

The Arg279Glu Substitution in the Adenovirus Type 11p (Ad11p) Fiber Knob Abolishes EDTA-Resistant Binding to A549 and CHO-CD46 Cells, Converting the Phenotype to That of Ad7p

Dan J. Gustafsson,* Anna Segerman, Kristina Lindman, Ya-Fang Mei, and Göran Wadell

Department of Virology, Umeå University, SE-901 85 Umeå, Sweden

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The major determinant of adenovirus (Ad) attachment to host cells is the C-terminal knob domain of the trimeric fiber protein. Ad type 11p (Ad11p; species B2) in contrast to Ad7p (species B1) utilizes at least two different cellular attachment receptors, designated sBAR (species B adenovirus receptor) and sB2AR (species B2 adenovirus receptor). CD46 has recently been identified as one of the Ad11p attachment receptors. However, CD46 did not seem to constitute a functional receptor for Ad7p. Although Ad7p shares high knob amino acid identity with Ad11p, Ad7p is deficient in binding to both sB2AR and CD46. To determine what regions of the Ad11p fiber knob are necessary for sB2AR-CD46 interaction, we constructed recombinant fiber knobs (rFK) with Ad11p/Ad7p chimeras and Ad11p sequences having a single amino acid substitution from Ad7p. Binding of the constructs to A549 and CHO-CD46 BC1 isoform-expressing cells was analyzed by flow cytometry. Our results indicate that an Arg279Glu substitution is sufficient to convert the Ad11p receptor-interaction phenotype to that of Ad7p and abolish sB2AR and CD46 interaction. Also a Glu279Arg substitution in Ad7p rFKs increases CD46 binding. Thus, the lateral HI loop of the Ad11p fiber knob seems to be the key determinant for Ad11p sB2AR-CD46 interaction. This result is comparable to another non-coxsackie-adenovirus receptor binding Ad (Ad37p), where substitution of one amino acid abolishes virus-cell interaction. In conjunction with previous results, our findings also strongly suggest that sB2AR is equivalent to CD46.

Human adenoviruses (Ad) are nonenveloped, double-stranded DNA viruses that have been classified into six species (A to F), currently containing 51 serotypes in total (4). Human Ad have a broad tropism, causing infection in respiratory, gastrointestinal, urinary tract, kidney, eye, and lymphoid tissue (39). The outer protein capsid structure mainly consists of three polypeptides: the penton base, the hexon, and the fiber proteins (15, 19). One penton base with a protruding trimeric fiber is localized at each of the 12 vertices of the viral particle, while most of the icosahedral capsid is made up of hexons (15, 41). The major determinant for Ad attachment to host cells is the C-terminal (knob) domain of the trimeric fiber protein (5, 26, 36, 41). The fiber consists of a tail, shaft, and knob domain (8). Trimerization of the fiber protein is noncovalent and dependent on β -sheet structures in the shaft, while the globular knob domain consists of an eight- to nine-stranded antiparallel β -sandwich connected by loops (9, 34). Each of the eight or nine strands is named A to H or A to J, respectively, while the loops are referred to as AB, CD, EF, etc. (10, 35). The Ad knob domain forms a three-bladed propeller-like structure, where each subunit consists of antiparallel β -sheets with loops pointing laterally from the center (10, 13, 45).

Most serotypes belonging to species A, C, D, E, and F appear to attach cells through binding the coxsackie-adenovirus receptor (CAR), which belongs to the immunoglobulin

family (29). However, species D serotypes 8, 19, and 37 (causing epidemic keratoconjunctivitis) have been shown to bind sialic acid instead of CAR (1, 3, 9, 11, 17). Recently it has been suggested that Ad37 can also bind to CD46 (44). It has been suggested that the majority of species B serotypes use CD46 as a cellular receptor (12, 31, 34).

CD46, or membrane cofactor protein, is a single transmembrane complement regulatory protein, expressed on all nucleated cells. Its natural ligands are C3b and C4b, and by acting as a cofactor for plasma serine protease factor I, CD46 mediates their breakdown and thus protects host cells from homologous complement attack (25). It serves as an attachment receptor for a number of pathogens including human herpesvirus 6, the Edmonston strain of measles virus, bovine viral diarrhea virus, *Nisseria gonorrhoeae*, *Nisseria meningitides*, and *Streptococcus pyogenes* (7). Human CD46 is alternatively spliced into several isoforms, resulting in various numbers of extracellular domains and two different cytoplasmic tails (33).

Species B Ad can be further divided into subspecies B1 (Ad 3, 7, 16, 21, and 50) and B2 (Ad 11, 14, 34, and 35) (4, 40) based on DNA homology. These differ in tropism; while most B1 serotypes cause acute respiratory tract infections, the B2 serotypes 11, 34, and 35 have mainly been associated with persistent urinary tract infections (6, 20, 24, 38, 39, 43). In a recent paper we have shown that the serotypes Ad3p (B1) and Ad7p (B1) differ not only in tropism from Ad11p (B2) and Ad35 (B2) but also in receptor usage (31). Our findings indicated that there are at least two different species B attachment receptors, which has been confirmed in a recent study (37). Ad3p

* Corresponding author. Mailing address: Department of Virology, Umeå University, SE-901 85 Umeå, Sweden. Phone: 46 90 785 8449. Fax: 46 90 12 9905. E-mail: dan.gustafsson@climi.umu.se.

and Ad7p mainly use a receptor that is sensitive to trypsin and chelating agents such as EDTA (31). This receptor was also bound by Ad11p and has been designated the species B adenovirus receptor (sBAR). The other receptor site was not affected by trypsin or EDTA and was found to be used by Ad11p and Ad35 but not by Ad3p or Ad7p. It was therefore designated sB2AR (species B2 adenovirus receptor) (31). We recently identified one of the Ad11p receptors as CD46 (32). Interaction of Ad11p with CD46-expressing cells showed the same characteristics as interaction of Ad11p with sB2AR, suggesting that CD46 and sB2AR are the same entity (32). In line with this conclusion, CD46 does not function as a receptor for Ad7p since expression of CD46 on CHO cells did not render these cells susceptible to Ad7p infection. Moreover, competition with anti-CD46 antibodies did not affect binding of Ad7p to human A549 cells (32). In this study, we set out to define regions and amino acid residues of the Ad11p fiber knob that are critical for cation-independent receptor interaction or interaction with sB2AR. Despite distinct differences in receptor usage, Ad11p and Ad7p show 94.3% amino acid identity in the knob domain (22). Insertion of Ad7p regions (chimeras) and single amino acid residues into the Ad11p recombinant fiber knob (rFK) backbone appeared to us to be an optimal strategy for identification of residues involved in sB2AR-CD46 interaction. Our results, in conjunction with previous studies, show sB2AR to be equivalent to CD46. We found the HI loop of the Ad11p fiber knob, more specifically Arg279, to be critical for sB2AR-CD46 interaction. An Arg279Glu substitution in the Ad11p knob converted the sB2AR-CD46 binding to EDTA-sensitive binding, thus giving a phenotype similar to that of Ad7p.

