Integrin $\alpha v\beta 5$ Selectively Promotes Adenovirus Mediated Cell Membrane Permeabilization

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Abstract. Human adenovirus type 2 (Ad2) enters host cells by receptor-mediated endocytosis, an event mediated by the virus penton base binding to cell surface integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. While both αv integrins promote virus internalization, $\alpha v\beta 5$ is involved in the subsequent event of membrane permeabilization. Cells transfected with the $\beta 5$ or $\beta 3$ subunit, expressing either $\alpha v\beta 5$ and $\alpha v\beta 3$, respectively, were capable of supporting Ad2 infection to varying degrees. In this case, cells expressing $\alpha v\beta 5$ were significantly more susceptible to Ad2-induced membrane permeabilization, as well as to Ad2 infection, than cells expressing $\alpha v\beta 3$. Adenovirus-mediated gene delivery was also more efficient in cells expressing $\alpha v\beta 5$. These results suggest that the interaction of $\alpha v\beta 5$ with Ad2 penton base facilitates the subsequent step of virus penetration into the cell. These studies provide evidence for the involvement of a cellular receptor in virus-mediated membrane permeabilization and suggest a novel biological role for integrin $\alpha v\beta 5$ in the infectious pathway of a human adenovirus.

A crucial step in virus infection of host cells is penetration/permeabilization of the cell plasma membrane, a past-internalization event required for delivery of the viral genome into the cytoplasm. Although a substantial amount of knowledge exists on cell entry by enveloped viruses, the mechanism(s) by which nonenveloped viruses penetrate cells is not well understood. Adenovirus, a nonenveloped DNA virus that is a major cause of respiratory and gastrointestinal infections of children (3, 14), has proved useful for studying cell entry by nonenveloped viruses.

Of the over 40 different serotypes of human adenovirus, the majority of cell interaction studies have been performed with serotype 2 (human adenovirus type 2; Ad2)[1]. Initial attachment of Ad2 to host cells is mediated by the fiber protein (13, 17), an elongated 62-kD protein that is present on each of the 12 vertices of the virion capsid (28). The fiber receptor, which is broadly distributed on a variety of cells, has not yet been identified. After Ad2 attachment to the fiber receptor, virus particles are rapidly internalized into clathrin-coated vesicles by receptor-mediated endocytosis (4, 33). The fiber protein is dissociated from the virion particle early in the entry pathway (12). Ad2 internalization is mediated by either of two secondary host cell receptors, integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ (38). Ad2 binding to αv integrins is mediated by five Arg-Gly-Asp (RGD) sequences present in a separate virus coat protein known as the penton base (1, 38). Mutations introduced into the penton base RGD sequence abolish interaction with αv integrins and also inhibit efficient virus infection (1). Thus, Ad2 cell entry requires sequential steps involving virus attachment to a primary cell receptor via the fiber protein, followed by internalization mediated by penton base binding to αv integrins.

Following virus internalization, Ad2 causes disruption of cell endosomes by a pH-dependent mechanism (2, 26) which is still poorly understood. Ad-mediated endosome disruption requires a low pH environment since agents such as monensin that inhibit endosome acidification also block Ad penetration but not virus internalization (12). The ability of adenovirus to disrupt cell endosomes efficiently has facilitated the use of this virus as a vector for gene therapy (20).

Ad2 also induces cell membrane permeabilization at pH 6.0 (2, 23), an activity associated with virus disruption of the cell endosome (penetration). Ad2-mediated membrane permeabilization has been demonstrated by the release of fluorescent dyes from liposomes (2) or by release of ^{51}chromium or [^{3}H]choline from human epithelial cells (23). A number of earlier studies indicated that Ad2 penton base has an important role in membrane permeabilization. Polyclonal antibodies directed against the Ad2 penton base but not fiber or hexon protein block Ad2-mediated membrane permeabilization, as measured by the release of ^{51}chromium (26) or by enhanced susceptibility to *Pseudomonas* toxin (22). The penton base also has a propensity to bind to nonionic detergents at low pH compared to other adenovirus coat proteins (25), suggesting that membrane-reactive hydrophobic regions in the penton base may become exposed upon genera-

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[1] *Abbreviations used in this paper*: Ad2, adenovirus type 2; FFU, fluorescent focus unit; MOI, multiplicities of infection; RLU, relative light units.

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tion of the low pH in the endosome. The penton base is released from Ad2 virions shortly after virus internalization (12), further suggesting the penton base is involved in membrane interactions within the cell endosome.

In these studies, we established cell lines expressing either $\alpha v\beta 3$ or $\alpha v\beta 5$ to analyze the role of each of these receptors in Ad2 penton base binding, internalization, and cell membrane permeabilization. The results demonstrate that although integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ both mediate Ad2 penton base binding and internalization, integrin $\alpha v\beta 5$ preferentially promotes virus-induced cell membrane permeabilization and gene delivery as well as adenovirus infection.

Materials and Methods

Cell Culture and Generation of Cell Lines

HEp-2, HeLa, and H2981 cell lines were obtained from American Type Culture Collection (Rockville, MD). The CS-1 hamster melanoma cell line (9), a generous gift of Dr. Carolyn Damsky (University of California at San Francisco, San Francisco, CA), was propagated in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml gentamicin. CS-1 cells do not adhere to vitronectin due to their failure to express αv integrins (32). These cells express neither $\alpha v\beta 3$ nor $\alpha v\beta 5$ heterodimers on their surface yet maintain an intracellular pool of the αv integrin subunit. To generate CS-1 sublines expressing either $\alpha v\beta 3$ or $\alpha v\beta 5$ integrins on their surface, cDNAs encoding full-length human $\beta 3$ (10) or $\beta 5$ (19) subunit proteins were subcloned into the pcDNA-1/NEO expression vector (Invitrogen, La Jolla, CA) and introduced into CS-1 cells via lipofectin-mediated transfection (GIBCO BRL, Gaithersburg, MD). Stably transformed cells were selected by growth in 500 μg/ml Geneticin (Sigma Chemical Co., St. Louis, MO) and enriched from the neomycin-resistant population for the expression of either $\alpha v\beta 3$ or $\alpha v\beta 5$ by selectively propagating the adherent cell population and discarding the unattached cells during passage of the cell line.

