The Identification of an Adenovirus Receptor by Using Affinity Capture and Mass Spectrometry

Sunia A. Trauger,[a] Eugene Wu,[b] Steve J. Bark,[a] Glen R. Nemerow,*[b] and Gary Siuzdak*[a]

A tandem mass spectrometry-based approach is demonstrated for detecting a receptor for Ad37, one of the causative agents for epidemic keratoconjunctivitis. Partial purification of membrane glycoproteins was performed by using lectin-affinity chromatography and SDS-PAGE. Gel bands that were shown to bind Ad37 by using Viral Overlay Protein Blot Assay (VOPBA) were excised, proteolyzed and analyzed by using nanoLC-MS/MS to identify putative receptors contained in a mixture of proteins. Four candidate receptors were identified among approximately 50 proteins based on a search against a protein database. Inhibition of gene delivery mediated by an Ad37 vector, with antibodies against the glycoproteins identified by tandem mass spectrometry, strongly indicated that Membrane Cofactor Protein (MCP), a member of the complement regulatory family of proteins, is the receptor. This rapid and sensitive MS/MS-based strategy is perceived to have wide potential applications for the detection of viral receptors.

Introduction

Identifying the cellular receptors involved in virus–host cell interactions is an important first step in developing new and effective antiviral therapies. However, the identification of viral receptors requires a considerable undertaking when using traditional methods. These methods can include the generation of function-blocking monoclonal antibodies, construction of cDNA expression libraries from receptor-bearing cells, and assaying viral infectivity in receptor-negative host cells following transfection with receptor cDNA. It can take months to years to perform the necessary analyses to identify the functional properties of putative receptors. For example, the characterization of the receptors and coreceptors involved in HIV infection took years and required the combined efforts of numerous labs.[1]

Adenoviruses type 37, 19, and 8 (Ad37, Ad19, and Ad8 from group D) cause a severe eye infection called epidemic keratoconjunctivitis (EKC) also known as "pink eye". The onset of infection is characterized by the association of the adenovirus fiber protein with the conjunctival cell membrane.[2–4] Ad37 binds to a 50 kDa cell-surface protein that is distinct from the 46 kDa coxsackievirus adenovirus receptor (CAR), which mediates infection of other adenovirus serotypes as well as coxsackie B viruses.[5–7] By using a virus overlay protein blot assay (VOPBA), calcium-dependent and sialic acid-independent binding was observed between Ad37 and a 50 kDa protein on permissive conjunctival Chang (Chang C) cells. Ad37 also bound to a 60 kDa protein (calcium dependent) in both permissive and nonpermissive cell lines.[5] Therefore, these earlier studies suggested that Ad37 uses a receptor that is distinct from that used by other adenovirus serotypes. Arnberg et al. also reported that Ad37 recognizes sialic acid on an unspecified membrane glycoprotein in A549 lung epithelial and Chinese hamster ovary cells.[8] However, these two cell lines support reduced levels of Ad37 infectivity compared to conjunctival cells.[9] Together these observations support the concept that Ad37 uses distinct cell receptor(s).

Here, a direct, sensitive, and rapid approach for identifying a viral receptor is described that employs mass spectrometry as a key methodology. Nanoscale reverse-phase chromatography combined with nanoelectrospray tandem mass spectrometry (nanoLC-MS/MS) and genomic-database searching has become a proven technology for identifying proteins in cellular and extracellular fractions.[10] By using this powerful technique, fragmentation data obtained on proteolytic peptides is compared to theoretical fragments predicted from the proteins contained in a database, such as the human genome. Established statistical criteria are then used to determine significant hits. This method of identifying proteins by using a database search is being widely used since the completion of the human and other genomes. However, to our knowledge, it has yet to be exploited for virus-receptor identification.

Our approach involved pooling and purifying glycoproteins contained in a membrane fraction from human conjunctival epithelial cells (Chang C) by using affinity chromatography, followed by nanoLC-MS/MS. Since many cell-surface proteins are glycosylated, we anticipated that the Ad37 receptor would bind to a lectin-affinity column (i.e., lentil lectin). The eluted

[a] *Dr. S. A. Trauger, Dr. S. J. Bark, Prof. G. Siuzdak*
The Department of Molecular Biology and
The Scripps Center for Mass Spectrometry
The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-9496
E-mail: siuzdak@scripps.edu

[b] *Dr. E. Wu, Dr. G. R. Nemerow*
Immunology, The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-8472
E-mail: gnemerow@scripps.edu

