

## Regulation of Adenovirus Membrane Penetration by the Cytoplasmic Tail of Integrin $\beta 5$ †

KENA WANG,<sup>1</sup> TINGLU GUAN,<sup>2</sup> DAVID A. CHERESH,<sup>1</sup> AND GLEN R. NEMEROW<sup>1\*</sup>

*Departments of Immunology<sup>1</sup> and Cell Biology,<sup>2</sup> The Scripps Research Institute, La Jolla, California 92037*

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**Adenovirus (Ad) cell entry involves sequential interactions with host cell receptors that mediate attachment (CAR), internalization ( $\alpha\beta 3$  and  $\alpha\beta 5$ ), and penetration ( $\alpha\beta 5$ ) of the endosomal membrane. These events allow the virus to deliver its genome to the nucleus. While integrins  $\alpha\beta 3$  and  $\alpha\beta 5$  both promote Ad internalization into cells, integrin  $\alpha\beta 5$  selectively facilitates Ad-mediated membrane permeabilization and endosome rupture. In the experiments reported herein, we demonstrate that the intracellular domain of the integrin  $\beta 5$  subunit specifically regulates Ad-mediated membrane permeabilization and gene delivery. CS-1 melanoma cells expressing a truncated integrin  $\beta 5$  or a chimeric ( $\beta 5$ - $\beta 3$ ) cytoplasmic tail (CT) supported normal levels of Ad endocytosis but had reduced Ad-mediated gene delivery and membrane permeabilization relative to cells expressing a wild-type integrin  $\beta 5$ . Thin-section electron microscopy revealed that virion particles were capable of being endocytosed into cells expressing a truncated  $\beta 5$ CT, but they failed to escape cytoplasmic vesicles and translocate to the nucleus. Site-specific mutagenesis studies suggest that a C-terminal TVD motif in the  $\beta 5$ CT plays a major role in Ad membrane penetration.**

A unique feature of human adenovirus (Ad) is the efficiency with which it delivers its nucleic acid payload to the host cell nucleus. This is reflected in the low virus particle/infectious unit ratio observed with many host cell types (11, 27). Based on this property, as well as its broad tissue tropism, replication-defective Ad vectors are currently under evaluation for in vivo gene therapy (4, 37). Ad has also facilitated investigation of different gene products in vitro, as well as uncovered several important host cell functions including RNA processing (2, 9) and cell cycle regulation (8, 45).

Although there is relatively little information on how Ad penetrates the barrier of the host cell plasma membrane, viral entry involves a sequence of distinct virus-host cell interactions. High-affinity virus binding to cells is mediated by the fiber protein interaction with a 46-kDa cell receptor known as CAR (1, 39). Following attachment, Ad type 2 (Ad2) particles are rapidly internalized via the penton base capsid protein interaction with cell integrins  $\alpha\beta 3$  and  $\alpha\beta 5$  (43). Ad2 internalization also requires dynamin (40), a GTPase involved in the formation of clathrin-coated pits, as well as several signaling molecules including phosphoinositide-3-kinase (26) and the Rho family of small GTPases (25) which promote cortical actin polymerization.

In order to penetrate the barrier of the host cell membrane, Ad particles disrupt cell endosomes (20), allowing partially uncoated virions to be released into the cytoplasm where they transit to nuclear pore complexes (6). While the precise mechanism by which Ad penetrates the endosome has not been delineated, several features of this process have recently come to light. Ad induces the release of small molecules from cells at pH 6.0 (33, 34) and promotes the formation of channels in artificial lipid bilayers (3, 32). Ad-mediated membrane permeabilization requires the interaction of the penton base protein with  $\alpha\beta$  integrins (33, 34), and interaction of the Ad3 penton

base with cell surface  $\alpha\beta$  integrins alone facilitates DNA delivery into cells (16). Activation of the 22-kDa Ad-encoded cysteine protease, a molecule which participates in virus penetration and uncoating (10), has been reported to require integrin interactions with the virus (20).

In a previous study, we demonstrated that integrin  $\alpha\beta 5$  promotes Ad-mediated membrane permeabilization in a CS-1 melanoma cell model (42). CS-1 cells expressing  $\alpha\beta 5$  also have increased susceptibility to Ad infection compared to cells expressing integrin  $\alpha\beta 3$ . In further experiments reported herein, we identify a region in the  $\beta 5$  integrin subunit that selectively mediates Ad cell entry.

### MATERIALS AND METHODS

**Ad, antibodies, and recombinant penton base.** Ad2 was obtained from the American Type Culture Collection, and a recombinant Ad encoding green fluorescent protein (GFP), Ad.RSV.GFP, was generated as previously described (23). Virions were isolated by sedimentation on CsCl density gradients (13). Ad2 was radiolabeled with  $\text{Na}^{125}\text{I}$  to a specific activity of  $10^7$  cpm/ $\mu\text{g}$  (Iodogen; Pierce). The P1F6 function-blocking monoclonal antibody to  $\alpha\beta 5$  has been previously described (7, 41). Recombinant penton base was produced in Tn5B insect cells and purified as previously described (44).

