Integrin $\alpha V \beta 5$ Is Not Involved in Adeno-Associated Virus Type 2 (AAV2) Infection

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$\alpha V \beta 5$ integrin was recently proposed as a coreceptor for adeno-associated virus type 2 (AAV2) infection (Summerford et al., 1999, Nat. Med. 5, 78–82), based mainly on the direct binding of AAV2 to denatured $\beta 5$ by virus overlay assay. In studies using purified natural or recombinant human integrin $\alpha V \beta 5$ we were unable to demonstrate AAV2 binding, either by virus overlay or by liquid binding assay. Furthermore, neither purified integrin $\alpha V \beta 5$, nor RGD peptides, nor functional blocking monoclonal antibody blocked rAAV2 transduction. These data strongly suggest that integrin $\alpha V \beta 5$ is not involved in AAV2 infection.

INTRODUCTION

Integrins are heterodimers composed of $\alpha$ and $\beta$ subunits that specify interactions with extracellular ligands and mediate cell migration, cell adhesion, cell growth and differentiation, wound repair, and phagocytosis (Hynes, 1992; Savill et al., 1990). Integrins are also used by a number of different viruses in order to gain entrance to host cells (Bergelson et al., 1992; Mason et al., 1994; Roivaninen et al., 1991; Vogel et al., 1993; Wickham et al., 1993). Adenoviruses use the vitronectin-binding integrins $\alpha V \beta 3$ and $\alpha V \beta 5$ to promote viral internalization (Bai et al., 1993; Wickham et al., 1993), rather than viral attachment (Bergelson et al., 1997), through an RGD sequence on the penton base protein (Bai et al., 1993). Foot-and-mouth disease virus (FMDV) also has been shown to use the RGD-binding integrin $\alpha V \beta 3$ as a receptor for viral internalization (Berinstein et al., 1995). Direct interaction of $\alpha V \beta 5$ with the adenovirus penton base protein and $\alpha V \beta 3$ with the FMDV VP-1 G-H loop has been demonstrated by solid-phase binding assay (Mathias et al., 1998; Jackson et al., 1997). Viral binding and infection are EDTA sensitive, can be blocked by the purified integrins and RGD-containing peptides (Mathias et al., 1998; Jackson et al., 1997), and are conformationally dependent; adenoviruses cannot bind to denatured integrins by adenoviral overlay assay (G. R. Nemerow, personal communication).

In recent years, AAV2, a dependovirus, has emerged as an attractive viral vector for gene therapy, but although AAV2 vectors have shown great success in the transduction of many cell types, not all cell types can be transduced by rAAV2, in part due to some cells lacking the host cell receptors that support initial viral binding, absorption, and internalization of AAV2 (Ponnazhagan et al., 1996; Mizukami et al., 1996). Recently, heparan sulfate proteoglycan was proposed as a receptor for AAV2 (Summerford and Samulski, 1998), with $\alpha V \beta 5$ integrin acting as a coreceptor (Summerford et al., 1999). The evidence suggesting the role of $\alpha V \beta 5$ integrin was based on (a) the ability of EDTA treatment of cells to decrease AAV2 infection, suggesting the possibility that an integrin mediates infection; (b) a 100-kDa AAV2-binding protein identified in cell membrane fractions by virus overlay assay identified to be $\beta 5$; and (c) cells transduced to express $\beta 5$ integrin showed a twofold increased susceptibility to AAV2 infection. No evidence was presented to indicate the role of $\alpha V$.

In this paper, we use both viral overlay assay and solid-phase liquid binding assay to explore the direct interaction of AAV2 with integrin $\alpha V \beta 5$ and a rAAV2-LacZ transduction assay to look into the inhibitory effects by integrin $\alpha V \beta 5$ and related molecules.