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glycoproteins from the affinity column were further fractionated by molecular weight (M_W) by using SDS-PAGE. A virus overlay protein blot assay was subsequently used to identify protein bands that bound to Ad37. Briefly, this method involves blotting proteins separated by SDS-PAGE onto a polyvinylidene fluoride (PVDF) membrane and incubating these with a virus suspension. The virus-binding region is detected by using a technique similar to a Western Blot. The 50 and 60 kDa bands that bound to the virus were excised and digested, and the proteolytic peptides were extracted and analyzed. The list of approximately 50 candidate proteins obtained from this analysis was narrowed to four, based on their predicted size and subcellular localization (i.e. plasma membrane): CD46, CD87, CD98, and CD147.

An experiment involving the inhibition of virus-mediated gene delivery by using antibodies directed against CD46, CD87, CD98, and CD147 strongly supported the identity of the receptor as CD46. Furthermore, immunodepletion and virus binding analysis reported elsewhere have provided additional evidence for CD46 as the receptor for Ad37.[11] An overall representation of the mass spectrometry-based approach is shown in Figure 1.

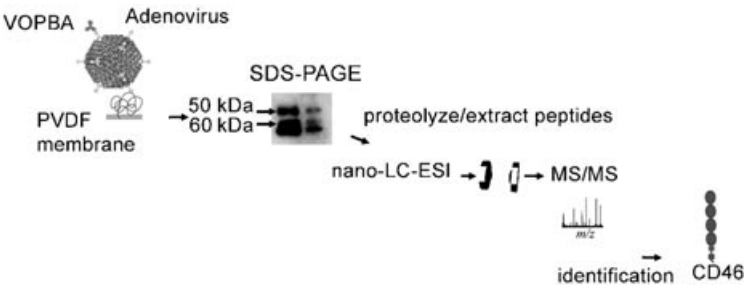

Figure 1. *A representation of the mass spectrometry-based detection of the viral receptor following partial purification by lentil lectin chromatography and SDS-PAGE. The reconstructed cryoelectron microscope image of Ad37 was taken from ref. [30] with permission.*

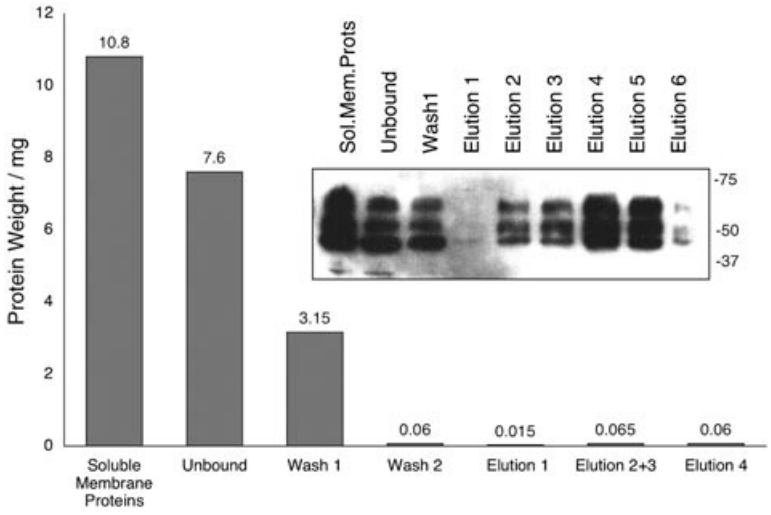

Figure 2. *Lentil lectin chromatographic purification of Ad37 receptors. 10.8 mg of protein were extracted from the membrane fraction of Chang C conjunctival cells with a buffer containing 1% CHAPS detergent and separated by lentil lectin chromatography as described in the Experimental Section. Protein weights in various fractions are displayed above each bar. An Ad37 virus overlay protein blot assay indicates the relative amounts of Ad37 receptors in each fraction (inset).*

