Title
CAR is Not Requred for Adenovirus Infection: Integrin alpha v beta 5 Mediates Binding to CAR-Negative Cells

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CAR is Not Required for Adenovirus Infection: Integrin alpha v beta 5 Mediates Binding to CAR-Negative Cells

by

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I would like to thank Frank McCormick, my mentor. He is inspiring and I have truly enjoyed working with him. Many thanks to the virus bay—Clodagh O'Shea, Conrado Soria, Serah Choi, Demetris Iacovides, Abigail Miller, Jamie Smyth, and Mike Nehil—for all of the support and fun through the years. Sang Lee and Pablo Rodriguez-Viciana helped me many times with their unending technical knowledge. My baymates, Amy Young and Irma Rangal, added so much fun to my experience by so whole-heartedly embracing my obsession with decorating. I would like to thank Jesse Lyons, who became one of my very best friends and helped me tremendously with all of the challenges that arise during graduate school (and life). I would also like to thank Liz Sharp, who started as a classmate but became a lifelong friend, for laughter and camaraderie through the years. My family, especially my wonderful parents Clara and Len Lyle, has always been so encouraging in everything I have pursued in life, even the long process of graduate school. And last but by no means least, I would like to thank my husband, Michael Mysinger, for his support, his love, and his confidence in me.

CAR is Not Required for Adenovirus Infection: Integrin alpha v beta 5 Mediates Binding to CAR-Negative Cells

Cynthia Myinger

Abstract

Adenovirus (Ad) is the most commonly used vector in gene therapy trials worldwide. Therefore, understanding the interaction between the virus and the cell surface and how this interaction impacts cell infection is of great importance to both the analysis of current trials using Ad vectors and the design of next generation Ad vectors. Cancer is in particular an important target of Ad-based therapeutics. Therefore, we have measured the ability of a replication incompetent subgroup C adenovirus (Ad5-GFP) to infect a panel of cancer cell lines. Infection across this panel was highly variable. Coxsackie and Adenovirus Receptor (CAR) is the known cellular receptor for subgroup C adenoviruses so we hypothesized that varying levels of CAR on these cell lines would explain the varying infections. However, neither CAR mRNA levels, as measured by both Affymetrix array and QT-PCR, nor CAR protein levels, as measured by both FACS and western, correlated with infection. One cell line, MDA MB 435, is CAR negative by all criteria that we have measured, but is one of the most infectible cell lines on the panel. Additionally, MCF7 cells and WM278 cells have minimal surface CAR but are infectible. Ad5 binds to these cell lines via a high affinity (0.16 nM) interaction. Surprisingly, the infection of CAR-negative cells is fiber-independent, as determined by competition experiments using soluble fiber. Because the penton base of the Ad virion is known to interact with RGD-binding integrins, we examined the ability of an RGD

peptide to block the binding of Ad5 to CAR-negative cells. We found that Ad5 is using an RGD-binding integrin as a primary receptor for binding and infection. We then utilized blocking antibodies to determine which integrins are involved. We found that a blocking antibody to integrin αvβ5 blocks Ad5 from binding to CAR-negative cells lines. We conclude that integrin αvβ5 is an alternate attachment receptor for Ad5, representing a previously unidentified entry pathway for Ad5 that is both CAR and fiber-independent.

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Introduction

Viruses bind to specific cellular receptors in order to infect their hosts. Which receptors a specific virus uses are important factors in determining host range, cellular tropism, and pathogenesis. Over the past few decades a number of viral receptors have been identified and several trends have emerged from this data. Multiple viruses, often ones with very different structures and pathologies, converge on the same cellular receptor. For example, both Coxsackie B viruses, which are the viruses most frequently associated with acute heart infections, and subgroup C Adenoviruses, which cause respiratory disease, are proposed to use Coxsackie and Adenovirus Receptor (CAR) as a receptor [1]. Viruses from diverse families, including Adenoviridae, Picornaviridae, Bunyaviridae, Reoviridae, and Herpesviridae all use integrin molecules as receptors [2-6]. Additionally, an individual virus can bind to multiple receptors: Herpes Simplex Virus (HSV) binds to heparin sulphate chains on proteoglycans, a tumor necrosis factor (TNF) receptor family member, and two immunoglobulin superfamily members [7]. Viruses frequently use these multiple receptors for distinct, independent functions [8].

