JOURNAL OF VIROLOGY, May 1995, p. 2850–2857 Vol. 69, No. 5
0022-538X/95/$04.00+0

Human Adenovirus Serotypes 3 and 5 Bind to Two Different Cellular Receptors via the Fiber Head Domain

SUSAN C. STEVENSON, MICHELE ROLLENCE, BRIAN WHITE, LINDA WEAVER, AND ALAN MCCLELLAND*

Department of Molecular and Cell Biology, Genetic Therapy, Inc., Gaithersburg, Maryland 20878

Received 30 November 1994/Accepted 1 February 1995

The adenovirus fiber protein is responsible for attachment of the virion to cell surface receptors. The identity of the cellular receptor which mediates binding is unknown, although there is evidence suggesting that two distinct adenovirus receptors interact with the group C (adenovirus type 5 [Ad5]) and the group B (Ad3) adenoviruses. In order to define the determinants of adenovirus receptor specificity, we have carried out a series of competition binding experiments using recombinant native fiber polypeptides from Ad5 and Ad3 and chimeric fiber proteins in which the head domains of Ad5 and Ad3 were exchanged. Specific binding of fiber to HeLa cell receptors was assessed with radiolabeled protein synthesized in vitro, and by competition analysis with baculovirus-expressed fiber protein. Fiber produced in vitro was found as both monomer and trimer, but only the assembled trimers had receptor binding activity. Competition data support the conclusion that Ad5 and Ad3 interact with different cellular receptors. The Ad5 receptor distribution on several cell lines was assessed with a fiber binding flow cytometric assay. HeLa cells were found to express high levels of receptor, while CHO and human diploid fibroblasts did not. A chimeric fiber containing the Ad5 fiber head domain blocked the binding of Ad5 fiber but not Ad3 fiber. Similarly, a chimeric fiber containing the Ad3 fiber head blocked the binding of labeled Ad3 fiber but not Ad5 fiber. In addition, the isolated Ad3 fiber head domain competed effectively with labeled Ad3 fiber for binding to HeLa cell receptors. These results demonstrate that the determinants of receptor binding are located in the head domain of the fiber and that the isolated head domain is capable of trimerization and binding to cellular receptors. Our results also show that it is possible to change the receptor specificity of the fiber protein by manipulation of sequences contained in the head domain. Modification or replacement of the fiber head domain with novel ligands may permit adenovirus vectors with new receptor specificities which could be useful for targeted gene delivery in vivo to be engineered.

The adenovirus fiber protein is responsible for attachment of the virion to cellular receptors (21). The sequences of the fiber genes from several different adenovirus serotypes including adenovirus type 2 (Ad2), Ad5, Ad3, Ad12, Ad40, and Ad41 are known (3, 13, 14, 22–25). The fiber protein can be divided into three domains as proposed in the original model of Green et al. (7). The conserved N terminus contains the sequences responsible for association with the penton base as well as a nuclear localization signal (9, 19, 20). A rod-like shaft of variable length contains repeats of a 15-amino-acid beta structure, with the number of repeats ranging from 6 in Ad3 to 22 in Ad5 (6, 24). A conserved stretch of amino acids which includes the sequence TLWT marks the boundary between the repeating units of beta structure in the shaft and the globular head domain (28). The C-terminal head domain ranges in size from 157 amino acid residues for the short fiber of Ad41 to 188 residues in Ad5 fiber (6, 12). The fiber spike is a homotrimer, and there are 12 spikes per virion which are attached via association with the penton base complex (26, 27). On the basis of the architecture of the viral capsid and of the fiber polypeptide, it is likely that the head domain of the fiber contains the receptor attachment site. Recently, the Ad5 and the Ad2 fiber head domains have been shown to interact with HeLa cell viral receptors (8, 17).

The cellular receptors for adenoviruses have not been identified at the molecular level. However, the existence of distinct receptors for the group C (Ad5 and Ad2) and group B (Ad3) adenoviruses has been suggested on the basis of competition binding experiments using intact adenovirus particles (5, 18); those authors showed that the infection of A549 cells by wild-type Ad2 could be blocked by the addition of excess noninfectious Ad2 particles derived from a temperature-sensitive mutant or by the addition of purified Ad2 fiber protein. Ad2 particles did not block the infectivity of Ad3 in the same assay, suggesting that this virus uses a different receptor. However, the reciprocal competition binding experiments were not performed, and the two virus preparations differed significantly in the ratio of particle number to infectious titer. Consequently, the relative amount of competing virus was much lower in the case of Ad3 than in the homotypic competition with Ad2, leaving open the possibility that this difference rather than binding to different receptors could explain the results. Since purified fiber protein competes with intact virus for receptor attachment (21, 30), we approached the question of receptor specificity by examining directly the binding of the Ad5 and Ad3 fiber proteins to cellular receptors. Our data support the conclusion that Ad5 and Ad3 recognize different receptors. In addition, we present evidence that the receptor specificity of the adenovirus fiber can be altered by exchanging the head domains of the Ad5 and Ad3 proteins. These studies provide a basis for targeting adenoviral vectors to specific cells and tissues by engineering the fiber protein to contain novel receptor specificities.

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, Genetic Therapy, Inc., 938 Clopper Rd., Gaithersburg, MD 20878. Phone: (301) 248-4800. Fax: (301) 590-2638.

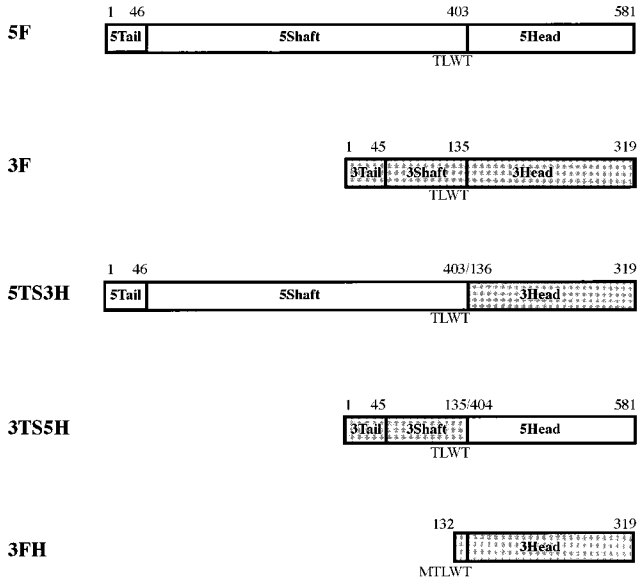

FIG. 1. Full-length, chimeric, and truncated fiber constructs. The full-length Ad5 (5F; open boxes) and Ad3 (3F; shaded boxes) fiber cDNAs were cloned by PCR. The chimeric fibers 3TS5H and 5TS3H were generated by PCR to join the tail (T), shaft (S), and head (H) segments of the Ad5 and Ad3 fiber cDNAs, as described in Materials and Methods. Sequences encoding amino acid residues 1 to 403 of the Ad5 fiber protein were joined to sequences encoding residues 136 to 319 of the Ad3 fiber protein to generate the 5TS3H chimeric fiber. Sequences encoding amino acid residues 1 to 135 of the Ad3 fiber protein were joined to sequences encoding residues 404 to 581 of the Ad5 fiber protein to generate the 3TS5H chimeric fiber. The Ad3 fiber head region (3FH) was generated by PCR to place an initiation ATG codon immediately in front of sequences encoding residue 132 of the Ad3 fiber protein. Amino acids are indicated by their one-letter designations.

