of wtAAV-2 in 3T3 cells is limited at one or more of these stages and if coinfection of MAV-1 helps wtAAV-2 to overcome these obstacles.

Infection of 3T3 and Hela cells 4 °C and 37 °C revealed that number of wtAAV-2 particles binding on the cell surface and their internalization is comparable for both cell lines. This data is in agreement with previous studies (Qing et al., 1999) wherein it was observed that even though rAAV vector could bind to both Hela and 3T3 cells efficiently, the transduction in 3T3 cells was much lower than Hela cells. The amount of binding detected in our assays is likely due to virus–receptor interactions rather than non-specific binding to other areas of the cell surface. Two published studies have shown that in the absence of virus receptor or co-receptor in four different cell lines, virus binding is dramatically reduced or negligible (Qing et al., 1999; Summerford and Samulski, 1998). These experiments helped us rule out the possibility of inefficient AAV-2 receptor binding or internalization as a reason for the difference in the AAV DNA replication in 3T3 cells compared to Hela cells.

Observing similar levels of AAV-2 particles bound and internalized in both Hela and 3T3 cells we questioned whether AAV-2 travels from the cell membrane to the nucleus in 3T3 cells. As seen in Fig. 7b, 3T3 cells coinfected with MAV-1 and AAV-2 showed Cap staining in the perinuclear region at the highest MOI. However, this was not the case with Hela cells. PCR analysis for detecting AAV-2 DNA in nuclear versus cytosolic fraction in each cell line showed that in 3T3 cells, AAV-2 DNA remained in the cytosolic fraction. These results support earlier observation (Hansen et al., 2000) that even 48 h after infection of 3T3 cells with rAAV, the majority of vector DNA was in the cytoplasmic fraction as compared to 293 cells that showed nuclear localization. The observation of Cap signal in 3T3 cells only at the higher MOI and lower levels of AAV-2 DNA may be explained by rapid degradation of AAV particles in 3T3 cells as compared to Hela cells (Zhong et al., 2008). We observed AAV-2 Cap signal in the cytosol up to 48 h post-infection, even though we had coinfected cells with MAV-1. Adenovirus has been shown to help AAV-2 traffic from cell membrane to nucleus (Xiao et al., 2002).

Bypassing the trafficking stage of AAV infection by transfection of plasmid for wt AAV-2 genome did not allow the levels of AAV DNA replication in 3T3 cells to be comparable to AAV-2 DNA levels in Hela cells. This lower level of DNA replication in 3T3 cells could be due to differences in the support provided by host nuclear factors in 3T3 versus Hela cells. These cellular differences may cause inefficient single-stranded to double-stranded conversion of genomes of internalized AAV-2 particles leading to decreased AAV-2 DNA replication. These results indicate that the intracellular milieu in Hela cells is more conducive for AAV-2 replication than 3T3 cells. It has been demonstrated that processing of AAV-2 vectors is impaired in 3T3 cells as compared to 293 cells (Hansen et al., 2001). It would be interesting to observe whether endocytic processing of AAV-2 in 3T3 cells after coinfection with MAV-1 gets altered and to identify the cytosolic compartment in which AAV-2 is obstructed in 3T3 cells after coinfection with MAV-1. Further, since EGFR inhibitors can increase transduction by rAAV vectors in non-permissive cell lines by phosphorylating ssDBP (Mah et al., 1998; Smith et al., 2003) it would be interesting to determine if treatment with these chemical agents increase AAV DNA replication in 3T3 cells. Previously it has been observed that treatment of 3T3 cells (Qing et al., 1999) with tyrphostin 1 did not increase transduction by rAAV-2 vector. We would like to analyze the effects of EGFR inhibitors when 3T3 cells are coinfected with wild type AAV-2 and MAV-1. It has been demonstrated by Zhong et al. (2008) that tyrosine phosphorylation may lead to proteosome mediated degradation of AAV particles. We have observed Cap signal in 3T3 cells only at the higher MOI and lower levels of AAV-2 DNA in 3T3 cells as compared to Hela cells. Hence, it would be worth to observe if there is an increased phosphorylation of AAV-2 capsid in 3T3 cells as compared to Hela cells thereby leading to a rapid degradation of AAV-2 Cap in 3T3 cells.