**Construction of  $\beta 5$  integrin expression vectors and CS1/ $\beta 5$  transfected cell lines.** A plasmid (pCl/ $\beta 5$ ) encoding the entire  $\beta 5$  integrin subunit (30) was used as a template in PCRs to create altered forms of the  $\beta 5$  subunit. Wild-type  $\beta 5$  integrin ( $\beta 5$ wt) cDNA for eucaryotic cell expression was generated by PCR (30) using a pair of oligonucleotide primers, primers 1 (5'-CTAGGATCCGGCGCC CCACCATGCCGCGG-3') and 2 (5'-CTAGAATTCCTATCAGTCCACAGT GCCATTGTA-3') using *Taq* polymerase. cDNA containing a chimeric integrin with a  $\beta 5$ - $\beta 3$  cytoplasmic domain was made by a two-step PCR. The first step was performed with primers 1 and 3 (5'-CGTGATATTGGTGAAGGTAGACGTG GCCTCTTTGTATAATGGATTGTAAGC-3'). The DNA fragment generated from this PCR was then used as the template in a second PCR using primers 1 and 4 (5'-CTAGAATTCCTATCAAGTGCCTCCCGGTACGTGATATTGGTGA AGGT-3'). To generate  $\beta 5$  deletion mutants, PCRs were performed with primer 1 and primer 5 (5'-CTAGAATTCCTATCAAGTGCCTCCCGGTACGTGATATTGGTGA AGGT-3') to create  $\beta 5\Delta 2$  or with primer 6 (5'-CTAGAATTCCTATCAGTCCACAGT TGTGTAACCTTGT-3') to create  $\beta 5\Delta 5$ , with primer 7 (5'-CTAGAATTCCTA TCAGTCCACAGTGTGCGTGGAGAT-3') to create  $\beta 5\Delta 15$ , with primer 8 (5'-CTAGAATTCCTATCAAGTGTGCGTGGAGATAGGCT-3') for  $\beta 5\Delta 17$ , and with primer 9 (5'-CTAGAATTCCTATCAGTATAATGGATTGTAAGCC AT-3') for  $\beta 5\Delta 25$ . Primers 10 (5'-CTAGAATTCCTATCAGGCCACAGTGGC ATTGTA-3') and 11 (5'-CTAGAATTCCTATCAGTCCACAGGCCATTG A-3') were used to generate the point mutants D799A and T797A, respectively. The PCR-generated DNA fragments encoding each  $\beta 5$  subunit was inserted into the mammalian expression plasmid pcDNA3 (Invitrogen, Carlsbad, Calif.) be-

\* Corresponding author. Mailing address: Department of Immunology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (858) 784-8072. Fax: (858) 784-8472. E-mail: gnemerow@scripps.edu.

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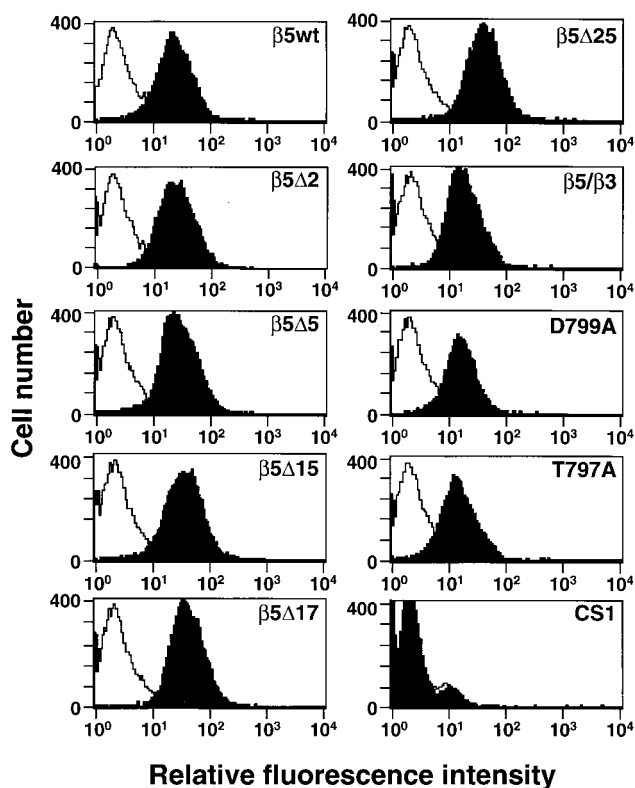


FIG. 2. Analysis of  $\alpha v \beta 5$  integrin expression on CS-1 cells by flow cytometry. CS-1 cells were transfected with cDNA constructs encoding the indicated  $\beta 5$  subunits and following drug selection were analyzed for  $\alpha v \beta 5$  integrin expression using the P1F6 function-blocking monoclonal antibody (black profiles). Control cell samples were incubated with fluorescein isothiocyanate-labeled secondary antibody alone (white profiles).

of a 200-fold-excess unlabeled virions. For Ad2 internalization, the cells were incubated at 37°C for various times. Uninternalized virus particles were removed by incubating the samples with 10 $\times$  trypsin-EDTA (Gibco BRL) at 37°C for 7 min, followed by washing with PBS at 4°C. The remaining radioactivity associated with the cell pellets, attributed to internalized virions, was then counted.

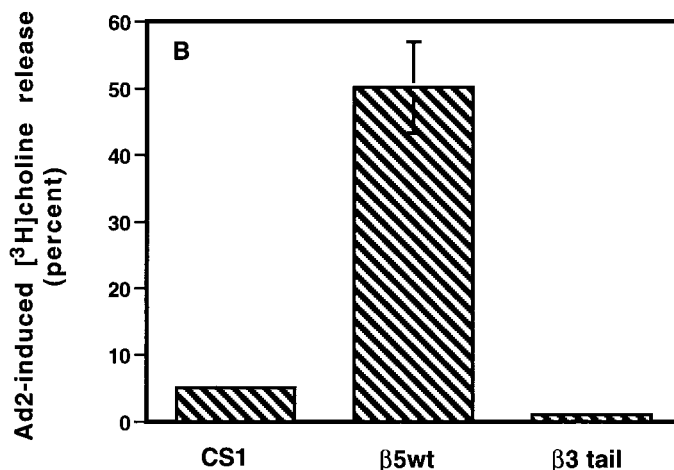
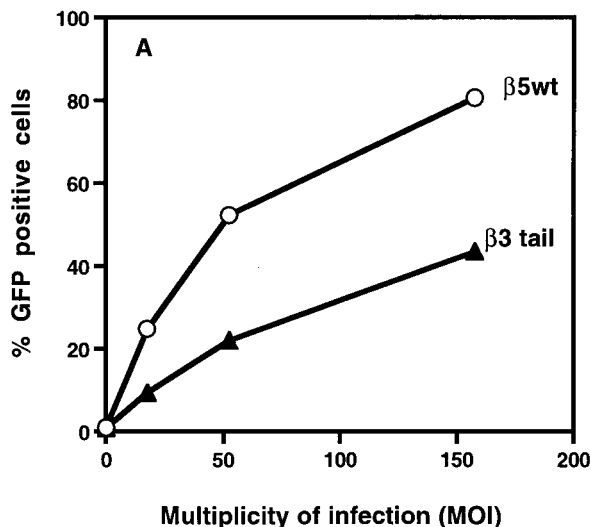


