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Comparative Analysis of Adenovirus Fiber-Cell Interaction: Adenovirus Type 2 (Ad2) and Ad9 Utilize the Same Cellular Fiber Receptor but Use Different Binding Strategies for Attachment

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We have analyzed the binding of adenovirus (Ad) serotypes from subgroups B, C, and D through fiber-virus and fiber-fiber cross-competition experiments. Since viruses in these distinct subgroups display markedly different tropisms, it was unexpected that the subgroup C viruses Ad2 and 5 and the subgroup D virus Ad9 cross-competed for the same cellular fiber receptor. The subgroup B serotype Ad3 recognized a receptor distinct from the Ad2, 5, and 9 fiber receptor. However, despite sharing the same fiber receptor, Ad2 and Ad9 displayed markedly different binding characteristics that appeared to result from direct Ad9 binding to cells via α_v-integrins. Unlike Ad2, Ad9 binding to many cell lines was not abrogated by competition with the fiber 9 knob (F9K). Ad9 binding to fiber receptor-deficient cells was blocked by a monoclonal antibody to α_v-integrins. In contrast, Ad9 binding to α_v-deficient cells that express fiber receptor was blocked by F9K. Transfection of an α_v-integrin-deficient cell line with a plasmid that expresses $\alpha_v\beta_5$ resulted in Ad9 binding that was not significantly blocked by F9K but was blocked with a combination of F9K and penton base. These results imply that the shorter length of fiber 9 (11 nm) relative to fiber 2 (37 nm) permits fiber-independent binding of Ad9 penton base to α_v-integrins. The difference in fiber length may explain the different binding characteristics and tissue tropisms of each virus despite both utilizing the same fiber and penton base receptors.

Human adenoviruses are a major cause of respiratory, eye, and gastrointestinal infections (16, 36, 37). Currently, human adenoviruses are classified in six subgroups (A to F) with over 49 serotypes (16, 29). Several criteria are in use for the characterization and classification of members of the family *Adenoviridae*, such as oncogenic and/or transforming potential (38), genetic analysis of the adenoviral genomes (10, 16) and hemagglutination patterns, and titers with erythrocytes of 14 different species, including human, rat, and rhesus monkey erythrocytes (13, 26).

Cellular receptors for adenovirus type 2 (Ad2) have been shown to be involved in the attachment, internalization, and penetration steps of the infection process (42). The trimeric Ad2 fiber protein and the pentameric penton base protein each interact with separate and distinct host cell receptors (42). The fiber protein mediates Ad2 attachment to an as yet unidentified cellular receptor (24, 42). Binding of the Ad2 penton base protein to α_v-integrins then mediates virus internalization and membrane permeabilization (38, 41, 42). Penton base binds to α_v-integrins through a tripeptide motif, Arg Gly Asp (RGD), most likely present as part of a protrusion of its tertiary peptide structure (21, 32). The penton proteins of adenovirus serotypes 2 and 5, 3, 4, and 12 from subgroups C, B, E, and A, respectively, all contain the RGD motif and likewise utilize α_v-integrins during viral entry into cells (2, 21).

Attempts to identify the Ad2 fiber receptor have met with limited success. A candidate for the Ad2 receptor with an apparent molecular mass of 40 to 42 kDa was isolated, but not identified, by sucrose gradient sedimentation assays and affinity chromatography (34). A similar approach identified three major Ad2 fiber-recognizing polypeptides with sizes of 78, 42, and 34 kDa (11). Affinity labeling of cell plasma membrane receptors by adenovirions in a ligand overlay blotting assay revealed a number of potential Ad2 and Ad3 receptor proteins of different molecular masses (7). Early studies with Ad3 virions have shown that a 47-kDa protein is the major adenovirus binding protein for this serotype (7). Recent studies with Ad3 virions and Ad3 fiber have suggested that a 130-kDa protein in the membrane of HeLa cells may be a candidate for the fiber receptor (9).

It has been demonstrated that the Ad2 fiber can block binding of both Ad2 and Ad5 (24), and it has been generally assumed that such homotypic (same serotype) and heterotypic (same subgroup) inhibition would also occur in other subgroups like, e.g., subgroup B with Ad3 and Ad7 (3, 4, 7). It has been shown that the fibers of two viruses, Ad3 and Ad5, from different subgroups bind to different receptors (12, 20, 31). In the present paper, we confirm that heterotypic inhibition between Ad2 and Ad5 occurs and is mediated by the fiber protein. The purified fiber and fiber knob of Ad3 (subgroup B) did not compete with Ad2 (subgroup C) or vice versa, which confirmed that the fibers of these two serotypes recognize distinct cellular receptors (7, 12, 20, 31). Surprisingly, the fiber and fiber knob of Ad9 (subgroup D) cross-competed with Ad2 for the cellular receptor on cell lines such as A549 and Hs 700T. We show that this cross-competition is mediated by the Ad9 fiber protein through recognition of the Ad2 fiber receptor. Furthermore, through competition experiments, it is shown that neither attachment to nor infection of A549 and Hs 700T cells by Ad9 can be significantly inhibited by excess amounts of fiber knob 5 (F5K) or F9K. In order to clarify these paradoxical results, we performed binding studies with a panel of cell lines expressing various levels of cellular fiber receptor with or without α_v-integrins. Our results show that Ad9 can make use of its

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fiber receptor for attachment in those human cell lines that are nonadherent and that lack α_v-integrins. Furthermore, upon reintroduction of functional $\alpha_v\beta_5$-integrins in hamster melanoma CS-1 cells, Ad9 employs a cell binding strategy that involves both fiber-receptor and penton–α_v-integrin interactions.

MATERIALS AND METHODS

General methods. All DNA manipulations were performed according to standard protocols (1). Protein analysis through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) was performed as described previously (1, 18).

