αVβ5 integrin: a co-receptor for adeno-associated virus type 2 infection

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Understanding the primary steps of viral entry can have important implications for strategies to prevent infection of known viral pathogens as well as determining parameters for efficient gene delivery using viral vectors. Recently, a two-step process for viral infection involving attachment of virus to a primary receptor (coxsackievirus adenovirus receptor and heparan sulfate proteoglycan) and subsequent mediation of virus entry by a co-receptor (αV integrins and HVEM) has been determined for both adenovirus and HSV, respectively[1–4]. Heparan sulfate proteoglycan serves as a primary attachment receptor for adeno-associated virus type 2 (AAV-2)(ref. 5). Here we determined that αVβ5 integrin plays a part in efficient AAV infection. Experiments using the chelating agent EDTA to disrupt integrin function resulted in a corresponding decrease in AAV infection, consistent with the possibility that integrin mediates infection. Viral overlay experiments on purified plasma membrane proteins as well as immunoprecipitated integrin β5 subunit demonstrated that AAV directly associates with the β5 subunit of αVβ5 integrin. Genetically defined cells expressing αVβ5 integrin showed increased susceptibility to AAV infection, demonstrating a biological role of this integrin in AAV infection. Finally, viral binding and internalization studies indicate that αVβ5 integrin is not a primary attachment receptor for AAV-2, but is instead involved in facilitating virus internalization. This study supports the idea that αVβ5 integrin serves as a co-receptor for AAV-2 virions, and should have a substantial effect on the use of AAV vectors in human gene therapy.

Members of the integrin family are responsible for a variety of events, including cell–cell adhesion, cell adhesion to extracellular matrix proteins, internalization and degradation of extracellular matrix molecules, as well as the induction of signal transduction cascades that modulate cellular proliferation, morphology, migration and apoptosis[6]. Integrins have also been implicated as receptors for many non-enveloped viruses, including echoviruses 1 and 8, rotavirus, papillomavirus, coxsackievirus A9, foot-and-mouth disease virus and hantavirus[7]. For infection, adenovirus attaches to host cells through the coxsackievirus adenovirus receptor[1] and subsequently interacts with αV integrins that facilitate virus internalization[2,8]. Adenovirus interaction with αVβ5 integrin induces membrane permeabilization, and thus may play a part in both adenovirus internalization and endosome 'escape'[8]. This is important when considering adenovirus as a vector for human gene therapy. For adenoviral gene therapy for cystic fibrosis, there is a direct correlation between the presence of integrin on airway epithelia cells and efficient transduction[9,10]. Because a similar interest in AAV as a vector for cystic fibrosis has emerged, understanding the initial steps of viral entry will be essential to providing effective gene delivery. So far, all wild-type (wt) isolates of AAV have been identified in conjunction with human adenovirus infections, demonstrating an intricate relationship between these two viruses. Because αVβ5 integrin is used by adenovirus to mediate both internalization and membrane permeabilization, we examined the possibility that this integrin may serve as a common factor in determining host cell permissivity for AAV.

To evaluate the role of αVβ5 integrin in AAV infection, we first determined the effect of EDTA on viral infection. αV integrins require divalent cations for their proper folding and function. Chelation of divalent cations with EDTA inhibits adenovirus infection[11] and this inhibition is in part due to the disruption of αV integrins[2]. In the presence of 20 mM EDTA, there was a 40% inhibition of recombinant adenovirus–LacZ(rAd-LacZ) transduction (Fig. 1*a*), which is in agreement with published results[11], and a greater than 90% inhibition of AAV infection (Fig. 1*b*). Although EDTA can have many effects on cells, these findings were consistent with a possible role for integrin in AAV-2 infection, and warranted further investigation.

Because adenovirus has a physical interaction with integrin molecules[2,8], we tested gradient-purified, adenovirus-free AAV-2 for its ability to interact with purified plasma membrane proteins after fractionation on 5–20% gradient gels using a 'triple western' technique. Using this assay, AAV-2 bound a protein at 100 kDa (Fig. 1*c*, lane 2), which migrated at the same position as integrin β5 subunit[12] (Fig. 1*c*, lane 3). To confirm that the β5-specific monoclonal antibody and AAV were each recognizing a protein that migrates at the same molecular weight, the viral overlay was stripped and re-probed with anti-β5 monoclonal antibody. The results from this analysis indicated that the β5 monoclonal antibody recognize the same protein (data not shown), and that AAV may directly interact with the β5 subunit of the αVβ5 integrin complex on the cell surface. The absence of bands for the 'no virus' control (Fig. 1*c*, lane 1) demonstrates that the 100-kDa protein detected by this technique was the result of AAV binding and was not due to non-specific binding of either the anti-AAV-2 antibody or the horseradish peroxidase-conjugated secondary antibody. To confirm that the common 100-kDa species (Fig. 1*c*) identified in purified plasma membranes was the β5 subunit of αVβ5 integrin, we immunoprecipitated β5 protein from HeLa cells, transferred the material to nitrocellulose, and did a viral overlay