MATERIALS AND METHODS

Cells, viruses, antibodies and rFKs. (i) **Cells.** Human respiratory epithelial A549 cells were grown in Dulbecco's modified Eagle medium (Sigma Chemical Co., St. Louis, MO) containing 10% fetal calf serum, HEPES, and penicillin-streptomycin (all from Sigma). Chinese hamster ovary (CHO) cells and CHO cells expressing the human CD46 isoform BC1 have been described elsewhere (25). CD46 expression was confirmed by flow cytometry using a previously described method (32).

(ii) **Antibodies.** Polyclonal antibodies were prepared by immunizing rabbits with soluble CD46 (lacking the tail, transmembrane, and anchor domain but preserving the BC ectodomain and all four CCP domains). Soluble CD46 was produced in CHO cells. CD46 polyclonal antibodies were a kind gift from John P. Atkinson, Division of Rheumatology, Washington University School of Medicine, St. Louis, MO.

(iii) **rFKs.** Isolation and manipulation of DNA was performed by standard techniques. DNA fragments encoding the fiber knobs of Ad11p and Ad7p were amplified by PCR with the following primers (DNA Technology A/S, Aarhus, Denmark), preserving the last shaft motif and the trimerization signal (13, 14): fk 11/7p forward (5'-GTA CTG GCC ATG GGA CTT ACA TTC AAT TC-3'), fk 11p reverse (5'-GC TAC GCA GGA TCC TCA GTC GTC TTC TCT GAT-3'), and fk 7p reverse (5'-GGG CAT GAA ACC TTT AGC ATT AGT AAG GGC ACC AGT AGC CAT GTT-3'), fk 7p forward flag 3 (5'-AAT GCT AAA GGT TTC ATG CCC AGC ACA ACT GCC TAT CCT TTC AAT-3'), fk 11p forward flag 2 (5'-AAT GCT AAA GGT TTC ATG CCC AGC ACG ACT GCC TAT CCT TTC AAT-3'), and fk 11p reverse flag 4 (5'-GGG CAT GAA ACC TTT AGC ATT AGT AAT GGC ACC AGT AGC CAT GTT-3'). Constructs 11-274-325-7-FK and 11-294-325-7-FK were created using the unique restriction sites for *Drd1* and *Ava2* (enzymes from New England Biolabs, Beverly, Mass., used according to the guidelines of the manufacturer) found at the same positions in

fk 11p and fk 7p. Construct 7-Q279R-L282I-N284D-FK was created using the unique restriction sites for *Drd1* and *Ava2* (enzymes from New England Biolabs, Beverly, Mass., used according to the guidelines of the manufacturer) found at the same positions in fk 11p and fk 7p. Construct 7-Q279R-FK was created by using the following primers: 5'-TGC TCT TCG GTT AAG CAT GAC AGA TAT GTC-3', 5'-TCT CGT AAT TTA TTA CTC TGT AGT ATA-3', 5'-GTA CTG GCC ATG GGA CTT ACA TTC AAT TC-3', 5'-GCT AGT TAT TGC TCA GCG G-3, and the unique restriction site for *Drd1* (New England Biolabs, Beverly, Mass., used according to the guidelines of the manufacturer) found at the same positions in fk 11p and fk 7p. Point-mutated constructs of wild-type (wt) fk 11p were created using a QuickChange kit (Stratagene, La Jolla, CA) according to the directions of the manufacturer and using the following primers: fk 11p Q-R forward (5'-CTT AAC CAA AGA GCA ATA AAT GAC GAC ACA-3'), 11p Q-R reverse (5'-TGT GTC GTC ATT TAT TGC TCT TTG GTT AAG-3'), 11p I-L forward (5'-CCA AAG AGC AAT AAA TGA CGA GAC ATC-3'), 11p I-L reverse (5'-GAT GTC TCG TCA TTT AAT GCT CTT TGG-3'), 11p D-N forward (5'-CCA AAG AGC AAT AAA TAA CGA GAC ATC-3'), 11p D-N reverse (5'-GAT GTC TCG TTA TTT ATT GCT CTT TGG-3'), 11p I-V forward (5'-TCA TAT TGT ATT CGT GTA ACT TGG TCC TGG-3'), and 11p I-V reverse (5'-CCA GGA CCA AGT TAC ACG AAT ACA ATA TGA-3'). The primers for generating rFK 5 and rFK 37 have been described elsewhere (27). All PCR products were cloned into a pET-3d expression vector (Novagen, Madison, WI) containing an N-terminal His₆ tag motif by using *NcoI*-*Bam*HI restriction sites (enzymes from New England Biolabs). The constructs were confirmed by nucleotide sequence analysis. Proteins were expressed in *Escherichia coli* (strain BL21) and purified by using Ni-nitrilotriacetic acid agarose according to the directions of the manufacturer (QIAGEN, Hilden, Germany). All recombinant proteins were visualized by Coomassie brilliant blue staining of both denaturing (sodium dodecyl sulfate) and native polyacrylamide gels.