Viruses and cell lines. Ad2, 3, 5, and 9 were obtained as stocks from the American Type Culture Collection (Rockville, Md.) and were passaged on A549 or HeLa cells in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, Md.) supplemented with 5% fetal calf serum (FCS). The HepG2, Hs 700T, A172, U118, and Tera 2 cell lines were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 5% FCS. The Ramos and Y79 cell lines were also obtained from American Type Culture Collection and maintained in RPMI with 5% FCS. Human aortic smooth muscle cells (HASMC) were purchased from Cell Systems Corp. (Kirkland, Wash.) and maintained in MCDB with 5% FCS. Human melanoma ACA19 cells were a gift from John Hilkens (Netherlands Cancer Institute, Amsterdam, The Netherlands) and were maintained in DMEM with 5% FCS. Hamster melanoma CS-1 cells were a gift from Caroline Damsky (Schools of Dentistry and Medicine, University of California—San Francisco, Calif.). The β_5-integrin subunit gene was obtained from Sarah Bodary (Genentech Inc., South San Francisco, Calif.). The CS-1 cells and derivatives were maintained in DMEM with 5% FCS.

[^{3}H]methyl thymidine-labeled adenoviruses were obtained by infecting A549 monolayers (approximately 12 million cells per flask) at a multiplicity of infection of 5. At 24 h postinfection, 0.5 mCi of [^{3}H]methyl thymidine was added in a volume of 10 ml. After overnight incubation in this small volume, 15 ml of medium was added, and cells were harvested at 55 to 96 h postinfection. The cells were pelleted, washed two times, and resuspended in DMEM with 10 mM Tris (pH 8.0). Virus was released from the cells by three freeze-thaw cycles, and the cell debris was removed by centrifugation. The supernatant was layered onto a CsCl gradient and centrifuged for 5 h at 22,500 rpm. Banded virus was collected and dialyzed overnight against 10 mM Tris (pH 8.0)–150 mM NaCl–10 mM $MgCl_2$–10% glycerol. The dialysate was aliquoted and stored at −80°C until further use.

Production and purification of adenovirus proteins. The Ad3 fiber sequence (30), the Ad5 fiber sequence (5, 6), and the Ad9 fiber sequence (GenBank accession numbers M12411, M18369, and X74659, respectively) were used for the design of primers for amplification of fiber knobs from adenovirus genomic DNA. The primers were designed such that they included a *Bam*HI site (sense primer) and a *Kpn*I site (antisense primer) to facilitate cloning of the PCR product after digestion with the appropriate restriction enzymes. The sense primers were designed to hybridize such that, upon amplification, the product would include the ultimate repeat of the shaft of each target fiber gene, the conserved shaft-knob junction TLWT (33), and the receptor-recognizing knob (12, 20, 31). PCRs were performed with Ultma DNA polymerase (Perkin-Elmer, Foster City, Calif.) with a standard PCR protocol. The products were cloned as *Bam*HI-*Kpn*I fragments in the bacterial expression vector pQE32 (Qiagen, Inc., Chatsworth, Calif.). The DNA sequence of the fiber knobs was determined with an automated 373 DNA sequencer (Applied Biosystems, Foster City, Calif.). No sequence differences were found in comparison with the original sequences reported in the GenBank database. Since typical attempts at production in *Escherichia coli* yielded little soluble, yet active, knob protein and massive amounts of insoluble knob protein, the fiber knob inserts from the pQE32 vector were excised with the enzymes *Eco*RI and *Kpn*I, which left the complete insert intact, and cloned in the baculovirus transfer vector pAcSG2 (Pharmingen, San Diego, Calif.). Recombinant baculoviruses containing the Ad3, Ad5, or Ad9 fiber knob constructs were isolated and amplified according to standard protocols (23). Recombinant Ad2, 3, 5, and 9 fibers were produced and purified as described earlier (42). The knob proteins were produced in Tn*5* B1-4 insect cells (Invitrogen, San Diego, Calif.) and were purified to homogeneity with Ni^{2+} nitrilotriacetic acid agarose columns as described by the manufacturer (Qiagen). ^{35}S-labeled Ad3, Ad5, and Ad9 fiber knobs were produced as previously described (42).

Competition experiments. Approximately 10^6 A549 cells in 250 μl of Dulbecco's phosphate-buffered saline (PBS) with 3 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS^{++}) were preincubated for 1 h at 4°C with increasing numbers of viral particles in Eppendorf tubes precoated with 5% bovine serum albumin in PBS^{++}. Preliminary experiments had shown that preincubation with inhibitors and competitors at 4°C yielded the same results as incubation at 37°C but took longer to reach equilibrium. We therefore performed all preincubations, except where noted otherwise, with proteins at 37°C. Recombinant fiber knobs were added to the cells at final concentrations of 10 μg/ml and incubated at 37°C for 1 h and then chilled for 15 min at 4°C. Labeled virus or labeled fiber protein was then added to the cell suspension and incubated at 4°C for an additional hour. After incubation, the cells were pelleted, the inoculum was removed, and the pellet was washed twice with PBS^{++}. The pellet was resuspended in 100 μl of PBS^{++}, added to scintillation fluid, and counted directly in a Beckman scintillation counter.

Fluorescent focus assay. Approximately 10^6 Hs700T cells per well were seeded and left to attach. The medium was removed, and the cells were washed twice with PBS^{++}. F5K and F9K and Ad2 penton base were then added at final concentrations of 10, 10, and 50 μg/ml, respectively, in DMEM without FCS and incubated for 2 h at 4°C. Virus was added at dilutions previously determined to generate statistically reliable numbers of foci and incubated for another 1.5 h in the cold. The inoculum was removed, and the wells were washed twice with cold DMEM without FCS. Two milliliters of DMEM with 10% FCS was added, and the cells were incubated for 24 to 48 h. The adenovirus infection was quantitated as described by Wickham et al. (42) with the following modifications. In brief, cell monolayers were fixed with 2% paraformaldehyde in PBS for 20 min, followed by two washes with PBS and a 5-min incubation with 0.2% Triton X-100. The cells were washed twice with 0.5% fish gelatin (Sigma, St. Louis, Mo.) in PBS and then blocked with the same solution for 1 h. Primary antibodies directed against the adenoviral DNA-binding protein and the hexon protein (both provided by Douglas Brough, GenVec, Inc.) or the penton protein (provided by Glen Nemerow, the Scripps Institute) were used in dilutions of 1:100 to 1:250 in PBS with 0.5% fish gelatin and incubated for 1 h. Cells were washed three times and incubated overnight with a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, Ind.) in PBS with 0.5% fish gelatin. After three washes, 1 ml of PBS was put over the stained cells, and foci were counted with a Nikon Diaphot 200 fluorescence microscope.