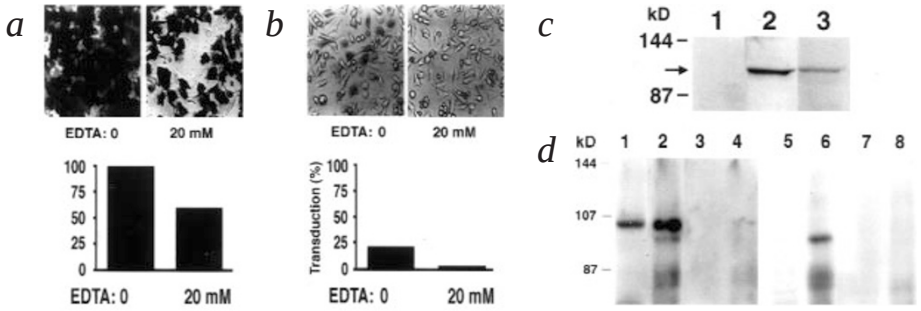

Fig. 1 Effect of EDTA on adenovirus and AAV infection and direct association of AAV with the β5 subunit of αVβ5 integrin. ***a*** and ***b***, HeLa cells were infected with recombinant virus (a, rAd-LacZ; b, rAAV-LacZ) in the presence or absence of EDTA, and stained for β-galactosidase activity (upper panels). Lower panels, Transduction quantitated from a single experiment, represented as the percentage of HeLa cells transduced in the presence or absence of EDTA. ***c***, Viral overlay and western blot analysis of plasma membrane proteins. Purified HeLa cell plasma membrane proteins were probed with either no virus (lane 1), purified AAV-2 virions (lane 2) or B5-IVF2 monoclonal antibody against the β5 subunit of αVβ5 integrin (lane 3). ***d***, Virus overlay of immunoprecipitated β5 subunit of αVβ5 integrin. Purified plasma membrane proteins (lanes 1 and 5); immunoprecipitated β5 subunit of αVβ5 integrin (lanes 2 and 6); and control immunoprecipitations, isotype-matched IgG1 antibody (lanes 4 and 8) or rabbit anti-mouse antibody (lanes 3 and 7) were probed with (lanes 1–4) or without (lanes 5–8) purified AAV-2 virions.

analysis (Fig. 1*d*). This antibody is known to be specific for the β5 subunit of αVβ5 integrin and has been well characterized for anti-β5 activity[13]. AAV-2 showed a direct association with the immunoprecipitated β5 integrin subunit (Fig 1*d*, lane 2). As expected, the signal generated with immunoprecipitated β5 integrin was greater than that obtained from the total plasma membrane proteins (Fig. 1*d*, lanes 1 and 2). In the absence of virus, the 100-kDa protein was not detected in either HeLa membranes (Fig. 1*d*, lane 5) or immunoprecipitated β5 subunit extract (Fig. 1*d*, lane 6). Therefore, the detection of the 100-kDa species was virus-specific. Furthermore, controls demonstrated that immunoprecipitation of the 100-kDa species was specific to anti-β5 monoclonal antibody (Fig. 1*d*, lanes 3, 4, 7 and 8). Because AAV interacts with the reduced form of integrin β5 subunit and AAV-2 does not contain an RGD amino acid motif like adenovirus, these data indicate that if AAV interacts with membrane αVβ5 integrin, it does so in a 'non-RGD-dependent' manner. Both HIV tat (ref. 14) and hantavirus[7] have been shown to interact with αVβ5 integrin in a non-RGD-dependent manner, supporting the idea of this type of interaction. In addition, adenovirus may interact with αVβ5 integrin through a non-RGD motif[8].

