

A 50-kDa Membrane Protein Mediates Sialic Acid-Independent Binding and Infection of Conjunctival Cells by Adenovirus Type 37

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The ocular tropism of adenovirus type 37 (Ad37) does not correlate with the wide distribution of the 46-kDa coxsackievirus and adenovirus receptor (CAR), the major receptor for most adenovirus serotypes. We previously found that Ad37 infects and binds well to conjunctival cells (Chang C), but poorly to lung epithelial (A549) cells that express CAR and hypothesized that this serotype uses a distinct receptor that is selectively expressed on conjunctival cells. To test this, we produced particles of a fiber-deleted Ad5 vector containing the Ad37 fiber protein. The "pseudotyped" vector infected Chang C cells better than A549 cells using a CAR-independent pathway. Ad37 binding was calcium-dependent and was abolished by protease digestion of cell surface proteins. Using a virus overlay protein blot assay (VOPBA), we detected calcium-dependent Ad37 binding to 50- and 60-kDa membrane proteins on permissive Chang C cells. In contrast, calcium-dependent binding was detected with only the 60-kDa protein on nonpermissive A549 cells. Ad19p, a closely related serotype that failed to bind to conjunctival cells, recognized the 60-kDa, but not the 50-kDa, protein. Ad37 has been reported to use sialic acid instead of CAR as a cell receptor on A549 cells. Pretreatment of Chang C cells with neuraminidase abolished Ad37 binding to only the 60-kDa protein, suggesting that sialic acid mediates Ad37 binding to the 60-kDa protein. The pseudotyped Ad37 vector was also able to infect neuraminidase-treated Chang C cells. Thus, subgroup D adenoviral binding to the 50-kDa protein is calcium-dependent and cell type- and serotype-specific, whereas binding to the 60-kDa protein is not necessary for infection of conjunctival cells. Together, these data suggest that the 50-kDa protein is the major receptor for Ad37 on conjunctival cells. © 2001 Academic Press

INTRODUCTION

Several members of adenovirus subgroup D, Ad8, 19a, and 37, are infectious agents that cause particularly severe cases of epidemic keratoconjunctivitis (EKC) (Arnberg *et al.*, 1997; Curtis *et al.*, 1998; Ritterband *et al.*, 1998; Takeuchi *et al.*, 1999). At the present time, there is no effective treatment for this debilitating and contagious disease and EKC continues to be a problem in ophthalmology clinics worldwide (Curtis *et al.*, 1998; Lukashok *et al.*, 1998). Characterization of the receptors that govern the tropisms of these adenovirus serotypes will aid the development of therapeutic measures against adenovirus-associated EKC.

The adenovirus fiber protein is a major determinant of adenovirus tropism (Gall *et al.*, 1996; Stevenson *et al.*, 1997). The fiber protein extends from the capsid and mediates viral binding to the cell surface by binding to cell surface receptors (Philipson *et al.*, 1968). The fiber protein consists of an N-terminal tail domain that interacts with the adenovirus penton base, a central shaft domain of varying length, and a C-terminal knob domain

that contains the cell receptor binding site (Ruigrok *et al.*, 1990; Stevenson *et al.*, 1995; Chroboczek *et al.*, 1995). Fiber proteins of all adenovirus subgroups, except subgroup B, have been shown to bind to the 46-kDa coxsackievirus-adenovirus receptor (CAR) (Bergelson *et al.*, 1997; Roelvink *et al.*, 1998). Northern blot analysis showed that CAR is expressed in a variety of human tissues, including the lung, at various levels (Bergelson *et al.*, 1997), but the unusual tropism of Ad37 for the eye and its poor binding to lung epithelial cells (Huang *et al.*, 1999) suggest that the tropism of this serotype may be influenced by host factors independent of CAR expression.

Structural and biochemical data also suggest that Ad37 uses a distinct receptor. Mutagenesis of the Ad5 fiber knob (Roelvink *et al.*, 1999) and crystallographic studies of the Ad12 fiber knob (Bewley *et al.*, 1999) located the CAR binding site at and around the loop joining β -strands A and B (AB-loop) on the side of the adenovirus fiber knob. We recently reported that a lysine residue at position 240 of the Ad37 fiber, located in the C-terminal half of the CD-loop, is important for receptor binding (Huang *et al.*, 1999). The C-terminal half of the CD-loop is oriented toward the top of the knob, while the AB-loop faces the side of the knob. The cocrystal structure of the Ad12 knob and the N-terminal domain of CAR (Bewley *et al.*, 1999) shows that the distal region of the

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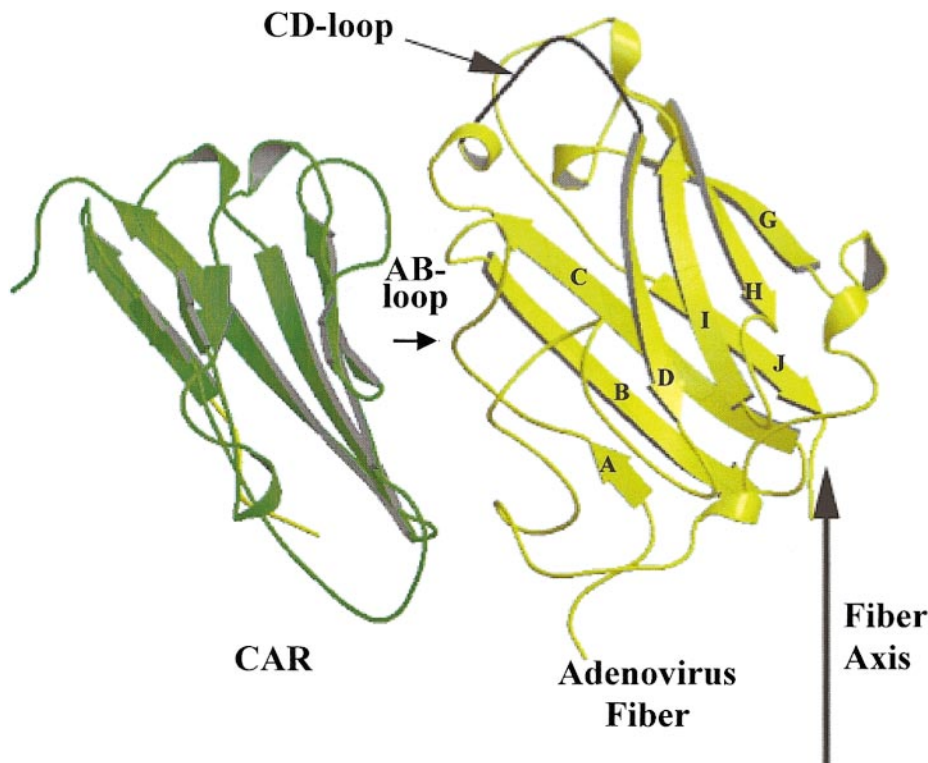


FIG. 1. Receptor interactions with distinct regions of the adenovirus fiber knob. The Ad12 knob in complex with the extracellular domain of CAR (CAR D1), viewed from the side of the knob, is shown (Bewley *et al.*, 1999). The axis of the fiber shaft is shown by a black arrow. The β -strands of the fiber knob are labeled in bold (Xia *et al.*, 1994). The CAR-binding site has been localized to and around the AB-loop of the fiber knob. The C-terminal half of the CD-loop proposed for Ad37 receptor interaction, in solid black, lies in a distinct region on the fiber knob. This image was generated using Molscript (Kraulis, 1991) and Raster3d (Merritt *et al.*, 1997) from coordinates obtained from the crystal structure of the Ad12 knob in complex with CAR D1 (Bewley *et al.*, 1999). Only one monomer of Ad12 knob and one molecule of CAR D1 are shown.

