

## Integrin $\alpha v \beta 1$ Is an Adenovirus Coreceptor†

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**The human embryonic kidney (HEK293) cell line, commonly used for recombinant adenovirus (Ad) propagation, does not express the Ad coreceptor  $\alpha v \beta 3$  or  $\alpha v \beta 5$  integrins, yet these cells are efficiently infected by Ad vectors. Here we demonstrate that Ad binds to HEK293 cells via the fiber receptor CAR and is subsequently internalized via interaction with integrin  $\alpha v \beta 1$ . Function-blocking antibodies directed against  $\alpha v$  or  $\beta 1$ , but not  $\beta 3$ ,  $\beta 5$ , or  $\alpha 5$ , integrin subunits block Ad infection and viral endocytosis. Therefore,  $\alpha v \beta 1$  serves as a coreceptor for Ad infection, and the lack of  $\beta 3$  and/or  $\beta 5$  but the relatively high expression of  $\alpha v \beta 1$  integrins on certain tumor cell types may explain why these cells are readily transduced by Ad vectors.**

Adenovirus (Ad) host cell entry requires initial attachment to cells mediated by the fiber interaction with its cellular receptor CAR. The subsequent association of penton base with  $\alpha v \beta 3$  or  $\alpha v \beta 5$  integrins promotes Ad entry (1, 21, 23). Integrins are heterodimeric receptors for extracellular matrix proteins and cell surface counterreceptors.  $\alpha v$  integrins ( $\alpha v \beta 1$ ,  $\alpha v \beta 3$ ,  $\alpha v \beta 5$ ,  $\alpha v \beta 6$ , and  $\alpha v \beta 8$ ) mediate cell adhesion to various matrix proteins, including fibronectin, vitronectin, and fibrinogen, that contain an arginine-glycine-aspartic acid (RGD) sequence (8, 19, 24). The interaction of the Ad penton base and  $\alpha v$  integrin triggers the activation of several signaling molecules (14) that promote actin cytoskeletal reorganization and enhance Ad internalization. Although HEK293 cells have been widely used for propagation of recombinant Ad vectors, the integrin repertoire of these cells has not been clearly established. For example, several reports indicate that HEK293 cells do not express  $\alpha v \beta 3$  and  $\alpha v \beta 5$  integrins (10, 18), while another report indicates that they do (6). Furthermore,  $\beta 5$  integrin-deficient mouse fibroblast cells support Ad infection, suggesting that other integrins play a role in Ad infection (11). HEK293 cells were also reported to express  $\alpha 5 \beta 1$  and  $\alpha v \beta 1$  integrins (2), which have been identified as RGD-dependent receptors for both fibronectin and vitronectin (2, 15, 22). Considering the fact that soluble fibronectin or RGD-containing peptide reduced Ad infection (23) and that an  $\alpha 5 \beta 1$ -activating antibody has been reported to enhance Ad-mediated gene delivery to certain melanoma cells (4), it was of interest to determine whether Ad uses either  $\alpha v \beta 1$  and/or  $\alpha 5 \beta 1$  integrins as alternative receptors for virus internalization.

**The role of CAR and  $\alpha v$  integrins in Ad infection of HEK293 cells.** To investigate whether CAR or integrins promote Ad attachment, we preincubated HEK293 cells with an excess of recombinant Ad type 2 (Ad2) fiber protein or with anti-CAR monoclonal antibody (MAb) (RmcB), with a function-blocking Fab fragment of a penton base MAb (DAV-1) (20), or with function-blocking  $\alpha v \beta 5$  (P1F6),  $\alpha 5$  (P1D6), or nonfunction-

blocking  $\alpha v$  (LM142) control MAb prior to measuring the specific binding of  $^{125}\text{I}$ -labeled Ad5 particles (Fig. 1A) as previously described (13). Pretreatment of cells with recombinant Ad fiber protein completely blocked  $^{125}\text{I}$ -Ad5 binding to HEK293 cells, and the RmcB antibody also partially blocked binding. These findings indicated that Ad binding to HEK293 cells is likely mediated by fiber association with CAR.