These studies are an assessment of AAV replication in mouse fibroblast cells using MAV-1 as a helper virus. Our results indicate that while there are some limitations to AAV replication, a full and productive infection occurs in mouse cells. These results pave the way for future investigations in whole animals as a rodent model of AAV infection.

Materials and methods

Cells and viruses

Hela and mouse NIH 3T3 cells were grown in MEM and DMEM respectively supplemented with 10% FBS, glutamine and antibiotics. All cells were maintained as monolayer cultures at 37 °C in a 5% CO₂ atmosphere.

Mouse adenovirus (MAV-1) was kindly provided by Dr. K.R. Spindler (University of Michigan). MAV-1 was prepared by infection of fifteen 10-cm dishes of NIH 3T3 cells. 96 h later cells were harvested by scraping in the medium and low speed centrifugation. Cells were resuspended and lysed by 3 freeze–thaw cycles. The crude lysate was cleared of cell debris by centrifugation at 10,000 rpm for 10 min at 4°C and the supernatant containing the virus was saved. Physical particle and infectious titer of MAV-1 was determined by real time PCR and western blotting for MAV-1 E1A protein after limiting dilution infection of 3T3 cells. Titer of Ad-5 was determined in Hela cells as previously described (Winters and Russell, 1971).

AAV was prepared by transfecting 293 cells with pNT2424 plasmid (Laughlin et al., 1983) and infecting them with Ad-5. 40 h later cells were harvested, subjected to three freeze–thaw cycles, and treated with DNase I. Extracts were fractionated by heparin-agarose column chromatography and the infectious titer of AAV was determined by dot blot analysis as described previously (Casper et al., 2005).

Infections and transfections

Ad-5 or MAV-1 were used at multiplicity of infection (MOI) 5 and AAV-2 was used at the MOI indicated in the text and figure legends. Cells were infected when they reached 90% confluence. Infections were performed in serum free medium for 2 h after which medium was replaced with complete medium. Cells were plasmid transfected at 90% confluence using Lipofectamine 2000 (Invitrogen) in serum free medium for 4 h according to manufacturer’s recommendations. The DNA to Lipofectamine 2000 ratio used was 1 μg:2.5 μl and 4 μg was transfected in 6 well plates. Four hours after transfection, medium was replaced with complete medium.

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Ad-5 or MAV-1 were used at multiplicity of infection (MOI) 5 and AAV-2 was used at the MOI indicated in the text and figure legends. Cells were infected when they reached 90% confluence. Infections were performed in serum free medium for 2 h after which medium was replaced with complete medium. Cells were plasmid transfected at 90% confluence using Lipofectamine 2000 (Invitrogen) in serum free medium for 4 h according to manufacturer’s recommendations. The DNA to Lipofectamine 2000 ratio used was 1 μg:2.5 μl and 4 μg was transfected in 6 well plates. Four hours after transfection, medium was replaced with complete medium.
Real time PCR

Genome copy numbers for AAV-2, Ad-5 and MAV-1 were determined by quantitative real time PCR. The forward primer for AAV-2 was 5′ AAC TGG TTC GCG GTC ACA A 3′ (AAV-2 nt 708) and the reverse primer was ACC CGA CCA GCT CTA TGT AC (AAV-2 nt 1008). These primers amplify a 301 base pair fragment. The forward primer for MAV-1 was 5′ ATG TCG GGG CTC CTA CG 3′ and the reverse primer was 5′ CAA CGA ACC ATA AAA AGA CAT CAT 3′. These primers amplify a 512 base pair fragment of the MAV-1 E1A gene. Primers were used at a final concentration of 0.3 μM. Applied Biosystems Power SybrGreen master mix was used to perform PCR. Amplification was performed at 95 °C for 10 min to activate the polymerase followed by 40 cycles at 95 °C for 30 s, 54 °C for 45 s and 72 °C for 45 s. Genome copy number was determined by comparison to a standard curve plotted after amplification of the same fragment from plasmid pNTC244 or from a MAV-1 E1A fragment cloned into pGEM-T (Promega) at 10 fold serial dilutions from 1×1010 to 1×102 copies. All experimental and serial dilution templates were run in triplicate. Data was analyzed using 7500 system SDS software (Applied Biosystems).