RESULTS

Cross-competition of Ad2, Ad5, and Ad9 for the same cellular fiber receptor. Differential coagulation of erythrocytes from diverse animal species by adenovirus virions of different serotypes suggests that their fiber proteins have distinct receptors. Three major hemagglutination patterns have been observed for adenovirus serotypes (13): those, including Ad3 (subgroup B), which strongly hemagglutinate rhesus erythrocytes but not rat erythrocytes; those, including Ad9 (subgroup D), which strongly hemagglutinate human, rat, and mouse erythrocytes, but not rhesus erythrocytes; and those, including Ad2 and Ad5 (subgroup C), which weakly hemagglutinate rat erythrocytes only. These distinct hemagglutination patterns suggested that cross-competition between serotypes for the same receptor site would be limited to distinct subgroups (3, 7). Moreover, while Ad2 fiber has been shown to compete with both Ad2 and Ad5 for the same receptor site (24), it has not been shown that both proteins can indeed be used interchangeably in competition and binding experiments, nor has the Ad5 fiber been shown to compete for the binding site of Ad2 and Ad5.

Cross-competition experiments were used to examine the binding of serotypes Ad2 and Ad5 to A549 cells. Increasing amounts of full-length recombinant fiber 2 and fiber 5 had a comparable inhibitory effect on the binding of both Ad2 (Fig. 1A) and Ad5 (Fig. 1B). In both cases, saturation of the receptor sites, with a maximum inhibition of labeled virus binding of 95%, occurred between final concentrations of 0.1 and 0.3 μg/ml. Therefore, fiber-mediated homotypic and heterotypic cross-competition occurred between serotypes Ad2 and Ad5, which suggested that the Ad2 and Ad5 fibers recognized the same receptor. This also suggested that the fibers could be used interchangeably as competitors in Ad2 and Ad5 virus binding studies.

Cross-competition between serotypes of different subgroups, i.e., Ad2 (subgroup C), Ad3 (subgroup B), and Ad9 (subgroup D), was next examined by using receptor-recognizing recombinant fiber knobs instead of the complete fiber proteins as competitors. His-tagged proteins of F3K, F5K, and F9K were generated by the baculovirus expression system. Expression of the fiber knobs by the resulting recombinant baculoviruses yielded large amounts of soluble proteins that could be purified easily. Protein gel analysis revealed bands that migrated at the expected sizes of 24.1 kDa for F3K, 23.5 kDa for F5K, and

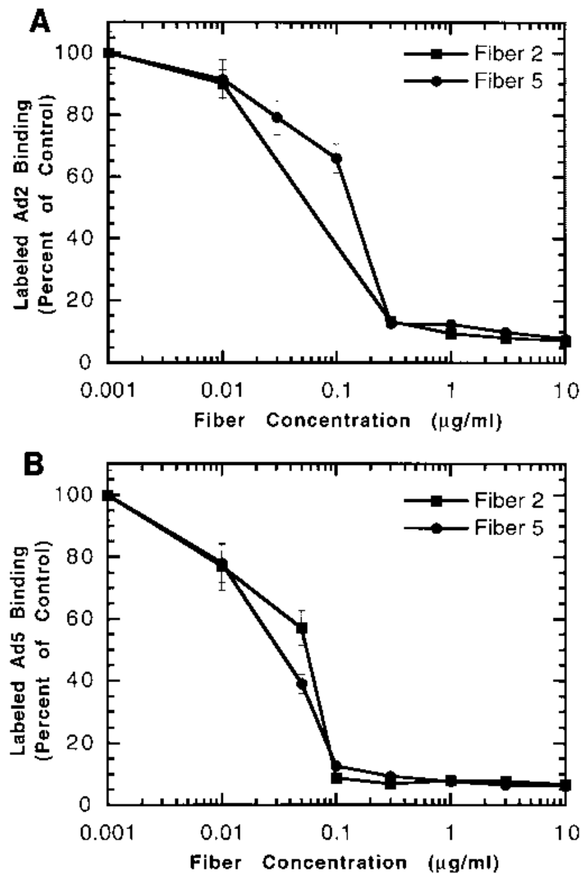


FIG. 1. Cross-competition of labeled Ad2 and 5 virions with unlabeled fibers 2 and 5. A total of 10^6 A549 cells in 250 μl of PBS^{++} were preincubated with the indicated concentrations of unlabeled fiber for 1 h at 4°C, followed by a further incubation with 1×10^4 to 3×10^4 cpm of labeled Ad2 or Ad5 for 1 h at 4°C. Cells were then pelleted and washed two times with cold PBS^{++}. Cell-associated counts per minute were then determined in a scintillation counter. Values are the average of triplicate determinations. Error bars indicate the standard deviation. (A and B) Competition of labeled Ad2 (A) and Ad5 (B) with unlabeled fibers 2 and 5.

24.3 kDa for F9K (Fig. 2). Western blot analysis showed that both F5K and F9K cross-reacted with a rabbit polyclonal antibody raised against the fiber 2 protein, whereas F3K did not (data not shown). Competition experiments with the purified knobs showed that preincubation of A549 cells with increasing amounts of F3K, F5K, or F9K resulted in a dose-dependent inhibition of labeled Ad2 binding with the latter two knobs (Fig. 3A). At a concentration of 1 μg/ml, more than 90% of Ad2 binding was inhibited. These experiments suggested that the F9K protein competed for the same receptor site as the F5K protein. The F3K protein, which was used as a control in this binding experiment, had no effect on Ad2 binding. Preincubation of A549 cells with increasing amounts of F3K, F5K, or F9K, followed by incubation with labeled Ad3, showed that only the homologous fiber knob had a maximum inhibitory effect of 85% on Ad3 binding at the highest doses tested (Fig. 3B). Further preincubation of cells with a combination of F3K and penton base protein or the monoclonal antibody (MAb) L230, which is directed against α_v-integrins (39), led to complete inhibition of Ad3 binding (data not shown). Neither F5K nor F9K had any effect on Ad3 binding. These results confirm that Ad3 recognized a receptor different from the Ad2 and Ad5 receptor (12, 20, 31) and also showed that it can make use of its penton base to interact directly with α_v-integrins.