Virus, Antibodies, and Proteins

Ad2 was propagated in HeLa or H2981 cells and was purified and stored as previously described (8). Briefly, Ad2 was banded on cesium chloride gradients and then dialyzed against 10 mM phosphate-buffered saline, pH 8.0 containing 10% glycerol, and 1 mM $MgCl_2$. Purified virus was stored at −70°C and dialyzed into the appropriate buffer just prior to use. Ad2 was labeled with [^{3}H]thymidine as previously described (30) or with NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) as recommended by the manufacturer. Recombinant adenovirus type 5 containing the gene encoding β-glucuronidase (Ad5-Glu) was kindly provided by Dr. Imi Kovesdi (GenVec, Rockville, MD). Ad5-glu was grown in 293 cells and purified by banding on cesium chloride gradients.

The LM142 mAb directed against a nonfunctional epitope of αv integrins (5) and the function-blocking mAbs LM609, P3G2, and P4C10 directed to $\alpha v\beta 3$, $\alpha v\beta 5$, and $\beta 1$, respectively, were produced as previously described (6, 37).

Recombinant penton base was produced in Tn5B insect cells using baculovirus as previously described (38). The hexon protein, the major capsid protein of Ad2, was purified by FPLC from Ad2-infected cells using a Resource Q ion exchange column (Pharmacia Fine Chemicals, Piscataway, NJ) (36). Polyclonal antibodies directed against the Ad2 fiber and penton base were produced in rabbits (38). The synthetic hexapeptides GRGDSP and GRGESP were obtained from Telios Pharmaceuticals, Inc. (San Diego, CA).

Cell Adhesion and Infection Assays

For cell adhesion assays, cells were labeled overnight with 0.5 μCi/ml of [^{3}H]thymidine and then removed from monolayer cultures using 10 mM EDTA and resuspended in adhesion buffer (DME supplemented with 2 mM $MgCl_2$, 1% BSA, and 20 mM Hepes). Labeled cells (1×10^5/well) were added to individual wells of 48-well nontreated cluster plates (Costar Corp., Cambridge, MA) which had been pre-coated overnight with 0.5 mg/ml penton base or 3 mg/ml vitronectin and then blocked with 5% BSA in PBS, pH 7.4 for 1 h. Cells were preincubated with 100 μg/ml of mAbs or 150 μg/ml of synthetic peptides in adhesion buffer for 1 h at 4°C, and then allowed to attach to wells at 37°C for 30–60 min. Unattached cells were removed by rapid washing with PBS, and the amount of cell-associated radioactivity remaining in each well was determined by addition of detergent and counting the cell lysates. The percentage of attached cells was calculated from the total cell cpm added to each well.

Ad2 virus infection was quantitated using the fluorescent focus assay (31). Briefly infected cell monolayers were fixed with 1% formaldehyde for 15 min and then permeabilized with a 0.2% Triton X-100 in PBS. After washing with PBS, the cells were preblocked with 0.2% gelatin and incubated with a 1:500 dilution of polyclonal antibodies to the Ad2 fiber or penton base in PBS containing 0.2% gelatin. Cells were then washed and incubated in PBS/gelatin containing 1:500 dilution of rhodamine-labeled polyclonal goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 2–12 h. Infection was expressed in fluorescent focus units (FFU) which represent the number of fluorescent cells counted with a fluorescent microscope. To analyze the effect of soluble mAbs on Ad infection, cells in suspension were incubated with these agents for 1 h at 4°C followed by the addition of the appropriate amount of adenovirus at 4°C. After 1 h, the cells were then warmed to 37°C for 30 min, trypsinized for 15 min, and then plated on poly-D-lysine–coated wells. CS-1 were infected in suspension with varying amounts of Ad2 for 1 h at 37°C, trypsinized for 15 min, and then washed and plated onto poly-D-lysine–coated chamber slides. For all experiments, plated cells were incubated in DME (plus 10 mM Hepes, 10% FBS) at 37°C for 2 d prior to determining the number of FFU/well.

Virus and Penton Base Binding and Internalization Assays

Binding of [^{3}H]thymidine-labeled Ad2 or [^{35}S]penton base to CS-1/$\beta 3$ and CS-1/$\beta 5$ cells was carried out as previously described (38). Nonspecific binding was determined in the presence of 50-fold excess unlabeled Ad2 or penton base.

Ad2 internalization into CS-1/$\beta 3$ and CS-1/$\beta 5$ cells was performed with biotinylated Ad2 using a capture ELISA as previously described by Smythe et al. (27). Briefly, 60 μg of biotinylated Ad2 was added to 1×10^7 CS-1/$\beta 3$ or CS-1/$\beta 5$ cells for 1 h at 4°C in adhesion buffer. The unbound virus was removed by washing, and cell samples of 1×10^6 cells each were warmed to 37°C for varying lengths of time. Uninternalized virus particles remaining on the cell surface were then "quenched" by the addition of 100 μg/ml of soluble avidin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 60 min at 4°C in Hepes-buffered saline containing 10 mM EDTA. Internalized Ad2 was then released by solubilizing the cells with 1% NP-40 in PBS/0.2% of BSA. Cell lysates containing biotin-Ad2 were then added to ELISA wells which had been precoated with 1 μg/well of polyclonal anti-penton base IgG. Biotin-Ad2 was then detected by addition of strepavidin-alkaline phosphatase (Boehringer-Mannheim Biochemicals) diluted 1:1,000 in PBS/0.5% nonfat dry milk (Blotto) followed by chromogenic substrate. The total amount of Ad2-biotin bound to the cells was determined at 4°C in the absence of soluble avidin. Nonspecific binding (<10% of total) was determined by analyzing cells incubated in the absence of biotin-Ad2.

To analyze soluble penton base internalization, radiolabeled penton base was added to cells at 4°C and then immediately warmed to 37°C. At varying times, samples were taken and diluted 10-fold in ice-cold HBSE, (Hepes-buffered saline containing 10 mM EDTA) washed twice in this buffer, and then resuspended in a small volume of HBSE containing 2 mg/ml subtilisin (Sigma Chemical Co.) and incubated at 37°C for 15 min. Finally, the cells were washed in ice-cold adhesion buffer, and the remaining protease-resistant Ad2-associated cpm were measured. To determine the total cell associated penton base, the samples were washed three times in HBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Subtilisin-treated cells remained >95% viable.