FIG. 3. Analysis of Ad-mediated gene delivery and membrane permeabilization in CS-1 cells expressing a wild-type integrin  $\alpha v \beta 5$  or  $\alpha v \beta 5$ - $\beta 3$ CT ( $\beta 3$ tail). (A) CS-1 cells expressing  $\beta 5$ wt or a  $\beta 5$  integrin with a  $\beta 3$ CT were infected at different MOIs with Ad encoding GFP and then analyzed for expression 48 h postinfection by flow cytometry. (B) CS-1 cells and CS-1 expressing  $\beta 5$ wt or a  $\beta 5$ - $\beta 3$ CT were analyzed for Ad-mediated membrane permeabilization by [ $^3$ H]choline release as described in Materials and Methods.

Ad-mediated cell membrane permeabilization assays were performed as previously described (42) with some modifications. Cells were incubated with [ $^3$ H]choline chloride (2  $\mu$ Ci/ml; NEN Life Science Products, Inc., Boston, Mass.) in complete medium at 37°C for 1.5 h. The cells were then chilled on ice and washed three times with ice-cold virus binding buffer (10 mM HEPES-buffered saline [pH 7.0], 0.2% BSA, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 50 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>). Twenty micrograms of purified Ad2 (approximately  $8 \times 10^{10}$  particles) was added to 10<sup>6</sup> cells, and the samples were incubated at 4°C for 1 h. Then the cells were washed once with ice-cold membrane permeabilization buffer (50 mM morpholineethanesulfonic acid [MES]-buffered saline [pH 6.0], 0.2% BSA, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 50 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>), resuspended to 400  $\mu$ l in the same buffer, and then incubated at 37°C for 1 h. After the cells were pelleted by centrifugation, the radioactivity in the supernatants was counted. Cells incubated in the absence of Ad2 were used as a control for nonspecific release of [ $^3$ H]choline. The percentage of [ $^3$ H]choline release specifically mediated by Ad particles was determined by the following formula: [(release with Ad - release without Ad)/release without Ad]  $\times$  100.

**Subcellular localization of Ad particles by thin-sectioning electron microscopy.** CS-1 cells ( $4 \times 10^6$ ) were incubated with Ad2 particles at a multiplicity of infection (MOI) of 5,000 in DMEM containing 10 mM HEPES (pH 7.5) and 0.5% BSA for 60 min at 4°C and then warmed to 37°C for 2 to 15 min. The cells were then washed with ice-cold PBS and then collected by low-speed centrifugation. The samples were then fixed with 2% glutaraldehyde in PBS for 30 min at 22°C, washed three times for 10 to 15 min each, and then washed once with PBS and then with H<sub>2</sub>O. The cells were then postfixed with 1% osmium tetroxide for 1 h at 22°C, washed with H<sub>2</sub>O, and then stained with 1% uranyl acetate overnight at 4°C. The cells were dehydrated with serial increasing concentrations of ethanol and proxypropane and then embedded with Epona (Ted Pella, Inc.). Thin sections were stained with 2% uranyl acetate for 1 min and examined with a Philips EM-208 transmission electron microscope. A minimum of 100 virus particles in approximately 20 randomly selected sections were quantitated, and their location, either free in the cytoplasm or inside vesicles, was noted.

## RESULTS

**Ad entry and infection of CS-1 cells expressing wild-type or mutated  $\alpha v \beta 5$  integrins.** To identify the mechanism of  $\alpha v \beta 5$ -dependent Ad cell entry, we investigated the role of the  $\beta 5$  integrin cytoplasmic tail (CT). CS-1 cells express endogenous  $\alpha v$  integrin subunits but do not produce  $\beta 3$  or  $\beta 5$  integrin mRNA (38). We therefore expressed  $\alpha v \beta 5$  integrins in CS-1 cells by transfecting them with a cDNA plasmid encoding a wild-type  $\beta 5$  integrin subunit consisting of its ectodomain, transmembrane (TM) anchor, and a full-length  $\beta 5$ CT. Alternatively, cells were transfected with cDNA encoding the  $\beta 5$  ectodomain-TM, a  $\beta 3$ CT, or a  $\beta 5$ - $\beta 3$ CT or a truncated  $\beta 5$ CT (Fig. 1). Cells expressing the different  $\beta 5$  integrins were se-