MATERIALS AND METHODS

Plasmid fiber constructs. The fiber genes from Ad5 (ATCC VR-5) and Ad3 (ATCC VR-3) were cloned by PCR and are referred to as 5F and 3F, respectively. The published Ad5 or Ad3 fiber DNA sequences (GenBank accession numbers M18369 and M12411, respectively) were used to design oligonucleotide primers for amplification with purified adenovirus genomic DNA as a template. Primers were designed to amplify the entire coding sequence of the full-length fiber genes, starting from the start codon, ATG, and ending with the termination codon, TAA. For cloning purposes, the majority of the sense primers contained a *Pst*I restriction site and the majority of the antisense primers contained an *Eco*RI restriction site. Amplified products of the expected size were obtained and were cloned into pVL1392 (Invitrogen, San Diego, Calif.) for baculovirus expression and into pGEM4Z (Promega, Madison, Wis.) for expression in the in vitro fiber receptor binding assay. The nucleotide sequence of the cloned insert was determined, and in each case a clone having a perfect match with the published sequence was selected. Two chimeric fiber constructs were prepared by PCR gene overlap extension (10). The Ad5 fiber tail and shaft regions (5TS; amino acids 1 to 403) were connected with the Ad3 fiber head region (3H; amino acids 136 to 319) to form the 5TS3H chimera, and the Ad3 fiber tail and shaft regions (3TS; amino acids 1 to 135) were connected with the Ad5 fiber head region (5H; amino acids 404 to 581) to form the 3TS5H chimera. The fusions were made at the conserved TLWT sequence at the fiber shaft-head junction. The fiber head regions of each serotype were also cloned by PCR with primers designed to amplify the head regions of each fiber gene starting at the fiber shaft-head junction designated by the amino acid sequence of TLWT. An ATG codon was placed immediately in front of this sequence for expression purposes. The constructions of the full-length 5F and 3F, chimeric 5TS3H and 3TS5H, and truncated 3FH fiber proteins are shown schematically in Fig. 1.

Expression of fiber constructs in baculovirus. The baculovirus expression system (Clontech, Palo Alto, Calif.) has been used to generate fiber proteins for receptor binding studies. The fiber constructs were each cloned into the baculovirus expression vector pVL1392 (Invitrogen), downstream of the baculovirus polyhedrin promoter, and recombinant baculovirus vectors expressing the fiber proteins were prepared. *Spodoptera frugiperda* cells (Sf21) were cultured as monolayers at 27°C in Grace's supplemented insect cell medium containing 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 2.5 μg of amphotericin B per ml. Sf21 cells were cotransfected with each of the pVL1392 plasmids and with *Bsu*36I-linearized viral DNA (Clontech) by using Lipofectin. The transfection medium containing the recombinant baculovirus was plaque purified, and individual plaques were screened for fiber expression. Large-scale infections with each recombinant fiber baculovirus were carried out. Three to 4 days postinfection, the Sf21 cells were harvested and the cells were lysed by sonication in 10 mM Tris, pH 7.4, containing 100 mM PefablocSC (Boehringer Mannheim, Indianapolis, Ind.), 2 mM EDTA, and 10 μg of aprotinin per ml. The cellular debris was removed by centrifugation, and the cell lysate supernatant was collected. The cell lysate protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) as the assay standard.

The expression of each fiber protein in the Sf21 cell lysates was verified by nondenaturing sodium dodecyl sulfate (SDS)–4 to 15% polyacrylamide gel electrophoresis (PAGE) and Western immunoblot analysis. Sample preparation for nondenaturing SDS-PAGE was carried out without boiling so that the trimeric

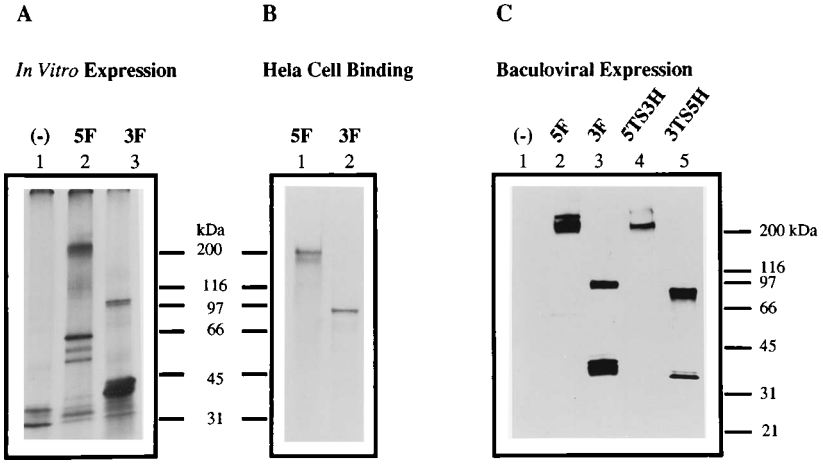

FIG. 2. Fiber expression and receptor binding assay. The ^{35}S-labeled 5F or 3F proteins were synthesized in vitro and analyzed directly by SDS–4 to 15% PAGE and fluorography (A) or applied to HeLa cell monolayers, and then the labeled cell bound proteins were analyzed by SDS–4 to 15% PAGE and fluorography (B). Molecular mass markers are indicated. (A) In vitro expression of pGEM4Z, the negative control plasmid (−; lane 1), pGEM5F (lane 2), and pGEM3F (lane 3). (B) HeLa cell binding activity of in vitro-expressed pGEM5F (lane 1) and pGEM3F (lane 2). (C) Recombinant baculoviral vectors expressing each of the full-length fiber proteins (Fig. 1) were prepared as described in Materials and Methods. Sf21 insect cells were infected with each of the recombinant baculoviral vectors, and the cells were harvested 3 to 4 days postinfection. An aliquot of each of the Sf21 cell lysates (CL) was subjected to SDS–4 to 15% PAGE and Western analysis. The membrane was developed with the antifiber monoclonal antibody 4D2-5 and an anti-mouse IgG horseradish peroxidase-conjugated antibody by chemiluminescence. The positions of molecular mass markers are indicated. Lane 1, uninfected (−) Sf21 CL (20 μg of protein); lane 2, 5F/CL (4 μg); lane 3, 3F/CL (9 μg); lane 4, 5TS3H/CL (9 μg); lane 5, 3TS5H/CL (4 μg).