Given the physical interaction between AAV-2 virions and the β subunit of αVβ5 integrin, we next determined if this interaction was biologically important. Cell lines that lacked αVβ5 (CS-1) or specifically expressed αVβ5 (CS1/β5) were tested for rAAV transduction[8]. These cell lines have been used to assess the role of αVβ5 in adenovirus infection[8], and were first FACS-analyzed using a monoclonal antibody against the αVβ5 heterodimer to ensure that homogeneous cell populations were used for viral infection experiments (Fig. 2*a*). We next used recombinant adenovirus (rAd) to infect cells, as this virus has been established to use αVβ5 integrin as a secondary viral receptor. Cells were infected with rAd-LacZ or rAAV-LacZ and assayed for β-galactosidase activity. The increase in rAAV transduction (260% in CS1/β5 compared with parental CS-1 cells; Fig 2*b*) is similar to the 320% increase seen with rAd infection[2,8] (Fig. 2*c*). Although the increase was similar for AAV and adenovirus, the level of galactosidase activity differed by 100-fold[15]. The rAAV used here was generated by an adenovirus-free packaging procedure[16], therefore, this result was not unexpected, as optimum AAV gene expression depends on adenovirus early genes[15,17]. An adenovirus-free packaging procedure was used to preclude the possibility that adenovirus virus or adenovirus structural proteins could influence interaction of AAV with cell surface integrins or augment rAAV gene expression. After re-introduction of adenovirus early genes, the overall rAAV β-galactosidase activity increased 100-fold, as described[15,17] (data not shown). These data demonstrate that the presence of αVβ5 integrin renders cells more susceptible to AAV-2 infection, similar to published results for adenovirus infection[2,8] and indicate that this integrin may play the same part in both adenovirus and AAV infections.

Viral overlay analysis and transduction studies provided evidence for αVβ5 integrin promoting AAV-2 infection most likely through a direct interaction. Heparan sulfate proteoglycan (HSPG) is a primary attachment receptor for AAV-2 virions[5]. To identify a potential mechanism for the involvement of αVβ5 in-

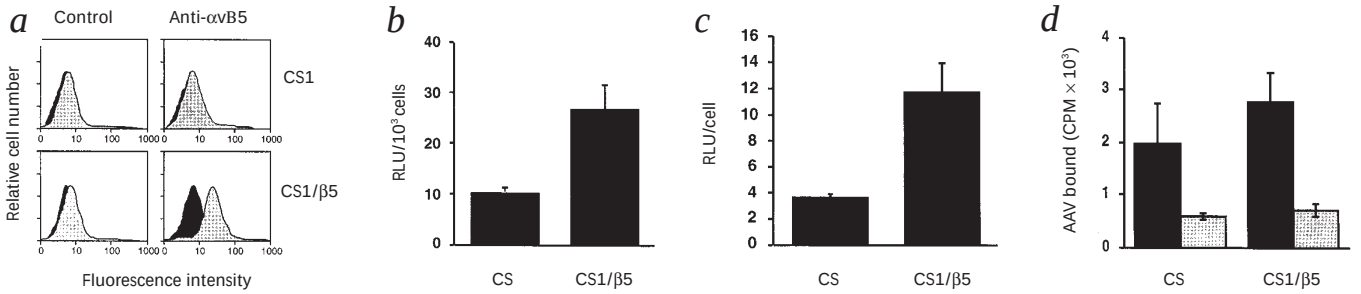

Fig. 2 αVβ5 expression and vector transduction of CS-1 and CS1/β5 cells. ***a***, FACS analysis of αVβ5 expression on CS-1 and CS1/β5 cell lines. ***b*** and ***c***, Transduction of CS-1 and CS1/β5 cell lines with rAAV (b), or rAd (c). RLU (vertical axes), relative light units. Data represent the mean and standard deviation of an experiment done in triplicate. ***d***, Binding of ^{3}H-wtAAV-2 to CS-1 and CS1/β5 cells. Filled bars, total binding; shaded bars, nonspecific binding. Data are the mean and standard deviation of two experiments done in duplicate.