CD-loop does not contact CAR (Fig. 1). The separate receptor-binding sites suggest the use of distinct receptors by Ad5 and Ad37.

Arnberg *et al.* (2000) recently reported that Ad37 does not use CAR, but instead uses a glycoprotein that contains sialic acid as its primary receptor on human lung epithelial cells and Chinese hamster ovary cells. A previous study, however, showed that Ad37 binds and infects conjunctival cells well, but lung epithelial cells poorly (Huang *et al.*, 1999), suggesting that Ad37 uses an alternate receptor on conjunctival cells. In this study, we report the characterization of a membrane protein receptor for Ad37 that is expressed on conjunctival cells, but not lung epithelial cells. We also provide evidence that sialic acid on a separate glycoprotein supports Ad37 binding, but is not the major receptor on conjunctival cells.

RESULTS

Comparison of adenovirus infection of human conjunctival and lung epithelial cells with virus particles retargeted with Ad5 or Ad37 fiber proteins

We recently described a method for the production of "pseudotyped" Ad5 vector particles containing fiber pro-

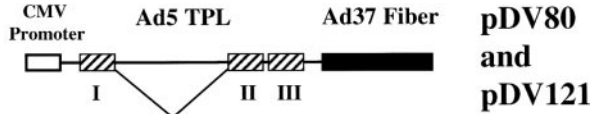
teins from a different adenovirus serotype (Von Seggern *et al.*, 2000). This system was used to assess receptor usage by particles containing the Ad37 fiber.

We first generated packaging cell lines producing the Ad37 fiber protein. The N-terminal amino acid sequences of the Ad5 and Ad37 fiber proteins differ significantly, and to ensure that the Ad37 fiber would be efficiently incorporated into Ad5 vector particles, we mutated several residues in the wild-type Ad37 fiber to more closely match the Ad5 sequence (Fig. 2A). Stable cell lines producing this fiber under control of the CMV promoter and the adenovirus type 5 tripartite leader (Fig. 2B) were then generated as previously described (Von Seggern *et al.*, 2000) and screened for fiber expression by indirect immunofluorescence. One clone (line 705) which expressed the Ad37 fiber at a high level (data not shown) was selected for further study. Cells from line 633, which expresses the wild-type Ad5 fiber protein (Von Seggern *et al.*, 2000), and line 705 were infected with a fiber-deleted Ad5 vector (Ad5. β gal. Δ F/0) carrying a β -galactosidase reporter gene. The resulting vector particles contained the Ad5 fiber protein (Ad5. β gal. Δ F/5F) or the Ad37 fiber protein (Ad5. β gal. Δ F/37F). Incorporation of the correct fiber protein into viral particles was verified by western blotting (Fig. 2C). Preparations of vectors containing

A

Ad37 MSKRLRVEDDFNPVYPY
 ↓ ↓ ↓ ↓ ↓
 KRARPS
 Ad5 MKRARPSEDTFNPVYPY

B



C

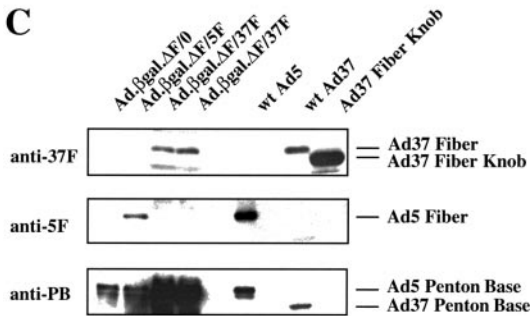


FIG. 2. Production of adenovirus particles pseudotyped with Ad5 or Ad37 fiber proteins. Construction of cell lines expressing Ad5 fiber was previously described (Von Seggern *et al.*, 1998, 2000). (A) Site-directed mutations made to the Ad37 fiber gene make the tail sequence more closely match that of Ad5 and facilitate Ad37 fiber binding to the Ad5 penton base. (B) The plasmid for the expression of the Ad37 fiber protein, pDV80 and pDV121 contains the CMV promoter, the adenovirus type 5 tripartite leader (TPL), and the modified Ad37 fiber gene sequence. pDV80 was electroporated into E1-2a S8 cells and stable lines were selected while pDV121 was transiently transfected into 293T cells as described under Materials and Methods. (C) The fiber-deleted vector Ad5.βgal.ΔF was grown in 293 cells, which does not express fiber (Ad5.βgal.ΔF/0), in cells from line 633, which express the Ad5 fiber (Ad5.βgal.ΔF/5F), or in cells from line 705, which express the Ad37 fiber (Ad5.βgal.ΔF/37F) and was CsCl-purified. A 10-μg sample of the purified particles was electrophoresed and transferred to a nylon membrane. As controls, 10 μg of wild-type Ad37 or the fiber gene-containing vector Ad5.βgal.wt or a sample of purified recombinant Ad37 fiber knob was also run. The blot was probed with polyclonal antisera against recombinant Ad2 fiber protein, which cross-reacts with the Ad5 fiber protein, or against recombinant Ad37 fiber protein. As a loading control, the same filter was reprobed with an antibody against the Ad2 penton base, which cross-reacts with the Ad5 and Ad37 penton base proteins. Ad5.GFP.ΔF/5F and Ad5.GFP.ΔF/37F vectors were grown and purified in a similar fashion.

a GFP reporter gene (Ad5.GFP.ΔF/0, Ad5.GFP.ΔF/5F, and Ad5.GFP.ΔF/37F) were created in the same fashion. Ad37 binds and infects lung epithelial (A549) and embryonic kidney cells (293) poorly compared to conjunctival (Chang C) cells (Huang *et al.*, 1999), so plaque assays of the Ad37 retargeted vector were not performed on 293 cells or A549-derived cells. Because Chang C cells do not contain the E1 gene, the E1-deleted vectors do not form plaques on Chang C cells. Typical particle to

plaque-forming unit (PFU) ratios for preparations of similar pseudotyped vectors, as assayed by infection of 293 cells, are approximately 1000 to 5000 (Von Seggern *et al.*, 2000). Two point mutations in the Ad37 fiber gene of line 705, S356 → P356 and I362 → T362, were discovered by DNA sequencing. Although the mutations are not located at known receptor binding regions and should be buried in the knob trimer interface, we recloned the Ad37 fiber, with the correct sequence, into an expression plasmid to confirm that these mutations did not alter phenotype. 293T cells were transfected with the virus and subsequently infected with Ad5.GFP.ΔF to produce Ad37 pseudotyped virus. Ad5.GFP.ΔF/37F experiments shown were performed with Ad37 pseudotyped virus produced from line 705. Infection trends are identical with the pseudotyped virus from transiently transfected 293T cells.