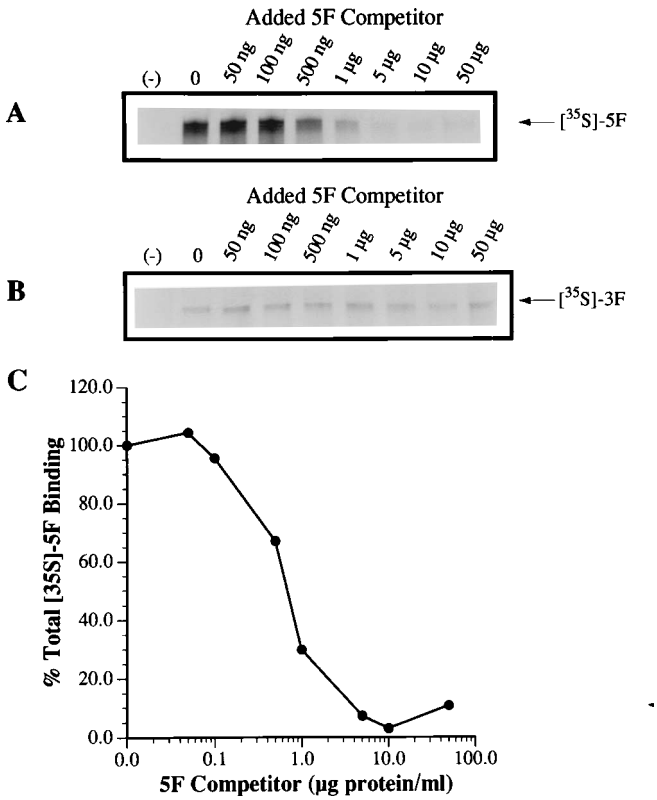

FIG. 3. Competition of labeled 5F and 3F binding to HeLa cells with the purified 5F protein. The pGEM5F or pGEM3F transcription/translation reaction mixtures were mixed with increasing amounts of purified 5F protein, from 50 ng/ml to 50 μg/ml. The labeled cell-bound proteins were analyzed by SDS–4 to 15% PAGE and fluorography. The negative control (−) was the pGEM4Z plasmid translation mixture applied to HeLa cell monolayers. (A) ^{35}S-5F binding in the presence of added 5F competitor. (B) ^{35}S-3F binding in the presence of added 5F competitor. (C) The ^{35}S-5F band intensity from each lane in panel A was quantitated with a phosphoimaging system and expressed as a percentage of the total binding in the absence of competitor. The percent ^{35}S-5F binding at each concentration of 5F competitor is plotted. Each point is the average of two determinations.

state of the fiber would remain intact (19). The proteins were transferred to a nitrocellulose membrane with a minitransblot apparatus (Bio-Rad, Hercules, Calif.) for 30 min at 100 V. After the transfer was completed, the nitrocellulose membrane was transiently stained with Ponceau red and the molecular weight standards were marked directly on the membrane. The molecular mass standards used ranged from 200 to 14 kDa (Bio-Rad). The membrane was blocked for at least 1 h at room temperature in 10 mM Tris (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 0.04% Tween 20, and 5% dried milk. The membrane was incubated for 1 h with a 1:10,000 dilution of the primary anti-Ad2 fiber monoclonal antibody 4D2-5 (ascites was kindly provided by J. Engler, University of Alabama). The membrane was then developed with a 1:10,000 dilution of the secondary goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase-conjugated antibody (Amersham Lifesciences, Arlington Heights, Ill.), using an enhanced chemiluminescence system (Amersham Lifesciences). The membrane was exposed to film for approximately 1 to 10 s.

The Ad5 fiber protein was purified from the infected Sf21 cell lysates by a modification of the procedure of Devaux et al. (6). The Sf21 cell lysate containing the Ad5 fiber was applied to a DEAE-Sepharose column equilibrated with 20 mM Tris, pH 8.0, containing 2 mM EDTA. The bound fiber protein was eluted with the same buffer containing 70 mM NaCl. The fiber-containing fractions were pooled and concentrated with a Centriprep 100-kDa molecular mass cutoff membrane concentrator (Amicon, Beverly, Mass.). The Ad5 fiber trimer was of approximately 90% or greater purity.

Transcription and translation of fiber constructs. The [^{35}S]methionine-labeled fiber proteins (either Ad5 or Ad3 fibers) were produced in vitro with the T7 coupled transcription/translation reticulocyte lysate system (Promega). A 1-μg aliquot of plasmid DNA (either pGEM5F or pGEM3F) was incubated with the system components, including 40 μCi of L-[^{35}S]methionine in a total volume of 50 μl for 20 h at 30°C (19). A 1- to 2-μl aliquot of the labeled protein mixture was analyzed by nondenaturing SDS–4 to 15% PAGE and fluorography with Amplify (Amersham Lifesciences). The gels were stained in Coomassie blue R-250 for fixation and were incubated in the Amplify solution for 30 min. The gels were dried and were exposed to film (Kodak) at −70°C for 1 to 7 days.

Fiber receptor binding assay. The HeLa cell binding assay was carried out with either labeled Ad5 (^{35}S-5F) or Ad3 (^{35}S-3F) fiber proteins produced in vitro by a modification of the procedure of Leone et al. as described for the reovirus sigma protein (15). The medium was removed from confluent HeLa cell monolayers in six-well plates, and the nonspecific sites were blocked with 10 mg of BSA (Sigma, St. Louis, Mo.) per ml in phosphate-buffered saline (PBS) for 10 min at room temperature. The blocking solution was removed and the ^{35}S-labeled protein mixture was applied to each well. A 50-μl aliquot of the transcription/translation reticulocyte lysate reaction mixture was diluted to 1 ml with PBS containing 10 mg of BSA per ml and 2.5 mM methionine and was then added to each well. The plates were incubated at room temperature for 1 h with intermittent rocking. The reaction mixture was removed, and the monolayers were washed extensively with PBS. The cells were then gently scraped off the dish. The cells were lysed in 100 μl of a 10 mM Tris (pH 7.4) buffer containing 1% Triton X-100, 2 mM EDTA, 2 μg of aprotinin per ml, and 100 mM PefablocSC. The cell-associated ^{35}S-labeled proteins were analyzed by nondenaturing SDS–4 to 15% PAGE and fluorography.