Flow cytometry binding assay. Adherent A549 or CHO cells, split the previous day, were harvested with phosphate-buffered saline (PBS) containing 0.05% (wt/vol) EDTA. The cells were either allowed to recover for 1 h in Dulbecco's modified Eagle medium containing 10% fetal calf serum under constant agitation (designated "recovered") or were directly resuspended in FLOW buffer containing 1% (wt/vol) bovine serum albumin and 0.01% NaN₃ (wt/vol) in PBS, pH 7.4 (designated "EDTA treated"). Both recovered and nonrecovered (EDTA-treated) cells were seeded at 1×10^5 cells per well in 96-well plates. After centrifugation at $290 \times g$ for 5 min at 4°C, the supernatant was removed, and fiber knob protein was added at the following concentrations: 1×10^{-4} μ g, 1×10^{-3} μ g, 1×10^{-2} μ g, and 0.1 μ g in a total volume of 100 μ l per well. Alternatively, only one concentration was used. Cells and fiber knobs were resuspended and incubated under constant agitation (at 4°C for 60 min). After incubation, the plates were centrifuged at $290 \times g$ for 5 min at 4°C, and the pellets were washed with 150 μ l of FLOW buffer. Mouse monoclonal penta-His Alexa Fluor 488-conjugated antibody (QIAGEN, Hilden, Germany) was added to washed cell pellets (1:200 in FLOW buffer) at 100 μ l per well. The pellets were then resuspended and incubated for 30 min (in darkness) as described above. The cells were again washed (as described above), resuspended in 300 μ l of FLOW buffer, and analyzed in a FACScan (Becton Dickinson) flow cytometer. Throughout the experiment, the samples were kept on ice so that they never reached a temperature higher than 4°C. The samples were evaluated by a FACScan (Becton Dickinson) flow cytometer, and the measurements consisted of 10,000 events per sample. The data were analyzed with the LYSYS II software program (Becton Dickinson).

Antibody inhibition assay. Adherent A549 cells, split the previous day, were harvested with PBS containing 0.05% (wt/vol) EDTA. Cells were directly resuspended in FLOW buffer containing 1% (wt/vol) bovine serum albumin and 0.01% NaN₃ (wt/vol) in PBS, pH 7.4. Cells were seeded at 1×10^5 cells per well in 96-well plates. After centrifugation at $290 \times g$ for 5 min at 4°C, the supernatant was removed, and rabbit polyclonal anti-CD46 serum was added to the cells (1:200; 90 μ l of FLOW buffer per well). Cells were then preincubated with CD46 antiserum for 30 min at 4°C under constant agitation prior to the addition of fiber knobs (0.1 μ g in 10 μ l of FLOW buffer). Cells and fiber knobs were resuspended and incubated under constant agitation (at 4°C for 60 min). After incubation, the plates were centrifuged at $290 \times g$ for 5 min at 4°C and the pellets were washed with 150 μ l of FLOW buffer. Mouse monoclonal penta-His Alexa Fluor 488-conjugated antibody (QIAGEN, Hilden, Germany) was added to washed cell pellets (1:200 in FLOW buffer) at 100 μ l per well. The pellets were then resuspended and incubated for 30 min (in darkness) as described above. The cells were again washed (as described above), resuspended in 300 μ l of FLOW buffer, and analyzed in a FACScan (Becton Dickinson) flow cytometer. Throughout the experiment, the samples were kept on ice so that they never reached a temperature higher than 4°C. The samples were evaluated by a FACScan (Becton

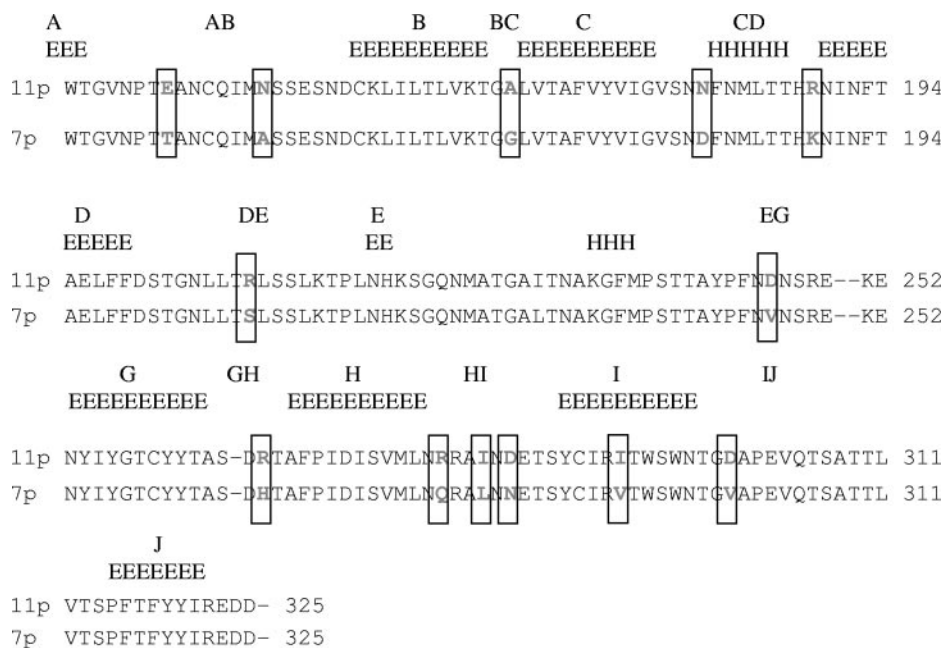


FIG. 1. Amino acid sequence alignment. Sequence alignment of the C-terminal knob domain between the Ad11p and Ad7p fiber. Nonhomologous residues are boxed. Strands and loops are marked according to Durmort et al. and van Raaji et al. (10, 42). The letters E and H denote β -strands.

Dickinson) flow cytometer, and the measurements consisted of 10,000 events per sample. The data were analyzed with the LYSYS II software program (Becton Dickinson).

Fiber knob model. The model of the Ad11p fiber knob was downloaded from the Swiss-Model Repository (<http://swissmodel.expasy.org>; SPROT/TrEMBL accession code P35774). The model is based on the structure of the crystallized Ad3 fiber (1h7zA.pdb) which is also available at the Swiss-Model Repository. Image manipulation was done with Swiss-PdbViewer 3.7 software.

RESULTS

Recombinant fiber knobs mimic viral cell-receptor binding phenotype. We have employed a flow cytometry-based assay to detect His-tagged rFK binding to A549 cells and to CHO cells that were transfected with cDNA encoding the BC1 isoform of CD46 (CHO-CD46 cells). Ad7p and Ad11p FKs show high amino acid identity (Fig. 1). A number of N-terminal His-tagged rFKs were constructed. The rationale was to insert consecutive Ad7p FK amino acid sequence or a single Ad7p FK amino acid into an Ad11p FK backbone (Fig. 2). To establish a reliable experimental system, we determined the time point for saturation to be 60 min at 4°C (data not shown) and confirmed that the wt fiber knobs showed saturation in binding (Fig. 3). The time point for equilibrium was determined in kinetic experiments using the same experimental system as above (data not shown).

wt Ad11p rFK (Fig. 3, 11 wt-FK) generated a typical dose-response curve, reaching saturation at approximately 0.1 μ g of rFK per 1.0×10^5 EDTA-treated A549 cells, indicating specific receptor interaction (Fig. 3). wt Ad7p rFK remained at the control level independently of concentration (Fig. 3, 7 wt-FK).