RESULTS AND DISCUSSION

Using the virus overlay assay with AAV2 we have previously reported the presence of a 150-kDa glycoprotein with a $pI$ of 4.5–5.0 in cell membrane fractions (Mizukami et al., 1996), and using a modified sensitive assay we detected a second but weaker AAV2-binding band at about 100 kDa. This 100-kDa AAV2-binding protein was identified as human nucleolin (Qi and Brown, 1999). To confirm whether the 100-kDa AAV2-binding protein in cell membrane fractions was integrin $\beta 5$ or human nucleolin, a virus overlay assay was performed using purified natural integrin $\alpha V \beta 5$ (2 $\mu$g), copurified human nucleolin (4 $\mu$g), and a lectin-purified cell membrane protein fraction (containing the 150-kDa AAV2-
binding protein, 50 µg) as control (Fig. 1). Despite a clearly visible β5 band after Coomassie blue staining and β5 antibody Western blotting, there was no evidence of AAV2 binding by viral overlay. However, as previously reported there was a strongly AAV2-binding 150-kDa protein in the membrane fraction (Mizukami et al., 1996), and AAV2 bound to the copurified human nucleolin (Qiu and Brown, 1999). The lack of binding of AAV2 to the integrin bands suggested that AAV2 was unable to bind to either denatured αV or β5.

To explore the possibility that AAV2 interacted with the αVβ5 heterodimer in a manner similar to adenovirus, a solid-phase liquid binding assay was performed in microtiter plates. Both natural integrin αVβ5 and recombinant αVβ5 bound to adenovirus and mAb P1F6, but not to purified AAV2 (Fig. 2), indicating that unlike adenovirus, AAV2 had no direct interaction with the αVβ5 heterodimer.

αVβ5 integrin is involved in adenovirus internalization (Wickham et al., 1993; Mathias et al., 1998), and purified αVβ5, RGD peptides, and mAb P1F6 can significantly inhibit recombinant adenovirus gene delivery (Mathias et al., 1998; Goldman and Wilson, 1995). We therefore used rAAV2-LacZ to identify whether rAAV2 transduction was also inhibited by αVβ5 in a similar manner. There was no inhibition of transduction with purified integrin αVβ5 in CHO cells and HeLa cells or by RGD peptides, mAb P1F6 (Fig. 3), and vitronectin, a ligand of αVβ5 (data not shown), although in both experiments heparin reduced rAAV-LacZ transduction (Summerford and Samulski, 1998).

Overall, we were unable to confirm that AAV2 binds directly to αVβ5 integrin, either by viral overlay or by solid-phase liquid binding assay. However, several viruses are thought to use αV integrins as a receptor or coreceptor: adenoviruses (αVβ5/β3) (Wickham et al., 1993; Mathias et al., 1998), and purified αVβ5, RGD peptides, and mAb P1F6 can significantly inhibit recombinant adenovirus gene delivery (Mathias et al., 1998; Goldman and Wilson, 1995). We therefore used rAAV2-LacZ to identify whether rAAV2 transduction was also inhibited by αVβ5 in a similar manner. There was no inhibition of transduction with purified integrin αVβ5 in CHO cells and HeLa cells or by RGD peptides, mAb P1F6 (Fig. 3), and vitronectin, a ligand of αVβ5 (data not shown), although in both experiments heparin reduced rAAV-LacZ transduction (Summerford and Samulski, 1998).

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FIG. 1. Viral overlay and Western blotting of integrin αVβ5. Protein molecular weight marker (lane 1), purified natural human integrin αVβ5 (2 µg, lane 2), lectin-purified membrane protein (50 µg, lane 3), and purified AAV2 with nucleolin (4 µg, lane 4) were separated by SDS–7% PAGE. The gel was stained with Coomassie blue, or was transferred to nitrocellulose membrane for AAV2 viral overlay, or was Western blotted with anti-β5. The arrow indicates the nucleolin band.

FIG. 2. Interaction of soluble integrin αVβ5 with AAV2 and adenovirus. Microtiter wells were coated with purified adenovirus (Ad5), AAV2, mAb P1F6, or BSA, blocked, and washed. The wells were incubated with purified natural integrin αVβ5 (■) or purified recombinant integrin αVβ5 (○). Integrin binding was detected with anti-β5 rabbit polyclonal antibody.
1993); coxsackievirus A9 \((\alpha V\beta 3)\) (Roivaninen et al., 1991); foot-and-mouth disease virus \((\alpha V\beta 3)\) (Mason et al., 1994); and hantaviruses \((\alpha V\beta 3)\) (Gavrilovskaya et al., 1998). Apart from hantaviruses, in all other cases the binding requires divalent metal cations and is EDTA sensitive, RGD peptides block both binding and infection, and binding occurs through the RGD motif, which is presented in the capsid viral protein. However, there is no RGD sequence in the AAV2 capsid sequence (Ruffing et al., 1994), and we were unable to inhibit transduction using RGD peptides, specific monoclonal antibody, or vitronectin. In addition, in contrast to the data for adenovirus, even at high concentration, we were unable to inhibit transduction of rAAV2 with purified \(\alpha V\beta 5\) integrin. Together, these data do not support a role of \(\alpha V\beta 5\) in AAV2 infection.