Ad-induced Cell Permeabilization Assay

Cell permeability was assayed by [^{3}H]choline release as previously described (23) with minor modifications. Experiments using CS-1 cells were performed in suspension, while permeability studies with other cell lines were performed in monolayer cultures. The appropriate number of cells in adhesion buffer containing 1–5 mCi/ml [^{3}H]choline (Amersham Corp.) were incubated for 1 h at 37°C. Cells (1×10^5 cells/sample) were then washed three times with ice-cold virus binding buffer 5 mM (Hepes-buffered saline, pH 7.0 containing 0.2% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM NaN_3 to prevent virus internalization during warming) (29) and incubated for 1 h at 4°C with the appropriate virus concentration (0–50 μg/ml for dose response experiments and 0–10 μg/ml for pH and kinetic experiments). After virus binding, the cell samples were washed once with saline and then incubated for 1 h at 37°C with 200 μl permeability buffer 50 mM MES (Sigma Chemical Co.)-buffered saline, pH 6.0 containing 0.2% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM NaN_3). For pH studies, a combination of 25 mM MES and 25 mM Hepes was used to adjust the permeability buffer to the desired pH. After incubation in permeability buffer, the cell samples were incubated with or without adenovirus and then gently centrifuged. The percent [^{3}H]choline release was determined by measuring the counts released into the permeability buffer and the counts remaining in the cell pellet. For experiments using adherent cells, 48-well untreated polystyrene plates were precoated with 10 mg/ml of laminin, vitronectin, or penton base in a volume of 0.2 ml. Wells were then blocked with 5% BSA and then 10^5 HEp-2 or CS-1 cells in adhesion buffer containing 1–5 mCi/ml [^{3}H]choline were added to each well. Cells became adherent within 1 h, after which they were washed three times with virus-binding buffer and then incubated with adenovirus for 1 h at 4°C. Cells were then washed once with saline and incubated with permeability buffer for 1 h at 37°C. [^{3}H]choline release was determined by measuring the radioactivity in the buffer and in the adherent cells which were solubilized in 0.2 ml of 1% SDS. For blocking experiments, antibodies or other soluble proteins were incubated with adherent cells for 1 h prior to virus binding and in the subsequent virus binding and low pH incubations.

Adenovirus-mediated Gene Delivery in CS-1 Cells

CS-1 cells, 2 × 10^5 in 50 μl, were infected with Ad5-Glu at multiplicities of infection (MOI) of 0.1, 1.0, and 10.0 at time zero. Infected cells were incubated for 5 h in DME media and subsequently harvested and washed three times in Hepes-buffered saline. To measure expression of β-glucuronidase, the cells were lysed by the addition of 60 μl of Reporter lysis buffer (Promega Biotec, Madison, WI) for 15 min at 4°C. Following high-speed centrifugation, the cell lysates were analyzed for β-glucuronidase activity using a chemiluminescence reporter assay performed according to the manufacturer's specifications (Tropix, Bedford, MA). The amount of chemiluminescence, expressed as relative light units (RLU), was measured in a LUMAT luminometer (Berthold Systems, Inc., Pittsburgh, PA).

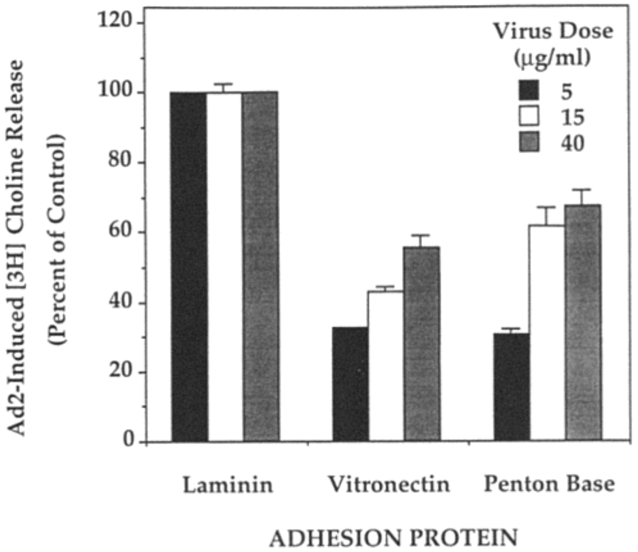

Figure 1. Adenovirus-induced permeabilization of HEp-2 cells adhered to cell matrix proteins or penton base. [^{3}H]choline-labeled HEp-2 cells were adhered to laminin, vitronectin, or penton base-coated wells and then incubated with varying amounts of purified Ad2 at 4°C for 60 min. The cells were then washed and incubated at 37°C for 60 min in permeabilization buffer at pH 6.0. The amount of [^{3}H]choline released into the buffer or remaining with the cells was measured in a scintillation counter. Nonspecific [^{3}H]choline release (cells not exposed to Ad2) was ~10% of the total cell-associated cpm and was independent of the cell matrix protein used. The amount of [^{3}H]choline corresponding to 100% was 1,954, 6,029, and 13,881 cpm for 5, 15, and 40 μg of Ad2, respectively. The total cell-associated [^{3}H]choline was 51,000 cpm. The results represent the average of duplicate samples.