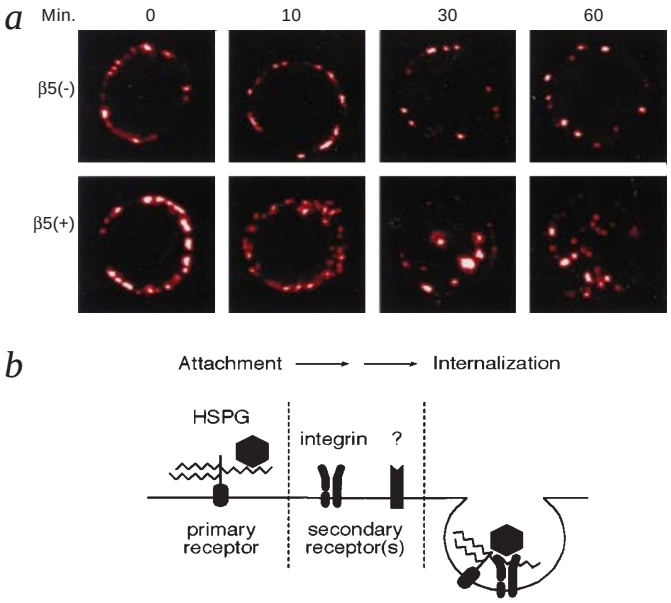

Fig. 3 ***a***, Internalization of Cy3-AAV2 by CS-1 and CS1/β5 cells. Images were obtained by confocal microscopy from cross-sections representative of the cells' centers. Times are indicated above each image. The CS1/β5 cell line shows an increase in the rate of internalization of the fluorescent virus compared with that of the CS1 cell line. Independent experiments yielded similar results. ***b***, Model for AAV-2 entry showing primary attachment of AAV to HSPG and interaction of AAV with αVβ5 integrin and/or other co-receptors that mediate efficient AAV-2 entry.

tegrin in AAV-2 infection, we did viral binding assays using ^{3}H-labeled virus (Fig 2*d*). Labeled AAV-2 bound specifically to both the CS-1 and CS1/β5 cell lines. Although slightly more AAV-2 bound to the CS1/β5 cell line, this level of binding was not substantially different from that in the parental cell line CS-1, indicating that integrin is not acting as a primary receptor for AAV-2. Consistent with results demonstrating that HSPG serves as a primary attachment receptor for AAV-2 (ref. 5), the similar binding of virus to CS-1 and CS1/β5 cell lines correlates with cell surface expression levels of HSPG (data not shown). The inability of integrin to mediate primary AAV-2 attachment indicates that there is another role for αVβ5 integrin in AAV-2 infection. Because attachment of AAV-2 to cell surface HSPG is necessary to initiate efficient infection by the virus (Fig. 3*b*), and given the data here on αVβ5 integrin, this molecule may serve as a secondary receptor for AAV-2 infection (Fig. 3*b*). Moreover, viral infection is more sensitive to the amount of cell surface HSPG than can be explained by the level of virus binding[5]. Thus, the density of HSPG on the cell surface, a factor that could influence the probability of a bound virus interacting with a second cell surface receptor, might influence AAV entry. Given what is known to occur during adenovirus infection, αVβ5 integrin may facilitate virus internalization.

To determine whether αVβ5 promotes AAV-2 entry, we investigated the rate of virus internalization in the CS-1 and CS1/β5 cell lines. Internalization was monitored using AAV-2 tagged with fluorescent (Cy3) dye. Fluorescent AAV-2 was incubated with cells at 4 °C, and unbound virus was removed before internalization was initiated at 37 °C. At various times after the temperature shift (0, 10 and 30 min and 1 h), the cells were fixed and analyzed by confocal microscopy. The CS1/β5 cell line internalized virus at a substantially faster rate than did the parental cell line (Fig. 3*a*). The enhanced rate of virus entry into the CS1/β5 cell line was specific for AAV-2, as internalization of a unrelated molecule (FITC–transferrin) was equivalent for both cell lines (data not shown). The substantial difference in uptake of AAV into these genetically defined cell lines indicates that αVβ5 integrin promotes infection by facilitating viral internalization. The interaction of AAV with αVβ5 integrin may represent a mechanism by which virus can be efficiently internalized, whereas the ability to infect CS1 cells may be related to other entry processes (for example, HSPG serving as a binding and internalization receptor, and/or the presence of an alternative low abundant/low affinity co-receptor). In addition, experiments assessing factors that influence AAV infection have suggested that many parameters (such as duration of viral exposure and non-specific uptake mechanisms) have a positive effect on virus infection[18,19].