We next examined infection of different cell types using the retargeted adenovirus particles. As expected for adenovirus particles lacking fibers, 10,000 particles per cell Ad5.GFP.ΔF/0 infected less than 1% of human conjunctival cells (data not shown). Ad5.GFP.ΔF/5F exhibited good gene delivery to both A549 and Chang C cells (Fig. 3A). In contrast, Ad5.GFP.ΔF/37F infected Chang C cells efficiently, but A549 cells very poorly. These data are in good agreement with previous cell attachment studies using ¹²⁵I-labeled wild-type Ad37 and Ad2 (Huang *et al.*, 1999). Although CAR is expressed on the surface of A549 cells, as indicated by Ad5.GFP.ΔF/5F infection, Ad5.GFP.ΔF/37F was unable to infect these cells efficiently. Thus, the Ad37 fiber protein confers preferential infection on human conjunctival cells, but not CAR-expressing human lung epithelial cells, suggesting that the Ad37 fiber protein does not bind to CAR.

Recent studies reported that expression of CAR on the surface of chinese hamster ovary (CHO) cells did not improve Ad37 binding (Arnberg *et al.*, 2000), also suggesting that Ad37 does not use CAR. To verify whether this was the case for human conjunctival cells, we pre-treated A549 and Chang C cells with RmcB (Hsu *et al.*, 1988), a function-blocking monoclonal antibody against CAR (Fig. 3B). As expected, the RmcB antibody inhibited infection of A549 cells by Ad5.GFP.ΔF/5F, but it had little effect on infection of Chang C cells by Ad5.GFP.ΔF/37F, confirming that the Ad37 fiber protein does not bind to CAR on Chang C conjunctival cells. Heparin, an inhibitor of Ad2 and Ad5 binding to heparin sulfate glycosaminoglycans (Dechecchi *et al.*, 2000), also did not inhibit Ad5.GFP.ΔF/37F infection (data not shown).

Ad37 binding to conjunctival cells requires divalent cations

Adenoviruses require the presence of either calcium or magnesium ions for internalization (Wickham *et al.*, 1993), but little is known about the roles divalent cations

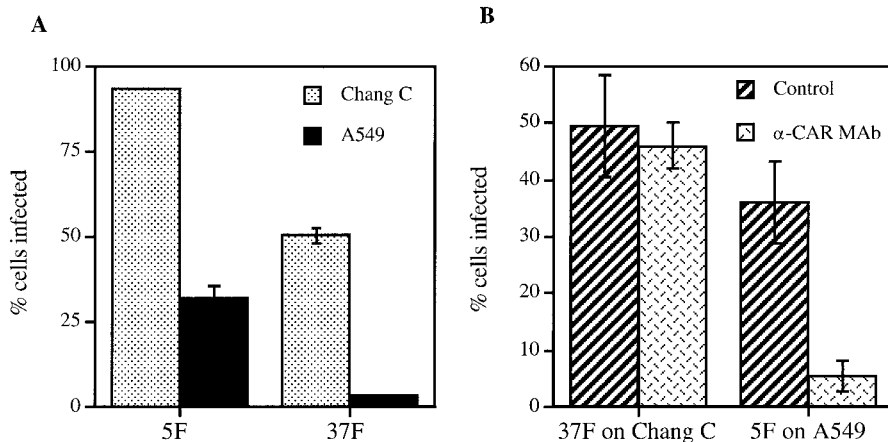


FIG. 3. Infection of A549 and Chang C by pseudotyped adenovirus vectors. (A) A549 human lung carcinoma cells or Chang C human conjunctival cells were infected with Ad5.GFP. Δ F/5F (/5F) or Ad5.GFP. Δ F/37F (/37F), both carrying a GFP reporter gene, at 10,000 particles per cell. (B) To determine the contribution of CAR binding to infection, A549 and Chang C cells were pretreated with 180 μ g/ml RmcB, a function-blocking anti-CAR antibody, then infected with Ad5.GFP. Δ F/5F and Ad5.GFP. Δ F/37F, respectively. Infection was determined at 24 h by calculating the percentage of cells detected above a threshold set by the fluorescence of uninfected Chang C cells. Errors are represented as the standard deviation of three experiments. Errors of Ad5.GFP. Δ F/37F infection on A549 and Ad5.GFP. Δ F/5F infection on Chang C were too small to show on the graph.

play in adenovirus binding to conjunctival cells. To investigate the potential role of divalent cations in Ad37 receptor binding, 125 I-labeled Ad37 binding to Chang C cells was examined in the absence or presence of EDTA. EDTA inhibited Ad37 binding to conjunctival cells (Fig. 4A), suggesting a requirement for divalent metals for Ad37 binding.

The metal-dependent binding could be attributed to either fiber-receptor interaction and/or penton base-integrin interaction. However, lung epithelial A549 cells express abundant α_v -integrins (Mathias *et al.*, 1998) but were unable to support efficient binding of Ad37 (Huang *et al.*, 1999). Moreover, recombinant Ad37 fiber knob can

inhibit >95% of Ad37 binding to Chang C conjunctival cells (Huang *et al.*, 1999), indicating that the fiber protein, but not the penton base, mediates Ad37 virus binding. Thus, Ad37 primarily binds to conjunctival cells using the fiber protein, not the penton base, and this binding is likely cation-dependent.

To further test the potential role of divalent cations in Ad37 cell attachment, 125 I-labeled Ad37 binding to Chang C cells was measured in the presence of varying concentrations of calcium or magnesium chloride. Magnesium ions had little effect on Ad37 binding to Chang C cells. In contrast, calcium ions dramatically enhanced Ad37 binding to Chang C cells (Fig. 4B). The optimal

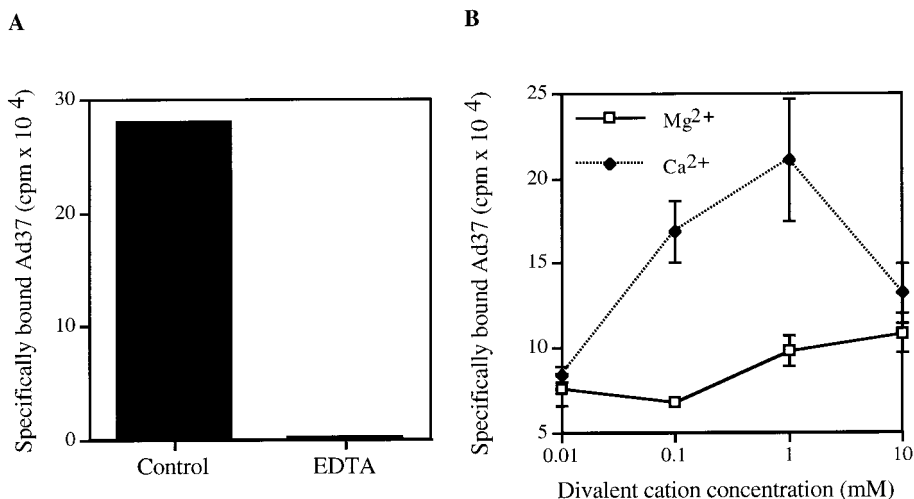


FIG. 4. Ad37 binding to conjunctival cells is calcium-dependent. Specific 125 I-labeled Ad37 binding to Chang C cells was measured in the presence of 10 mM EDTA (A) and in the presence of varying concentrations of calcium chloride or magnesium chloride (B). Specific binding was determined by subtracting the nonspecific counts in the presence of 100-fold excess unlabeled virus from the total counts. Results shown are the averages of two experiments. Errors shown are the average deviations of two experiments.