Experiments were next performed to determine if CAR and/or integrins regulate Ad-mediated gene delivery (Fig. 1B). Cells were infected with Ad5.CMV.LacZ at a multiplicity of infection (MOI) of 1 and then assayed for  $\beta$ -galactosidase expression at 20 h postinfection. As expected from the binding studies, soluble fiber protein and anti-CAR antibody significantly inhibited Ad infection. Furthermore, anti-penton base antibody (L230) inhibited Ad-mediated gene delivery by approximately 40%. In contrast, the function-blocking  $\alpha v \beta 3$  (LM609) and  $\alpha v \beta 5$  (P1F6) integrin antibodies failed to significantly inhibit infection. These results suggested that other members of the  $\alpha v$  integrin family, such as  $\alpha v \beta 1$ , may facilitate Ad infection of HEK293 cells.

**HEK293 cells express  $\alpha v \beta 1$  integrins.** Since conflicting results have been reported with regard to  $\alpha v$  integrin expression on HEK293 cells (6, 10, 18), we next sought to determine the repertoire of different  $\alpha v$  integrins expressed on HEK293 cells using flow cytometry. These studies demonstrated that HEK293 cells expressed significant levels of  $\alpha v$ ,  $\alpha 5$ , and  $\beta 1$  integrins (Fig. 2). In contrast,  $\alpha v \beta 3$  and  $\alpha v \beta 5$  integrin expression was undetectable on HEK293 cells, whereas these  $\alpha v$  integrins were expressed on A549 cells. To verify integrin expression, cell surface proteins of HEK293 cells were biotinylated and then solubilized with 1% Nonidet P-40. Cell lysates were then immunoprecipitated with anti- $\alpha v$ , anti- $\alpha 5$ , or anti- $\beta 1$  or with anti- $\alpha v \beta 1$  integrin antibodies and then analyzed on a 6% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions, followed by immunoblotting with a horseradish peroxidase-conjugated antibiotin antibody (Sigma, St. Louis, Mo.). As shown in Fig. 3A, the anti- $\alpha v$  antibody (L230) immunoprecipitated two proteins of approximately 155 and 121 kDa. In contrast, the anti- $\alpha v \beta 5$  (P1F6) and anti- $\alpha v \beta 3$  (LM609) antibodies failed to immunoprecipitate these integrins, although both antibodies immunoprecipitated the corresponding  $\alpha$  and

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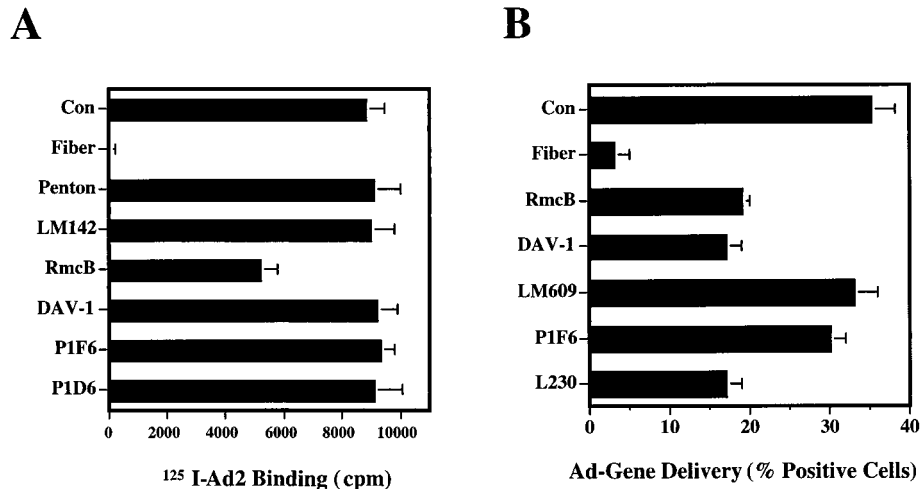