Results and Discussion

Membrane proteins were fractionated from cultured Chang C cells, solubilized by 1% CHAPS detergent, and separated by lentil lectin chromatography. Analyses of fractions by a Bradford protein assay and an Ad37 VOPBA showed the enrichment of the 50 and 60 kDa candidate receptors by lentil lectin chromatography (Figure 2). Purified proteins with apparent molecular weights of 50 and 60 kDa were then proteolyzed by trypsin and deglycosylated. By using nanoLC-MS/MS, peptides from approximately 50 unique proteins were identified in the membrane fraction for each of the 50 and 60 kDa bands. Annotated collision-induced dissociation (CID) mass spectra of two of the seven peptide ions used for identifying CD46 are shown in Figure 3. The identified proteins included many 50 and 60 kDa isoforms of keratin. This is not surprising since keratin is highly expressed on the ocular epithelium.[12,13] Based on their subcellular localization, plasma membrane proteins were identified as putative cell-surface receptors for Ad37: CD46, or membrane cofactor protein (MCP), CD87, CD98, and CD147. Interestingly, peptides from isoforms of CD46 were identified in both the 50 and 60 kDa gel slices that corresponded to proteins that bind Ad37. Flow cytometric analysis (FACS) with antibodies specific to CD46, CD87, CD98, or CD147 confirmed that all four identified glycoproteins are expressed on Chang C cells (Figure 4a).

To identify the Ad37 receptor, Chang C cells were preincubated with antibodies directed against the extracellular domains of CD46, CD87, CD98, and CD147 and then infected with Ad37.GFP, an adenovirus vector carrying a green fluorescent protein (GFP) transgene, and the Ad37 fiber. After being cultured, the cells were analyzed for expression of GFP by flow cytometry. In contrast to antibodies against CD87, CD98, or CD147, an antibody against CD46 significantly blocked Ad37.GFP-mediated gene delivery to conjunctival cells (Figure 4b), indicating that CD46 plays a key role in Ad37 entry. In addition, in a series of experiments reported elsewhere,[11] CD46 expression in Chinese hamster ovary (CHO) cells conferred increased permissiveness to Ad37 infection, supporting its role as the primary Ad37 receptor. These experiments also indicated that Ad5 does not use CD46 as a receptor. The identification of CD46 as a receptor for Ad37 infection implies that it is also the receptor for Ad19a, which has an identical knob sequence to Ad37 and also causes EKC.[14]

The sequence coverage for CD46 (~25%) is shown in Figure 5. Two peptides detected (GTYLTDETHR and ADGGAEYATYQTK) are from two different isoforms of CD46. The presence of these two peptides from the cytoplasmic tail of

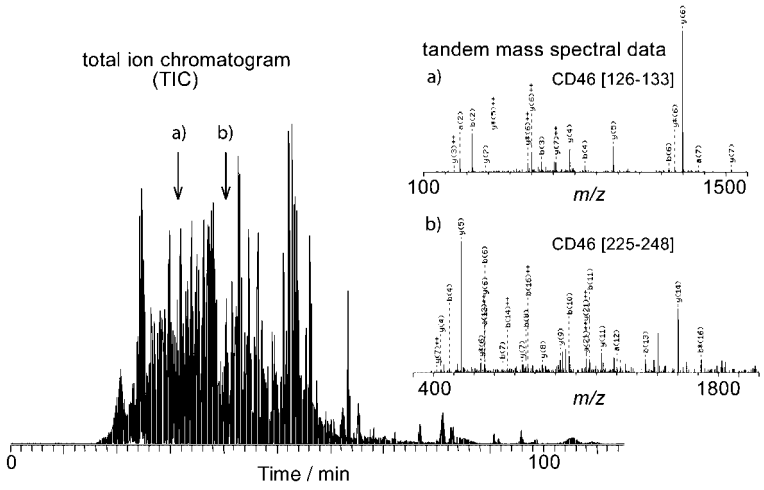

Figure 3. *Base peak chromatograms observed from the nano-LC MS/MS of the 50 kDa fraction show a highly complex mixture containing hundreds of peptides. a) and b) are annotated tandem mass spectra showing MS/MS of* $[M+2H]^{2+}$ *ions of the proteolytic peptides VLCTPPPK [126–133], and GFYLDGSDTIVCDSNSTWDPPVPK [225–248], respectively, two of the seven peptides observed from the putative receptor CD46 (MCP).*