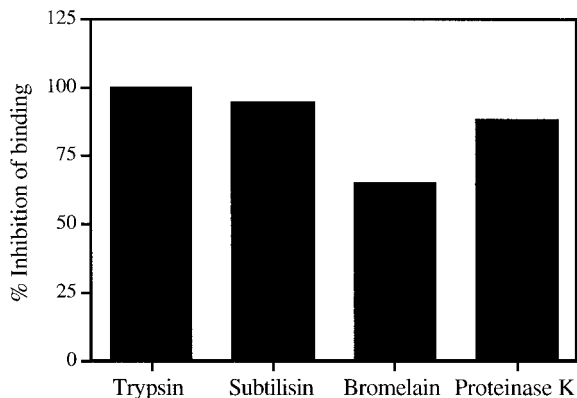


FIG. 5. Pretreatment of conjunctival cells with proteases inhibits Ad37 binding. Chang C cells were pretreated with various proteases for 1 h before binding ^{125}I -labeled Ad37 to the cells. Nonspecific binding was measured by adding 100-fold unlabeled Ad37 to cells with ^{125}I -labeled Ad37 and subtracting from total counts for specific binding. Percentage inhibition represents the difference in specific binding of untreated cells and pretreated cells as a percentage of the specific binding of untreated cells.

concentration of calcium chloride for Ad37 binding was 1 mM, while higher concentrations of calcium actually decreased virus binding to cells. The reduction in viral binding at high calcium concentrations suggests regulation by multiple calcium binding sites on the Ad37 fiber or its receptor. The precise role of calcium in Ad37 receptor binding has not yet been determined.

Wild-type Ad37 particles bind to three conjunctival membrane proteins

Recent studies reported that protease treatment of sialic acid positive CHO cells abolished Ad37 binding (Arnberg *et al.*, 2000), implying that Ad37 bound to a sialoconjugate protein receptor on CHO cells. To determine whether the Ad37 binding site on human conjunctival cells is also a protein, we treated Chang C cells with different proteases prior to measuring binding of ^{125}I -labeled Ad37. Digestion of surface proteins by all four proteases inhibited Ad37 binding to Chang C cells by more than 50% (Fig. 5), demonstrating that Ad37 binds to a protein receptor on Chang C cells.

Virus overlay protein blot assays (VOPBAs) have been effectively used to characterize candidate viral protein receptors (Borrow *et al.*, 1992; Cao *et al.*, 1998), including receptors for another adenovirus serotype (Tarassishin *et al.*, 1997). This modified western blot technique uses intact viral particles in place of antibodies to probe viral-receptor interactions. We used the VOPBA to identify Chang C membrane proteins that bind to Ad37 (Fig. 6). In the absence of Ad37 particles, no signal was observed (lanes 1 and 3), while addition of virus in the absence of calcium (lane 2) revealed binding to a single 45-kDa protein (Fig. 6A). In the presence of 1 mM calcium chloride, Ad37 bound to three proteins with approximate

molecular weights of 45, 50, and 60 kDa (Fig. 6A). The same three proteins were detected using a recombinant Ad37 fiber knob alone (Fig. 6B), indicating that Ad37-receptor interactions are fiber mediated and do not require interactions of other capsid proteins such as the penton base. The size of the calcium-independent protein (45 kDa) is very similar to the known molecular weight of CAR. A direct comparison of the Ad37 VOPBA and a CAR western blot showed that the 45-kDa protein comigrates with CAR on SDS-PAGE (data not shown).

To determine whether the 50- or 60-kDa protein is the receptor for Ad37 on conjunctival cells, we examined the serotype- and cell type-dependence of adenovirus binding to these proteins. A comparison of Ad37 and the closely related Ad19p fiber knob sequences shows that only two residues (K^{240} and N^{340} in Ad37 and E^{240} and D^{340} in Ad19p) are different (Arnberg *et al.*, 1997). The mutation of K^{240}E in the Ad37 fiber abolishes Ad37 binding to Chang C cells, whereas the reverse mutation in the Ad19p fiber confers Ad19p binding to Chang C (Huang *et al.*, 1999). Wild-type Ad19p particles, which do not efficiently bind to Chang C cells (Huang *et al.*, 1999), bound to the 45- and 60-kDa proteins in the VOPBA, but did not bind to the 50-kDa protein (Fig. 7, lane 4), suggesting that the single K^{240} to E^{240} alteration prevents Ad19p binding to the 50-kDa protein. Moreover, the 50-kDa receptor is expressed on Chang C cells, but not A549 cells, which

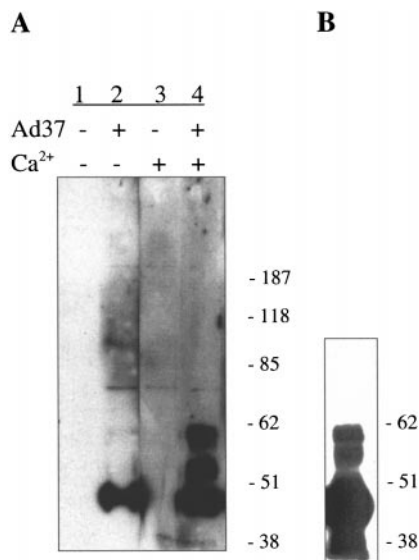


FIG. 6. VOPBA of human conjunctival membrane proteins probed with Ad37 in the presence of EDTA or calcium chloride. (A) Chang C membrane fractions were separated by 8% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was subsequently probed with (lanes 2 and 4) or without (lanes 1 and 3) whole Ad37 particles, a polyclonal antibody against Ad37 fiber, and finally a horseradish peroxidase conjugated anti-rabbit antibody, in the presence of EDTA (lanes 1 and 2) or calcium chloride (lanes 3 and 4). (B) Transferred Chang C membrane proteins were probed with recombinant Ad37 knob protein, instead of Ad37 knob, in the presence of calcium chloride.

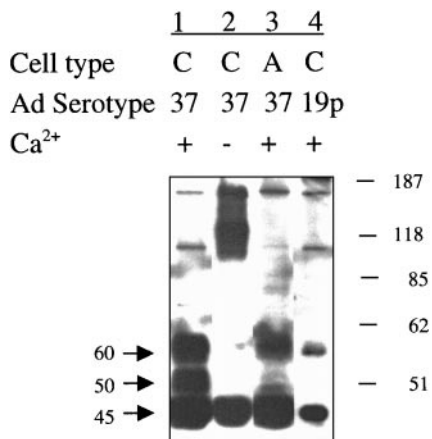


FIG. 7. VOPBA of different adenovirus serotypes reacted with A549 or Chang C cells. A549 (lane 3) or Chang C (lanes 1, 2, and 4) membrane fractions were separated by 8% SDS-PAGE and transferred to a PDVF membrane. The Chang C membrane fraction was probed with Ad37 in the presence of calcium chloride (lane 1) or EDTA (lane 2) or with Ad19p in the presence of calcium chloride (lane 4). The A549 membrane fraction was also probed with Ad37 in the presence of calcium chloride (lane 3). C = Chang C membrane fraction, A = A549 membrane fraction. The upper molecular weight bands in lane 2 have not been observed in other VOPBAs, including the VOPBA in Fig. 6A. The anti-Ad37 fiber antibody may not recognize the Ad19p fiber as well as the Ad37 fiber, resulting in a weaker signal in lane 4. No signal was observed for the 50-kDa band in lane 4, even on a much longer exposure.