Competition assays were set up such that 50 μl of the transcription/translation reticulocyte lysate reaction mixture was mixed with increasing amounts of competitor protein ranging from 50 ng/ml to 1,000 μg/ml. For each assay, all competitor concentrations were prepared in duplicate. The purified Ad5 fiber protein or Sf21 cell lysates containing fibers were used as cold competitors. The mixture was applied to HeLa cell monolayers for 1 h at room temperature, and the samples were processed as described above. The HeLa cell lysate protein was determined by the bicinchoninic acid protein assay (Pierce). To analyze the radioactively labeled bound proteins, a 30-μg aliquot of cell lysate protein was

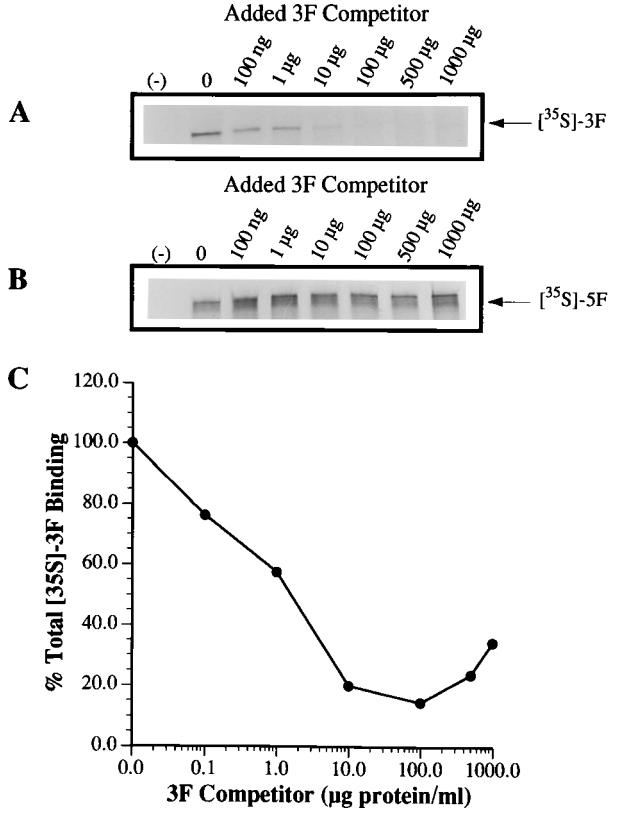

FIG. 4. Competition of labeled 5F and 3F binding to HeLa cells by the Ad3 fiber expressed in insect cells. The pGEM5F or pGEM3F transcription/translation reaction mixtures were mixed with increasing concentrations of the Ad3 fiber-containing insect cell lysates (3F/CL) from 100 ng/ml to 1,000 μg/ml. The negative control (−) was the pGEM4Z plasmid translation mixture applied to HeLa cell monolayers. (A) ^{35}S-3F binding in the presence of added 3F competitor. (B) ^{35}S-5F binding in the presence of added 3F competitor. (C) The ^{35}S-3F band intensity from each lane in panel A was quantitated with a phosphoimaging system and expressed as a percentage of the total binding in the absence of competitor. The percent ^{35}S-3F binding at each concentration of 3F competitor is plotted. Each point is the average of two determinations.

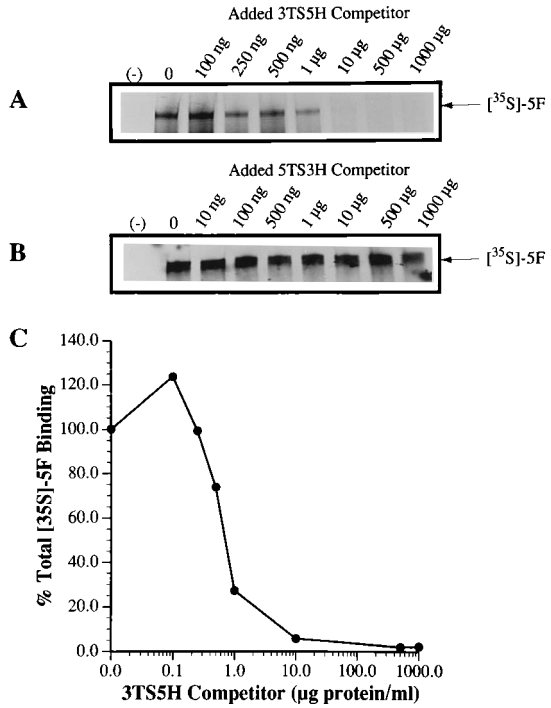

FIG. 5. Competition of labeled 5F binding to HeLa cells by the chimeric fiber proteins 3TS5H and 5TS3H. The pGEM5F transcription/translation reaction mixture was mixed with increasing concentrations of Sf21 cell lysate containing the chimeric fiber proteins 3TS5H/CL (A) or 5TS3H/CL (B). The negative control (−) was the pGEM4Z plasmid translation mixture applied to HeLa cell monolayers. (C) The ^{35}S-5F band intensity from each lane in panel A was quantitated with a phosphoimaging system and expressed as a percentage of the total binding in the absence of competitor. The percent ^{35}S-5F binding at each concentration of 3TS5H competitor is plotted. Each point is the average of two determinations.

analyzed by nondenaturing SDS–4 to 15% PAGE and fluorography. The gels were dried and exposed to film for 24 h for up to 7 days. If competition occurred, the ^{35}S-labeled band intensity from each lane was determined with a phosphoimaging system (Molecular Dynamics), and the average total ligand binding at each competitor protein concentration was determined and expressed as a percentage of the total ligand binding in the absence of competitor protein. Each competition experiment was carried out at least two to three times.

Flow cytometric fiber receptor binding assay. Cells to be tested for fiber binding, including HeLa and Chinese hamster ovary (CHO) cells, were obtained from the American Type Culture Collection and cultured in the recommended medium. A primary human diploid fibroblast (HDF) cell line was a gift of A. D. Miller, Fred Hutchinson Cancer Research Center. Cells were harvested by incubation in PBS containing 0.03% EDTA and resuspended in PBS containing 2% heat-inactivated fetal bovine serum and 0.01% sodium azide (buffer A). Approximately 10^6 cells were incubated with 1 μg of purified 5F protein or without fiber for 1 h at room temperature. After incubation, the cells were washed with 3 ml of buffer A and pelleted by centrifugation. The cells were resuspended in 20 μl of a 1:1,000 dilution of the antifiber monoclonal antibody 4D2-5 in buffer A and were incubated on ice for 30 min. After the cells were washed as described above, the cell pellet was resuspended with 20 μl of a 1:40 dilution of the secondary goat anti-mouse IgG R-phycoerythrin-conjugated antibody (Southern Biotech, Birmingham, Ala.) and was incubated on ice for 30 min. The cells were washed, pelleted, and resuspended in PBS containing 1.2% paraformaldehyde. Cell were analyzed by 488-nm excitation for log fluorescence emission at 575 nm on a Coulter Epics Elite flow cytometer. Analysis of Ad5 fiber binding to each cell line was carried out in triplicate.