Results

Ad2-induced Membrane Permeabilization Involves Penton Base Interaction with αv Integrins

A major event following adenovirus internalization into cell endosomes is penetration of the cell membrane allowing the virus nucleocapsid to enter the cytoplasm. The ability of adenovirus to permeabilize cell membranes at low pH has been used to study this event. Although our previous studies clearly showed that integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ were involved in Ad2 internalization, they did not examine whether either of these receptors also mediated virus interaction with the cell membrane. We reasoned that if integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ were involved in virus-induced membrane permeabilization, and then cells adhered to immobilized vitronectin or penton base protein should be resistant to Ad2-mediated membrane permeabilization since their αv integrins would be redistributed to the basolateral surface and thus unavailable to the virus. We have previously used this approach to demonstrate the role of αv integrins in Ad2 internalization (38). In the current studies, however, cell permeabilization studies were performed in the presence of 50 mM NaN_3 to prevent virus internalization, thus restricting virus interactions to the cell plasma membrane. As shown in Fig. 1, human epithelial Hep-2 cells adhered to immobilized vitronectin or penton base were significantly less susceptible to Ad2-induced membrane permeabilization at pH 6.0 compared to cells adhered to a control matrix protein, laminin which binds to non-αv integrins. In parallel experiments, we found that cells adhered to vitronectin or penton base showed a decrease in binding of ^{125}I-labeled mAb LM142 (634, 477 cpm, respectively) compared to cells adhered to laminin (1,007 cpm). These studies indicate that αv integrins were redistributed to the basolateral surface following adhesion to vitronectin and penton base. These studies strongly suggested that penton base binding to αv integrin promotes Ad2-induced cell permeabilization. To examine this possibility further, we performed inhibition studies using soluble adenovirus capsid proteins or cell matrix molecules (Fig. 2). Preincubation of HEp-2 cells with soluble penton base significantly inhibited Ad2 induced membrane permeabilization, while control proteins including the Ad2 hexon protein or the cell matrix protein had no effect on Ad2 membrane permeabilization. Interestingly, penton base alone did not induce [^{3}H]choline release from HEp-2 cells. These results suggested that penton base binding to αv integrins was required but not sufficient for Ad2-induced cell membrane permeabilization.

Adhesive Properties of CS-1 Cells Expressing αvβ3 or αvβ5

To systematically investigate the role of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ in the early events in Ad2 infection, a CS-1 cell model was used. CS-1 cells lack αv integrins due to the absence of

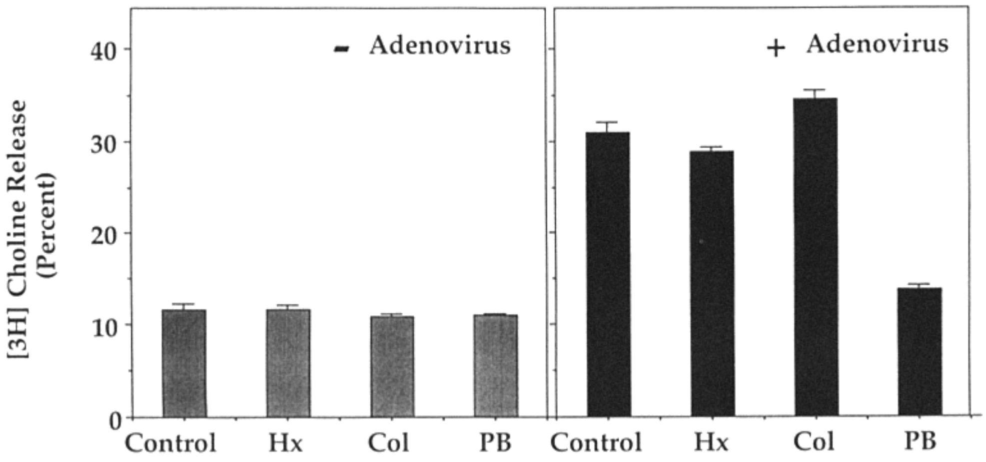

Figure 2. Inhibition of Ad2-induced membrane permeabilization by soluble penton base. [^{3}H]choline-labeled HEp-2 cells were adhered to laminin-coated wells and then incubated at 4°C for 60 min with 1 mg/ml of purified Ad2 hexon, penton base, or collagen followed by incubation for 1 h at 4°C in the absence (*left*) or presence of 20 μg/ml purified Ad2 for an additional 60 min at 4°C (*right*). The cells were then incubated for 60 min at 37°C in permeabilization buffer in the presence of the indicated proteins at 1 mg/ml at pH 6.0 and assayed for [^{3}H]choline release. The results represent the average of duplicate samples.

the β3 or β5 integrin subunit (32). Therefore, we transfected these cells with cDNAs encoding the β3 or β5 subunit. Each of these stably transfected cell lines were capable of adhering to immobilized vitronectin or penton base (Fig. 3). Adhesion to penton base or vitronectin was blocked by the appropriate monoclonal antibody to αvβ3 (LM609) or αvβ5 (P3G2) but not by a control antibody to β1 integrins (P4C10) (Fig. 3). The parental cell line, CS-1, failed to adhere to either penton base (not shown) or vitronectin (32).

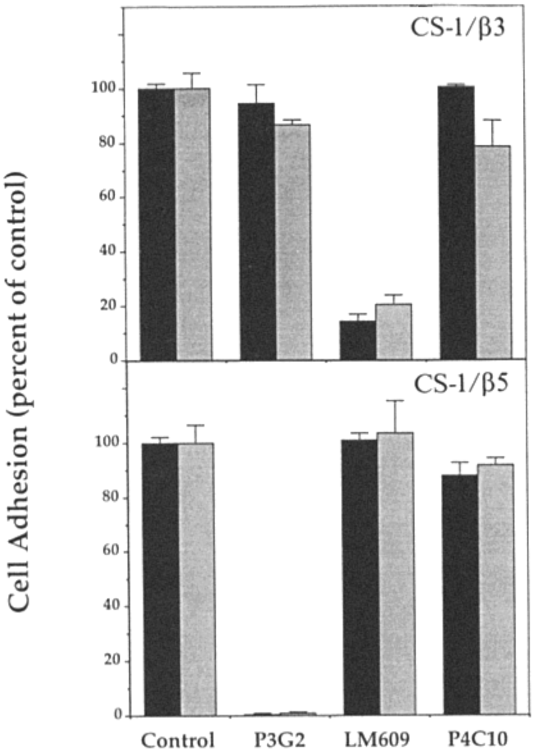

Figure 3. Adhesive properties of CS-1 cells expressing αvβ3 and αvβ5. [^{3}H]Thymidine labeled CS-1 cells (10^5) expressing αvβ3 (*CS-1/β3*) or αvβ5 (*CS-1/β5*) were incubated with function-blocking MAbs to αvβ3 (*LM609*) or αvβ5 (*P3G2*) or to β1 integrins (*P4C10*) and then allowed to adhere for 30 min at 37°C to vitronectin (*black bars*) or penton base (*gray bars*) coated polystyrene wells. Approximately 90% of CS-1/β3 or CS-1/β5 cells (71,000 cpm) incubated in the absence of mAb (controls) adhered to both vitronectin or penton base coated wells.