wt rFKs resemble wt virion receptor interaction. Ad11p binds sB2AR independently of divalent cations, and thus EDTA can be used to distinguish between interaction with

sBAR and sB2AR (31). We then ascertained that rFKs showed the same receptor interaction phenotypes as previously determined for wt virions (31) by incubation of wt Ad11p and wt Ad7p rFKs with either cells recovered from EDTA treatment or EDTA-treated A549 and CHO-CD46 cells (Fig. 4). The binding of Ad5p (Fig. 4, Ad5 wt-FK) and Ad37 (Fig. 4, Ad37 wt-FK) rFKs was assayed as tentative negative controls. The binding of Ad11p rFK was not affected significantly by EDTA treatment of the A549 and CHO-CD46 cells, while a reduction in Ad7p rFK binding to A549 cells was observed. Ad5p rFK did not bind CHO-CD46 cells, and its binding to A549 cells was unaffected by EDTA treatment. In principle, both Ad7p and Ad37 rFKs showed the same low binding levels to CHO and CHO-CD46 cells as Ad5. Somewhat surprisingly, there was no significant difference in the binding of Ad37 rFKs to CHO-CD46 or to CHO cells, even though Wu et al. have shown that the BC and C isoform of CD-46 can act as a receptor for a green fluorescent protein-Ad5 vector expressing Ad37 fiber knob (44). In summary, these experiments confirmed that wt rFKs resembled wt virions in their receptor interaction.

sB2AR corresponds to CD46. To determine whether sB2AR corresponds to CD46, we used polyclonal rabbit anti-CD46 serum to compete for binding of Ad5, 7, 11, and 37 rFKs to EDTA-treated A549 cells (Fig. 5). Apart from species B virus-receptor interaction, this polyclonal rabbit CD46 antiserum blocks C3b/C4b binding and cofactor activity of all four isoforms of CD46 (John P. Atkinson, personal communication). Thus, pretreatment with EDTA and anti-CD46 serum should block Ad11p rFK interaction to A549 cells if CD46 would correspond to sB2AR.

The addition of anti-CD46 serum almost abolished the binding of Ad11p rFK (95% reduction), yet the binding of Ad37

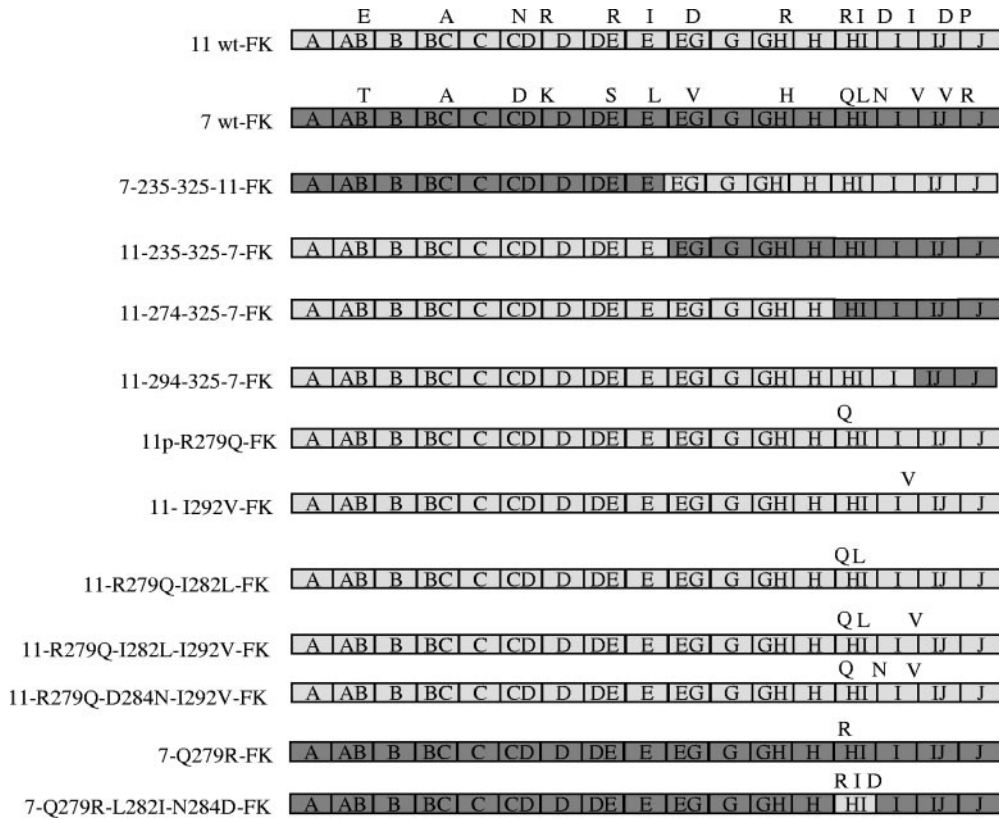


FIG. 2. rFK constructs. The discrepant amino acids between Ad11p and Ad7p rFKs are indicated at their relative positions in the wt fiber knobs. Boxed sections indicate strands or loops. The amino acid residues replaced by point mutations are also indicated.

rFK to A549 cells was unaffected by its presence (Fig. 5). As with Ad37, the binding of the Ad5 and Ad7 rFKs was not significantly affected by the presence of anti-CD46 (Fig. 5). Thus, the Ad11p cation-independent receptor, earlier defined

as sB2AR, appears to be equivalent to CD46, which is in accordance with our earlier results (32).