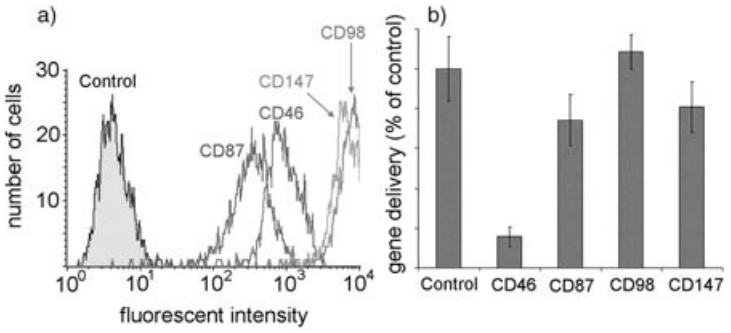

Figure 4. *Expression of identified membrane proteins on conjunctival cells and inhibition of Ad37 infection by CD46 antibody. a) Expression of CD46, CD87, CD98, and CD147 on Chang C cell surfaces was analyzed by flow cytometry. Secondary antibody alone showed no reactivity (control). B) PBS (control) and antibodies against CD46, CD87, CD98, and CD147 were tested for inhibition of Ad37.GFP infection as measured by gene delivery. Data represent the averages and standard deviations of quadruplicates. The CD46 antibody significantly decreased gene delivery (ANOVA,* $P < 0.001$*).*

CD46[15] demonstrates the presence of both isoforms in Chang conjunctival cells. Additionally, no peptides from the heavily *O*-glycosylated serine–threonine–proline-rich "STP" region were observed, since only a general *N*-linked deglycosylation step was adopted in the procedure. One peptide (N*HTWLPVSDDACYR) was identified from a *N*-glycosylated site;[16] this shows that the deglycosylation step with PNGase-F was effective in removing *N*-linked sugars. An additional deglycosylation with an *O*-glycosidase such as neuraminidase could result in the identification of more peptides from CD46 in the highly glycosylated STP domain.

CD46 is a membrane glycoprotein that serves as a cofactor of serine protease factor I for the deactivation of C3b and C4b through proteolytic cleavage. Importantly, it also is known to function as a receptor for the Edmonston strain of the measles virus,[17–20] human herpesvirus-6, *Streptococcus pyogenes*, and *Neisseria gonorrhoeae*.[21,22] In previously reported work with VOPBA, the Ad37-receptor binding has been shown to be sensitive to reducing agents and to be calcium dependent.[5] CD46 contains disulfide bridges and a calcium ion in crystal form;[13] this might help explain the sensitivity of virus-receptor binding to the presence of reducing agents and calcium ions. Furthermore, CD46 is extensively expressed in human corneal epithelium, stroma, and photoreceptor rods and cones.[23] Soluble CD46 is also present in ocular fluids.[24] Its availability on the exposed eye surface also makes it a good match for the putative receptor.

We have shown an alternative approach for identifying viral receptors that involves purifying cell surface glycoproteins, crudely fractionating proteins by molecular weight, performing a VOPBA to ascertain virus binding, followed by a proteolytic digest and identifying proteins based on a single reverse-phase separation and peptide sequence data obtained using tandem mass spectrometry. The identity of the receptor was confirmed through affinity-based experiments including the inhibition of gene delivery by using antibodies against the identified glycoproteins. This approach significantly reduces the number of receptor candidates that must be tested in further cell-based assays involving receptor expression and gauging susceptibility to viral infection. In addition, other lectins or chromatographic techniques can be applied to this approach. The application of mass spectrometry to receptor identification removes the need for pure protein from antibody or viral ligand chromatography. Partial purification of the receptor with lentil lectin chromatography was used to remove ~99% of protein weight from the initial cell extract (Figure 2). Therefore the crude membrane fraction would have been expected to contain many thousands of proteins in a single SDS band; this would make the mixture too complex for analysis by the nanoLC-MS/MS approach. Even when improved separation strategies such as multidimensional chromatography are employed to identify more proteins, a comprehensive list cannot be generated due to the presence of thousands of proteins. Multidimensional LC-MS/MS approaches have typically been used to identify several hundred to less than 1600 proteins in highly complex mixtures, like yeast-cell lysates.[25,26] It is also important to note that this partial purification is not feasible with techniques such as Edman sequencing or MALDI/TOF peptide mass mapping-based identification, since both require a highly purified protein sample and have a limited capacity to identify proteins in mixtures.

Recently, two reports have been published that identify CD46 as the cellular receptor for group B adenoviruses Ad11 and Ad35.[27,28] Segerman and co-workers hypothesized that CD46 or its homologue, CD55, was the Ad11 receptor based on similarities between adenovirus and picornavirus receptors