FIG. 1. Adenovirus infection of HEK293 cells is mediated by fiber and penton base proteins. (A) Fiber protein mediates Ad binding to HEK293 cells. HEK293 cells ( $1.5 \times 10^6$  cells/sample, in duplicate) in suspension were preincubated with recombinant Ad2 fiber, penton base ( $10 \mu\text{g/ml}$ ), the anti-CAR antibody (RmcB), anti-penton base antibody (DAV-1), or anti-integrin antibodies at  $20 \mu\text{g/ml}$  at  $4^\circ\text{C}$  for 60 min. Specific Ad binding to cells was determined by using  $^{125}\text{I}$ -labeled Ad2 as previously described (13). (B) Penton base interaction with  $\alpha\text{v}$  integrins promotes Ad-mediated gene delivery. HEK293 cells ( $10^6$  cells/sample) were preincubated with fiber protein, anti-CAR, anti-penton base, or anti-integrin antibodies ( $20 \mu\text{g/ml}$ ) as described above, followed by incubation with Ad.CMV.LacZ at an MOI of 1 particle/cell at  $4^\circ\text{C}$  for another 30 min. The cells were then warmed to  $37^\circ\text{C}$  for 15 min. Noninternalized virus was removed by trypsinization. Ad-mediated gene delivery was determined at 20 h postinfection by expression of  $\beta$ -galactosidase. Representative data from two independent experiments were plotted as the mean  $\pm$  standard deviation.

$\beta$  chains from A549 cells (Fig. 3B). The 155- and 121-kDa as well as a 200-kDa protein were also recognized by a pan-specific  $\beta 1$  antibody (P4C10). The 200-kDa protein is likely the  $\alpha 1$  integrin subunit, which is known to form a heterodimer with the  $\beta 1$  integrin subunit. An  $\alpha 5$ -specific antibody (P1D6) also immunoprecipitated a 155- and a 121-kDa protein. Integrin  $\alpha 5$  also associates with the  $\beta 1$  subunit and exhibits mobility similar to that of the  $\alpha\text{v}$  integrin subunit. These findings indicated that  $\beta 1$  integrins form heterodimeric receptors with  $\alpha\text{v}$  and  $\alpha 5$  but not with  $\beta 3$  and  $\beta 5$  subunits on HEK293 cells. Moreover, these

findings indicated that  $\alpha\text{v}\beta 1$  is the major  $\alpha\text{v}$  integrin on HEK293 cells. To verify this, we performed immunoprecipitation detection experiments with an  $\alpha\text{v}\beta 1$ -specific antibody (9EG7). As expected, this antibody also immunoprecipitated 155- and 121-kDa proteins on HEK293 cells.

**$\alpha\text{v}\beta 1$  integrins promote Ad infection of HEK293 cells.** We next analyzed whether the  $\alpha\text{v}\beta 1$  integrins expressed on HEK293 cells were capable of interacting with Ad by performing cell adhesion assays in the presence or absence of function-blocking integrin antibodies. HEK293 cells adhered to fi-