RESULTS

Binding of fiber proteins synthesized in vitro to HeLa cell receptors. The Ad3 and Ad5 fiber genes were isolated from adenovirus DNA by PCR and cloned into the plasmid vector pGEM4Z for in vitro expression. A coupled transcription/translation system was used to produce [^{35}S]Met-labeled protein which was analyzed by nondenaturing SDS-PAGE and fluorography (Fig. 2A). In order to permit the detection of assembled fiber trimers, the samples were prepared for electrophoresis without boiling, which permits fiber trimers to remain associated (19). Both monomeric and trimeric forms of Ad5 fiber of approximately 62 and 186 kDa, respectively, were produced (Fig. 2A, lane 2). Similarly, Ad3 fiber oligomers of approximately 35 and 105 kDa, representing monomer and trimer, respectively, were produced (Fig. 2A, lane 3). The ^{35}S-labeled proteins produced in vitro were tested for binding to HeLa cell receptors by an assay in which the labeled translation mixture was applied directly to cell monolayers (15). Only the trimeric forms of both Ad5 and Ad3 fibers had cell binding activity (Fig. 2B, lanes 1 and 2). These results indicate that fiber trimer assembly can occur in vitro, that material which is functional in terms of receptor binding can be produced, and that the trimer is the functional form of both Ad5 and Ad3 fiber proteins which binds to HeLa cell surface receptors.

Overexpression of wild-type and chimeric fiber proteins in insect cells. In order to assess the specificity of cellular receptor binding by the two fiber proteins, the Ad5 and the Ad3 fiber genes were cloned into the baculovirus expression vector pVL1392 to produce sufficient quantities of protein for competition binding studies. We also constructed two chimeric fibers in which the head domains of the Ad5 and Ad3 fiber

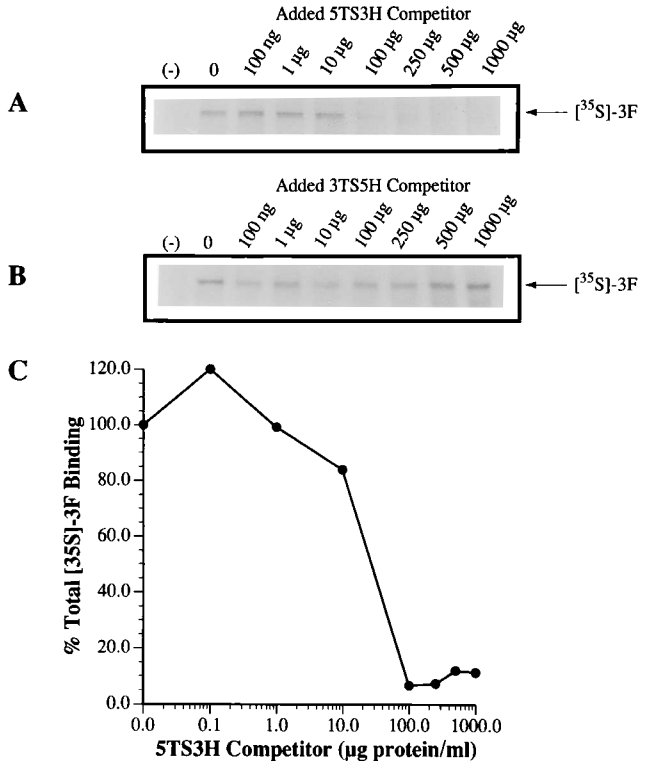

FIG. 6. Competition of labeled 3F binding to HeLa cells by the chimeric fiber proteins 3TS5H and 5TS3H. The pGEM3F transcription/translation reaction mixture was mixed with increasing concentrations of Sf21 cell lysate containing the chimeric fiber protein 5TS3H/CL (A) or 3TS5H/CL (B). The negative control (−) was the pGEM4Z plasmid translation mixture applied to HeLa cell monolayers. (C) The ^{35}S-3F band intensity from each lane in panel A was quantitated with a phosphoimaging system and expressed as a percentage of the total binding in the absence of competitor. The percent ^{35}S-3F binding at each concentration of 5TS3H competitor is plotted. Each point is the average of two determinations.

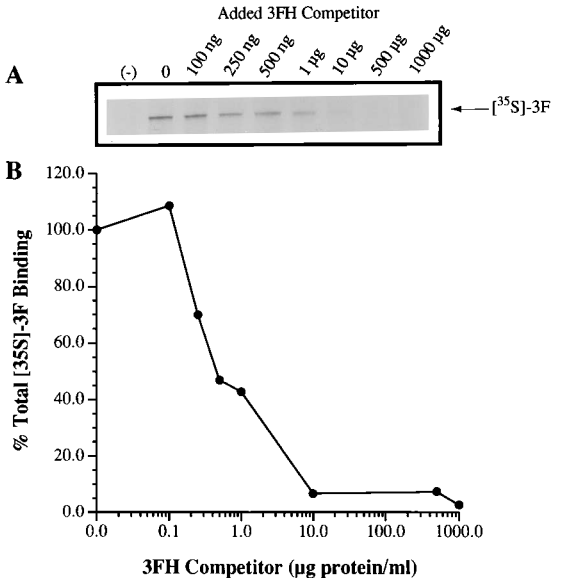

FIG. 7. Competition of labeled 3F binding to HeLa cells by the Ad3 fiber head domain (3FH/CL). The pGEM3F transcription/translation reaction mixture was mixed with increasing amounts of Sf21 cell lysate containing the Ad3 fiber head domain (3FH/CL) (A). The negative control (−) was the pGEM4Z plasmid translation mixture applied to HeLa cell monolayers. (C) The ^{35}S-3F band intensity from each lane was quantitated with a phosphoimaging system and expressed as a percentage of the total binding in the absence of competitor. The percent ^{35}S-3F binding at each concentration of 3FH competitor is plotted. Each point is the average of two determinations.

genes were exchanged by PCR fusion at the conserved TLWT sequence which marks the junction between the fiber shaft and the globular head domain (28). This produced the chimeric fibers 5TS3H and 3TS5H, as depicted in Fig. 1.

Expression of the wild-type and chimeric fiber proteins in insect cells was assessed by Western analysis of the infected Sf21 cell lysates with the antifiber monoclonal antibody 4D2-5 (Fig. 2C). The 4D2-5 antibody reacts with both native fiber trimers and SDS-denatured fiber monomer (9) and cross-reacts with both Ad5 (5F) and Ad3 (3F) fiber proteins (Fig. 2C). The Ad5 and 5TS3H fiber proteins were predominantly trimeric, while the Ad3 and 3TS5H proteins were present as both monomers and trimers. The Ad5 fiber was expressed at higher levels than the other fiber constructs, since it was readily detected by Coomassie blue R-250 staining of baculovirus-infected Sf21 cell lysates (data not shown). Since the chimeric fibers formed trimers of the expected molecular weights, we conclude that these proteins are likely to have adopted the normal conformation of the wild-type fiber polypeptide.