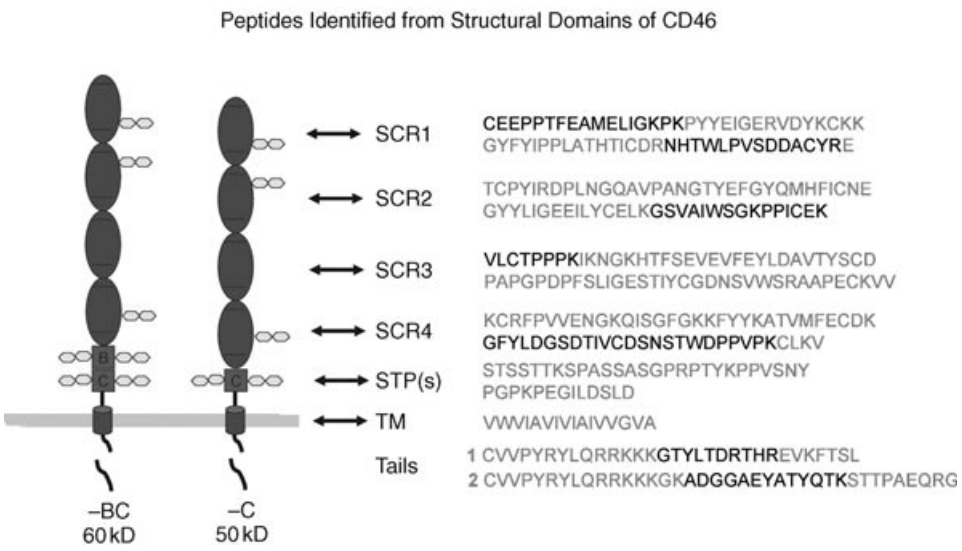

Figure 5. *A pictorial representation of the different domains of CD46 (adapted from ref. [31]). Domain regions are roughly indicated to show the location of peptides identified by MS/MS sequencing within the entire CD46 sequence. Five peptides were found from the short consensus repeat units (SCR1–4). Two peptides sequenced from the cytoplasmic tail domains indicated the presence of at least two isoforms of CD46 resulting from alternative splicing. No sequence coverage was obtained in the transmembrane (TM; cylinders) and serine–threonine-rich (STP; squares) regions. Hexagons in the diagram denote sites of glycosylation.*

and found that CD46 mediated Ad11 binding using antibodies and infectivity studies with CD46-expressing cells.[27] Unfortunately, this approach requires previous knowledge of several other receptors for separate virus families and cannot be generally adopted for other viruses. In comparison, our approach relied on the empirical identification of CD46, as well as other membrane proteins in the fractions containing 50 and 60 kDa proteins that were determined to bind the virus, by using VOPBA. In another work by Gaggar and co-workers, CD46 was directly determined to bind to Ad35 after the extensive purification of the knob fiber domain by using recently developed protocols.[29] In contrast to these reports, our approach for the identification of CD46 as the receptor for the specific case of group D adenovirus Ad37, does not depend on the development of specific protocols to purify the fiber knob domain and protein purification, and offers a rapid and more general method based on partial purification and tandem mass spectrometry that can potentially be applied to other cases.

It is important to note that this general approach to identifying receptors has some aspects that can limit applicability. These include, having enough copies of the receptor to identify it in the presence of other more abundant proteins, the availability of antibodies against putative receptors, and the availability of GFP fusions of viral proteins. The rapid and sensitive mass spectrometry-based approach described here used only a few picomoles of sample and required less than a week to identify candidate receptors. Thus, we anticipate that similar approaches may accelerate progress towards the identification of other viral receptors in cases in which the mode of cell entry is still poorly understood. The substantial list of viral diseases under this category includes SARS, which is caused by the coronoavirus. Identification of these receptors and the elucidation of the functional mechanisms involved are unequivocally linked to the development of effective antiviral therapies.

Experimental Section

Partial purification and VOPBA: Chang C cells were resuspended in sucrose (250 mM), HEPES (20 mM), pH 7.0, EDTA (1 mM), aprotinin, and leupeptin (2 $\mu g\,mL^{-1}$). Cells were then homogenized/disrupted, and the organelles and nuclei were pelleted at 500 *g* for 15 min. Plasma-membrane fragments were pelleted from the supernatant of cell lysates at 200 000 *g* for 1 h and then resuspended in Tris-HCL (10 mM), pH 8.1, aprotinin and leupeptin (10 $mg\,mL^{-1}$). The membrane fraction from Chang C cells ($\sim 5\times10^8$ cells) was then solubilized in 1% CHAPS (3-((cholamidopropyl)dimethylammonio)propanesulfonate), and membrane glycoproteins containing terminal mannose groups were separated on a lentil lectin sepharose affinity column. The glycoproteins were eluted initially with three 0.5 mL aliquots of α-methylmannoside (0.2 M), EDTA (1 mM), Tris-HCl (16 mM), pH 8.1, 0.4% CHAPS, NaCl (0.4 M; elutions 1–3) and then with three 0.5 mL aliquots of SDS-PAGE sample buffer (elutions 4–6). The eluted glycoproteins from this separation were pooled and concentrated by using a Centricon YM-30 spin column. Semi-native PAGE-VOPBA was performed as previously described.[5]