Expression of αvβ5 in CS-1 Cells Promotes Ad2 Infection

To examine the role of αvβ5 and αvβ5 in Ad2 infection independent of one another, we analyzed the susceptibility of CS-1/β3 an CS-1/β5 cells to virus infection. As shown in Fig. 4, CS-1/β5 cells were ∼5–10-fold more susceptible to Ad2 infection at multiplicities of infection from 10^2 to 10^4, than CS-1/β3 cells. The parental cell line, CS-1, showed a similar level of infection as CS-1/β3 (not shown). The enhanced susceptibility of CS-1/β5 to Ad2 infection was due to the expres-

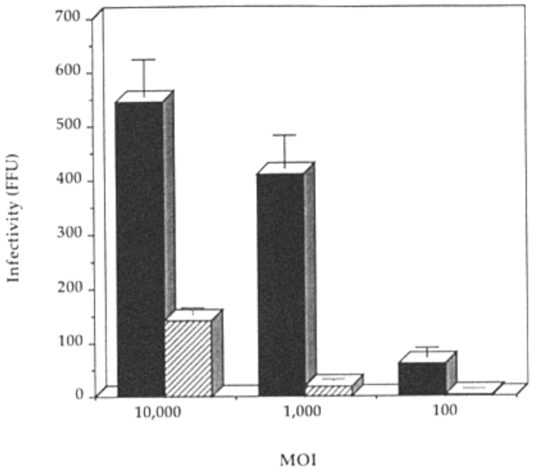

Figure 4. Susceptibility of CS-1/β3 and CS-1/β5 cells to adenovirus infection. CS-1/β3 and CS-1/β5 cells (2 × 10^4) were incubated with varying multiplicities of Ad2 (*MOI*). The relatively high MOI used for these experiments is due to the inability of human Ad2 to replicate efficiently in hamster cells (18). Virus infection was measured 48-h after infection using the fluorescent focus assay. ■, CS-1/β5; ▨, CS-1/β3.

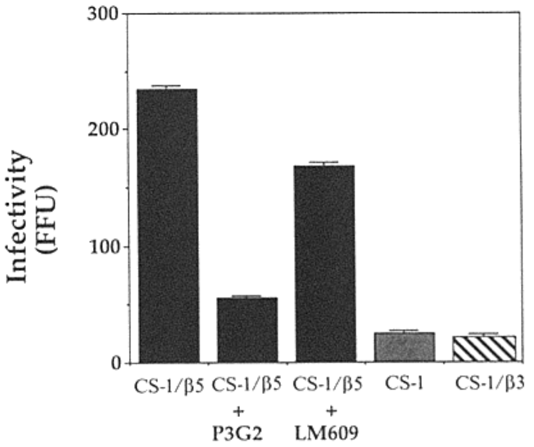

Figure 5. Inhibition of Ad2 infection of CS-1/β5 cells by function blocking mAbs to αvβ5. CS-1/β5 cells (2 × 10^4) were preincubated with 500 μg/ml mAbs to αvβ5 (*P3G2*), αvβ3 (*LM609*) or β1 integrins (*P4C10*) followed by incubation with Ad2 at an MOI of 100. Virus infection was assessed by the fluorescent focus assay.

sion of αvβ5, since infection of these cells could be reduced close to the level of CS-1 or CS-1/β3 cells by preincubation with the P3G2 (anti-αvβ5) monoclonal antibody but not by a control antibody, LM609 (anti-αvβ3) (Fig. 5). These results indicate that penton base binding to αvβ5 preferentially promotes Ad2 infection.

To examine the basis for the enhanced susceptibility of CS-1/β5 cells to Ad2 infection, we next analyzed Ad2 binding to and internalization into CS-1β/3 and CS-1β/5 cells. CS-1/β5 and CS-1/β3 cells bound similar amounts of ^{3}H-Ad2; an average of 5,100 and 6,200 cpm of ^{3}H-Ad2 virus was specifically bound to 10^6 CS-1/β3 and CS-1/β5 cells, respectively. CS-1/β3 and CS-1/β5 cells also supported Ad2 internalization (Fig. 6). Although the kinetics of Ad2 uptake into CS-1/β5 cells was somewhat more rapid than in CS-1/β3 cells, ~70% of the virus that had been internalized in CS-1/β5 cells had been internalized into CS-1/β3 cells by 15 min (Fig. 6). CS-1/β3 and CS-1/β5, but not αv integrin-negative CS-1 cells, also supported similar levels of penton base binding (Fig. 7 *A*) and internalization (Fig. 7 *B*). These studies thus further substantiate that αvβ3 and αvβ5 both promote Ad2 as well as penton base internalization.

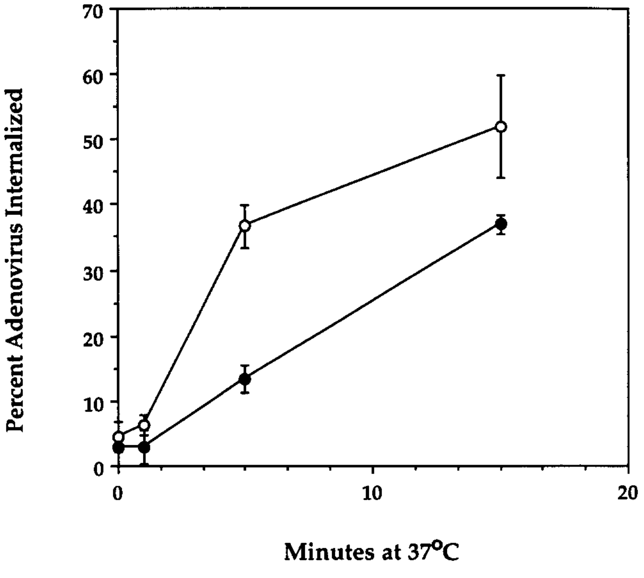

Figure 6. Adenovirus internalization into CS-1/β3 and CS-1/β5 cells. Internalization of biotinylated Ad2 was measured in a capture ELISA using immobilized anti-penton base antibody. Detection of internalized biotin-Ad2 in cell lysates was performed by the addition of strepavidin-alkaline phosphatase and chromogenic substrate. Total cell-associated Ad2 was measured at 4°C in the absence of soluble avidin. ○, CS-1/β5; ●, CS-1/β3.