support only low levels of Ad37 binding and infection (lane 3). VOPBA analysis of other human conjunctival cells (S. Ward, Gillette) confirmed that conjunctival cells

express the 45- and 50-kDa proteins, but interestingly, do not express the 60-kDa protein (data not shown). The presence of additional marker chromosomes from HeLa cells, which also express a 60 kDa protein bound by Ad2 (Belin and Boulanger, 1993) in Chang conjunctival cells (ATCC) may explain why of the 60-kDa protein is expressed on Chang C conjunctival cells. Taken together, the distribution and serotype specificity suggest that the 50-kDa protein is the primary receptor for Ad37 on human conjunctival cells. The identity of the 60-kDa molecule and its relationship to the 50-kDa protein, if any, have not yet been determined.

Role of sialic acid in Ad37 binding

Arnberg *et al.* (2000) reported that Ad37 uses sialic acid on a glycoprotein as a cell receptor. To determine the role of terminal sugars, especially sialic acid, in Ad37-receptor interactions, we performed a VOPBA on cells pretreated with neuraminidase, β -galactosidase, and α -mannosidase (Fig. 8A). Ad37 binding to only the 60-kDa protein decreased slightly after β -galactosidase pretreatment (lane 1), compared to untreated Chang C membrane proteins (lane 4). This may be the result of either minor loading differences (suggested by a comparison of the signal intensities of the 45-kDa protein) or contaminating exoglycosidases in the β -galactosidase extract (Roche). Pretreatment with α -mannosidase only slightly decreased Ad37 binding to both the 50- and 60-kDa receptors (lane 3). The relationship between

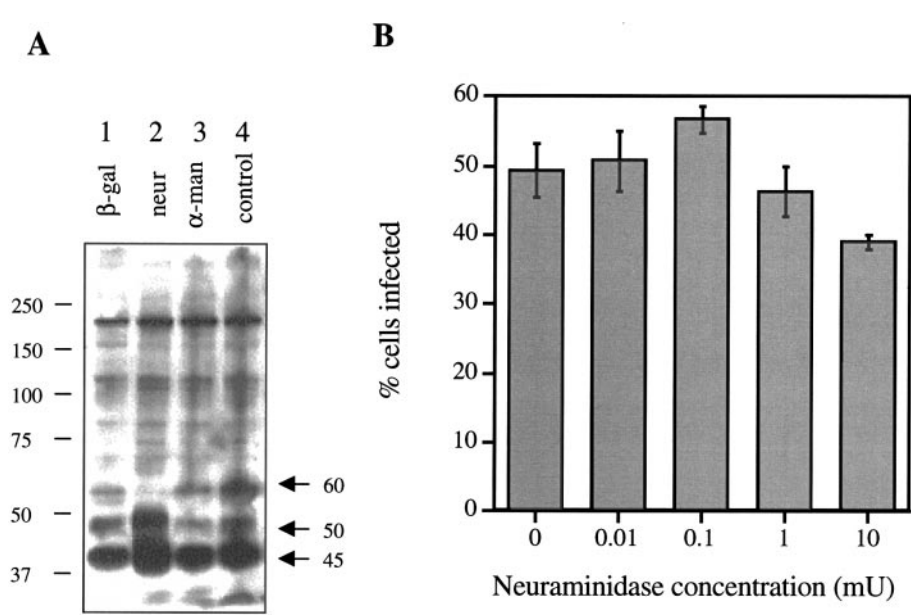


FIG. 8. Sialic acid is not required for Ad37 infection of Chang C cells or binding to the 50-kDa protein. (A) Membrane proteins of Chang C cells pretreated with 50 mU/ml β -galactosidase (lane 1), 10 mU/ml *Vibrio cholerae* neuraminidase (lane 2), or 10 mU/ml α -mannosidase (lane 3) were separated by nonreducing SDS-PAGE and analyzed by Ad37 VOPBA in the presence of 1 mM CaCl₂. Lane 4 contains membrane proteins of untreated Chang C cells. (B) Chang C cells (50,000) were treated with various concentrations of *Vibrio cholerae* neuraminidase in PBS, 2% FCS at 37° for 1 h and subsequently infected with Ad5.GFP. Δ F/37F at 10,000 particles per cell for 3 h. Infection was determined at 24 h by calculating the percentage of cells detected above a threshold set by the fluorescence of uninfected Chang C cells. Errors are represented as the standard deviation of three experiments.

Ad37 binding and terminal sugars other than sialic acid has not yet been determined. Importantly, the removal of terminal sialic acid residues by neuraminidase abolished Ad37 binding to the 60-kDa protein, but did not inhibit binding to the 50-kDa protein (lane 2). A549 cells, which were used to show that sialic acid is a cell receptor for Ad37 (Arnberg *et al.*, 2000), also express the 60-kDa protein. Thus, sialic acid appears to be required for Ad37 binding to the 60-kDa, but not required for binding to the 50-kDa protein, suggesting that Ad37 binding to sialic acid may not be necessary for infection of conjunctival cells.

To determine the relative importance of sialic acid and the 60-kDa protein in Ad37 infection, Chang C cells were pretreated with various concentrations of low-protease *Vibrio cholerae* neuraminidase in the presence of protease inhibitors and subsequently infected with Ad5.GFP.ΔF/37F (Fig. 8B). The removal of terminal sialic acids on Chang C cells by neuraminidase at low concentrations (0.01–0.1 mU) had no effect on infection. At the concentrations of neuraminidase required to inhibit Ad37 binding to A549 cells (Arnberg *et al.*, 2000), Ad37 infection of Chang C cells was inhibited by only 20.7% (10 mU neuraminidase). A high concentration of neuraminidase from *Clostridium perfringens* in the presence of protease inhibitors also failed to inhibit ¹²⁵I-labeled wild-type Ad37 binding to Chang C conjunctival cells (results not shown). These findings indicate that the binding of the Ad37 fiber protein to sialic acid is not necessary for infection of conjunctival cells.

DISCUSSION

In this study, we characterized the Ad37 interaction with the conjunctival cell surface using radioactive cell binding assays to measure virus binding while an Ad5 vector equipped with the Ad37 fiber protein was used to measure infectivity. These experiments demonstrated that Ad37 binding to conjunctival cells is calcium-dependent and CAR-independent. Combined with previous experiments showing that Ad37 preferentially bound and infected conjunctival cells while Ad2 and Ad19p did not (Huang *et al.*, 1999), these experiments demonstrated that Ad37 binds a distinct primary receptor expressed on conjunctival cells, but not on lung epithelial cells.