To determine AAV-2 DNAse resistant particles (DRP), crude viral extracts were prepared from harvested cells by 3 freeze–thaw cycles to lyse cells, DNAse I treatment for 30 min to remove any free viral DNA followed by inhibition of DNAse I by heating extracts at 65 °C. Viral DNA was extracted from these extracts and real time PCR was performed to amplify AAV-2 DNA, which would result in the number of DNAse resistant AAV-2 genomes produced per cell from both cell lines.

Southern blot

Low molecular weight viral DNA was extracted from Hela and NIH 3T3 cells 24 or 48 h post-infection respectively using Qiagen QIAmp DNA mini kit. DNA was extracted from transfection was subjected to DpnII digestion before electrophoresis to degrade unreplicated plasmid DNA. Samples were subjected to RNase treatment and extracted DNA was electrophoresed on a 1% agarose gel. After electrophoresis, the DNA was transferred to a NYTRAN membrane and hybridized to AAV-2-, MAV-1- or Ad-5-specific radioactive probe. The membrane was exposed to HyBlot Cl autoradiography film. For quantification of data the membrane was exposed to a phosphor screen and the signal was quantified using a Typhoon Phosphorimager and Image Quant Software.

The plasmid pNTC244 was digested with HindIII and the resulting 512 base pair fragment was amplified and a 512 base pair fragment cloned into pGEM-T (Promega) at 10 fold serial dilutions from 1×1010 to 1×102 copies. All experimental and serial dilution templates were run in triplicate. Data was analyzed using 7500 system SDS software (Applied Biosystems).

Immunoblotting

Hela cells or 3T3 cells were harvested at 24 or 48 h post-infection, respectively. For detecting expression of AAV-2 Rep, Cap and MAV-1 E1A proteins nuclear extracts were prepared. Infected cells were scraped in medium, pelleted and washed with PBS + 5mM MgCl2. Cell pellets were resuspended in STM-NP buffer [10 mM Tris (pH8.0), 0.25 mM sucrose, 10 mM MgCl2, 0.5% NP40, 1 mM PMSF and 0.1 mM DTT]. Cells were kept on ice for 15 min with intermittent vortexing. Nuclei were pelleted by centrifugation at 2000 rpm for 5 min. The supernatant (cytoplasmic fraction) was removed and pellets were resuspended in IPP buffer [50 mM Tris, 150 mM NaCl, 20 mM EDTA and 0.5% NP40 and 1 mM DTT supplemented with protease inhibitors (1 mM PMSF, 1 μM leupeptin, 1 μg/ml pepstatin and 1 mM benzamidin)]. Extracts were kept on ice for 1 h with intermittent vortexing. After 1 h, extracts were centrifuged at high speed (13000 rpm) for 5 min and the supernatant was saved. These nuclear extracts were separated on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA in Tris Buffered saline containing 0.5% Tween 20 (TBST). Rabbit anti E1A serum (provided by KR Spindler) diluted in 1% BSA-TBST (1:3000) was used to probe for MAV E1A protein. AAV Rep and Cap proteins were probed using affinity purified rabbit anti-Rep (Trempe et al., 1987) and anti-Cap antibody (unpublished data) diluted in 0.5% BSA-TBST (1:3000). After 1’ antibody treatment the membrane was washed 3 times with TBST and the membrane was then incubated with horseradish peroxidase (HRP) conjugated anti-rabbit 2’ antibody diluted in 0.5% BSA-TBST (1:10,000). Proteins were detected by ECL reaction and exposure of membrane to HyBlot Cl autoradiography film.

Nuclear and cytosolic fractionation of infected Hela and 3T3 cells

Two hours post-infection 3T3 and Hela cells were harvested and nuclear and cytosolic fractions to amplify AAV DNA were prepared as described (Smith et al., 2003).

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References