Arg279, in the HI loop, is a critical determinant for Ad11p rFK binding to sB2AR/CD46. The Ad11p wt rFK has earlier

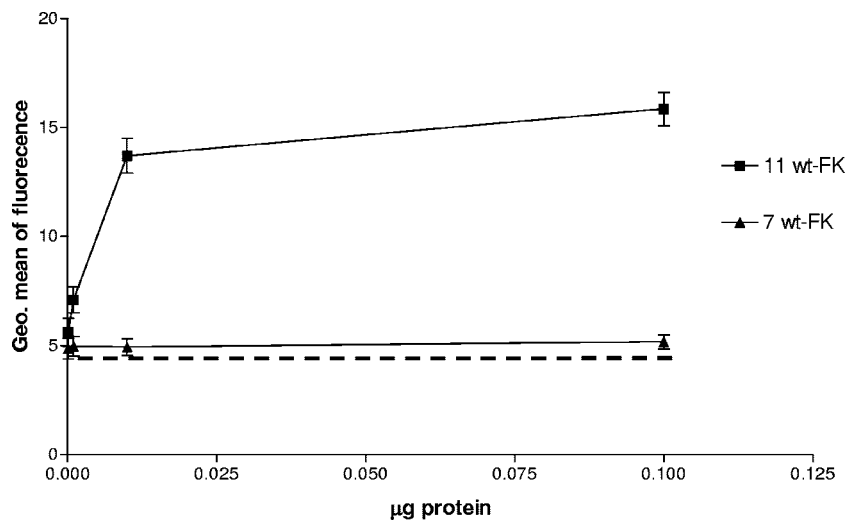


FIG. 3. EDTA-treated A549 cells incubated with wt Ad11p and Ad7p rFKs. EDTA-treated A549 cells were incubated for 60 min at 4°C with 0.1, 0.01, 0.001, or 0.0001 µg of Ad11p or Ad7p rFKs. The dotted line represents the mean level of negative control (cells incubated with antibody). The analysis was performed by flow cytometry. The results are geometric (Geo) means ± standard errors of means of duplicate data from three independent experiments. 11 wt-FK, wt Ad11p rFK; 7 wt-FK, wt Ad7p rFK.

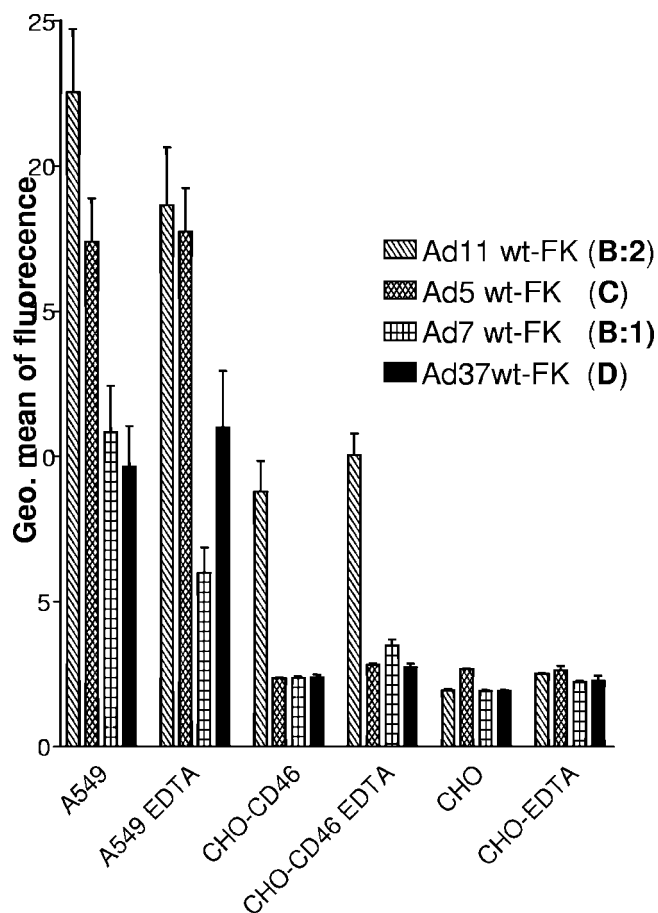


FIG. 4. Binding of Ad5, Ad7, Ad11, and Ad37 rFKs to A549, CHO-CD46, and CHO cells. A549, CHO-CD46, and CHO cells were treated with EDTA or recovered (see Materials and Methods). Subsequently, the cells were incubated with Ad5, Ad7, Ad11, or Ad37 rFKs (see Materials and Methods). Analysis was performed by flow cytometry. The results are geometric (Geo) means \pm standard errors of means of duplicate data from two independent experiments.

been shown to block the binding of viral particles to A549 and CHO-CD46 cells (32) and thus to compete for the same cellular receptors as whole virions. To define regions important for the interaction of Ad11p rFK with sB2AR, or most probably with CD46, we first constructed a number of chimeric Ad11p/Ad7p rFKs (Fig. 2).

Since substitution of the N-terminal Ad11p FK amino acids (aa) 235 to 325 with Ad7p FK amino acid sequence (construct 7-235-325-11-FK) only marginally affected cell binding (15%), we constructed three chimeras containing an Ad11p backbone with various lengths of the Ad7p C terminus (Fig. 2). Of these, only substitution of the last 31 C-terminal Ad11p FK amino acids with Ad7p FK amino acid sequence (construct 11-294-325-7-FK) showed a dose-response phenotype resembling that of wt Ad11p rFK (Fig. 6). Like wt Ad7p rFK, the other two chimeras, 11-235-325-7-FK and 11-274-325-7-FK, showed no cell binding activity. From these results, we concluded that the HI and I domains of Ad11p rFK, aa 274 to 294, mediate binding of the fiber to sB2AR (or CD46). Having narrowed down our Ad11p rFK region of interest to aa 274 to 294, we prepared a number Ad11p rFKs with amino acid substitutions

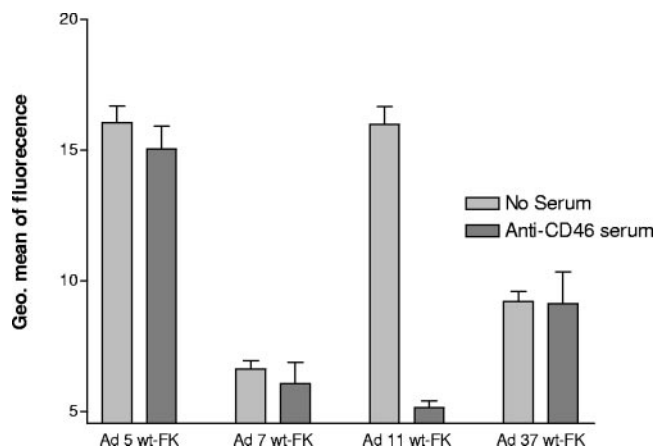


FIG. 5. Binding of Ad rFKs to EDTA-treated A549 cells after preincubation with polyclonal anti-CD46 serum. EDTA-treated A549 cells were preincubated with polyclonal rabbit anti-CD46 antibodies for 30 min at 4°C before the addition of 0.1 μ g of Ad rFKs (see Materials and Methods). The baseline was set at the mean values of negative controls (anti-His monoclonal antibody and CD46 CHO cells only). Analysis was performed by flow cytometry. The results are geometric (Geo) means \pm standard errors of means of duplicate data from two independent experiments.