Using the virus overlay protein blot assay, we identified a 50-kDa human conjunctival cell membrane protein as the primary receptor for Ad37. This protein is present on Chang C cells, but not A549 lung epithelial cells, and Ad37 binding to this receptor is calcium-dependent, consistent with Ad37 binding and infection experiments. Ad2, a subgroup C adenovirus, also binds 45- and 60-kDa proteins on HeLa cells (Belin *et al.*, 1993), suggesting that these two proteins serve as receptors for multiple adenovirus serotypes and subgroups on multiple cell types. Ad37, as well as Ad19p, bound to a 60-kDa protein

that is present on both human conjunctival and lung epithelial cells. It is worth noting that the molecular weights of the α and β subunits of integrin $\alpha_v\beta_5$ and the MHC class I heavy chain, which has been proposed as a receptor for Ad5 (Hong *et al.*, 1998), are distinct from the 50- or 60-kDa receptor characterized in this study.

The studies of virus–receptor interaction using the Ad37 virus and knob overlay protein blot assays are consistent with previous studies showing that subgroup D adenoviruses, such as Ad9 and Ad15, can bind to extracellular domain of CAR (Roelvink *et al.*, 1998). Recent biochemical and structural studies on knob–CAR interactions indicate that the CAR-binding site is located on the AB-loop of the fiber knob (Bewley *et al.*, 1999; Roelvink *et al.*, 1999). Alignment of the fiber sequences of Ad37 and other adenoviruses reveals that the AB-loop of Ad37 is very similar to that of both Ad12 and Ad5 (Bewley *et al.*, 1999). Moreover, a phylogenetic tree of adenovirus knobs (Roelvink *et al.*, 1998) shows that fiber proteins of subgroup D are most similar to those of subgroup C, which use CAR as their primary receptor. These sequence analyses suggest that the Ad37 fiber might bind to CAR.

If the Ad37 fiber knob can bind to CAR in the VOPBA, why is Ad37 unable to bind to CAR on the lung epithelial cell surface for infection? It should be remembered that the fiber–receptor interaction takes place in the context of the virus capsid and the cell surface *in vitro* and *in vivo*. The fact that Ad37 and Ad2 do not compete for the same receptor (Huang *et al.*, 1999) and Ad5.GFP.ΔF/37F infection of conjunctival cells is not inhibited by a function-blocking antibody against CAR suggests that Ad37 does not use CAR (Fig. 4) as a primary receptor on conjunctival cells. It is possible that the short shafts of subgroup D adenoviruses place the virus capsid too close to the cell surface once bound to CAR, but adenovirus fiber shaft sequences suggest another possible model. Comparison of subgroup D adenovirus fiber protein sequences against those of other subgroups suggests that subgroup D fiber proteins have a different architecture in the fiber shaft. Adenovirus fiber proteins contain a shaft domain consisting of a variable number of 15–23 residue β -repeats that connect the fiber knob to the tail domain. Members of all human adenovirus subgroups, except subgroup D, contain a nonconsensus repeat at the third repeat from the tail and the first full repeat from the knob (Chroboczek *et al.*, 1995). Negatively stained electron micrographs of the Ad2 fiber bound to intact penton showed that the fiber is often bent near the N-terminal tail, which correlates with the site of the nonconsensus third repeat from the tail (Chroboczek *et al.*, 1995). The third repeat from the tail may allow the fiber to bend, providing an explanation for why only a small portion of the fiber shaft could be seen from an image reconstruction from cryoelectron microscopy of Ad2 and Ad12 (Chiu *et al.*, 1999). A recent crystal struc-

ture of the fiber knob plus the four β -repeats of Ad2 showed that the threefold axis of the fiber knob was tilted from the axis of fiber shaft (van Raaij *et al.*, 1999). It is possible that this tilt results from the abnormal sequence in the first full repeat from the knob shared by all human adenovirus subgroups, except subgroup D. The fiber shafts of subgroup D adenoviruses, without these two flexible repeats, should be rigid. An image reconstruction from cryoelectron microscopy of Ad5.GFP. Δ F/37F showed this is the case, as the entire Ad37 shaft and knob could be visualized (Chiu *et al.*, manuscript in preparation).

It is therefore possible that the rigidity of the Ad37 fiber shaft and perhaps shafts of other subgroup D viruses could prevent knob interaction with CAR. If the knob-CAR interaction occurs at an acute angle, subgroup D adenoviruses with rigid fibers may not be able to interact with CAR as the virus capsid would collide with the cell surface. A flexible fiber, however, would allow the knob freedom to bend and bind CAR without the steric hindrance from the capsid and the cell surface. Such a model would explain the ability of Ad37 to bind to CAR on a PVDF membrane, where CAR is fixed in a random orientation, but not on the surface of a cell, where CAR may be fixed in one orientation. The binding of an alternate receptor at the tip of the knob near the CD loop instead of the side of the knob at the AB loop could allow subgroup D adenoviruses, such as Ad37, to bind and infect cells without using CAR.

Arnberg *et al.* (2000) reported that Ad37 uses sialic acid as a receptor on chinese hamster ovary (CHO) cells and human lung epithelial (A549) cells, but did not study human conjunctival cells. We previously showed that Ad37 preferentially infected and bound to conjunctival cells compared to lung epithelial cells (Huang *et al.*, 1999). Ad37 bound relatively poorly to transformed human corneal epithelial (HCE) cells, the only ocular cell line used in their study, compared to binding on A549 cells (Arnberg *et al.*, 2000). Our study indicates that sialic acid plays a role in Ad37 binding to the 60-kDa protein, but it is unclear what role, if any, this protein plays in Ad37 ocular cell infection. Instead, Ad37 uses a 50-kDa membrane protein as its major cell receptor for binding to conjunctival cells, and Ad37 binding to this protein does not require sialic acid.

The characterization and identification of the Ad37 receptor have potential therapeutic implications and can explain its different tropism. Ad19a, along with Ad8 and Ad37, are major causes of EKC. Ad19a and Ad37 have identical fiber proteins (Arnberg *et al.*, 1997) and have similar tropisms *in vivo*. Ad8, Ad19a, and Ad37 also agglutinated dog and guinea pig erythrocytes more efficiently than did four other serotypes that are associated with less-severe forms of conjunctivitis (Arnberg *et al.*, 1997), implying that the receptors of Ad8, Ad19a, and Ad37 have similar characteristics. The 50-kDa receptor

for Ad37 may be the receptor for other subgroup D adenoviruses that cause severe cases of EKC. If all three of these pathogenic serotypes indeed share a common receptor, this receptor would be a very attractive drug target against adenoviral EKC. Further understanding of host-cell interactions of subgroup D adenoviruses may allow therapeutic intervention of ocular diseases associated with these viruses.

The wealth of information on adenovirus structure and mechanism of infection, its efficient infection of nondividing cells, and its large genetic capacity make adenovirus a popular gene therapy vector. However, the wide expression of CAR in the human body makes targeting adenovirus vectors difficult. The identification of alternative adenovirus receptors may therefore allow improved targeting of adenovirus vectors to specific human ocular cell types.

MATERIALS AND METHODS

Cell lines and wild-type adenoviruses

Human 293 embryonic kidney cells (Graham *et al.*, 1977), 293T cells (DuBridge *et al.*, 1987), A549 lung carcinoma epithelial cells, human Chang C conjunctival cells [American Type Culture Collection (ATCC), Rockville, MD], and Ad5 fiber expressing 633 cells (Von Seggern *et al.*, 2000) were maintained in complete Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. E1-2a (Gorziglia *et al.*, 1996) cells were obtained from M. Kadan (Genetic Therapy/Novartis, Summit, NJ) and maintained in Richter's medium. Wild-type Ad19p and Ad37 (ATCC) were propagated in A549 cells because of poor viral yields in Chang C and purified by banding on CsCl density gradients as previously described (Everitt *et al.*, 1977). The identities of the viral strains used were confirmed by DNA sequencing of the fiber gene. Viral protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA) and was used to calculate the number of viral particles based on the known molecular weight of Ad2 virions ($1 \mu\text{g} = 4 \times 10^9$ particles).