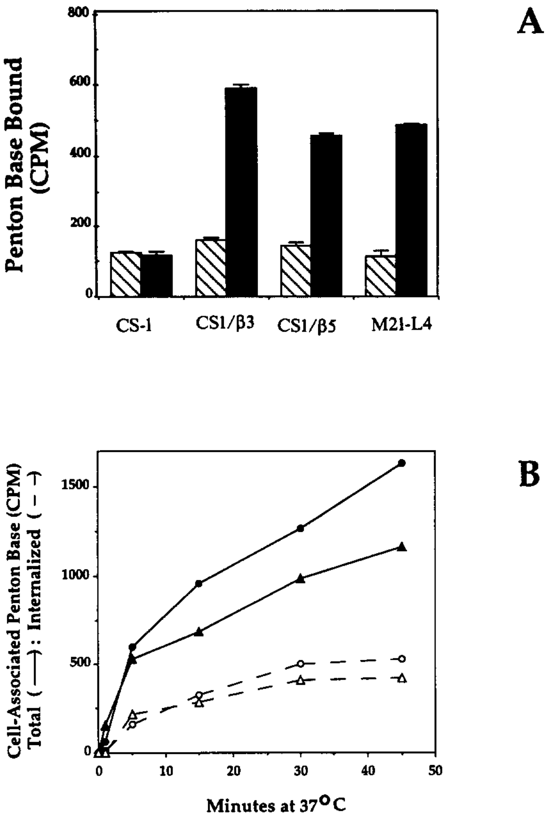

Figure 7. Analysis of penton base binding to and internalization into CS-1/β3 and CS-1/β5 cells. (*A*) ^{35}S-labeled penton base binding to CS-1/β3, CS1/β5, or CS-1 cells or M21-L4 cells (which express both αvβ3 and αvβ5). Binding was analyzed by incubating cells at 4°C for 60 min with labeled penton base in the presence or absence of a 50-fold excess of unlabeled protein to determine the nonspecific (▨) and total (■) cpm bound. The results represent the average of duplicate samples. (*B*) ^{35}S-penton base internalization into CS-1/β3 and CS-1/β5 cells. The total amount of labeled protein bound to each cell type was determined at 4°C while the amount of internalized penton base was determined at varying times at 37°C by resistance to treatment with 10 mM EDTA and subtilisin. ● and ○, CS-1/β3; ▲ and △, CS-1/β5.

Expression of αvβ5 in CS-1 Cells Promotes Ad2-induced Membrane Permeabilization

Since CS-1 cells expressing αvβ3 and αvβ5 were both capable of supporting Ad2 binding and internalization, we next asked whether these integrins were capable of supporting subsequent steps in infection including membrane permeabilization. As shown in Fig. 8 *A*, Ad2 caused a dose-dependent release of [^{3}H]choline from CS-1/β5 cells. In contrast, only a low level of marker release was detected in

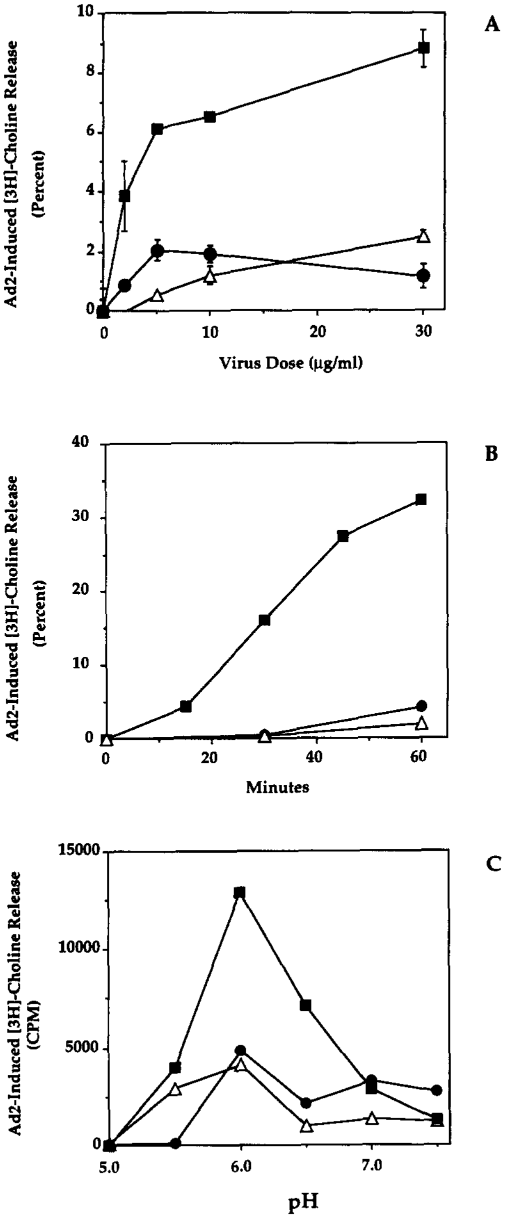

Figure 8. Analysis of Ad2-induced permeabilization of CS-1/β3 and CS-1/β5 cells. (*A*) Dose response of Ad2-induced [^{3}H]choline release from CS-1/β3, CS-1/β5, and CS-1 cells. [^{3}H]choline-labeled cells were incubated with 0–30 μg/ml of Ad2 (MOI of 10^4–10^5) for 1 h at 4°C in binding buffer, washed, and then incubated for 60 min at 37°C in permeabilization buffer at pH 6.0. Nonspecific [^{3}H]choline release was ~5% for each cell type. Results represent the average of duplicate samples. (*B*) Time course of [^{3}H]choline release from CS-1 cells. Ad2-stimulated [^{3}H]choline release was measured at varying times at 37°C at pH 6.0 following incubation for 1 h at 4°C in binding buffer containing 20 μg/ml of Ad2. (*C*) Ad2-induced [^{3}H]choline release from CS-1/β3 or CS-1/β5 cells as a function of Ph. [^{3}H]Choline release was measured after incubation with 20 μg/ml Ad2 for 60 min at 4°C, pH 7.4, and then for 60 min at 37°C in permeabilization buffer adjusted to different pH values. ●, CS-1; △, CS-1/β3; ■, CS-1/β5.