To test the hypothesis that the inhibition of Ad2 binding was due to a competition for the Ad2 and Ad5 fiber receptor, direct protein-protein competition experiments for the fiber receptors on A549 cells were set up with ^{35}S-labeled F5K and unlabeled F5K or F9K and vice versa. Both competitors competed for the binding sites of labeled F5K with identical affinity (Fig. 4A). Complete competition of labeled F5K binding occurred upon preincubation of the cells with concentrations of unlabeled F5K or F9K of between 300 ng/ml and 1 μg/ml. With labeled F9K, the unlabeled competitors also competed in a similar fashion (Fig. 4B). Also, in virus-virus competition experiments, preincubation of A549 cells with increasing numbers of unlabeled Ad9 virions per cell resulted in a dose-dependent decrease in binding of labeled Ad2 virions (data not shown). The virus-virus, fiber-virus, and fiber-fiber cross-competition data all strongly suggest that F5K and F9K recognized the same or a similar receptor.

Ad9 fiber does not significantly block Ad9 binding to Hs 700T cells. Competition experiments with labeled Ad9 and unlabeled F3K, F5K, and F9K (Fig. 3C) showed that the latter two knobs, at the highest concentration used, had a maximum inhibitory effect of 20% on viral binding. Indeed, in competition experiments with two epithelial cell lines, A549 and Hs 700T, with end concentrations of up to 25 μl/ml, no further inhibition was observed (data not shown). These results suggested that Ad9 can attach to A549 and Hs 700T cells by employing a fiber-independent mechanism. It has been shown that the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote the internalization of Ad2 (41, 42). Because the Ad9 fiber is much shorter than the Ad2 or 5 fiber (11 to 13 nm versus 37 nm [8, 28, 38]), which may make its penton more accessible and available, we hypothesized that, as is the case with Ad3 binding (data not shown), the Ad9 penton base might interact directly with α_v-integrins and facilitate binding. We therefore investigated Ad2 and Ad9 binding by preincubating Hs 700T cells with either F5K or F9K, the MAb L230, the Ad2 penton base, or a combination of the latter with the relevant fiber knob. Mab L230, which is directed against the α_v-integrins (39), will functionally block direct penton–α_v-integrin interactions. Next, labeled virus was added and incubated for 1 h at 4°C. The determination of bound virus showed that Ad2 binding was inhibited by more than 95% by F5K (Fig. 5A). Preincubation of the cells with saturating amounts of the MAb L230 or Ad2 penton base had no effect on Ad2 binding. A combination of penton base with F5K showed no additive inhibitory effect on Ad2 binding, which confirmed that only fiber mediates the initial Ad2 binding to Hs 700T cells and other (epithelial) cell lines, such as A549 (described below [Table 1]).

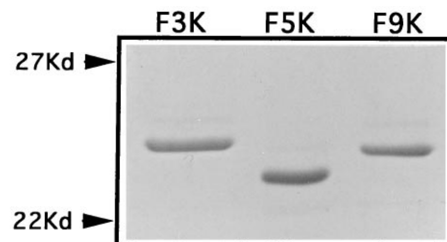
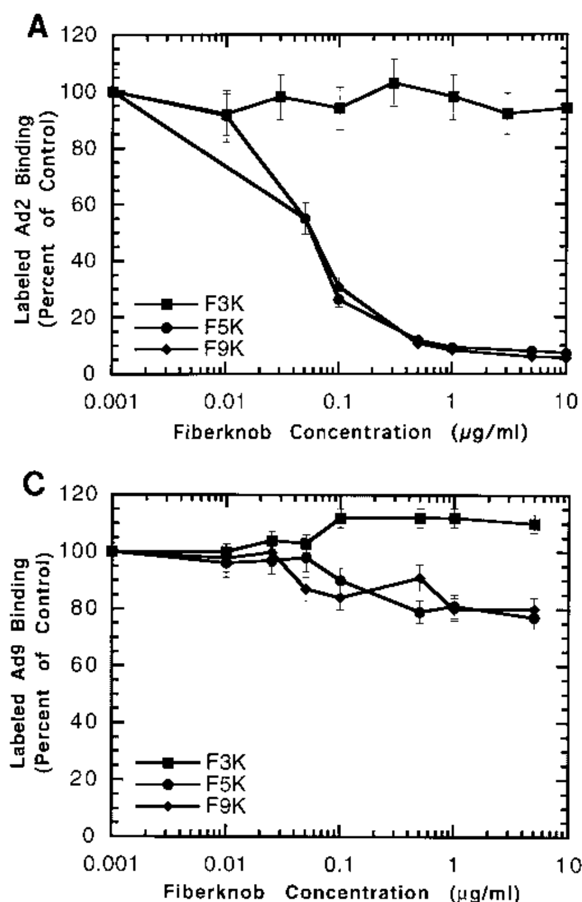


FIG. 2. Coomassie-stained SDS-polyacrylamide gel of the fiber knobs. The fiber knobs of Ad3, Ad5, and Ad9 were produced and purified as described and run on a 15% gel, followed by Coomassie staining. The sizes of the markers are indicated.



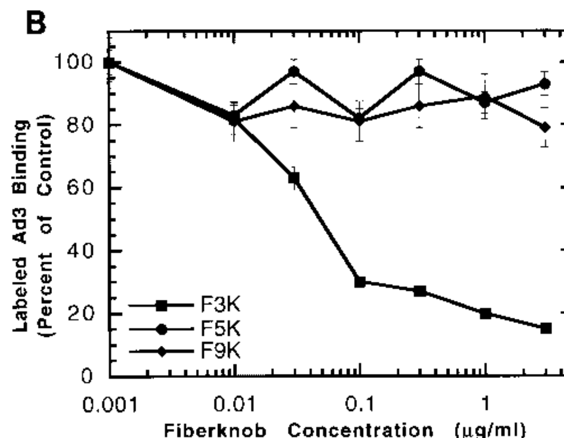


FIG. 3. Competition of purified fiber knobs with labeled adenovirus. A total of 10^6 A549 cells in 250 µl of PBS^{++} were preincubated with the increasing concentrations of F3K, F5K, or F9K for 1 h at 4°C, followed by a further incubation with 1×10^4 to 3×10^4 cpm of labeled Ad2, Ad3, or Ad9 for 1 h at 4°C. Cells were pelleted and washed twice with cold PBS^{++}. Cell-associated counts per minute were then determined in a scintillation counter. Values are the average of triplicate determinations. (A, B, and C) Competition of labeled Ad2 (A), Ad3 (B), and Ad9 (C) with F3K, F5K, and F9K.