The Ad5 and Ad3 fibers bind to different cellular receptors. To evaluate the receptor specificities of the Ad5 and Ad3 fiber proteins, binding experiments were carried out with radiolabeled fiber and the baculovirus-expressed fiber proteins as cold competitors. Figure 3 shows the results of a binding experiment in which labeled Ad5 (^{35}S-5F) and Ad3 (^{35}S-3F) fibers were incubated with increasing amounts of the purified Ad5 fiber protein. The binding of labeled Ad5 fiber decreased with increasing amounts of competitor, with maximal competition occurring between 1 and 5 μg/ml (Fig. 3A and C). This concentration of fiber protein has previously been shown to compete for binding of intact adenovirus particles (21, 30). In contrast, as shown in Fig. 3B, the purified Ad5 fiber trimer did not compete with labeled Ad3 fiber trimer for binding to HeLa cell receptors, indicating that Ad5 and Ad3 fibers were binding to different cell surface receptors.

The unfractionated insect cell lysate containing the Ad5 fiber also competed effectively with labeled Ad5 fiber for binding, and this competition was specific, since the uninfected cell lysate did not compete (data not shown). We therefore used insect cell lysates containing expressed fibers as competitors in subsequent binding experiments.

Competition analysis of labeled Ad3 and Ad5 fibers with the Sf21 cell lysate containing the Ad3 fiber (3F/CL) is shown in Fig. 4. Binding of the labeled Ad3 fiber decreased with increasing amounts of Ad3 fiber competitor (Fig. 4A and C), indicating that the Ad3 fiber expressed in insect cells was functional. The Ad3 fiber did not compete with labeled Ad5 fiber (Fig. 4B). These results support the conclusion that the two fiber proteins bind to different sites on HeLa cells and that the receptors for Ad5 and Ad3 are distinct.

The fiber head domain determines receptor specificity. Chimeric fiber genes composed of reciprocal exchanges of the Ad5 and Ad3 fiber head domains were constructed in order to further examine the interaction of the fiber with cellular receptors. Competition analysis was carried out with both chimeric fiber proteins. As shown in Fig. 5, the binding of labeled Ad5 fiber was blocked by the chimera containing the Ad5 fiber head domain, 3TS5H (Fig. 5A and C), but not by the Ad3 fiber head-containing protein, 5TS3H (Fig. 5B). Similarly, the chimeric fiber containing the Ad3 fiber head domain blocked the binding of labeled Ad3 fiber (Fig. 6A and C), but the binding of labeled Ad3 fiber was not blocked by the chimera containing the Ad5 fiber head domain, 3TS5H (Fig. 6B). These results indicate that the specificity for cellular receptor binding is located in the C-terminal head domain of the fiber protein and that the receptor binding properties of the fiber protein can be modified by altering the head domain.

The isolated head domain of the Ad3 fiber binds to adenovirus receptors. In order to determine whether the isolated head domain of the fiber protein was capable of trimerization and receptor binding, we inserted an initiator methionine codon upstream of the coding region for the head domain. Both Ad5 and Ad3 fiber head fragments were expressed in vitro and in the baculovirus system. In vitro, only the 19.8-kDa monomer of the Ad5 fiber head was produced, whereas both the 20.8-kDa monomer and 62.4-kDa trimer of the Ad3 fiber head were found (data not shown). One repeat of the shaft region of Ad5 fiber protein may be necessary for trimerization (8). Using the HeLa cell binding assay described for Fig. 2, we found that the trimeric form of the Ad3 fiber head interacts with HeLa cell receptors, while the monomer does not (data not shown). Competition analysis with the Ad3 fiber head (3FH/CL) expressed in the baculovirus system showed that the fiber head was an effective competitor of the labeled full-length Ad3 fiber protein (Fig. 7). These results support the conclusion that the receptor binding domain of the Ad3 fiber is localized to the C-terminal head domain, as has recently been shown for both the Ad2 and Ad5 fiber proteins (8, 17).

Fiber adenovirus receptor binding. Fiber binding to the adenovirus receptor was also determined by a flow cytometric assay with the antifiber monoclonal antibody 4D2-5 and a fluorescence-labeled secondary antibody. The Ad5 fiber protein bound to HeLa cell surface receptors, as indicated by a shift in the log fluorescence intensity of the cell population (Fig. 8A). Fiber binding to CHO or HDF cells was not detected (Fig. 8B and C). The absence of detectable fiber binding to CHO and HDF cells indicates that these cells either lack or express very low levels of the Ad5 receptor.