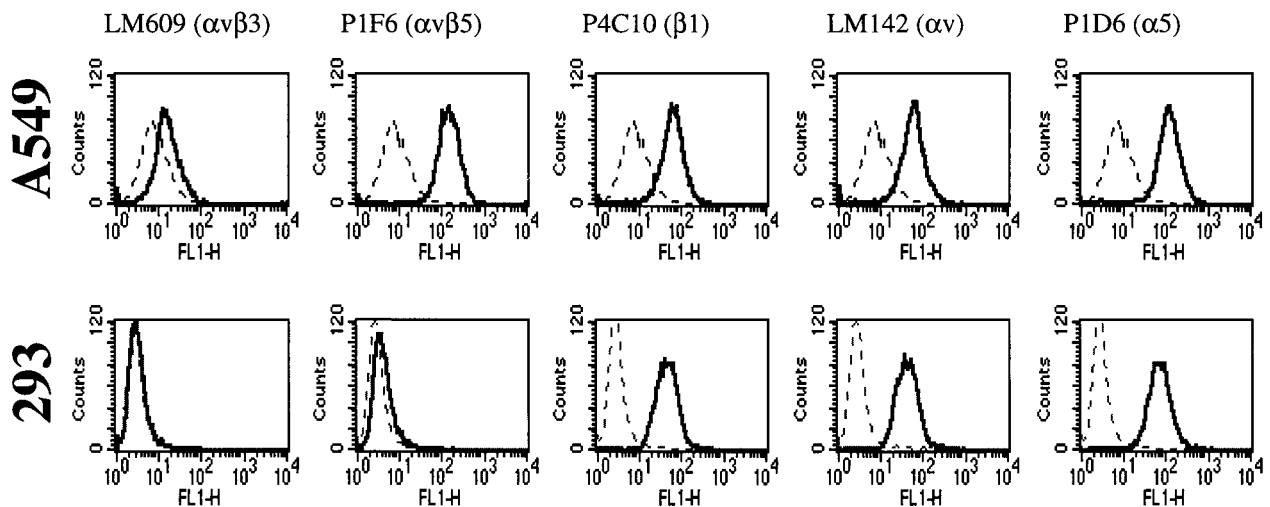


FIG. 2. Flow cytometric analysis of integrin expression on HEK293 cells. HEK293 or A549 cells were incubated with  $20 \mu\text{g}$  of anti-integrin antibodies/ml at  $4^\circ\text{C}$  for 60 min. Cell surface-bound antibody was detected by incubation with an R-phycoerythrin-labeled goat anti-mouse secondary antibody. Dashed line, secondary antibody control; solid line, indicated integrin antibodies.

### A. HEK293 Cells

### B. A549 Cells

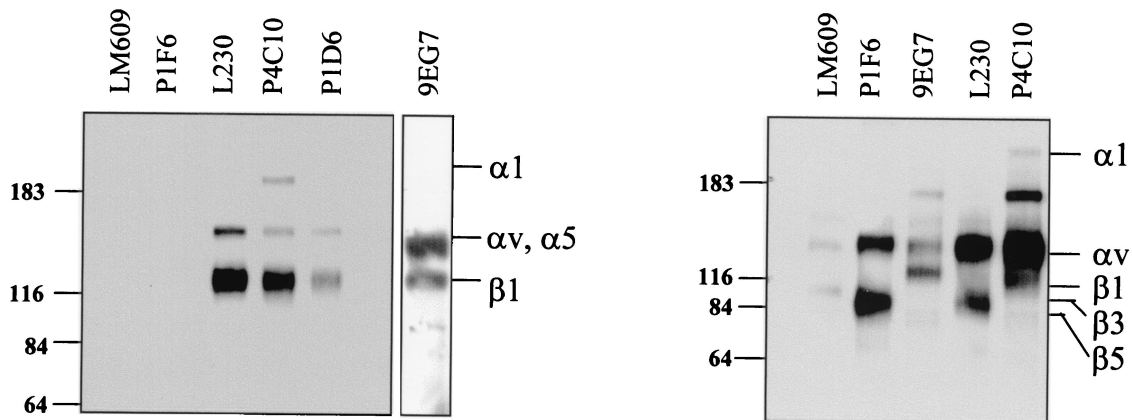


FIG. 3. Immunoprecipitation analyses of integrin expression on HEK293 cells. HEK293 (A) and A549 (B) cells ( $10^7$  cells) were detached with 1 mM EDTA and then biotinylated with 1 mg of sulfo-*N*-hydroxysuccinimide-biotin. After being washed with phosphate-buffered saline four times, labeled cells were then lysed in 1 ml of lysis buffer containing 1% Nonidet P-40 and protease inhibitors. Lysates from biotinylated cells (equal to  $10^6$  cells) were immunoprecipitated with 2  $\mu$ g of anti-integrin antibodies followed by capture with protein A/G beads. The immunoprecipitates were then separated on 6% sodium dodecyl sulfate gels under reducing conditions. After transfer to a polyvinylidene difluoride membrane, the filter was probed with a horseradish peroxidase-conjugated anti-biotin antibody.