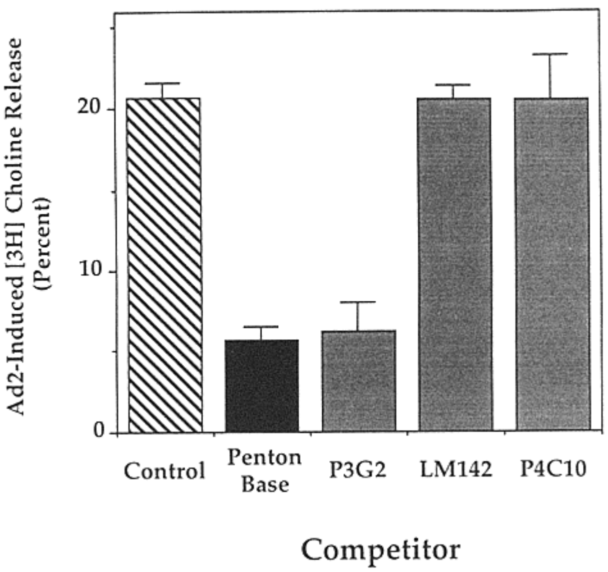

Figure 9. Inhibition of Ad2-induced permeabilization of CS-1/β5 cells by soluble penton base and anti-αvβ5 mAb. [^{3}H]Choline-labeled CS-1/β5 cells were adhered to laminin-coated wells and then incubated with 1 mg/ml soluble penton base, 200 μg/ml mAb P3G2 (anti-αvβ5), LM142 (a nonfunctional blocking mAb to αv integrins) or P4C10. The cells were then washed and incubated with 20 μg/ml Ad2 at 4°C for 1 h and then in permeabilization buffer for 1 h at 37°C. Nonspecific release of [^{3}H]choline was ~5%. The results represent the average of duplicate samples.

CS-1 cells or CS-1/β3 cells. Ad2-mediated release of [^{3}H]choline from CS-1/β5 cells was dependent on the time of incubation at 37°C (Fig. 8 *B*) and had a pH optimum of ~6.0 (Fig. 8 *C*). Ad2-mediated membrane permeabilization of CS-1/β5 cells required the interaction of penton base and αvβ5 since this activity was blocked by the function-blocking monoclonal antibody to αvβ5 (P3G2) and by soluble penton base but not by an irrelevant antibody LM142 (nonfunction blocking anti-αv) or by a control antibody P4C10 (anti-β1) (Fig. 9). These results indicate that αvβ5 binding to penton base preferentially promotes Ad2-induced cell membrane permeabilization. Thus, CS-1 cells lacking this integrin in CS-1/β3 cells expressing αvβ3 as their only αv integrin are relatively resistant to Ad2-induced membrane permeabilization.

Integrin αvβ5 Promotes Adenovirus-mediated Gene Delivery

To further examine whether specific αv integrins are capable of enhancing adenovirus-penetration into the cell, we analyzed adenovirus-mediated gene delivery into CS-1 cells using a recombinant adenovirus (Ad5-GLU) encoding the enzyme β-glucuronidase. The ability of adenovirus to enhance delivery of reporter genes into cells is facilitated by virus-mediated endosome disruption (7). Delivery of the β-glucuronidase gene and subsequent expression of the enzyme activity was measured at early times (5 h) after virus infection in the absence of virus replication.

As shown in Fig. 10, αvβ5 expressing CS-1 cells contained 5–10-fold more β-glucuronidase activity following infection with Ad5-GLU than cells expressing αvβ3 or the parental CS-1 cell line lacking either αvβ3 or αvβ5. These studies, therefore, provide further evidence for the selective role of αvβ5 in promoting adenovirus penetration into cells.

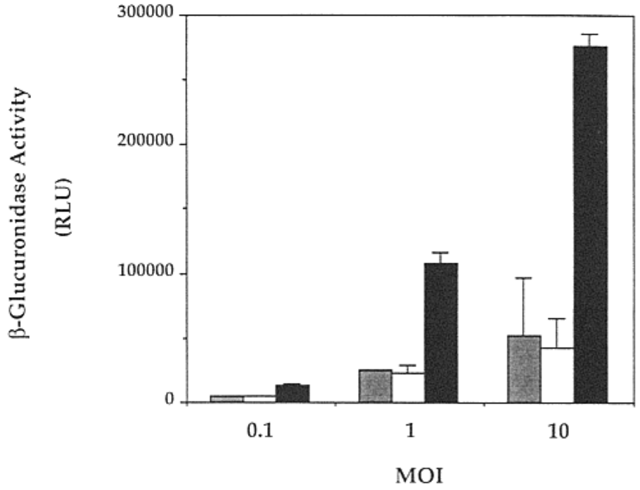

Figure 10. Adenovirus-mediated gene delivery in CS-1 cells. CS-1 cells (2 × 10^5) expressing $\alpha v\beta 5$ (*CS-1/$\beta 5$*), $\alpha v\beta 3$ (*CS-1/$\beta 3$*), or neither integrin (*CS-1*) were incubated with the replication-defective Ad5-Glu virus at multiplicities of infection of 0.1, 1.0, or 10 pfu/cell. After addition of virus, the cells were incubated at 37°C for 5 h in DME plus 20 mM Hepes. The cells were then harvested and washed three times with PBS. Expression of β-glucuronidase was measured at 5-h after infection in a chemiluminescent assay (see Materials and Methods). Results represent duplicate samples. ▨, CS-1; □, CS-1/$\beta 3$; ■, CS-1/$\beta 5$.

Discussion

A unique feature of human adenovirus is its ability to disrupt cell endosomes efficiently thus enabling the virus to rapidly enter the cytoplasm and be translocated to the cell nucleus (4, 12, 33). This attribute has facilitated the use of adenovirus as a vector for gene therapy (20, 21) since this virus also promotes the entry of foreign DNA into the cytoplasm before it can be degraded in lysosomes (7, 35), thus overcoming one of the major impediments to gene delivery. Although the precise mechanism by which adenovirus penetrates the cell endosome has yet to be elucidated, virus-induced enhancement of cell membrane permeability has been detected by the release of small molecules such as [^{3}H]choline phosphate from intact cells (23, 26). Enhancement of cell membrane permeability is thought to be indicative of events leading to virus penetration of the cell endosome since it occurs with increasing time of incubation at 37°C and also requires mildly acidic conditions (23).