from the corresponding positions in Ad7p rFK (Fig. 2). Substitution I292V (11-I292V-FK) did not affect the Ad11p rFK phenotype (Fig. 7), indicating that the I domain does not play a significant part in binding. On the other hand, mutation of Arg279 and Ileu282 (11-R279Q-I282L-FK) completely abolished binding relative to wt Ad11p rFK (Fig. 7). Construct 11-R279Q-FK showed a similar phenotype to that of 11-R279Q-I282L-FK, indicating that Arg279 is the critical determinant necessary for interaction of Ad11p rFK with sB2AR (or CD46). As a control, we made two constructs where we tried to rescue the Ad11p rFK phenotype by not substituting Ileu282 (11-R279Q-D284N-I292V-FK) and Asp284 (11-R279Q-I282L-I292V-FK) (Fig. 7). Neither 11-R279Q-D284N-I292V-FK nor 11-R279Q-I282L-I292V-FK showed a phenotype resembling that of wt Ad11p rFK. To further assess the role of Arg279 in receptor binding, we constructed 7-Q279R-FK. This construct showed significantly higher binding to CD46 than wt Ad7p rFK (Fig. 8). Substitution of the whole Ad7p HI loop with the corresponding amino acids in Ad11p (construct 7-Q279R-L282I-N284D-FK) gave an additional receptor binding effect compared to 7-Q279R-FK (Fig. 8).

Fiber knobs have the same binding phenotype toward both CHO-CD46 and A549 cells. To further verify that sB2AR corresponds to CD46, we tested the binding of chimeric and mutated rFKs to EDTA-treated CHO-CD46 cells (Fig. 9). Both Ad7p and Ad11p rFKs gave stronger signals at base and steady-state levels with CHO-CD46 cells than with A549 cells. As for A549 cells, Ad11p rFK showed a similar dose-dependent binding, but Ad7p wt rFK displayed no significant binding even at the highest rFK concentrations (Fig. 3 and 9). Chimeric rFKs 7-235-325-11-FK and 11-294-325-7-FK gave rise to phenotypes similar to the phenotype of Ad11p wt rFK (Fig. 6 and 9). Finally, substitution of Arg279 in the Ad11p FK (11-R279Q-FK) abolished the CHO-CD46 cell binding of Ad11p wt rFK and gave the same phenotype as Ad7p wt rFK (Fig. 8).

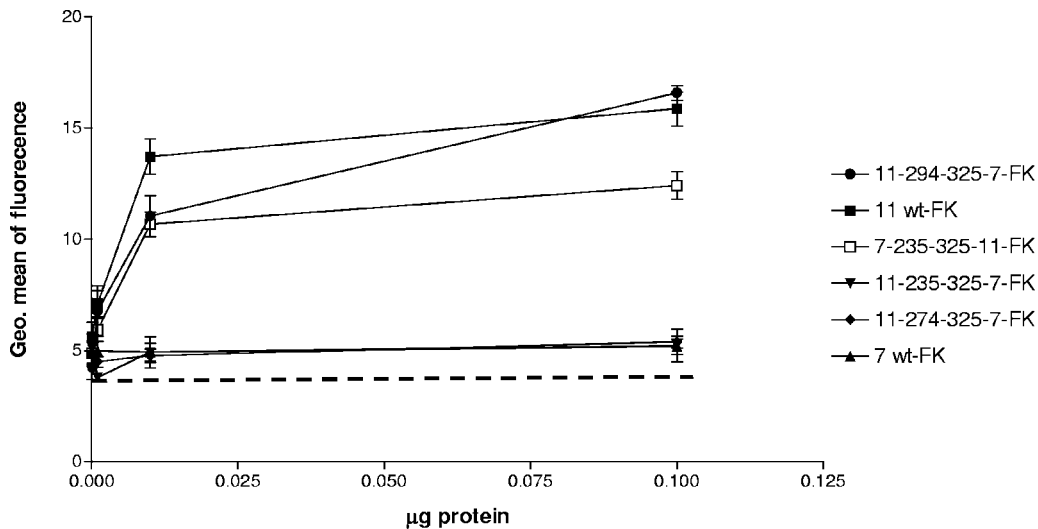


FIG. 6. Interaction of wt and chimeric rFKs with EDTA-treated A549 cells. EDTA-treated A549 cells were incubated for 60 min at 4°C with 0.1, 0.01, 0.001, or 0.0001 µg of wt or chimeric Ad rFKs. The dotted line represents the mean level of negative control (cells incubated with antibody). The analysis was performed by flow cytometry. The results are presented as geometric (Geo) means ± standard errors of means of duplicate data from three independent experiments. 11 wt-FK, wt Ad11p rFK; 7 wt-FK, wt Ad7p rFK.

In summary, the results obtained with EDTA-treated CHO-CD46 cells and A549 cells were almost identical (Fig. 7 and 9).

DISCUSSION

In previous studies, we have shown distinct differences in receptor usage between Ad7p and Ad11p virions (31). To determine the region of the fiber knob that is responsible for interaction of Ad11p with sB2AR, we performed binding studies, after pretreatment of cells with EDTA, using chimeric Ad11p and Ad7p rFKs and rFKs with single amino acid substitutions. CD46 has been identified as a receptor for several

group B Ad (12, 32, 34) and as one (of at least two) of the Ad11p attachment receptors (31, 32). Since competition with CD46 antiserum completely abolished the Ad11p rFK cation-independent receptor interaction with sB2AR, this strongly suggests that CD46 and sB2AR are the same entity. This is also in line with previous and present findings regarding interaction of Ad7 with CD46 (32). Regarding the cation dependency of the interaction with attachment receptors, our wt rFKs showed the same phenotypes as shown previously for wt virions (31, 32).