Adenovirus is one of the first viruses for which the use of unique receptors for binding and for internalization was proposed [6]. Adenovirus is a non-enveloped double stranded DNA virus associated with respiratory disease, ocular disease, and gastroenteritis [9]. Adenovirus infections are typically mild, although in individuals who are immunologically or nutritionally compromised more severe disease has been reported [9, 10]. Adenovirus has three major capsid proteins: hexon, which forms the bulk of the

capsid and is present in 240 copies, penton, which is present in five copies at each of the twelve vertices, and fiber, a homotrimeric protein that protrudes from each vertice, extending outward from the penton base. More than 50 human serotypes of Adenovirus have been identified to date [11]. The best studied of these are the subgroup C Adenoviruses, including Adenovirus Serotype 2 (Ad2) and Adenovirus Serotype 5 (Ad5). The primary receptor for subgroup C Adenoviruses is Coxsackie and Adenovirus Receptor (CAR), which binds to the globular knob domain of fiber [1]. This requisite high affinity interaction docks the virus to the cell, thus allowing secondary interactions to occur. Following fiber binding to CAR, the penton base engages cellular integrins, most notably αvβ3 and αvβ5, to initiate receptor mediated endocytosis and viral entry [6]. Adenovirus binds to integrins via a conserved RGD motif present in the penton base.

Adenovirus is one of the best characterized viruses. As a result of both the wealth of knowledge about its structure and mode of replication as well as technical considerations, such as the ease with which high titers of virus can be produced, Adenovirus has been frequently used as a model system [12, 13].

Chapter One:

Integrin αvβ5 mediates binding of Adenovirus Serotype 5 in the absence of CAR

Introduction

Viral entry is thought to be a multistep process involving the initial binding to the cell surface, mediated by a primary receptor, followed by internalization frequently mediated by independent coreceptors [8, 14]. The idea that virus entry is the result of distinct sequential events using multiple different receptors influences how we understand virus evolution, how we interpret new data about virus receptors, and how we design novel antiviral agents [8, 15, 16].

Adenovirus is one of the first viruses for which a multistep entry hypothesis, using unique receptors for binding and internalization, was proposed [6]. Adenovirus fiber first binds to CAR, the primary receptor, followed by penton base engaging integrins to initiate receptor mediated endocytosis [1, 6]. The penton-integrin interaction is proposed to be exclusively involved in virus internalization and not to contribute to virus binding [6].

Since the identification of CAR and αv integrins, a number of studies examining Adenovirus infection and receptor expression have been published. Some of this data has been confusing within the context of the current model. Studies using Adenoviruses with mutant fibers ablated for CAR binding found no change in the biodistribution of the virus compared to wildtype, in both mice and non-human primates [17-20]. Similarly, although overexpression of CAR does allow infection of non-permissive cells *in vitro*, a lack of correlation between CAR expression and Adenovirus infection of cell lines has

been observed [1, 21, 22]. These observations have led us to question the proposed two-step model for Adenovirus infection, in which Adenovirus must first bind to CAR, the primary receptor, in order to bind to integrins and trigger viral entry.

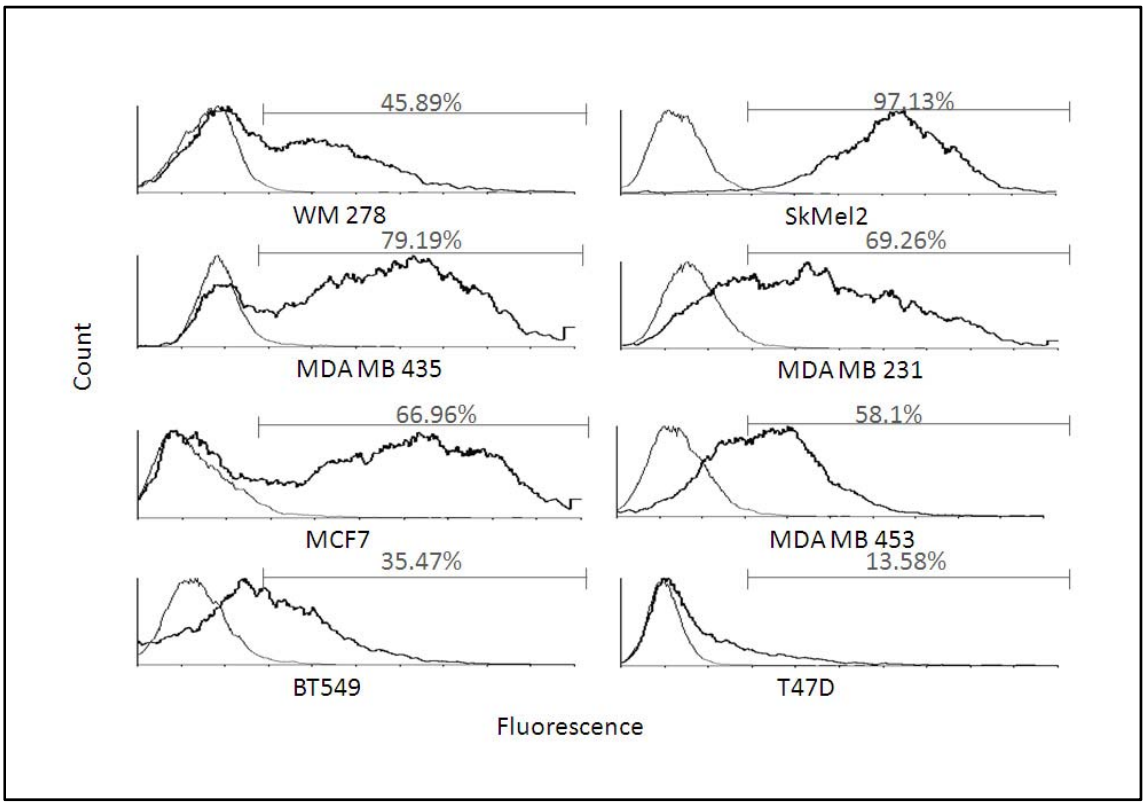
We report here that Ad5 can efficiently infect cells which do not express CAR. Further, Ad5 binds to these cells via Integrin αvβ5, a receptor previously thought to be exclusively used for internalization and thus classified as a secondary coreceptor.