Here we examined whether αVβ5 integrin, a secondary determinant for adenovirus tropism, might serve as a common factor in defining how susceptible cells are to AAV infection. Our data indicate that both adenovirus and AAV use αVβ5 integrin as a co-receptor to mediate viral entry, and demonstrate a role for integrin in a parvovirus infection. When considering the implications of this for AAV as a viral vector in human gene therapy, this study provides strong justification for using AAV vectors with target cells that express high levels of αVβ5 integrin[20,21]. In addition, use of reagents that upregulate integrin expression should also enhance AAV vector transduction, as demonstrated with adenovirus vectors[22,23]. These results also provide direct insight into AAV vector strategies now being used in clinical trials. For example, these results indicate that AAV gene therapy for cystic fibrosis will be influenced by the gradient of αVβ5 integrin expression documented in human airway cells[9,10].

The identification of αVβ5 integrin as a co-receptor is consistent with the fact that many viruses use secondary molecules to enhance viral infection. So far, three distinct co-receptors have been identified for the herpes simplex viruses[3,24], and data from Srivastava and colleagues[25] support the identification of an additional co-receptor for AAV. Because wt AAV optimally produces progeny virions in the presence of a helper virus, this places an evolutionary selective pressure for AAV virions that can co-infect the same host range as the helper viruses. As with AAV-2, HSPG is a primary receptor for HSV and CMV, and has recently been implicated as a receptor for vaccinia virus[5], all known helpers for AAV infection. Because HSV and adenovirus seem to be the most common helper viruses used for a productive AAV infection, each of these helpers (HSV/HSPG, and adenovirus/αVβ5 integrin) may have contributed to the evolution of AAVs pathway for entry.

Methods

Virus and cell culture. The CS-1 and CS1/β5 (ref. 8) cell lines were provided by D. Cheresh (Scripps Clinic & Research Foundation) with permission from C. Damsky (University of California at San Francisco), and were propagated as described[8]. Wild-type (wt) AAV-2, ^{3}H-AAV-2, Cy3-AAV2, rAAV-LacZ, adenovirus *dl*309 and rAd-LacZ viruses were prepared as described[5,26,27]. All virus preparations were purified by two successive bandings on CsCl gradients to ensure purity. Wild-type AAV particle numbers were determined by protein quantitation[5]. Recombinant virus titers were determined as described[26] and are expressed as transducing units per cell (MOI) and not particles per cell. The rAAV-LacZ was prepared with an adenovirus-free packaging system[16] .

Antibodies. Monoclonal antibody A-20, which is an IgG antibody specific for intact AAV capsids, was provided by J. Kleinschmidt (Deutsches Krebsforschungszentrum, Germany). Two mouse monoclonal IgG1 antibodies against human integrin β5 subunit were used: clones B5-IVF2 (Upstate Biotechnology, Lake Placid, New York) and B5-IA9. Clone B5-IA9, provided in the form of acites fluid, was used for immunoprecipitation and was a gift from M.E. Hemler[13] (Dana Farber Cancer Institute, Boston, Massachusetts). Goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, Pennsylvania) was used for chemiluminescent detection in both western and viral overlay analyses. Mouse IgG1 (MOPC 21; Sigma) was used as the isotype match control antibody in immunoprecipitation and flow cytometry experiments. Monoclonal antibody P1F6 against αVβ5 (Life Technologies) and anti-mouse IgG conjugated to fluorescein isothiocyanate (Caltag, Burlingame, California) were used for flow cytometric analysis.

Plasma membranes, viral overlay assay and western blot. Plasma membranes were prepared from HeLa S3 cells by a sucrose gradient flotation method as described[28]. Enrichment of plasma membrane proteins was 30-fold, as assessed by 5'-nucleotidase activity[29]. For the viral overlay analysis: plasma membrane proteins were separated under reducing conditions (50–100 μg/lane) by 5–20% SDS–PAGE, then electrophoretically blotted to nitrocellulose by semi-dry transfer (BioRad, Richmond, California). Nonspecific binding was blocked in phosphate-buffered saline/0.05% tween-20 (PBST) containing 10% (weight/volume) non-fat dried milk (12 hours at 4 °C). After being blocked, the membranes were incubated with AAV (6×10^{11} particles/ml, for 3 h at room temperature in PBST). Unbound virus was removed by successive washes with PBST. The blot was then incubated with a 1:300 dilution of monoclonal antibody A20 hybridoma supernatant against wt AAV for 1 h, washed three times (5 min each) in PBST, and incubated with a 1:10,000 dilution of goat/anti-mouse IgG–horseradish peroxidase for 1 h. Subsequent washes consisted of one brief wash, one 15-min wash and three 5-min washes in PBST. A final wash in PBS was done before chemiluminescence analysis (Pierce, Rockford, Illinois). Control reactions were done in the absence of virus. Western blot analysis was done with B5-IVF2 monoclonal antibody at a dilution of 1:100, and a 1:10,000 dilution of secondary antibody was used.