Construction of the Ad37 fiber expressing cell line and the recombinant Ad37 knob protein

The plasmid for the expression of the Ad37 fiber protein in mammalian cells was constructed in two steps. First, the Ad37 fiber open reading frame was amplified from Ad37 genomic DNA using synthetic oligonucleotide primers, L37 (5' TGT CCT **GGA TCC** AAG ATG AAG CGC GCC CGC CCC AGC GAA GAT GAC TTC 3') and 37FR (5' AAA CAC **GGC GGC CGC** TCT TTC ATT CTT G 3'). L37 contains nucleotides that differ from the Ad37 genomic sequence, to add a unique *Bam*HI site (bold) before the start codon (italicized) and create point mutations that make the N-terminal sequence of the fiber more closely

match the N-terminal sequence of the Ad5 fiber protein (underlined, see Fig. 2A). 37FR incorporates a unique *NotI* site (bold). The PCR product was inserted into the *Bam*HI and *NotI* sites of pCDNA3.1zeo(+) (Invitrogen, San Diego, CA) to create pDV78. Second, a 1.2-kb *Bam*HI/*Bgl*III fragment containing an adenovirus type 5 tripartite leader (TPL) was excised from pDV55 (Von Seggern *et al.*, 1999) and inserted into the *Bam*HI site of pDV78 to create pDV80.

The pDV80 plasmid was electroporated into the adenovirus-complementing cell line E1-2a as previously described (Von Seggern *et al.*, 1998) and stable clones were selected with 600 μ g/ml zeocin. Clones were screened for fiber expression by indirect immunofluorescence (Von Seggern *et al.*, 1998) using a rabbit polyclonal antibody raised by immunizing rabbits with recombinant Ad37 fiber protein (α -Ad37 fiber rabbit antibody). Two clones (lines 705 and 731) that expressed the protein at a uniformly high level were selected for further study.

Recombinant Ad37 knob protein containing an N-terminal T7-Tag was produced in *E. coli* using the pET expression system (Novagen, Madison, WI). Ad37 fiber DNA (GenBank accession number U69132) was PCR-amplified from wild-type Ad37 genomic DNA using the following primers: 5' GGATCCATGGGATACTTGGTAGCA 3' (*Bam*HI site underlined) and 5' GCAACTCGAGTCATCTTGGGCAATATAGG 3' (*Xho*I site underlined). The PCR reactions were performed at 94°C (denaturation), 55°C (annealing), and 72°C (extension, 30 cycles) using *Taq* DNA polymerase (Qiagen, Chatsworth, CA). The amplified DNA fragments, which contained residues 172 to 365 of the Ad37 fiber protein with the addition of an N-terminal start codon (italicized), were purified and subcloned into the pCR-TOPO vector using the TA-Cloning Kit (Invitrogen). No replication errors were found by DNA sequencing. The *Bam*HI/*Xho*I fragment was inserted into the bacterial expression vector, pET21a (Novagen), and transformed into (DE3)pLYS S expression cells (Invitrogen). The *Bam*HI site in pET21a is preceded by a short sequence coding for an 11 amino acid T7-Tag, resulting in the addition of the T7-Tag to the N-terminus of the Ad37 fiber knob. Expression was induced with 1 mM IPTG for 4 h at 37°C and knob expression was determined by SDS-PAGE. The clone displaying highest knob expression was used for large-scale knob expression and induced with 0.5 mM IPTG at 30°C for 4 h. The recombinant T7-tagged Ad37 knob protein was purified from sonicated bacteria using the T7-Tag Affinity Purification Kit was recommended by the manufacturer (Novagen). Recovered protein was analyzed for purity by SDS-PAGE followed by Coomassie staining or Western blotting with an HRP-conjugated α -T7-Tag monoclonal antibody as described by the manufacturer (Novagen) or an α -Ad37 fiber rabbit antibody (data not shown).

Pseudotyped adenoviral vectors

The construction of Ad5. β gal. Δ F was previously described (Von Seggern *et al.*, 1999). Ad5.GFP. Δ F was constructed by recombination in bacteria using a modification of the method of He *et al.* (1998). First, a fiber-deleted genomic plasmid was constructed by removing the fiber gene from pAdEasy1 (He *et al.*, 1998). pDV43 (Von Seggern *et al.*, 1999) was digested with *Pac*I, the ends blunted by treatment with the large fragment of *E. coli* DNA polymerase and dNTPs, and the product religated. The resulting plasmid, pDV76, is identical to pDV43 except for loss of the *Pac*I site and contains the right end of the Ad5 genome with E3 and fiber deletions. A 4.23-kb fragment was amplified from pDV76 using the oligonucleotide primers: 5' CGC GCT GAC TCT TAA GGA CTA GTT TC 3' (including the unique *Spe*I site in the Ad5 genome, bold) and 5' GCG CTT AAT TAA CAT CAT CAA TAA TAT ACC TTA TTT T 3' [including a novel *Pac*I site (bold) adjacent to the right Ad5 ITR]. This PCR fragment therefore contains nucleotides 27,082 to 35,935 of the Ad5 genome with deletions of nucleotides 28,133 to 32,743 (the E3 and fiber genes), and was used to replace the corresponding *Spe*I/*Pac*I fragment of pAdEasy 1 to create pDV77.

E. coli strain BJ5183 was electroporated with a mixture of pDV77 and *Pme*I-linearized pAdTrack as described (He *et al.*, 1998), and DNA was isolated from kanamycin-resistant colonies. The resulting plasmid, pDV83, contains a complete Ad5 genome with E1-, E3-, and fiber-deletions. The full-length Ad chromosome was isolated by *Pac*I digestion, and transfected to the E1- and fiber-complementing 633 cells (Von Seggern *et al.*, 2000). The recovered virus was then plaque-purified by plating on 633 cells and virus stocks were prepared by freeze-thawing cell pellets.

Ad37 fiber-producing cells (line 705) were infected at approximately 1000 particles/cell with Ad5. β gal. Δ F or with Ad5.GFP. Δ F. Viral particles were isolated and purified over CsCl gradients. For analysis of viral proteins, 10 μ g of purified Ad5. β gal. Δ F particles with no fiber (grown in 293 cells), the Ad5 fiber (grown in 633 cells), or the Ad37 fiber (grown in 705 cells) were electrophoresed by 8–16% polyacrylamide gradient SDS-PAGE, and the proteins were transferred to nylon membranes. The blot was then probed with α -Ad37 fiber rabbit antibody. Ad5 fiber and penton base were detected by reprobing the blot with polyclonal antibodies raised against recombinant Ad2 fiber or penton base expressed in baculovirus-infected cells (Wickham *et al.*, 1993).