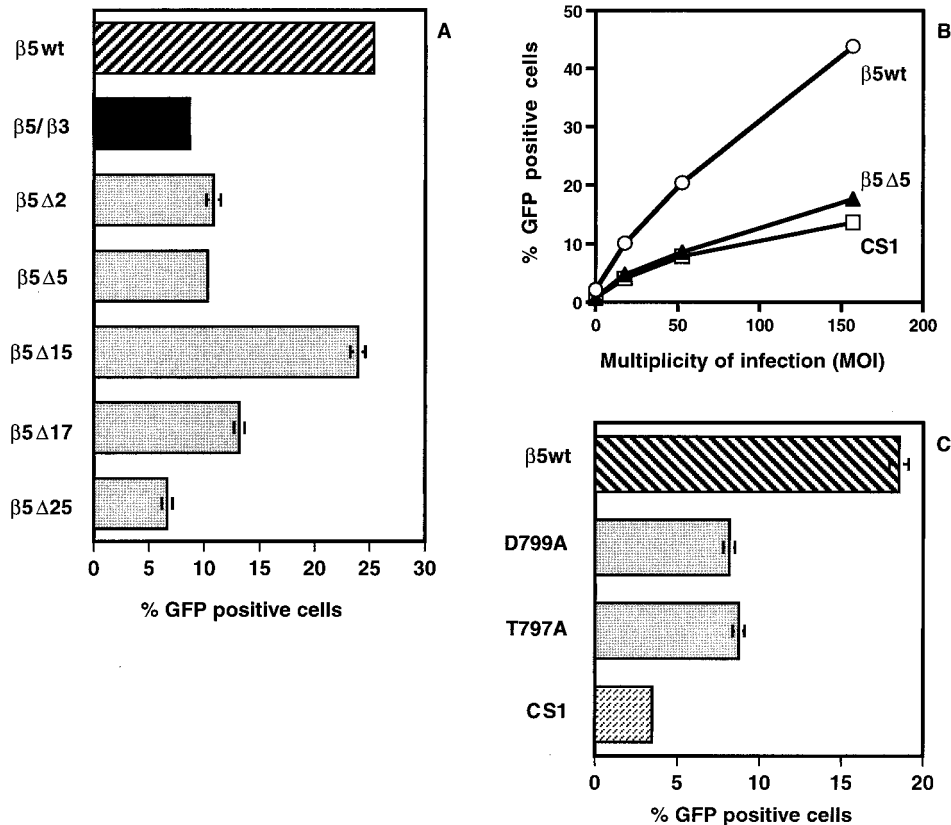


FIG. 4. Ad-mediated gene delivery in cells expressing different  $\alpha v\beta 5$  integrins. Ad-mediated gene delivery to CS-1 cells expressing deletion or chimeric forms of  $\alpha v\beta 5$  integrins (A and B) or alanine substitutions (C) was measured 48 h postinfection. The MOI for the experiments shown in panels A and C is 50.

lected by growth in G418 and also by adhesion in tissue culture plates. Each of the transfected cell types expressed similar levels of  $\alpha v\beta 5$  integrin as determined by flow cytometric analysis (Fig. 2).

Next, we compared Ad2 interactions with CS-1 cells expressing the wild-type  $\beta 5$  integrin or the chimeric integrin containing a  $\beta 5$  ectodomain and a  $\beta 3$ CT ( $\beta 3$  tail). Each cell type showed very similar Ad binding and internalization (data not shown). However, CS-1 cells expressing the wild-type  $\beta 5$  integrin supported two- to fourfold-higher levels of Ad-mediated gene delivery than cells expressing the  $\beta 3$  tail (Fig. 3A). The  $\beta 5^{wt}$ -expressing cells also supported 10-fold-higher levels of Ad-induced membrane permeabilization (Fig. 3B). Together, the results of these initial experiments suggest that the CT of the  $\beta 5$  integrin subunit specifically regulates Ad cell entry.

**Identification of a region in the  $\beta 5$ CT that regulates Ad-mediated gene delivery.** To identify the amino acid residues in the  $\beta 5$ CT that promote Ad cell entry, we expressed a number of truncated  $\beta 5$  and chimeric  $\beta 5$ - $\beta 3$  integrins in CS-1 cells and assessed their ability to support Ad-mediated gene delivery. We focused on the C-terminal 25 amino acids of the  $\beta 5$  CT, as this region displays the greatest divergence among the different  $\beta$  integrin subunits (17). Cells expressing a  $\beta 5$ - $\beta 3$  chimeric integrin in which the C-terminal 24 amino acid residues of  $\beta 5$ CT were replaced by the corresponding residues in  $\beta 3$ CT supported smaller amounts of Ad-gene delivery relative to cells expressing the wild-type  $\beta 5$  integrin (Fig. 4A). These findings suggested that the C terminus of the  $\beta 5$  integrin CT regulates Ad-mediated gene delivery in CS-1 cells. To more precisely identify the amino acids responsible, we examined

Ad-mediated gene delivery in cells expressing integrin  $\beta 5$  truncation or site-specific mutants. Removal of as few as two or five amino acid residues from the C terminus of the  $\beta 5$ CT significantly decreased Ad-mediated gene delivery, similar to the

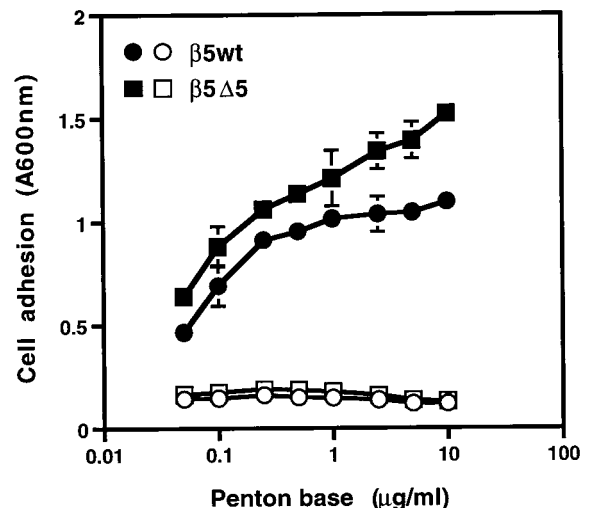


FIG. 5. Analysis of integrin-mediated cell adhesion to the Ad penton base. Adhesion of CS-1 cells expressing wild-type  $\alpha v\beta 5$  or a truncated  $\beta 5$ CT ( $\beta 5\Delta 5$ ) to immobilized Ad2 penton base was measured in the absence (filled symbols) or presence (open symbols) of 10 mM EDTA as described in Materials and Methods.