Immunnoprecipitation. HeLa cells were lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 50 mM Tris-HCl pH 7.5; protease inhibitors: 1 mM PMSF, 1 mM iodoacetamide, 25 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM EGTA). Next, extracts were 'pooled' before the lysate was clarified by centrifugation. The 'pooled' lysate was then pre-cleared by a primary incubation with mouse IgG1 antibody (MOPC 21) and a secondary incubation with protein A/protein G sepharose beads, each for 1 h at 4 ºC (Santa Cruz Biotechnology, Santa Cruz, California). Each imunoprecipitation reaction was done using 500 μl of aliquoted pre-cleared lysate and 5 μg of antibody (incubation for 12 h at 4 ºC). Rabbit anti-mouse IgG (2.5 μg; Jackson ImmunoReseach, West Grove, Pennsylvania) was then added to each reaction to maximize adsorption to sepharose beads[13]. The reaction was incubated for 1 h at 4 ºC before the addition of sepharose beads to capture the antibody–protein complexes (1 h at 4 ºC). After extensively washing the beads, the antibody–protein complexes were eluted by boiling in reducing SDS sample buffer and separated by 7.5% SDS–PAGE.

Infectivity and virus binding assays. Infectivity and binding assays were done in HEPES-buffered saline (HBS) containing 1% BSA (HBSB). Adherent cells were first detached with 10 mM EDTA, then washed in PBS containing 8.8 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and then in HBS. To assess the effect of EDTA on AAV transduction, HeLa cells were incubated with rAAV or rAd at an MOI of 2 in the presence of absence of 20 mM EDTA in HBS for 1 h at 4 ºC. Cells were washed in HBS (± EDTA) to remove unbound virus, resuspended in HBS with or without 5 mM EDTA, and incubated at 37 ºC for 30 minutes. Cells were then treated with trypsin (0.5 mg/ml) for 10 min to remove uninternalized virus, washed, resuspended in media, and transferred to 6-well tissue culture dishes for growth. Cells were fixed and stained for β-galactosidase activity 24 h (rAd) or 36 h (rAAV) after infection. For transduction of CS1 and CS1/β5 cell lines, either rAAV (MOI 2) or rAd (MOI 1) was bound to cells at 4 ºC in HBSB. After 1 h, cells were washed extensively, resuspended in HBSB, and incubated at 37 ºC for 40 min. Cells were then plated in media containing 2% FBS (Life Technologies). After 24 h (rAd infection) or 48 h (rAAV infection), cells were collected and assayed for β-galactosidase activity with a Galacto-Light Plus kit (Tropix, Bedford, Massachusetts) as described by the manufacturer. Each experimental condition was done in triplicate, and independent experiments yielded similar results.

For direct binding assays, 4×10^{11} particles of ^{3}H-labeled wt AAV were incubated with 3×10^5 cells for 90 min at 4 °C in HBSB either in the absence or presence of a 50-fold excess of unlabeled wt AAV. After extensive washing with ice-cold HBSB, cell-associated radioactivity was determined by counting in a scintillation counter.

Internalization assay. To monitor virus internalization, Cy3-labeled AAV-2 was incubated with cells for 1 h at 4 ºC in HBSB at a concentration of 1×10^5 AAV particles/cell. After thorough washing with ice cold HBSB, cells were resuspended in HBSB and placed in a 37 ºC water bath. At various times, cells were placed on ice, washed once with ice-cold HBSB, and resuspended in PBS containing 2% paraformaldehyde. Cells were then distributed to coverslips treated with Cell-Tak (Collaborative Biomedical Products, Bedford, Massachusetts) and mounted on slides. Internalized virus was visualized by confocal microscopy. A series of optical sections were made through a single cell, and images representative of the cell's center were compared to assess viral entry.

Acknowledgments

We acknowledge F. Ferrari and X. Xiao for their technical help with the adenovirus-free production system. This work was supported in part by NIH-grants NHLBI 539490, 533016 and CF foundation grant MARZLU 96PO.

RECEIVED 18 SEPTEMBER; ACCEPTED 2 DECEMBER 1998

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