Our findings implicate Arg279 in the lateral HI loop (Fig. 10) as being the critical determinant for interaction between Ad11p rFK and CD46. The substitution Arg279Glu changed

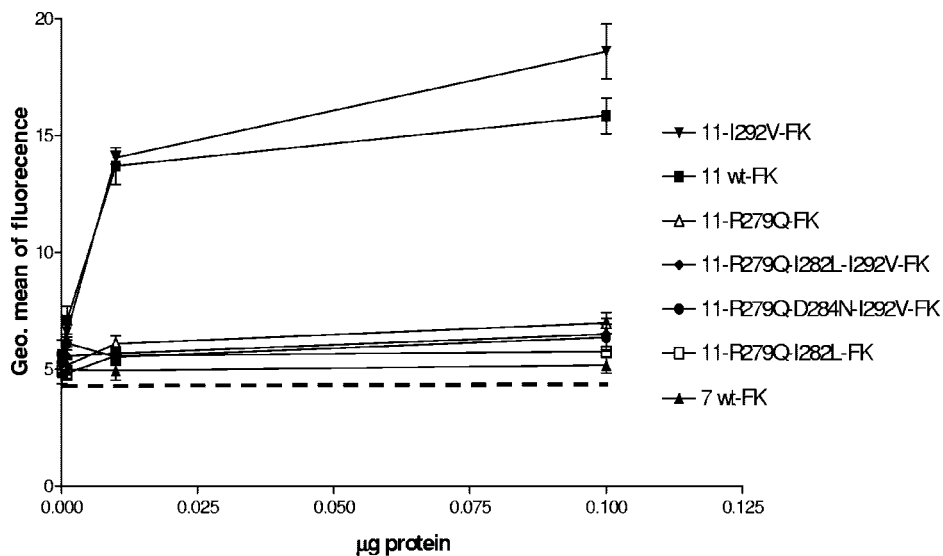


FIG. 7. Binding of Ad11p rFKs with Ad7p amino acid substitutions to EDTA-treated A549 cells. EDTA-treated A549 cells were incubated for 60 min at 4°C with 0.1, 0.01, 0.001, or 0.0001 µg of point-mutated Ad11p rFKs. The dotted line represents the mean level of negative control (cells incubated with antibody). Analysis was performed by flow cytometry. The results are geometric (Geo) means ± standard errors of means of duplicate data from two independent experiments. 11 wt-FK, wt Ad11p rFK; 7 wt-FK, wt Ad7p rFK.

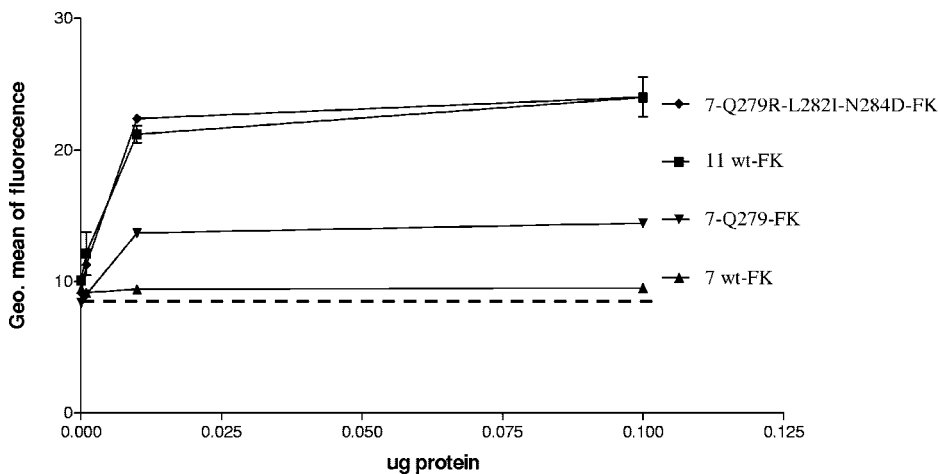


FIG. 8. Binding of Ad7p rFKs with Ad11p amino acid substitutions to EDTA-treated A549 cells. EDTA-treated A549 cells were incubated for 60 min at 4°C with 0.1, 0.01, 0.001, or 0.0001 µg of point-mutated Ad11p rFKs. The dotted line represents the mean level of negative control (cells incubated with antibody). Analysis was performed by flow cytometry. The results are geometric (Geo) means ± standard errors of means of duplicate data from two independent experiments. 11 wt-FK, wt Ad11p rFK; 7 wt-FK, wt Ad7p rFK.

the Ad11p rFK-receptor interaction from being EDTA insensitive to being EDTA sensitive. This also abolished the binding of Ad11p rFK to CD46-expressing cells and was thus sufficient to create a phenotype corresponding to that of wt Ad7p rFK. Further, the reverse mutation Glu279Arg in Ad7p created a phenotype resembling that of Ad11p. In Ad5, single substitutions of four different amino acids, Ser 408 and Pro 409 in the AB loop, Tyr477 in the DG loop, and Leu 485 in beta-strand F, have been shown to each abolish high-affinity interaction with CAR (18, 45). The crystal structure of the Ad12 fiber knob in complex with CAR indicates that the AB loop mediates the majority of the CAR interactions and forms an anchor

for the complex (5). The results from the chimeric Ad7 and Ad11 rFKs suggest that the HI loop of Ad11p rFK is critical for interaction with CD46. One amino acid in this loop, Arg279, appears to be necessary for fiber-receptor interaction. Arg279 may function as a contact residue anchoring the fiber to CD46, which would be analogous to the role of the AB loop in CAR-binding Ad (5, 18, 30). The increased binding efficacy of 7-Q279R-L282I-N284D-FK compared to 7-Q279R-FK further indicates the importance of the HI loop. This observation suggests that the immediate molecular environment around Arg279 is of importance in receptor binding. Arg279Glu substitution in Ad11p rFK might also disrupt local conformation,