Results

Adenovirus infection is variable across a panel of cancer cell lines.

Adenovirus fiber binding to the cellular membrane protein CAR has been proposed to be the required first step of adenovirus infection [1]. CAR is a cell adhesion molecule and, like other cell adhesion molecules, is downregulated during cancer progression [23, 24]. Additionally, several reports have shown that the ability of Adenovirus to infect different cancer cell lines is variable [25-27]. Therefore, we chose a panel of cancer cell lines to study the requirement for a CAR-mediated binding event in Adenovirus infection. We first measured the ability of Ad5 to infect this panel of cancer cell lines. Because we are specifically interested in the initial steps of infection and did not want to complicate our results with potential variability in replication, we infected cells with a non-replicating virus deleted for E1A that expresses GFP (Ad5-GFP) and used GFP expression as a measure of infection. Figure 1 shows that Ad5-GFP infected these cells with a wide range of efficiency. SkMel2 cells were most infectible with more than 95% of cells positive for GFP. MDA MB 435, MCF7, MDA MB 231, and MDA MB 453 were infected to similar levels, ranging from 58-79% of cells GFP-positive. On the lower end of the spectrum, BT549 and WM278 cells were infected around 40%. And finally, T47D cells show a very small shift in fluorescence after infection, indicating these cells are resistant to Ad5 infection.

Figure 1. Ad5 infection is variable across a panel of cancer cell lines.



Cells were infected with Ad5-GFP at MOI 25 and incubated overnight. Infection was quantified using flow cytometry analysis of cells infected with Ad5-GFP (black line) compared to an uninfected control (grey line) for each cell line. The percentage of cells positive for GFP is quantified. Data shown is representative of at least two independent experiments.

Surface CAR levels do not explain differences in Ad5 infection.

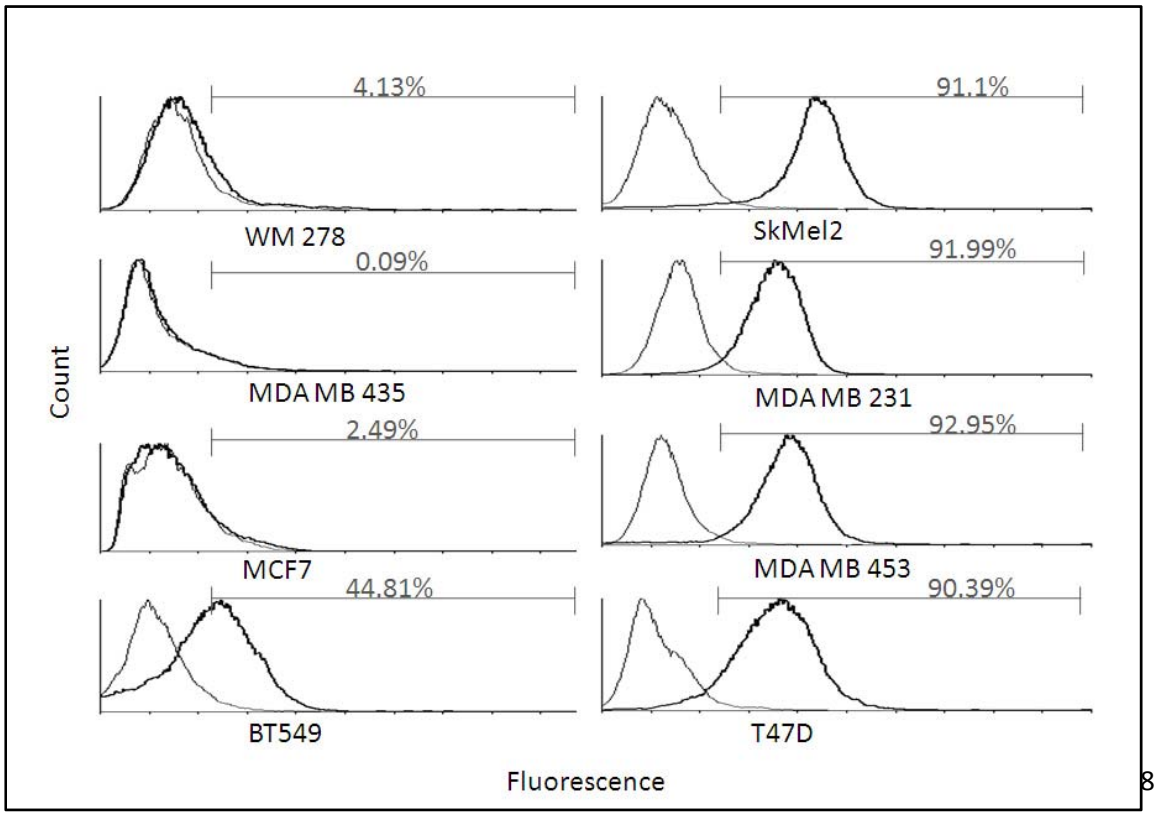
CAR expression is thought to be required for Ad5 infection. To investigate whether the variability in infection could be explained by differences in CAR levels, as would be expected within the framework of the current model for Adenovirus infection, we next measured surface CAR expression using FACS. Figure 2a shows that most of the cell lines, including SkMel2, MDA MB 231, MDA MB 453 and T47D cells, expressed CAR