A number of previous studies indicated that the penton base protein is involved in early events leading to disruption of the cell endosome. However these earlier studies did not clearly distinguish between penton base–mediated virus internalization from virus penetration into the cytoplasm. Our recent studies demonstrated that the penton base mediates Ad2 internalization via interaction with integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. In the studies reported here, while both αv integrins promote Ad2 internalization, penton base binding to $\alpha v\beta 5$ was also shown to be involved in subsequent steps in virus infection, including cell membrane permeabilization. Redistribution of αv integrins to the basolateral surface of cultured cells inhibited Ad2-induced membrane permeabilization (Fig. 1). Membrane permeabilization was also blocked by preincubation of cells with soluble penton base (Fig. 2) implicating a role for αv integrin interaction with penton base in cell membrane permeabilization.

In previous studies with M21-L4 cells expressing both $\alpha v\beta 3$ and $\alpha v\beta 5$, function-blocking monoclonal antibodies to both $\alpha v\beta 3$ and $\alpha v\beta 5$ were required to block Ad2 internalization. However, these cells express ~20-fold more $\alpha v\beta 3$ than $\alpha v\beta 5$, thus, it was not possible to assess the precise role of each integrin in later steps of virus infection. To analyze the individual roles of $\alpha v\beta 3$ and $\alpha v\beta 5$ in Ad2 infection, we developed a cell model comprised of CS-1 cells expressing either $\alpha v\beta 3$ or $\alpha v\beta 5$. It was of interest that CS-1/$\beta 5$ cells were significantly more susceptible to Ad2 infection than CS-1/$\beta 3$ cells, although both cell types supported similar levels of Ad2 and penton base binding and internalization (Fig. 6, 7). CS-1/$\beta 5$ cells also showed enhanced susceptibility to Ad2-induced membrane permeabilization compared to CS-1/$\beta 3$ or CS-1 cells which were relatively resistant to Ad2 (Fig. 8). The enhanced susceptibility of CS-1/$\beta 5$ cells to Ad2-induced membrane permeabilization was due to expression of $\alpha v\beta 5$ since it could be abrogated by function blocking antibodies to $\alpha v\beta 5$ or by soluble penton base (Fig. 9). We have also observed similar differences in susceptibility to Ad2-induced membrane permeabilization in a number of other human cell lines. For example, M21-L4 human melanoma cells, which have relatively low levels of $\alpha v\beta 5$, showed a low level of Ad2-induced membrane permeabilization while HEp-2 cells, which have significantly higher levels of $\alpha v\beta 5$, were highly susceptible to [^{3}H]choline release.

The enhanced susceptibility of CS-1/$\beta 5$ cells to Ad2-induced cell membrane permeabilization also correlated with the enhanced susceptibility of these cells to virus infection (Fig. 4), as well as adenovirus-mediated gene delivery (Fig. 10). CS-1/$\beta 5$ cells were ~5–10-fold more susceptible to virus infection and supported higher levels of gene delivery compared to CS-1/$\beta 3$ cells, and infection could be abrogated by preincubation with the P3G2 mAb (anti-$\alpha v\beta 5$) or soluble penton base. These results demonstrate that penton base binding to $\alpha v\beta 5$ facilitates efficient virus penetration into the cell cytoplasm, an event required for efficient virus infection. However, these studies do not rule out the possibility that $\alpha v\beta 5$ mediates other cellular events which do not directly impact virus penetration but which, nonetheless, promote infection.

We do not yet know the basis for the selective role of $\alpha v\beta 5$ for Ad2 infection. However, our preliminary studies show that penton base binding to $\alpha v\beta 3$ and $\alpha v\beta 5$ is qualitatively different since binding to $\alpha v\beta 5$ occurs at both low and neutral pH while $\alpha v\beta 3$ binding is significantly reduced at low pH (not shown). Moreover, $\alpha v\beta 3$ but not $\alpha v\beta 5$ could be eluted from a penton base affinity column with RGD peptides, suggesting that $\alpha v\beta 5$ may bind to penton base in a non-RGD–dependent manner. Previous studies have shown that a non-RGD–containing basic region in HIV-Tat protein preferentially binds to $\alpha v\beta 5$ (34). Whether a similar region is also involved in penton base to $\alpha v\beta 5$ binding has yet to be determined. Of particular relevance for the current studies, our preliminary studies show that penton base is capable of binding to CS-1 cells expressing $\alpha v\beta 5$ but not $\alpha v\beta 3$ at pH 5.5, suggesting that the low pH environment of the endosome favors an interaction of $\alpha v\beta 5$ with the Ad2 penton base. The preferential interaction of $\alpha v\beta 5$ with Ad2 penton base appears to have physiological significance since human bron-

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chial epithelial cells have been reported to express high levels of $\alpha v\beta 5$ and/or $\alpha v\beta 6$ while they express low or undetectable levels of $\alpha v\beta 3$ (16).

Although penton base binding to $\alpha v\beta 5$ was shown to be required for Ad2-mediated membrane permeabilization, the penton base protein alone did not induce membrane permeabilization (Fig. 2). A possible explanation for the inability of penton base to permeabilize cells is that other adenovirus capsid proteins (28) mediate the penetration event. However, it is also possible that penton base has an indirect role in endosome disruption through a cell-signaling pathway. Previous studies have shown that Ad2-induced cell membrane permeabilization involves ATPase activity (24), a multisubunit enzyme involved in endosome acidification (11). Cell surface integrins, including $\alpha v\beta 3$, are also known to stimulate a rise in intracellular pH and an influx of calcium (15). Whether penton base binding to αv integrins has a role in activating this ATPase signaling pathway remains to be determined.

These studies demonstrate the participation of a specific cellular receptor integrin $\alpha v\beta 5$ in virus-host cell interactions occurring after internalization and, therefore, provide further insight into how a nonenveloped virus penetrates the cell membrane. These findings also have important implications for the future use of adenovirus to target and deliver foreign genes into host cells.

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