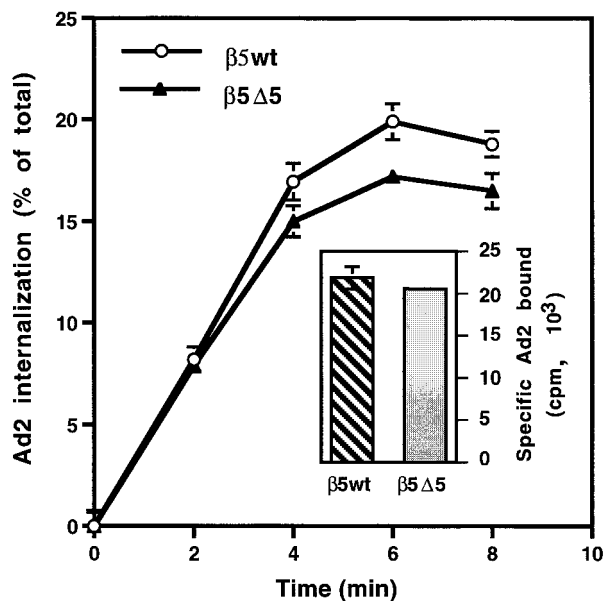


FIG. 6. Comparison of Ad binding and internalization in CS-1 cells expressing wild-type or a truncated  $\beta 5$ CT. The specific binding of radiolabeled Ad particles to each cell type is shown in the inset. Virus internalization was measured by resistance to trypsin digestion following warming of infected cells to 37°C for various lengths of time.

level seen in CS-1 cells lacking  $\alpha v$  integrins (Fig. 4A and B). Interestingly, deletion of an additional 10 residues from the C terminus of the  $\beta 5$ CT ( $\beta 5\Delta 15$ ) which results in a new C-terminal TVD sequence (residues 782 to 784) identical to the one present on the native C-terminal  $\beta 5$ CT (residues 797 to 799) (Fig. 1), restored Ad-mediated gene delivery nearly to the level seen in cells bearing the wild-type  $\beta 5$  integrin (Fig. 4A). Deletion of two additional residues,  $\beta 5\Delta 17$ , significantly decreased Ad-mediated gene delivery. These findings suggested that the C-terminal TVD motif (residues 782 to 784) regulates Ad-mediated gene delivery and that exposure of the "internal" TVD sequence (residues 782 to 784) can partially compensate for the C-terminal TVD deletion.

To explore further the potential role of the C-terminal TVD motif, we expressed mutants of  $\beta 5$ CT with two different alanine substitution (T797A or D799A) in CS-1 cells. As shown in Fig. 4C, the D799A and T797A mutations caused a significant decrease in Ad-mediated gene delivery. Together, these results suggest that the C-terminal TVD motif specifically regulates Ad-mediated gene delivery.

**Mutations in  $\beta 5$ CT selectively impair Ad-mediated membrane permeabilization.** Efficient Ad-mediated gene delivery is mediated in part by the interaction of the virus penton base protein with  $\alpha v$  integrins. However,  $\alpha v$  integrin-mediated gene delivery regulates both virus internalization and efficient endosome disruption. To examine whether cells expressing mutated  $\beta 5$  integrins were still capable of interacting with the Ad penton base protein, we performed cell adhesion assays. Cells expressing the  $\beta 5\Delta 5$  integrin exhibited dose-dependent binding to the Ad2 penton base, similar to cells expressing the  $\beta 5$ wt integrin, and adhesion required the presence of divalent metal cations (Fig. 5). These results indicated that truncations of  $\beta 5$ CT did not impair receptor functional interactions with the Ad penton base protein.

We next asked whether cells expressing  $\beta 5\Delta 5$ CT were capable of supporting Ad internalization. <sup>125</sup>I-labeled Ad2 showed

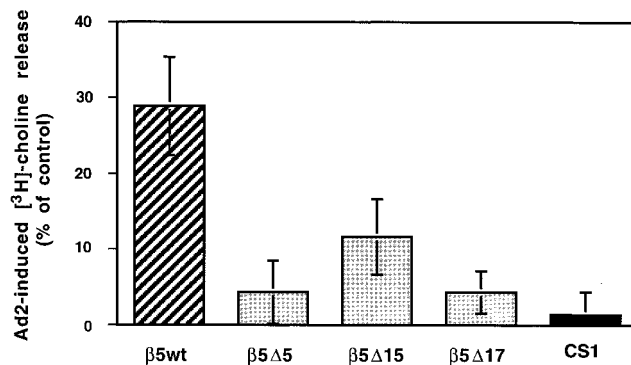


FIG. 7. Ad-mediated membrane permeabilization of CS-1 cells. CS-1 cells expressing wild-type  $\beta 5$  or a truncated  $\beta 5$ CT or cells lacking these integrins (CS-1) were loaded with [<sup>3</sup>H]choline, and Ad-mediated membrane permeabilization was measured at pH 6.0 for 1 h at 37°C in the presence of 50 mM sodium azide to prevent viral internalization. Nonspecific [<sup>3</sup>H]choline release was determined for each cell type in the absence of Ad.

very similar levels of binding to cells bearing  $\beta 5$ wt or  $\beta 5\Delta 5$ CT. Virions were also capable of being internalized into both cell types (Fig. 6). These findings indicate that deletions of  $\beta 5$ CT which reduced Ad-mediated gene delivery did not alter either virus binding or endocytosis.

Following internalization, viral particles must escape the early endosome in order to transit to the cell nucleus (21). Ad-mediated cell membrane permeabilization has been used as a surrogate assay for virus penetration of the early endosome. Membrane permeabilization requires reduced pH and is not affected by the presence of 50 mM sodium azide, which inhibits ligand internalization. We therefore tested whether Ad2 particles were capable of permeabilizing CS-1 cells expressing wild-type or mutated  $\beta 5$  integrins. Cells expressing  $\beta 5\Delta 5$ CT had a markedly reduced ability to support Ad-mediated membrane permeabilization compared to cells bearing the wild-type  $\beta 5$  integrin (Fig. 7). Cells expressing the  $\beta 5\Delta 15$  integrin with a C-terminal TVD sequence also showed reduced Ad-mediated membrane permeabilization activity, although they supported higher permeabilization activity than cells expressing the  $\beta 5\Delta 5$  or  $\beta 5\Delta 17$  integrin. Together, these findings indicate that C-terminal  $\beta 5$ CT mediates Ad-mediated membrane permeabilization.