The addition of chelating agents such as EDTA was also claimed to be able to block rAAV transduction (Summervold et al., 1999), similar to their role in blocking adenovirus infection (Svensson and Persson, 1984). However, recently Duan et al. (1999) reported that, in contrast, application of EGTA, another chelating agent, at the time of basolateral infection with rAAV2 did not affect transduction and even enhanced mucosal rAAV2 transduction sevenfold (Duan et al., 1998). These findings suggest that in contrast to adenoviral infection, divalent cations are not required for AAV2 infection, again indirectly supporting our data that AAV2 and adenovirus do not share binding to \(\alpha V\beta 5\).

At the same time that it was suggested that AAV2 binds to integrin \(\alpha V\beta 5\), a separate group also proposed basic fibroblast growth factor receptor (bFGFR) as a coreceptor for AAV2 infection (Qing et al., 1999), similar to that previously hypothesized for HSV infection (Kaner et al., 1990). However, subsequent research has shown FGFR is not required in HSV infection (Muggeridge et al., 1992), and we have shown in similar studies that cell lines lacking FGFR are still permissive for AAV2 infection (Qiu et al., 1999). Previously, our group identified a 150-kDa AAV2-binding glycoprotein in cell membrane fractions of AAV2 permissive cells, absent by virus overlay assay in AAV2 nonpermissive cells (Mizukami et al., 1996) and present on the external surface of susceptible cells (unpublished observations). Although this protein remains a good candidate as a receptor for AAV2, the identity of this protein remains unknown. Further studies are required on this and the other candidate receptors to understand the initial events of AAV2 binding and infection.

**MATERIALS AND METHODS**

**Viruses and cells**

Seed viruses (AAV2 and adenovirus type 5) and cells (KB, HeLa, and CHO-K1 cells) were purchased from the American Type Culture Collection (ATCC, Manassa, VA). All the cells were maintained as monolayers in Dulbecco’s modified Eagle’s minimal medium (DMEM), supplemented with 10% fetal bovine serum and penicillin–streptomycin, and maintained at 37°C in a humidified atmosphere with 5% CO₂.

**Virus preparations**

Confluent KB cells were coinfected with AAV2 (multiplicity of infection (m.o.i.) = 10) and adenovirus type 5 (m.o.i. = 5), and infected cells were harvested 48 h later. Wild-type AAV2 was purified by CsCl ultracentrifugation performed as previously reported (Qiu and Brown, 1999) without sonication treatment of the cell lysates, to prevent copurification of nucleolin (Qiu and Brown, 1999). Purified AAV2 was demonstrated to be free of adenovirus and nucleolin by Western blotting. For adenovirus production, KB cells were infected with adenovirus type 5.
equivalent to 3.0 that were positive 48 h later. The infectious titer was vector stock and determining the percentages of cells obtained by infecting 293 cells with serial dilution of AAV Southern blot. The infectious titer of rAAV2-LacZ was methylated to be 6.5 \times 10^{11} genomic copies per milliliter by Southern blot. The infectious titer of rAAV2-LacZ was obtained by infecting 293 cells with serial dilution of AAV vector stock and determining the percentages of cells that were positive 48 h later. The infectious titer was equivalent to 3.0 \times 10^7 infectious particles per milliliter. The AAV vector was further confirmed to be adenovirus free by Western blotting using a polyclonal antibody to adenovirus (Biodesign International, Kennebunk, ME).