Chromatography and mass spectrometry: The 50 and 60 kDa bands recognized by Ad37 were excised and treated with dithiothrietol (10 mM) to reduce disulfide linkages. Alkylation was performed with iodoacetamide (55 mM; Sigma–Aldrich) before digestion with trypsin (Promega) over night at 37 °C, with an estimated 1:30 enzyme/substrate ratio in ammonium bicarbonate (50 mM). Soybean Trypsin inhibitor (Sigma–Aldrich) was used in a 1:1 (*w*/*w*) ratio to trypsin in order to suppress proteolytic activity before deglycosylation. The extract was incubated for 1 h with the inhibitor at 37 °C and then for 2 h with PNGase-F (10 μL, 1.2 units per μL; Roche Applied Sciences). The treatment with PNGase-F, an *N*-glycosidase, was performed as a general deglycosylation step in order to simplify mass spectral interpretation, since the presence of complex carbohydrates on a protein tends to result in missed cleavages and difficulty in identification when using database searching. The LC separation was performed on a laser-pulled 100 μm ID C_{18} column with a tip of <5 μm that was also used as a nanoelectrospray emitter. Gradient elution was performed with 0.1% formic acid/acetonitrile as the mobile phase, and from 5% to 60% acetonitrile over 90 min. This was maintained for an additional 20 min with a flow rate of $\sim 200\,nL\,min^{-1}$. The MS/MS analysis was performed on a LCQ-DECA (Thermo Finnigan, San Jose, CA), as well as an Agilent LC/MSD Trap ion-trap mass spectrometer. Data-dependent scanning was used to maximize the number of peptides sequenced in the highly complex mixture. This mode of operation uses preset criteria to select unique peptides on-the-fly for undergoing MS/MS. Over 4000 MS/MS spectra were obtained during the

run. These were searched with MASCOT (Matrix Science, Limited) and Sequest (University of Washington, WA) search engine software by using the NCBInr (nonredundant database). To improve searching efficiency, the taxonomic category was limited to mammalian proteins. Only peptides producing good-quality fragmentation spectra and scoring higher than the threshold required for a 95% confidence level for Mascot were used for protein identification. We estimate that the analyzed SDS band contained approximately 5–10 pmol of receptor.

Flow cytometry: Chang C cells were incubated with Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum (DMEM-FBS) or 1:100 dilution of a monoclonal antibody specific for CD46, CD87, CD98, or CD147 (Becton Dickenson Pharmingen, San Diego, CA) in DMEM-FBS. Cells were washed twice and incubated with a 1:250 dilution of Alexa488-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) in DMEM-FBS. After the cells had been washed twice more, they were detached with ethylenediaminetetraacetic acid (5 mM) in DMEM-FBS and analyzed for fluorescence with a FACScan flow cytometer (Becton Dickenson, San Jose, CA).

Antibody inhibition of virus-mediated gene delivery: Goat antibodies raised against terminal peptides of extracellular domains of CD46, CD87, CD98, and CD147 (200 $\mu g\,mL^{-1}$ each; Santa Cruz Biotechnology, Santa Cruz, CA) were dialyzed in PBS (2 L) at 4 °C to remove any trace contaminants. 40000 Chang C cells were gently rocked with 2X 199 Medium (125 μL; Invitrogen, Carlsbad, CA), hydroxyethylpiperazine ethanesulfonate (HEPES; 25 μL, 1 M) pH 7.0, and phosphate-buffered saline (PBS; 80 μL) or dialyzed CD46, CD87, CD98, or CD147 antibody for 30 min at room temperature. Ad37.GFP, an adenovirus vector carrying a green fluorescent protein (GFP) transgene and the Ad37 fiber (20 μL of 2×10^6 particles per μL; 4×10^7 particles or 1000 particles per cell),[11] was added to each antibody/medium mixture. Cells were gently rocked for 1 h at room temperature, and unbound virus was removed by three washes with DMEM-FBS. Cells were cultured overnight and analyzed for GFP transgene expression by flow cytometry.

Statistical analysis: Antibody-inhibition data were tested with an ANOVA by using SPSS 11.0 (SPSS, Chicago, IL).

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