Binding of Ad9 is blocked up to 20 to 25% by F9K, 46% by Mab L230, 57% by Ad2 penton base, and 77% by Ad2 penton base plus F9K (Fig. 5B). These results confirmed that Ad9 could bind to cells independent of fiber-receptor interactions by employing a mechanism such as direct interaction of penton with α_v-integrins, which has been shown here to play a predominant role in Ad9 attachment and binding to Hs 700T cells and other (epithelial) cell lines like A549 (described below [Table 1]). Our results do not exclude the possibility that proteins other than fiber or penton play a minor role in virus attachment and binding to the cell surface.

Ad9 infection is not blocked by soluble fiber protein. To determine whether the observed fiber-independent binding represented an infectious route of attachment, a fluorescent focus assay similar to the binding assay was performed to study the effect of the competitors on the infection of Hs 700T cells by unlabeled virus. Infections by both Ad2 and Ad9 were analyzed with antibodies that detected proteins specific to both the early phase (α-DNA-binding protein) and late phase (α-hexon and α-penton base) of the adenoviral infection cycle. Analysis of the Ad2 infection with the three antibodies described showed total abrogation by preincubation of the cells with both fiber knobs (Fig. 6A). No effect was observed for preincubation with saturating amounts of Ad2 penton base protein.

Penton base itself, used here as a control to inhibit any possible direct interaction of the Ad2 penton with the α_v-integrins, had no discernible effect on the Ad2 infection. This is not contradictory to the demonstration that α_v-integrins promote the internalization of Ad2, which requires the continuous presence of penton base during a short warming period followed by trypsinization (42). In our experimental design, Ad2 penton base was only present during the attachment phase at 4°C, was removed by two washes with PBS^{++}, and therefore was not present to continue to block internalization of the adenovirus by internal pools or newly synthesized α_v-integrins upon incubation at 37°C. The results for Ad9 infectivity (Fig. 6B) showed no significant inhibitory effect for F5K, while preincubation of the cells with F9K resulted in a marginal, statistically insignificant inhibition of infectivity. Preincubation of the Hs 700T cells with Ad2 penton base resulted in a 45% decrease in the number of foci formed. These results show that Ad9 infection involves direct interaction of the penton base with the α_v-integrins. The fact that competition with a combination of penton base and fiber does not further decrease infectivity suggests that Ad9 penton may interact with other cellular receptors.

Ad9 can bind directly to α_v-integrins. The paradoxical binding characteristics of Ad9 and its fiber protein—that is, the fiber recognizes the same receptor as do Ad2 and Ad5 fibers, and yet the virus foregoes employment of fiber-receptor interactions—prompted us to further analyze Ad2 and Ad9 binding with a panel of human cell lines that displayed various levels of fiber receptor and expressed or lacked α_v-integrins (Table 1). Two cell lines, the hepatocarcinoma-derived HepG2 and Hs 700T, bound Ad2 at levels between 5 and 10% of the input dose of labeled Ad2 virus used. These levels dropped by more than 90% when the cells were first preincubated with saturating amounts of F5K protein, showing that the binding of Ad2 is fiber dependent and specific. No effect was observed for L230, indicating that the penton base does not play an appreciable role in Ad2 binding. Ad9 binding to HepG2 is not inhibited by F9K, but it was inhibited 25% by L230; 25% of Ad9 binding to Hs 700T can be inhibited by preincubation with F9K protein, and about 45% of the binding involves interaction with the α_v-integrins. Four other cell lines (A172 and U-118 MG, both human glioblastoma-derived cell lines; ACA19, a human melanoma cell line; and HASMC) all display low levels of Ad2 fiber receptor. Binding of Ad2 to these cell

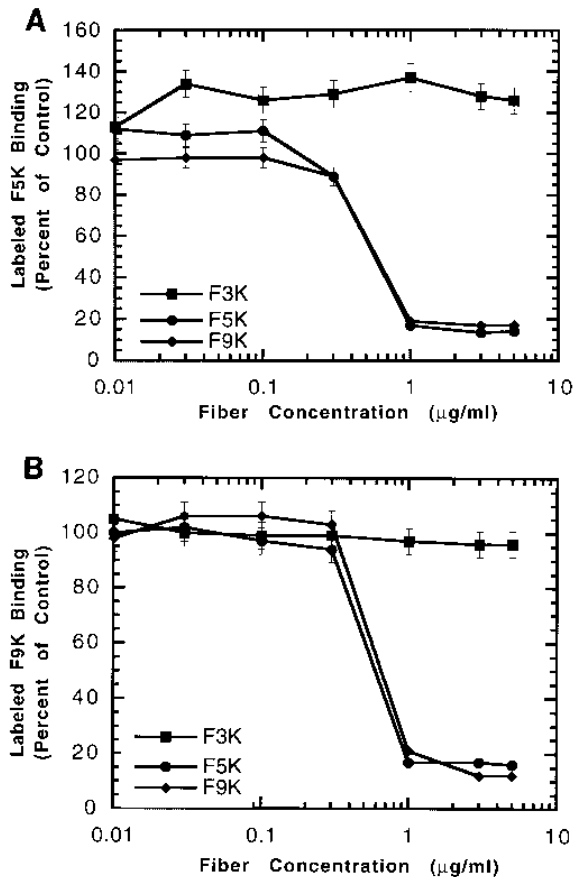


FIG. 4. Competition of unlabeled fiber knobs with labeled fiber knobs. Two million A549 cells in 250 μl of PBS^{++} were preincubated with increasing concentrations of unlabeled F5K or F9K for 1 h at 4°C, followed by a further incubation with 3×10^3 to 5×10^3 cpm of labeled F5K or F9K for 1 h at 4°C. Cells were pelleted and washed twice with cold PBS^{++}. Cell-associated counts per minute were then determined in a scintillation counter. Values are the average of triplicate determinations. (A and B) Competition of labeled F5K (A) and F9K (B) with unlabeled F5K and F9K.