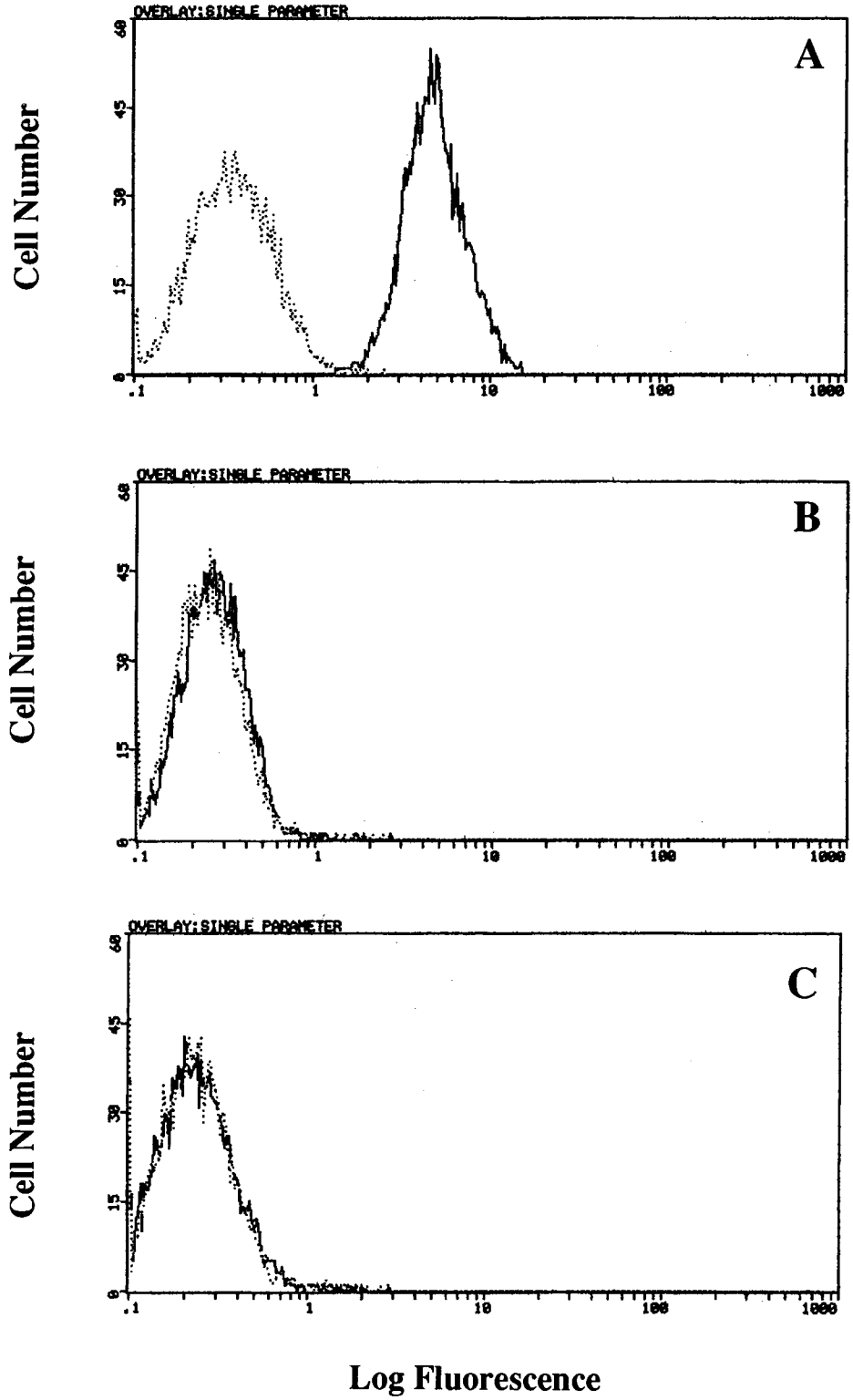

FIG. 8. Fiber binding properties of HeLa, CHO, and HDF cell lines. Approximately 10^6 HeLa (A), CHO (B), or HDF (C) cells were tested for the ability to bind the purified Ad5 fiber protein (solid line). Control samples (dotted line) were incubated without fiber. Detection of fiber binding was carried out with the antifiber monoclonal antibody 4D2-5 and a goat anti-mouse IgG R-phycoerythrin-conjugated antibody as described in Materials and Methods. For a representative binding experiment, the relative cell number is shown as a function of log fluorescence. Fiber receptor binding is indicated by a shift in the log fluorescence intensity of the cell population.

DISCUSSION

The role of the adenovirus fiber protein in mediating attachment of the virus to cellular receptors is well established (16, 21, 30). However, relatively little is known about this interaction, and the identity and nature of the cellular receptors are unknown. Recently, it has been shown that efficient internalization of the virion is mediated by an interaction between the penton base protein and members of the integrin class of cell adhesion molecules (1, 2, 18, 30). This interaction is a postbinding event which occurs after the initial attachment to cellular receptors mediated by the fiber protein. We are interested in developing a more detailed understanding of the viral and cellular components involved in the initial binding steps. In the experiments reported here, we have taken advantage of the fact that isolated fiber protein will bind to cellular receptors in the absence of other viral components. We have devised a relatively simple binding and competition assay which permits rapid assessment of the receptor binding characteristics of the fiber protein. The assay is an adaptation of the method employed by Leone et al. (15) to study the assembly and function of the reovirus sigma protein. The Ad3 and Ad5 fiber proteins were synthesized by in vitro transcription and translation, which produced a mixture of both monomers and trimers in approximately equal proportions, consistent with the findings of Novelli and Boulanger (19, 20). The labeled proteins were applied to HeLa cell monolayers and allowed to bind. The labeled material which remained cell associated was visualized by nondenaturing gel electrophoresis, which revealed that only the trimeric fiber had bound to the cells. It is not known whether each fiber trimer contains three individual receptor binding sites which bind cooperatively to multiple receptors or a single site comprising the three polypeptide chains. While our results do not distinguish between these alternative models, the data demonstrate that trimer assembly is required for functional receptor binding. Further analysis and mapping of the receptor binding site within the fiber head domain as well as the identification of the cellular receptors should shed further light on this question.

The absence of competition between recombinant Ad5 and Ad3 fibers for HeLa cell binding provides evidence for the existence of at least two distinct fiber receptors. This is consistent with the results obtained by Defer et al. (5) and Mathias et al. (18), who concluded that Ad3 and Ad2 bind to different cellular receptors, on the basis of binding experiments which showed a lack of competition between viral particles. The use of recombinant fiber proteins to measure receptor binding in this study eliminates possible artifacts due to differences in virus preparations or the contribution of other viral components such as the penton base. The relatively low degree of amino acid sequence homology between the Ad5 and Ad3 fiber proteins (37.6%) (12) is also consistent with the recognition of different cell surface receptors. Our data do not formally exclude the possibility that there is a single adenoviral receptor which contains two distinct binding sites for the Ad5 and Ad3 fiber proteins. Although this is unlikely, molecular cloning and identification of the adenovirus receptors will definitively resolve this issue.

We have also developed a flow cytometric assay which can be used to visualize the binding of Ad5 fiber to cellular receptors. Binding of the Ad5 fiber to HeLa cells was readily detectable with this assay. However, we found that CHO cells and an HDF line failed to bind the Ad5 fiber, suggesting that these cells lack the Ad5 fiber receptor. This is consistent with the observation that these cell types are very poorly infectable with an Ad5 vector containing the β-galactosidase gene (data not shown). A low level of transduction was obtained at a high multiplicity of infection, indicating that the cells are otherwise permissive. Virus entry in the absence of fiber receptor may reflect a low level of virus adsorption via the integrin pathway (30). Identification of fiber receptor-negative cell types will be of value in efforts to clone the adenovirus receptors.

As a first step towards identifying the specific determinants on the fiber protein which interact with cell surface receptors, we constructed fiber chimeras in which the head domains of the Ad3 and Ad5 fibers were exchanged. Both chimeric proteins were capable of trimer assembly when expressed in baculovirus, and competition analysis indicated that they were functional in a receptor binding assay. The specificity of the chimeras was consistent with the recognition of the Ad3 and Ad5 receptors by the fiber head domain. This conclusion was reached independently in two recent studies which showed that the isolated head domains of the Ad2 and Ad5 fibers have cell binding activity and compete for the binding of Ad2 or Ad5 virions to HeLa cells (8, 17). We show here that this is also true for the Ad3 fiber, which binds to a receptor different from that bound by Ad2 and Ad5. We have also expressed the Ad3 fiber head domain in vitro and in baculovirus and found that it is capable of trimerization and cell binding and competes with the full-length Ad3 fiber for receptor binding.