Proteins, peptides, and antibodies

Human integrin αVβ5 purified protein (95% purity by SDS–PAGE) from human placenta was purchased from Chemicon International, Inc. (Temecula, CA). Soluble recombinant human integrin αVβ5, expressed in the baculovirus system and purified using mAb P1F6 covalently linked beads (Mathias et al., 1998), was a gift from Dr. G. R. Nemerow (Scripps Research Institute, La Jolla, CA). Cell membrane protein was prepared from a HeLa S3 membrane fraction as previously reported (Mizukami et al., 1996), solubilized with 2% n-dodecylmaltoside (DMT) in 20 mM phosphate buffer, pH 7.2, with 1 M NaCl and protease inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany) and loaded on a PSA lectin-conjugated agarose (EY Laboratories, Inc., San Mateo, CA) column. Lectin-purified protein was eluted by 100 mM methyl-α-D-glucopyranoside and 100 mM methyl-α-D-mannopyranoside in the same buffer as described above with 0.1% DMT and dialyzed against 20 mM Tris–HCl, pH 7.2, 1 mM EDTA, 0.05% DMT. Copurified human nucleolin was prepared as previously reported (Qiu and Brown, 1999). Protein concentrations for all protein preparations were measured by BCA protein assay reagent (Pierce Co.).

RGD peptide (GRGDSP) and RGE peptide (GRGESP) were purchased from Life Technologies Inc. (Rockville, MD). Monoclonal antibody to αVβ5 integrin (P1F6, an integrin functional blocking antibody), rabbit anti-αVβ5 polyclonal antibody, and an IgG1 isotype control were all from Chemicon International Inc. Monoclonal antibody to integrin β5 (B5-IVF2) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal antibody to intact AAV2 particles (A20) was obtained from American Research Products Inc. (Belmont, MA).

Viral overlay assay and Western blotting assay

The virus overlay protein-binding assay was performed as previously described (Mizukami et al., 1996), with a slight modification. Briefly, proteins were separated by SDS–7% PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was blocked and incubated with purified AAV2 (20–50 μg/ml) in PBS with 0.1% Tween 20 and 1% BSA (PBST–BSA), after extensive washes and further incubated with monoclonal antibody A20 at 1:500 in PBST–BSA and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Biosource International Inc., Camarillo, CA) at 1:10,000 in PBST–BSA. The detection of binding was by chemiluminescence (Pierce Co.) and exposure to X-ray film.

Western blot was performed in a similar manner, but instead of incubation with AAV2 and A20 monoclonal antibody, the transferred membrane was incubated with monoclonal antibody.

Solid-phase liquid binding assay

The assay was carried out as previously described (Mathias et al., 1998) with some modifications. Purified AAV2, adenovirus, mAb P1F6, and BSA as control were coated onto microtiter plate wells (EIA/RIA strip plate-8, Costar Co., Cambridge, MA) in 200 mM sodium carbonate buffer, pH 9.6, at 10 μg/ml, 37°C for 1 h. The plate was blocked by PBST with 5% BSA for 1 h at RT, washed thoroughly with PBST, and incubated with either purified αVβ5 from human placenta or recombinant αVβ5 at 1–2 μg/ml in PBST–BSA for 1 h at RT with constant shaking. After washing, the strips were further incubated with a rabbit polyclonal antibody to integrin αVβ5 at 1:1000 in PBST–BSA for 1 h at RT, followed by further washing. The strips were finally incubated with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:10,000 in PBST–BSA for 1 h at RT. After washing, the reaction was developed by addition of ABTS solution (Boehringer Mannheim GmbH, Mannheim, Germany) and absorbance was measured at 405 nm.

Recombinant AAV2 transduction and blocking assay

Confluent CHO cells and HeLa cells on microtitre wells were preincubated with RGD peptides (2 mg/ml), RGE peptides (2 mg/ml), mAb P1F6 (1 mg/ml), and isotype control IgG1 (1 mg/ml) in PBS at 37°C for 1 h, prior to infection with rAAV-LacZ (m.o.i. = 1.5). In separate experiments, rAAV-LacZ particles (m.o.i. = 1.5) were preincubated in equal volume with αVβ5 from human placenta (1 mg/ml), heparin (1 mg/ml) in PBS with 1 mM CaCl2 and 1 mM MgCl2 for 1 h at 37°C and then added...
to CHO cells or HeLa cells plated in microtiter wells. After 48 h, the monolayers were fixed with 2% glutaraldehyde in PBS and β-galactosidase stained (Beta-gal staining kit, Panvera Corp., Madison, CA), and the number of positive cells was counted by light microscopy.

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