lines is of low specificity and, in comparison to the other cell lines tested, only small amounts of Ad2 are bound. Likewise, Ad9 binding to these cell lines cannot be inhibited by preincubation with F9K protein. Instead, binding consists primarily of direct penton–α_v-integrin interactions, as evidenced by the drop in binding percentage upon preincubation of the cells with the MAb L230. A different picture emerges for Ad9 binding to the lymphocyte-derived Ramos cell line and retinoblastoma-derived Y79 cell line that grow in suspension and have been shown to express few or no α_v-integrins (17, 40). Although Ad9 binds here at low levels, 60 to 80% of the virus bound appears to bind through the fiber, as evidenced by the binding percentage obtained upon preincubation with F9K protein. Neither Ramos nor Y79 shows any effect on binding by preincubation with L230. The embryonic teratocarcinoma cell line Tera 2 does have α_v-integrins and, as evidenced by the binding data for Ad2, expresses extremely high levels of fiber receptor. The fact that binding of Ad9 to this cell line also goes primarily through the fiber receptor, despite the presence of α_v-integrins, may be a function of the cellular fiber receptor density. Preincubation of these cells with L230 has no effect on Ad9 binding. Our studies with these cell lines therefore support the hypothesis that α_v-integrins can act as a second attachment site for Ad9 virus. The presence of this alternative attachment site for Ad9 suggested why attachment to many cell lines was not blocked by soluble fiber.

We tested this hypothesis directly by analysis of Ad2 and Ad9 binding to the hamster melanoma cell line CS-1, which grows in suspension and has been shown to lack functional α_v-integrins (35, 40). Binding of Ad2 to this cell line follows the regular pattern (Fig. 7A), in that it binds well, can be inhibited more than 90% by preincubation with F5K, and shows no role for penton base. A similar pattern emerges for Ad9. It does bind at a level approximately five times lower than Ad2, can be inhibited up to 65% by preincubation with F9K, and shows no significant effect for penton base preincubation. Transfection of CS-1 cells with $\alpha_v\beta_5$-integrin has no effect on Ad2 binding, but it changes the binding characteristics of Ad9 dramatically (Fig. 7B). Whereas the total binding of Ad9 increases by a factor of 2 (data not shown), the inhibition induced by F9K preincubation drops to 25%, the effect of penton base prein-

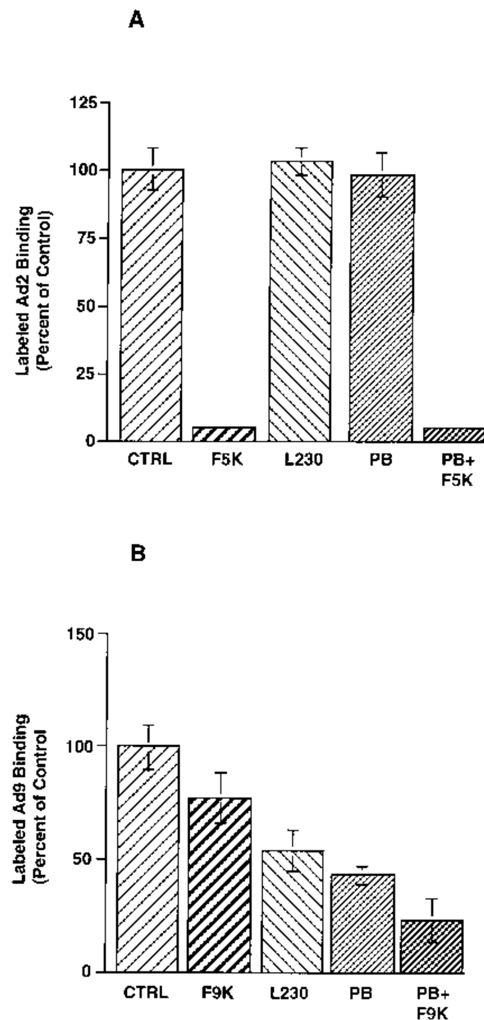


FIG. 5. Effect of competitors on labeled adenovirus binding. A total of 10^6 Hs 700T cells in 250 μl of PBS^{++} were preincubated with saturating concentrations of F5K, F9K, the MAb L230, the Ad2 penton base (PB), or a combination of PB with one of the knobs for 1 h at 4°C, followed by a further incubation with 1×10^4 to 3×10^4 cpm of labeled Ad2 or Ad9 for 1 h at 4°C. Cells were pelleted and washed twice with cold PBS^{++}. Cell-associated counts per minute were then determined in a scintillation counter. CTRL, control experiment (binding in the absence of competition). Values are the average of triplicate determinations. (A and B) Effect of the competitors on labeled Ad2 (A) and Ad9 (B) binding.

TABLE 1. Binding of Ad2 and Ad9 to cell lines expressing various levels of fiber receptor and α_v-integrins[a]

Cell line	Expression of:		% binding of[b]:					
			Ad2			Ad9		
	Fiber receptor	Integrins	Control	F5K	L230	Control	F9K	L230
A549	++	+	10.6	0.8	10.0	9.7	7.8	6.0
HepG2	++	+	10.0	0.9	10.0	1.5	1.8	1.1
Hs 700T	++	+	6.9	0.5	ND[c]	9.5	7.3	5.1
A172	+/−	+	3.0	1.7	3.0	2.4	2.4	1.1
ACA19	+/−	+	2.1	1.1	ND	2.3	2.3	0.4
HASMC	+/−	+	1.1	0.6	1.3	2.6	4.3	0.5
U-118 MG	+/−	+	2.2	2.0	ND	0.9	1.0	0.4
Ramos	++	−	11.6	1.0	10.5	1.4	0.2	1.4
Y79	+++	−	28.2	0.94	26.2	3.2	1.3	3.3
Tera 2	+++	+	43.4	1.3	41.2	6.4	1.9	3.3

[a] Integrins refers to the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Cells were (pre) incubated as described in Materials and Methods. Fiber knobs were used at a final concentration of 2 μg/ml, and the Mab L230 was used at 50 μg/ml. The values shown are the mean of three samples. The standard deviation of the mean in all samples shown was 7% or lower. The cell lines and deviations are as follows: A549, human lung carcinoma; HepG2, hepatocarcinoma; Hs 700T, metastasis of an intestinal or pancreatic carcinoma; A172 and U-118-MG, glioblastomas; ACA19, human melanoma cell line; Ramos, human lymphoma; Y79, retinoblastoma; and Tera 2, embryonic teratocarcinoma.