The ability to manipulate the receptor binding properties of adenovirus by modifying the fiber head domain may have practical utility in the development of adenovirus vectors for gene therapy. Modifications of viral receptor ligands have been carried out with retroviral vectors to incorporate novel receptor binding domains to target specific cell types (4, 11, 29). Kasahara et al. have recently demonstrated that a chimeric protein consisting of the polypeptide hormone erythropoietin and the retroviral envelope gp70 protein can be incorporated into retroviral particles which will specifically target and infect cells expressing the erythropoietin receptor (11). These studies demonstrate that it is possible to target viral particles to specific cell types. We have shown that it is possible to change the specificity of the adenovirus fiber protein by exchanging the head domain with another serotype which recognizes a different receptor. It should be possible, by modification or replacement of the fiber head domain with an appropriate ligand, to construct novel fiber proteins that interact with cellular receptors which have a defined and restricted pattern of expression on target cells or tissues. This may permit the targeting of adenovirus vectors to the specific cell type in which expression of the therapeutic gene product is required.

ACKNOWLEDGMENTS

We thank Paul Tolstoshev for critically reviewing the manuscript and J. A. Engler at the University of Alabama for providing the antifiber monoclonal antibody 4D2-5.

REFERENCES

1. **Bai, M., L. Campisi, and P. Freimuth.** 1994. Vitronectin receptor antibodies inhibit infection of HeLa and A549 cells by adenovirus type 12 but not by adenovirus type 2. J. Virol. **68:**5925–5932.
2. **Bai, M., B. Harfe, and P. Freimuth.** 1993. Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. J. Virol. **67:**5198–5205.
3. **Chroboczek, J., and B. Jacrot.** 1987. The sequence of adenovirus fiber: similarities and differences between serotypes 2 and 5. Virology **161:**549–554.
4. **Chu, T. T., I. Martinez, W. C. Sheay, and R. Dornburg.** 1994. Cell targeting with retroviral vector particles containing antibody-envelope fusion particles. Gene Ther. **1:**292–299.
5. **Defer, C., M. Belin, M. Caillet-Boudin, and P. Boulanger.** 1990. Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. J. Virol. **64:**3661–3673.
6. **Devaux, C., M. Adrian, C. Berthet-Colominas, S. Cusack, and B. Jacrot.**

1990. Structure of adenovirus fiber. I. Analysis of crystals from adenovirus serotypes 2 and 5 by electron microscopy and X-ray crystallography. J. Mol. Biol. **215:**567–588.
7. **Green, N. M., N. G. Wrigley, W. G. Russell, S. R. Martin, and A. S. McLachlan.** 1983. Evidence for a repeating cross-β sheet structure in the adenovirus fibre. EMBO J. **2:**1357–1365.
8. **Henry, L. J., D. Xia, M. E. Wilke, J. Deisenhofer, and R. D. Gerard.** 1994. Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. J. Virol. **68:**5239–5246.
9. **Hong, J. S., and J. A. Engler.** 1991. The amino terminus of the adenovirus fiber protein encodes the nuclear localization signal. Virology **185:**758–767.
10. **Horton, R. M., Z. Cai, S. N. Ho, and L. Pease.** 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques **8:**528–535.
11. **Kasahara, N., A. M. Dozy, and Y. W. Kan.** 1994. Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. Science **266:**1373–1376.
12. **Kidd, A. H., J. Chroboczek, S. Cusack, and R. W. H. Ruigrok.** 1993. Adenovirus type 40 virions contain two distinct fibers. Virology **192:**73–84.
13. **Kidd, A. H., and M. J. Erasmus.** 1989. Sequence characterization of the adenovirus 40 fiber gene. Virology **172:**134–144.
14. **Kidd, A. H., M. J. Erasmus, and C. T. Tiemessen.** 1990. Fiber sequence heterogeneity in subgroup F adenoviruses. Virology **179:**139–150.
15. **Leone, G., L. Maybaum, and P. W. K. Lee.** 1992. The reovirus cell attachment protein possesses two independently active trimerization domains: basis of dominant negative effects. Cell **71:**479–488.
16. **Longberg-Holm, K., R. L. Crowell, and L. Philipson.** 1976. Unrelated animal viruses share receptors. Nature (London) **259:**679–681.
17. **Louis, N., P. Fender, A. Barge, P. Kitts, and J. Chroboczek.** 1994. Cell-binding domain of adenovirus serotype 2 fiber. J. Virol. **68:**4104–4106.
18. **Mathias, P., T. Wickham, M. Moore, and G. Nemerow.** 1994. Multiple adenovirus serotypes use αV integrins for infection. J. Virol. **68:**6811–6814.
19. **Novelli, A., and P. A. Boulanger.** 1991. Assembly of adenovirus type 2 fiber synthesized in cell-free translation system. J. Biol. Chem. **266:**9299–9303.
20. **Novelli, A., and P. A. Boulanger.** 1991. Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fiber. Virology **185:**365–376.
21. **Philipson, L., K. Longberg-Holm, and U. Petterson.** 1968. Virus-receptor interaction in an adenovirus system. J. Virol. **2:**1064–1075.
22. **Pieniazek, N. J., S. B. Slemenda, D. Pieniazek, J. Velarde, and R. B. Luftig.** 1989. Sequence of human enteric adenovirus type 41 Tak fiber protein gene. Nucleic Acids Res. **17:**9474.
23. **Pieniazek, N. J., S. B. Slemenda, D. Pieniazek, J. Velarde, and R. B. Luftig.** 1990. Human enteric adenovirus type 41 (Tak) contains a second fiber protein gene. Nucleic Acids Res. **18:**1901.
24. **Signäs, C., G. Akusjärvi, and U. Pettersson.** 1985. Adenovirus 3 fiber polypeptide gene: implications for the structure of the fiber protein. J. Virol. **53:**672–678.
25. **Sprengel, J., B. Schmitz, D. Heuss-Neitzel, C. Zock, and W. Doerfler.** 1994. Nucleotide sequence of human adenovirus type 12 DNA: comparative functional analysis. J. Virol. **68:**379–389.
26. **Stewart, P. L., R. M. Burnett, M. Cyrklaff, and S. D. Fuller.** 1991. Image reconstruction reveals the complex molecular organization of adenovirus. Cell **67:**145–154.
27. **Stewart, P. L., S. D. Fuller, and R. M. Burnett.** 1993. Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. EMBO J. **12:**2589–2599.
28. **Stouten, P. F. W., C. Sander, R. W. H. Ruigrok, and S. Cusack.** 1992. New triple helical model for the shaft of the adenovirus fibre. J. Mol. Biol. **226:**1073–1084.
29. **Valsesia-Wittmann, S., A. Drynda, G. Deleage, M. Aumailley, J. M. Heard, O. Danos, G. Verdier, and F. L. Cosset.** 1994. Modifications in the binding domain of avian retrovirus envelope protein to redirect the host range of retroviral vectors. J. Virol. **68:**4609–4619.
30. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrins αvβ3 and αvβ5 promote adenovirus interalization but not virus attachment. Cell **73:**309–319.