Two mutations, S356 \rightarrow P356 and I362 \rightarrow T362, were discovered in the Ad37 fiber sequence from pDV80. The Ad37 fiber was recloned from Ad37 genomic DNA into pCDNA3.1zeo(+) and the Ad5 TPL was inserted as described above to create pDV121. The correct sequence of the Ad37 fiber protein, including predicted changes in

the fiber tail, was then confirmed by sequencing. 293T cells were transfected with pDV121 using the Calcium Phosphate Transfection System (Gibco BRL, Gaithersburg, MD), according to manufacturer's instructions. Transfected 293T cells were infected with Ad5.GFP. Δ F/5F as previously described (Jakubczak *et al.*, 2000) to produce Ad5.GFP. Δ F/37F. Ad5.GFP. Δ F particles containing the Ad37 fiber protein with the correct sequence were isolated and purified over CsCl gradients.

Adenovirus infection and cell binding assays

Adherent Chang C and A549 cells were infected with GFP expressing Ad5 vectors containing the Ad5 fiber (Ad5.GFP. Δ F/5F) or the Ad37 fiber (Ad5.GFP. Δ F/37F) at 10,000 particles per cell for 3 h at 37°C, 5% CO₂ in DMEM, 10% FBS. Cells were washed twice with saline and then cultured overnight for transgene expression. The next day, the cells were detached with buffer containing 0.05% (w/v) trypsin and 0.5 mM EDTA (Boehringer-Mannheim, Indianapolis, IN) for 5 min at 37°C. Suspended cells were washed once with phosphate-buffered saline (PBS), pH 7.4 and then resuspended in PBS. GFP fluorescence was measured with a FACScan flow cytometer. A threshold established by the fluorescence of uninfected cells was used to distinguish cells expressing GFP.

To assess the role of CAR in Ad infection, adherent cells were preincubated with 180 μ g/ml RmcB monoclonal antibody (Hsu *et al.*, 1988) in complete DMEM for 1 h at 4°C. A small volume containing Ad5.GFP. Δ F/5F or Ad5.GFP. Δ F/37F was then added at 10,000 particles per cell. The cells were infected and analyzed for GFP expression as described above.

To study Ad37 infection on neuraminidase- and heparin-treated cells, 50,000 Chang C cells were detached with EDTA, pelleted, and washed in PBS, 2% fetal calf serum (FCS). Pelleted cells were resuspended in 100 μ l of 10 μ g/ml heparin in PBS, 2% FCS, or various concentrations of *Vibrio cholerae* neuraminidase (Roche Molecular Biochemicals, Indianapolis, IN) and 10 μ g/ml aprotinin and leupeptin in PBS, 2% FCS and incubated at 37°C for 1 h. After the 1-h incubation, a small aliquot of cells was reserved to verify cell viability by the trypan blue method. The remaining cells were pelleted and resuspended in 300 μ l of 1.7×10^9 particles Ad5.GFP. Δ F/37F per ml (10,000 particles per cell) media with and without 10 μ g/ml heparin and plated in wells for 3 h at 37°C. After 3 h, the cells were washed with saline and cultured in media overnight. The following day, the cells were harvested and analyzed for GFP expression as described above.

To measure adenovirus binding to cells, wild-type Ad37 was labeled with ¹²⁵I using Iodogen (Pierce, Rockford, IL) according to the manufacturer's instructions and separated from free ¹²⁵I by gel filtration as described

(Huang *et al.*, 1999). Binding of radiolabeled wild-type Ad37 on Chang C cells was then quantitated as described (Huang *et al.*, 1999). Nonspecific binding was determined by incubating cells and labeled Ad37 particles in the presence of 100-fold concentration of unlabeled Ad37. Specific binding was calculated by subtracting the nonspecific binding from the total cpm bound.

To examine whether divalent cations are required for binding, 10 mM ethylenediaminetetraacetic acid (EDTA) or various concentrations of CaCl₂ or MgCl₂ were added to cells before incubation with labeled virus. To examine whether the receptor for Ad37 is a protein, cells were pretreated with 10 μ g/ml trypsin (GIBCO), subtilisin (Sigma, St. Louis, MO), proteinase K (Boehringer-Mannheim), and bromelain (Sigma) at 37°C for 1 h, then washed twice with complete DMEM before adding labeled virus. Cells were >95% viable after protease treatment.

Virus overlay protein blot assay (VOPBA)

Confluent monolayers of Chang C and A549 cells were detached by scraping, pelleted by centrifugation, and then resuspended in 250 mM sucrose, 20 mM HEPES, pH 7.0, 1 mM EDTA, and 2 μ g/ml aprotinin and leupeptin. Cells were transferred into a dounce homogenizer and disrupted with 30 strokes. Organelles and nuclei were pelleted at 500 *g* for 15 min. Plasma membrane fragments were then pelleted from the supernatant for cell lysates at 200,000 *g* for 1 h and then resuspended in 10 mM Tris-HCl, pH 8.1, 10 μ g/ml aprotinin and leupeptin.

Cell membranes of Chang C or A549 cells were incubated (1:1) with a 2% SDS, nonreducing buffer and separated on an 8% polyacrylamide gel without boiling. Membrane proteins were then electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P from Millipore, Bedford, MA) and blocked in 5% (w/v) milk in PBS, pH 7.4, 0.02% Tween-20 (PBS-T). After blocking, the membrane was incubated with 1 μ g/ml wild-type Ad19p or Ad37 in 0.5% (w/v) milk in PBS-T, 1 mM CaCl₂, for 1 h at room temperature. The membrane was then washed three times with PBS, 1 mM CaCl₂, and incubated with 1:500 dilution of α -Ad37 fiber rabbit antibody in 0.5% (w/v) milk in PBS-T, 1 mM CaCl₂, for 30 min at room temperature. The membrane was washed again with PBS, 1 mM CaCl₂, and incubated with 1:5000 dilution of horseradish peroxidase (HRP)-conjugated α -rabbit antibody (Sigma) in 0.5% (w/v) milk in PBS-T, 1 mM CaCl₂, for 30 min at room temperature. The membrane was washed four times in PBS, 1 mM CaCl₂, once with PBS-T, 1 mM CaCl₂, and once in 1 mM CaCl₂. The blot was developed with enhanced chemiluminescence reagents (Pierce) for 5 min and placed onto a piece of Biomax film (Kodak, Rochester, NY) for 5 s to 1 min. For divalent metal cation experiments, membranes were incubated in the pres-

ence of 2 mM EDTA instead of 1 mM CaCl₂ in all solutions.

To assay fiber knob binding to cell membrane proteins, transferred membrane proteins on PVDF were incubated with 1 μg/ml purified T7-tagged Ad37 knob protein in Tris-buffered saline, 0.1% Tween-20, 1 mM CaCl₂, for 1 h at room temperature. α-Ad37 fiber rabbit antibody and HRP-conjugated anti-rabbit antibody were applied and the membrane was developed with substrate solution as described above.

To determine the role of terminal sugars in Ad37 binding, monolayers of Chang C cells were treated with 50 mU/ml β-galactosidase (Roche), 10 mU/ml *Clostridium perfringens* neuraminidase (Roche), or 10 mU/ml α-mannosidase (Sigma) in PBS, 2% FCS for 1 h at 37°C. Cell membrane fractions were obtained immediately after exoglycosidase treatment and probed by VOPBA as described above.

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