**Electron microscopic visualization of Ad entry into CS-1 cells expressing wild-type  $\beta 5$  integrins or mutant  $\beta 5$ CT.** On the basis of these findings, we considered the possibility that cells bearing altered forms of  $\beta 5$ CT would internalize virus particles but would not allow Ad penetration of the cell endosome. To examine this possibility, we analyzed Ad entry into CS-1 cells expressing wild-type  $\beta 5$  or  $\beta 5\Delta 5$  integrin at various times by thin-section transmission electron microscopy. Each cell type was incubated with Ad particles at 4°C for 60 min to allow virus attachment and then warmed to 37°C for various times. The samples were then fixed, processed, and examined by transmission electron microscopy. At approximately 2 to 5 min after cells were heated to 37°C, Ad particles were seen in the process of being internalized into clathrin-coated invaginations in CS-1 cells expressing either wild-type  $\beta 5$  or  $\beta 5\Delta 5$ CT mutant integrin (Fig. 8). These findings were consistent with the similar rates of internalization of <sup>125</sup>I-labeled Ad for each cell type (Fig. 7). At later times after warming, Ad capsids were frequently seen in the cytoplasm in close proximity to the nuclear membrane in CS-1 cells expressing  $\beta 5$ wt integrin, indicating that Ad particles had successfully achieved endosome

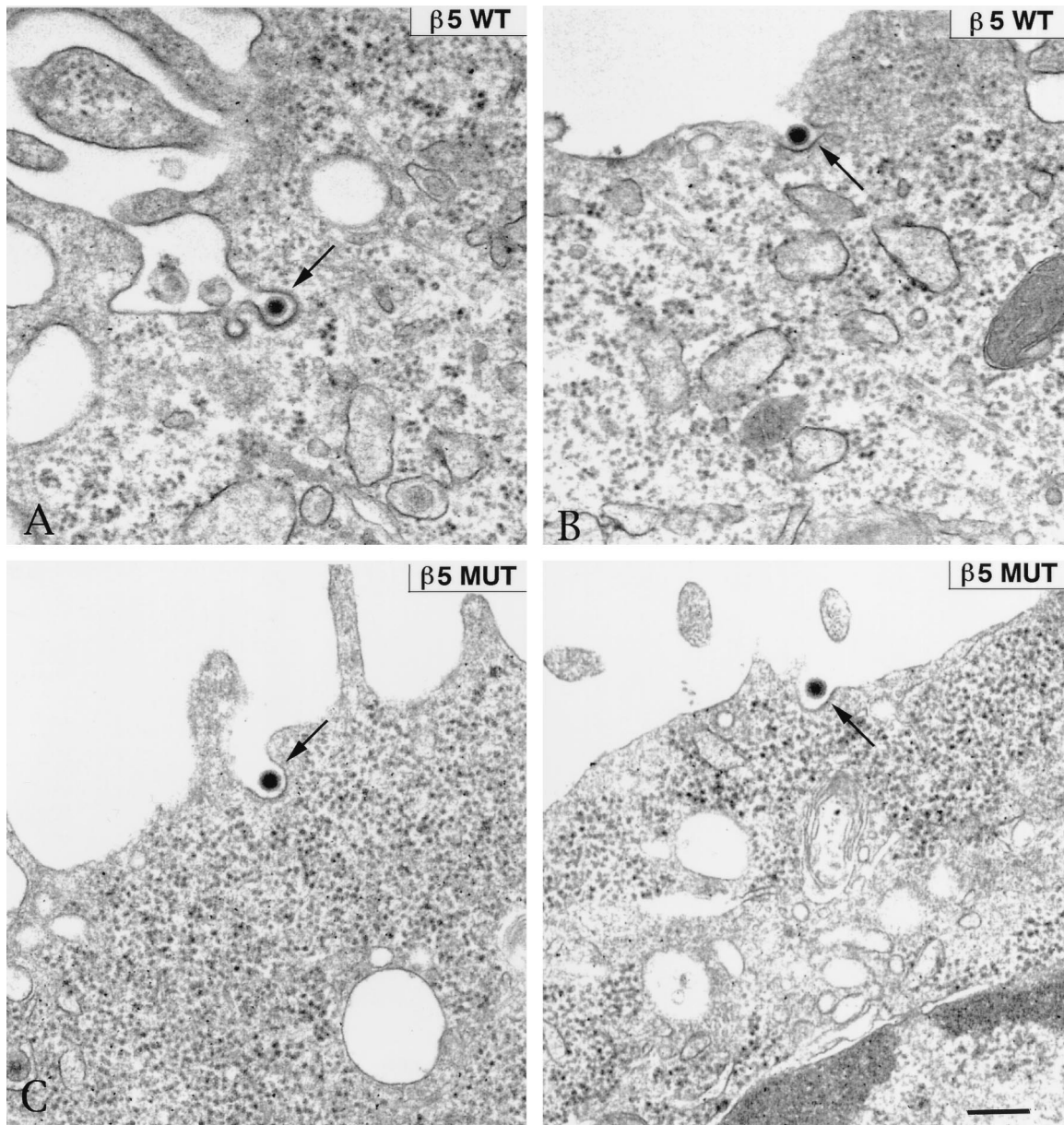


FIG. 8. Transmission electron micrographs of Ad internalization into CS-1 cells. Cells expressing a wild-type (WT) or mutant (MUT)  $\beta 5$  integrin with a deleted cytoplasmic tail ( $\beta 5\Delta 5$ ). Following attachment of Ad2 particles to cells at 4°C for 60 min, the cells were warmed to 37°C for 5 min and then fixed, embedded, and processed further for thin-sectioning transmission electron microscopy. Bar, 200 nm.

rupture (Fig. 9A). In striking contrast, virions inside CS-1 cells expressing  $\beta 5\Delta 5$ CT were rarely seen free in the cytoplasm but instead were found inside large vacuoles (Fig. 9B and C). Quantitative analysis of the electron micrographs substantiated these findings (Fig. 10). A progressive increase of virions located free in the cytoplasm was observed in  $\beta 5$ wt-expressing cells. In contrast, thin sections of cells expressing the  $\beta 5$  mutant integrin showed an accumulation of virions inside large vesicles with very few (<10% of total) particles observed free in the cytoplasm (Fig. 10). Thus, the mutations of  $\beta 5$ CT which impair membrane permeabilization also inhibit virion escape from cytoplasmic vesicles. These defects likely account for the decreased gene delivery seen in cells expressing altered  $\beta 5$ CT integrins.