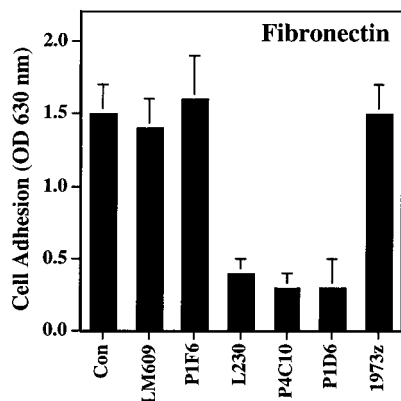
bronectin, and adhesion could be inhibited with a function-blocking anti- $\alpha 5$  (P1D6), anti- $\alpha v$  (L230), or anti- $\beta 1$  (P4C10) MAb (Fig. 4). HEK293 cells also adhered to penton base; however, adhesion to penton base could only be inhibited by anti- $\alpha v$  or the anti- $\beta 1$  antibodies. This finding indicated that integrin  $\alpha v \beta 1$  but not  $\alpha 5 \beta 1$  serves as a receptor for Ad penton base.

To determine whether Ad uses  $\alpha v \beta 1$  integrin for infection, we treated HEK293 cells with anti- $\alpha v$  integrin antibody (L230), anti- $\alpha 5$  antibody (P1D6), or anti- $\beta 1$  integrin antibody (P4C10)

and then measured Ad-mediated gene delivery to these cells (Fig. 5A). Pretreatment of HEK293 cells with anti- $\alpha v$ , anti- $\beta 1$ , or a combination of anti- $\alpha v$  and anti- $\beta 1$  antibodies significantly blocked Ad infection. In contrast, the anti- $\alpha 5$  antibody alone inhibited Ad infection by less than 10%. Pretreatment with a combination of anti- $\beta 1$  and anti- $\alpha 5$  antibodies did not further decrease gene delivery relative to that achieved with anti- $\beta 1$  alone (data not shown). These data indicated that  $\alpha v \beta 1$  integrin also facilitates Ad infection of HEK293 cells.

Further studies investigated whether penton base interac-

### A



### B

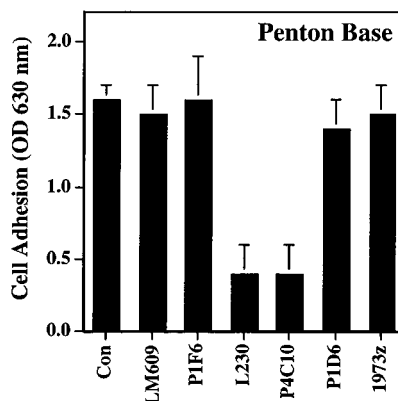


FIG. 4. Integrin-dependent cell adhesion to fibronectin (A) or penton base (B). Non-tissue culture-treated cluster plates were precoated with 10  $\mu$ g of recombinant penton base or fibronectin/ml. HEK293 cells were preincubated with 20  $\mu$ g of anti-integrin antibodies/ml before the cells were allowed to attach to coated plates for 15 min at 37°C. Attached cells were quantitated after fixation and stained with crystal violet. Data are presented as the mean  $\pm$  standard deviation of duplicate samples. Antibodies: LM609, anti- $\alpha v \beta 3$ ; P1F6, anti- $\alpha v \beta 5$ ; L230, anti- $\alpha v$ ; P4C10, anti- $\beta 1$ ; P1D6, anti- $\alpha 5$ ; 1973z, anti- $\alpha 1$ .