[b] Approximately 60,000 cpm of labeled virus was used per assay. The binding percentages shown here reflect the counts of the input dose bound to the cells divided by the precise counts per minute of the input dose.

[c] ND, not determined.

cubation increases to 40%, and the additive inhibitory effect of both competitors increases to about 65%. Transfection of CS-1 cells with $\alpha_v\beta_3$ had a similar yet less pronounced effect (data not shown). We conclude that the failure of F9K to block Ad9 binding to many cell lines correlates with the ability of Ad9, in contrast to Ad2, to bind independent of fiber to α_v-integrins.

DISCUSSION

The recognition of a specific cellular receptor by the fiber protein has been shown to be the first and crucial step in attachment and binding to a cell by an adenovirus (16). The subgroup classification of adenoviruses holds that the fibers of serotypes that are in different subgroups recognize different cellular receptors, thus defining tissue specificity and viral tropism (16, 22, 36). We have shown in this paper that the fiber protein of the subgroup D Ad9 virus recognizes the same cellular fiber receptor as the fibers of the subgroup C viruses Ad2 and Ad5. The subgroup B serotype Ad3 recognizes a cellular fiber receptor distinct from the Ad2, 5, and 9 fiber receptor.

Our data further demonstrate that binding of Ad9 to cells almost always involves two or more distinct protein-protein interactions at the virus-cell level. It is therefore no surprise that the binding profile of Ad9 differs by cell line and is correlated with the presence or absence of cellular fiber receptors and/or α_v-integrins. We have analyzed some of these cellular binding profiles, or fingerprints, in some detail (Table 1 and Fig. 7B) and have found that adenoviral attachment can involve more than just the fiber-cellular receptor interaction; i.e., the penton base can interact directly with α_v-integrins and, potentially, other integrins.

The epithelial cell lines we have analyzed (e.g., Hs 700T and HepG2) show that preincubation of the cells with saturating amounts of F9K protein has little or no effect on labeled Ad9 binding. Preincubation with L230 or Ad2 penton base shows that, depending on the cell line used, between 20 and 40% of Ad9 binding involves direct penton–α_v-integrin interactions. Cells that have been categorized as having little or no Ad2, 5, or 9 receptor show a different fingerprint for Ad9 binding. There is no competition effect for F9K whatsoever, and at least 50% of the binding goes through the penton base. Cells that lack α_v-integrins yield yet another fingerprint. Here Ad9 does show a marked competition for its cellular fiber receptor. Finally, a comparison of the Ad9 binding fingerprints obtained for the hamster melanoma CS-1 cells and the CS-1 cells transfected with the α_v-integrins shows (Fig. 7B) that the vitronectin receptors influence these fingerprints dramatically by eliminating the ability of Ad9 fiber to block Ad9 binding to its cellular

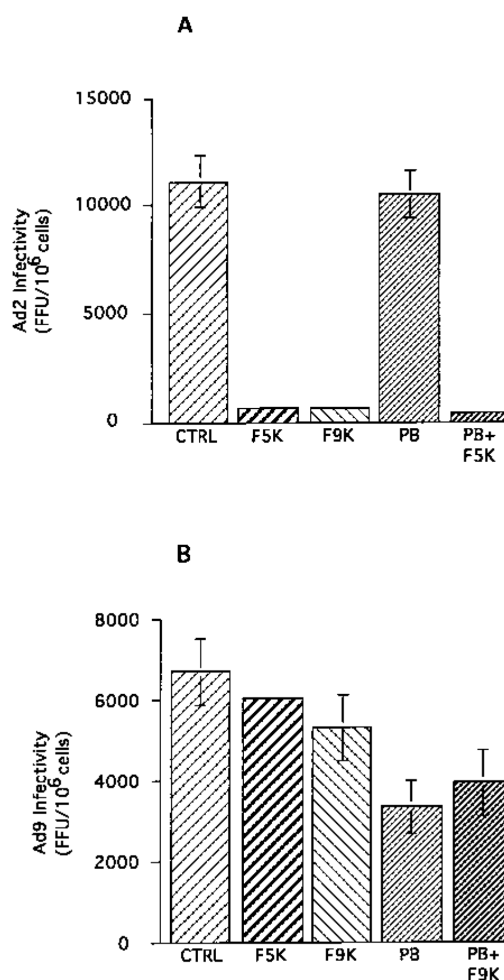


FIG. 6. Effect of competitors on adenovirus infectivity. A six-well plate with 10^6 Hs 700T cells per well was preincubated with saturating concentrations of F5K, F9K, the Ad2 penton base (PB), or combinations of PB with either fiber knob for 1 h at 4°C, followed by a further incubation with appropriate dilutions of unlabeled Ad2 or Ad9 virus stocks for 1 h at 4°C. The cells were washed twice with cold DMEM to remove competitors and unbound virus and then further incubated for 24 h with DMEM–5% FCS. The focus-forming assay was performed as described in Materials and Methods. The experiment was performed three times with antibodies raised against proteins produced during the early and/or late stages of virus infection. One typical experiment, performed in duplicate with detection by anti-penton base antibody, is shown here. (A and B) Effect of the competitors on Ad2 (A) and Ad9 (B) infectivity. FFU, focus-forming units; CTRL, control experiment.