## DISCUSSION

Ad entry into susceptible host cells is a multistep process requiring interaction of multiple outer virus capsid proteins with distinct cell receptors. Ad penton base interactions with integrins  $\alpha\beta 3$  and  $\alpha\beta 5$  facilitate virus internalization via a clathrin-coated pit pathway. Somewhat surprisingly, integrin  $\alpha\beta 5$  was previously shown to selectively facilitate Ad-mediated membrane permeabilization and Ad-mediated gene delivery. In light of these findings, it is interesting that  $\alpha\beta 5$  rather than  $\alpha\beta 3$  integrins predominate in the same tissue infected by Ad *in vivo*, including the upper respiratory tract (19) and eye (31).

In the current study, we sought to obtain further clues to the



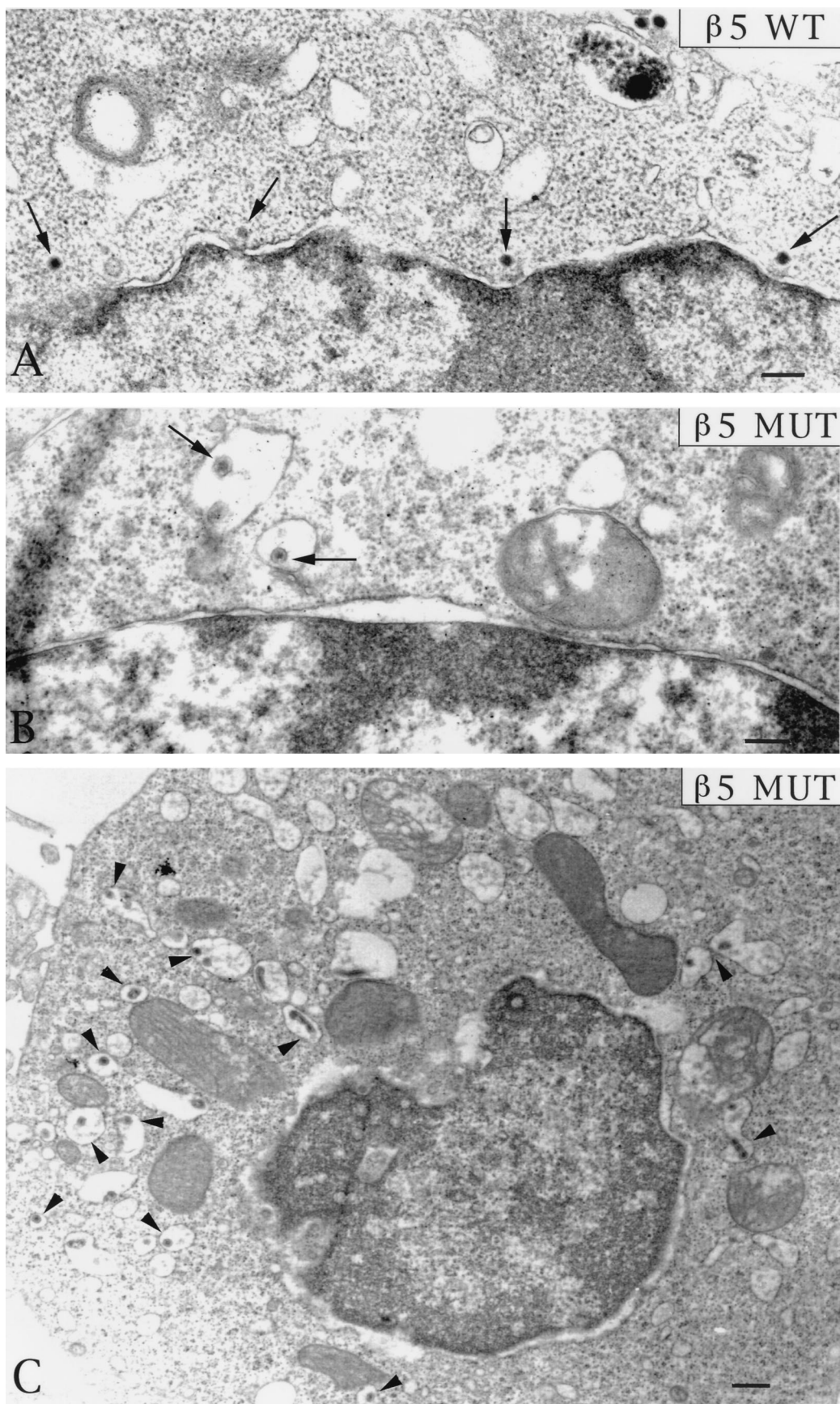


FIG. 9. Transmission electron micrographs of the late stages of Ad entry in CS-1 cells. (A) Ad particles (indicated by the arrows) were observed free in the cytoplasm in close proximity to the nucleus in  $\beta 5$ wt integrin-expressing cells at 10 to 15 min after warming to 37°C. In contrast, Ad particles were located inside large vacuoles in cells expressing a mutant (MUT)  $\beta 5$  integrin ( $\beta 5\Delta 5$ ) at 10 to 15 min (B) or at 30 min (C) after warming to 37°C.