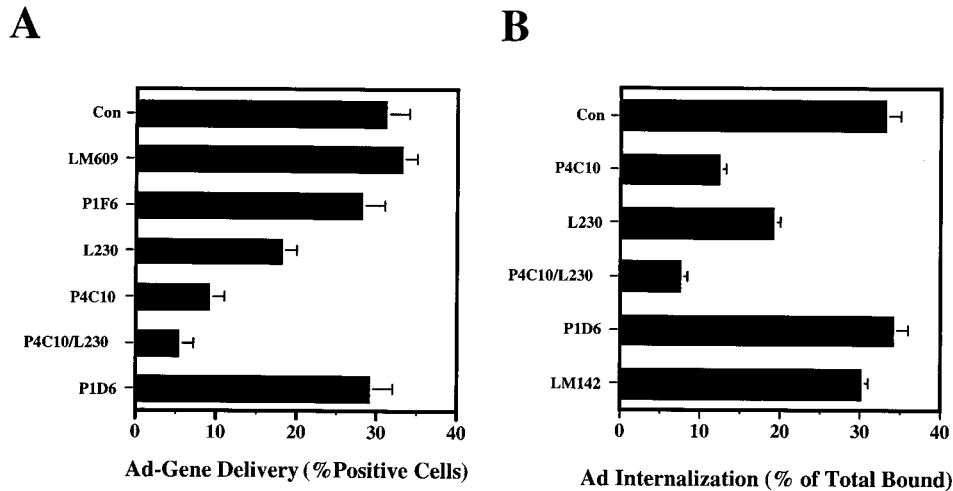


FIG. 5. Integrin  $\alpha\beta 1$  promotes Ad-mediated gene delivery and virus internalization. (A) HEK293 cells in suspension were preincubated with anti-integrin antibodies for 60 min at 4°C, followed by incubation with Ad.CMV.LacZ at an MOI of 1 viral particle/cell. After being warmed to 37°C for 15 min, noninternalized virus was removed by trypsinization. Cells were plated in 6-well plates, and  $\beta$ -galactosidase expression was determined at 20 h postinfection (mean  $\pm$  standard deviation). (B) HEK293 cells were pretreated with 20  $\mu$ g of anti-integrin antibodies/ml at 4°C for 60 min, followed by the addition of  $^{125}$ I-labeled Ad2 ( $2 \times 10^5$  cpm/cell). Bound virus particles were then allowed to internalize by warming the cells at 37°C for 15 min. Internalized virions were determined by measuring their resistance to trypsinization. The data represent the percentage of trypsin-resistant cpm/total cpm of specifically bound Ad  $\pm$  standard deviation of duplicate samples.

tion with integrin  $\alpha\beta 1$  promotes Ad internalization. Cells were preincubated with function-blocking anti-integrin antibodies prior to the addition of  $^{125}$ I-labeled Ad2 (Fig. 5B). Virus uptake was then assayed by measuring the resistance of Ad particles to trypsin treatment. Pretreatment with anti- $\beta 1$  or anti- $\alpha 5$  antibody significantly inhibited Ad internalization by 63 and 42%, respectively, compared to that of untreated control cells. The combination of anti- $\alpha 5$  and anti- $\beta 1$  antibodies inhibited Ad internalization by 76%. In contrast, the anti- $\alpha 5$  or LM609 antibody ( $\alpha\beta 3$ ) had little if any effect on virus entry. These studies demonstrated that  $\alpha\beta 1$  integrin promotes Ad infection by enhancing virus internalization.

Here we demonstrated that HEK293 cells use  $\alpha\beta 1$  integrin, instead of  $\alpha\beta 3$  or  $\alpha\beta 5$  integrin, for virus internalization and infection. The ability of  $\alpha\beta 1$  to promote Ad infection may also explain why mice genetically deficient in  $\beta 5$  integrin are susceptible to Ad infection (11). It will be of interest to determine whether other cell types that express  $\alpha\beta 1$  or perhaps other  $\beta 1$  integrins (3, 12) are also susceptible to Ad infection. In this regard, human melanoma, breast cancer, and neuroblastoma cells have been shown to express integrin  $\alpha\beta 1$  (5, 7, 9, 16). Integrin  $\alpha\beta 1$  may also regulate the migration of human oligodendritic cells in the central nervous system (17).

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