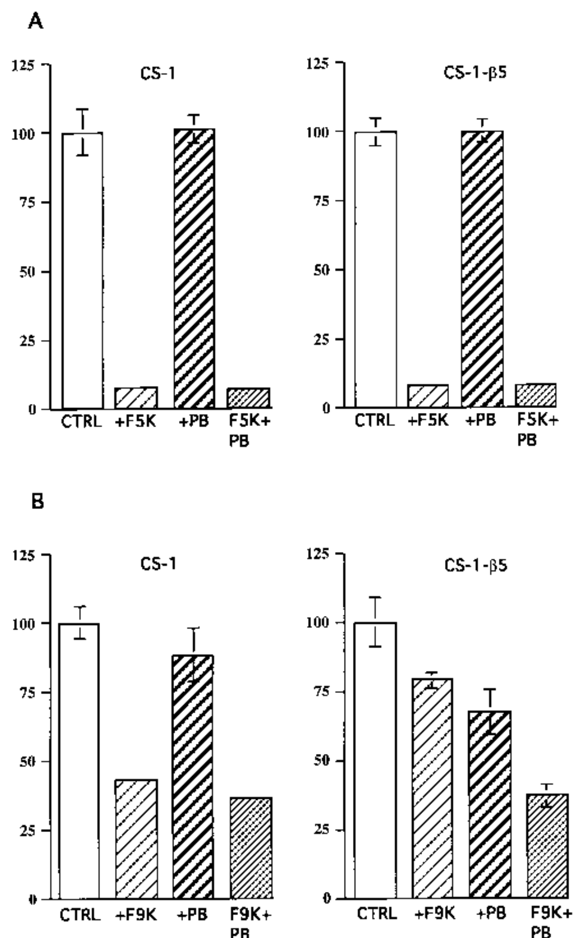


FIG. 7. Binding of Ad2 and Ad9 to hamster melanoma CS-1 cells. One million cells were preincubated with 2 μg of the appropriate fiber knob per ml, 50 μg of Ad2 penton base per ml, or a combination of both and incubated for 1 h at 4°C. A subsaturating quantity of labeled virus was added, and the incubation continued for 1 h. The cells were then pelleted, washed twice with cold PBS^{++}, and counted in a scintillation counter. The results shown are the mean of three samples and were standardized to the control (CTRL) at 100%. The standard deviation of the mean in all samples shown was 10% or lower. (A and B) Effect of competitors on labeled Ad2 (A) and Ad9 (B) binding to CS-1 and CS-1-$\alpha_v\beta_5$ (CS-1-β5) cells.

fiber receptor. Instead, a significant portion of binding is directed towards penton–α_v-integrin interactions.

How can two viruses whose fibers bind to the same receptor and whose penton base proteins both bind to α_v-integrins nevertheless display different binding strategies? We hypothesize that the different Ad9 binding fingerprints described here may be the result of two possibly cooperative factors: the length of the fiber protein shaft and the interaction with other virion coat proteins, i.e., penton and/or hexon with cell surface proteins. The length of the adenoviral fiber shaft varies from subgroup to subgroup: the fiber shaft of Ad3 (subgroup B) has a length of 10 to 11 nm, that of Ad9 (subgroup D) has a length of 12 to 13 nm, and that of Ad2 and Ad5 (subgroup C) has a length of 37 nm (8, 28, 38). The shortened Ad9 fiber may be precluded from interacting with the fiber receptor by glycogroups of highly glycosylated glycoproteins such as the mucins (14). Such glycogroups may cover and/or obstruct significant portions of certain cells and may tower 200 to 500 nm above the cellular glycocalyx, which reaches only 10 nm above the cell surface (14). In contrast, the extended Ad2 and 5 fiber structure (8, 28) may permit fiber-receptor interactions while avoiding the steric inhibition imposed by extended cellular receptor proteins. In contrast, the short fiber shaft length of both Ad3 and Ad9 results in a fiber-penton base complex in which the receptor-recognizing knob (20, 31) sits only very slightly above the edge of the penton base with its protruding RGD loops (32).

We hypothesize therefore that it is the short shaft of the fiber that permits other virion components such as the penton base and hexon to approach close to the cell surface, where they may interact directly with proteins such as the α_v-integrins. Preincubation of A549 cells with saturating amounts of the function-neutralizing L230 MAb, directed against α_v-integrins in combination with saturating amounts of F3K, resulted in complete abrogation of labeled Ad3 binding (data not shown). This showed that up to 15% of the binding observed for labeled Ad3 resulted from direct interaction of the virus with the α_v-integrins (data not shown). It is clear from the data presented for Ad9 (Table 1 and Fig. 7B) that both the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ can play a significant role in Ad9 attachment to mammalian cells. Why does Ad3, despite having a fiber shaft length comparable to that of Ad9, interact primarily with its fiber receptor and not with α_v-integrins? We hypothesize that the Ad3 fiber, unlike the Ad2, 5, and 9 fibers, recognizes a cellular receptor in a position that is elevated above the cell surface and may be part of the glycocalyx. In order to interact with its cellular fiber receptor, Ad3 would then not have to approach the cell surface, where the α_v-integrins are located, and would therefore be less prone to direct interactions with the α_v-integrins.

The observation that preincubation of Hs 700T cells with saturating amounts of penton base and F9K only blocks binding by 77% indicates that virus-cell interactions other than fiber-receptor and penton–α_v-integrin may play a role and in fact can at least partially replace these interactions as shown for the focus-forming unit analysis of Ad9 infection in Hs 700T cells (Fig. 6B). Interactions of penton base with cellular integrins other than α_v-integrins and hexon with cell surface proteins may explain why Ad9 can apparently bind to and enter cells by a fiber- and α_v-integrin-independent mechanism (Fig. 6B). Screening of phage display libraries has identified potential receptor sequences that bind penton base in an integrin-independent manner (15).

If the length of the Ad9 fiber shaft predisposes this virus toward using the attachment mechanisms that have been described above, generation of Ad5 recombinants with short shafts may allow us to target Ad vectors directly to the α_v-integrins. Adenoviruses, in particular Ad2 and Ad5 (subgroup C), have been utilized as vectors to deliver therapeutic genes in vivo (19, 25, 27). Knowledge of the receptor specificity of adenoviruses will allow the design of tissue-specific vectors that contain fiber proteins from other serotype viruses for use in gene therapy.

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