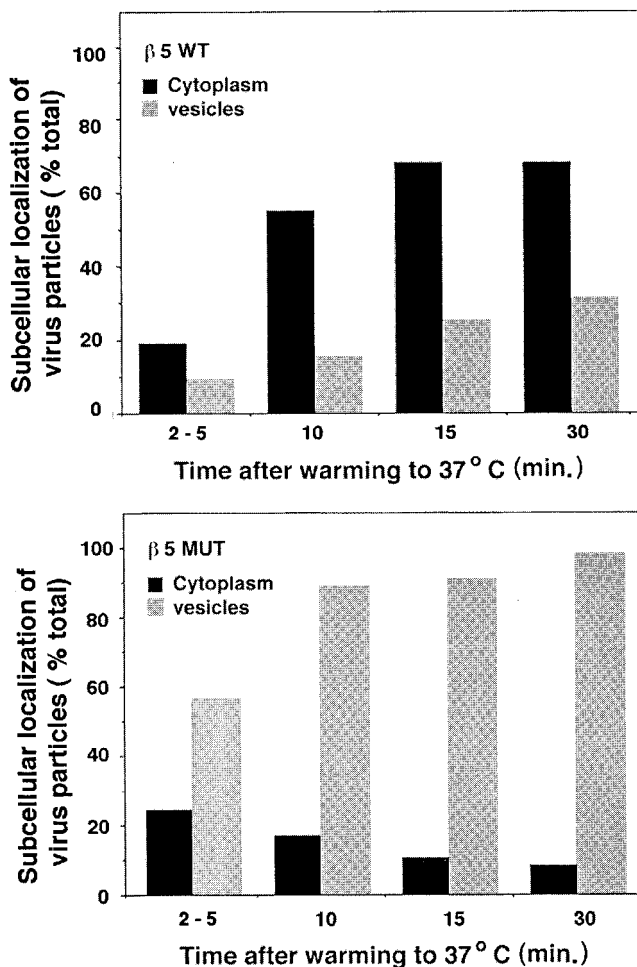


FIG. 10. Morphometric analysis of Ad entry into cells expressing a wild-type  $\beta 5$  integrin or an integrin with a mutated  $\beta 5$  CT. The location of Ad particles, at least 100 per time point in randomly selected cell sections, was determined directly from electron micrographs. Virus particles were identified as being free in the cytoplasm or inside large uncoated vesicles in cells expressing the wild-type  $\beta 5$  (upper panel) or the  $\Delta 5\beta 5$  integrin (lower panel). Differences in the amount of internalized Ad particles observed at the earliest time point (2 to 5 min) for each cell type are likely due to small variations in the warming times (1 to 2 min). Note that viruses accumulate in vesicles in cells expressing the mutant integrin relative to cells expressing the wild-type receptor. MUT, mutant.

mechanism(s) involved in Ad membrane penetration by comparing Ad infection in CS-1 cells expressing altered  $\beta 5$  integrins with those bearing the wild-type receptor. Initial experiments showed that replacement of the C-terminal 25 amino acids of  $\beta 5$ CT with those of a related integrin subunit,  $\beta 3$ , caused a significant reduction in Ad-mediated gene delivery, thus suggesting that  $\beta 5$ CT plays a major role in regulating virus infection. This finding was confirmed by analyzing a number of deletion and substitution mutants of  $\beta 5$ CT. Such mutants had significantly impaired Ad-mediated gene delivery. Interestingly, a TVD motif which is present in two separate regions of  $\beta 5$ CT, but not in any other  $\beta$  integrin subunit, plays a significant role in virus infection. Deletions or substitutions of the C-terminal TVD sequence negatively impacted Ad infection. While the more internal TVD sequence  $\beta 5$ CT could not compensate for small deletions or the alanine substitutions in the C-terminal TVD motif, removal of all of the C-terminal flanking residues adjacent to it restored Ad-mediated gene delivery

and partially reconstituted Ad-mediated membrane permeabilization. This finding suggests that exposure of this internal TVD sequence reconstitutes viral function. We cannot rule out the possibility, however, that other amino acid residues flanking the TVD motif or located further upstream of this sequence could influence  $\beta 5$  integrin function. The addition of a VD sequence to the C terminus of  $\beta 3$ CT by site-directed mutagenesis (thus creating a TVD motif) did not confer on this integrin the ability to support efficient Ad-mediated gene delivery (data not shown). Thus, further experimentation will therefore be necessary to more precisely define the sequences in  $\beta 5$ CT that regulate Ad-mediated gene delivery.

A major finding of the current study was that  $\beta 5$ CT is selectively involved in mediating membrane penetration rather than other steps of viral entry. Thus deletions or substitutions of the C-terminal  $\beta 5$ CT which reduced Ad-mediated gene delivery did not impair cell adhesion to the penton base or alter virus attachment or internalization into cells. This result is consistent with those of previous mutagenesis studies showing that an NPXY motif, present in  $\beta 5$  and  $\beta 3$  integrin subunits (and in all forms of  $\beta 5$ CT used for our studies), regulates cell migration (17) as well as  $\beta 1$ -mediated bacterial invasion (24). Instead, alterations of the C-terminal  $\beta 5$ CT inhibited Ad-mediated membrane penetration as measured by release of [ $^3$ H]choline at low pH. Ad-mediated membrane permeabilization is thought to correspond to the ability of Ad to disrupt cell endosomes at low pH. Consistent with this notion, CS-1 cells bearing mutant  $\beta 5$  integrins had numerous virions inside large cytoplasmic vacuoles, whereas cells expressing wild-type  $\beta 5$  integrins contained virions free in the cytoplasm and translocated to the nuclear pore complex (Fig. 9 and 10). These results confirm that the reduced Ad-mediated gene delivery in cells expressing mutant  $\beta 5$ CT occurs as a consequence of a defect in virus penetration.

An obvious question arising from these experiments is how does  $\beta 5$ CT orchestrate Ad-mediated membrane penetration? One possibility is that  $\beta 5$ CT interacts with another host cell factor, perhaps via the C-terminal TVD motif, and this interaction is required for membrane penetration. Although a co-factor for the  $\beta 5$  integrin subunit has not yet been reported, host cell molecules which interact with other integrin subunits have been identified. For example, the NXXY motif mediates the association of  $\beta 1$ CT with ICAP-1 protein (5) and between  $\beta 3$ CT and endonexin (12).  $\beta 1$  integrin interaction with CD98 (15), an early marker of T-cell activation, has also been shown to enhance cell fusion by Newcastle disease virus (29) and human immunodeficiency virus (28). Moreover, membrane fusion during fertilization involves integrin interactions (35). While the precise mechanism(s) involved in  $\beta 5$ -mediated Ad entry requires further investigation, the unanticipated findings reported herein may help to unravel the complex events associated with virus penetration of host cells.

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