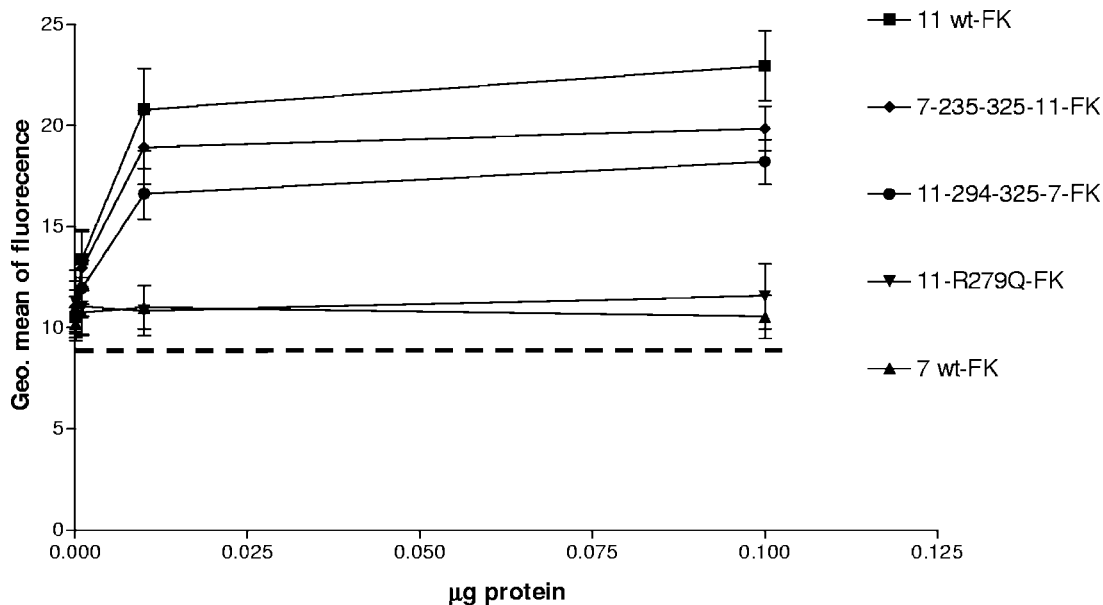


FIG. 9. Interaction of wt, chimeric, and point-mutated Ad rFKs with EDTA-treated CHO-CD46 cells. EDTA-treated CHO-CD46 cells were incubated for 60 min at 4°C with 0.1, 0.01, 0.001, or 0.0001 µg of wt, chimeric, or point-mutated Ad rFKs. The dotted line represents the mean level of negative control (cells incubated with antibody). The results are geometric (Geo) means ± standard errors of means of duplicate data from two independent experiments.

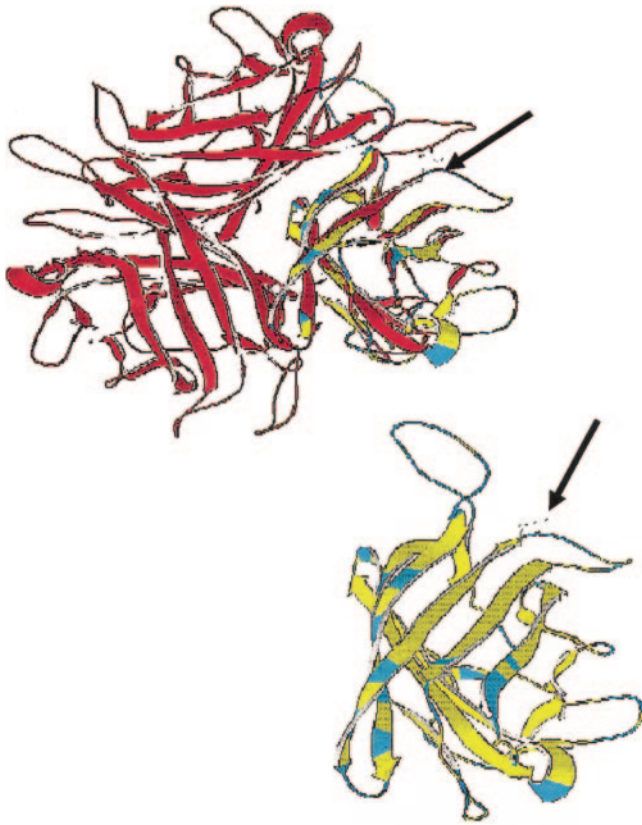


FIG. 10. Position of Arg279 in the trimeric fiber knob. This model of the Ad11p fiber knob monomer is based on the Ad3 crystal structure retrieved from the Swiss-Model database. The fiber knob is seen from the top with the shaft in the middle. The Ad11p FK monomer is depicted in red both alone and superimposed on the Ad3 FK trimer (yellow). Arg279 is indicated (arrows) at the beginning of the HI loop. Magenta blue areas represent accessible amino acid residues.

thereby altering the phenotype without being a contact residue. However, since a Glu279Arg substitution alone alters the CD46 binding phenotype of Ad7p rFK to one resembling Ad11p rFK, this seems to suggest that Arg279 is a contact residue to CD46 or significantly alters the conformation of the HI loop. The roles of Arg279 as a critical mediator of CD46 interaction or Glu 279 as a block to fiber-receptor interaction remain to be elucidated, preferably by crystallization studies. Although complete parallels between Ad37 and Ad11p cannot be drawn with respect to fiber-receptor interaction, a Glu240Lys substitution in the Ad37 fiber knob abrogated binding to Chang C cells, which are of HeLa origin (16). Thus, the situation of having one amino acid residue that is critical for determination of receptor usage is not unique to species B Ad but has also been observed in two closely related species D serotypes (Ad37 and Ad19p) which also differ in tropism (1, 2).

Ad11p recombinant fibers display hemagglutination (HA) activity with monkey erythrocytes which, in contrast to their human counterparts, express CD46 (21, 23). HA activity was demonstrated to be conformation dependent and to lie between aa 260 to 280 in the Ad11p fiber knob (21). Interestingly, Ad11p HA activity on monkey erythrocytes, like Ad11p CD46 interaction, is significantly reduced by the Arg279Glu muta-

tion, indicating that the same fiber knob region mediates both interactions (20). This, in turn, suggests that Ad11p HA activity as well as attachment to various cell lines is mediated by the same receptor; reasonably, that receptor would correspond to CD46.

The questions of whether and how CD46 interaction influences the tropism of species B adenoviruses remain to be addressed. Whether CD46 serves as a strict attachment receptor, by analogy with CAR for several other adenoviruses or CD4 for human immunodeficiency virus (HIV), is also unknown. CD46 is capable of initiating intracellular signal transduction (28) and of modulating immune regulatory functions, which may be one answer to why CD46 is such a magnet for pathogens (7). This aspect of CD46 function may be important regarding the capability of Ad11p and Ad35 to establish persistent infections. In addition, CD46 is ubiquitously expressed, which may be crucial for species B2 Ad, in order to accomplish systemic or hematogenic spread and reach the kidney and urinary tract. Ultimately, it would be interesting to determine in an *in vivo* model whether the Arg279Glu substitution alters Ad11p tropism and pathology.

Some studies have suggested that CD46 also serves as a receptor for Ad37 (44). We observed no significant increase in binding of Ad37 rFK to CHO-CD46 compared to CHO cells, and, in addition, CD46 antisera failed to block attachment of Ad37 rFK to A549 cells. These are remarkable findings if Ad37 really does use CD46 as an attachment receptor. Since CD46 is spliced into multiple isoforms and the extracellular domain is posttranslationally modified, the contradictory results may perhaps be explained by usage of different cellular expression systems. In summary, our results suggest that the lateral HI loop of the Ad11p fiber knob, and more specifically Arg279, is crucial for binding of Ad11p to CD46.

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