on the majority of cells. Interestingly, T47D cells, which are resistant to Ad5 infection, also express CAR on the cell surface. In contrast, WM278, MDA MB 435, and MCF7 cells, all of which are infectible with Ad5-GFP, do not express CAR on the cell surface. These data suggests CAR binding is neither sufficient nor necessary for Ad5 infection.

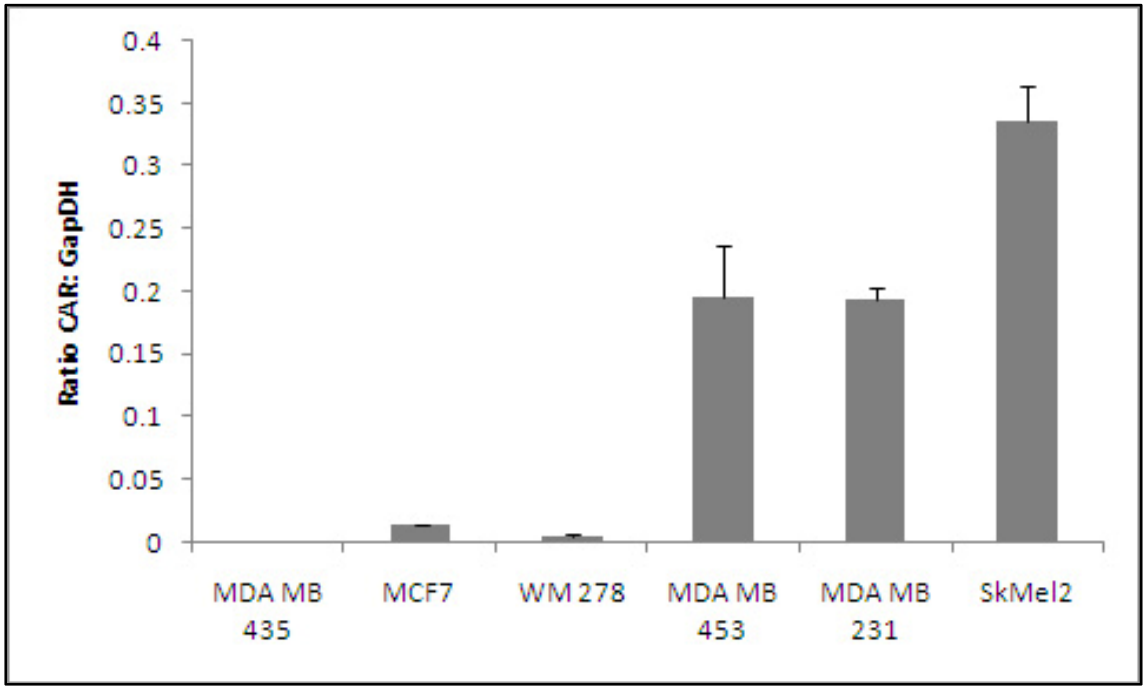
We next verified CAR expression by measuring mRNA levels in these cell lines using Taqman analysis. Figure 2b shows that CAR mRNA levels correlated with surface protein levels (Figure 2b). The three cell lines which show undetectable surface CAR expression also had very little to, in the case of MDA MB 435 cells, no detectable CAR mRNA expressed (Figure 2b).

Figure 2. CAR expression levels.

A



B



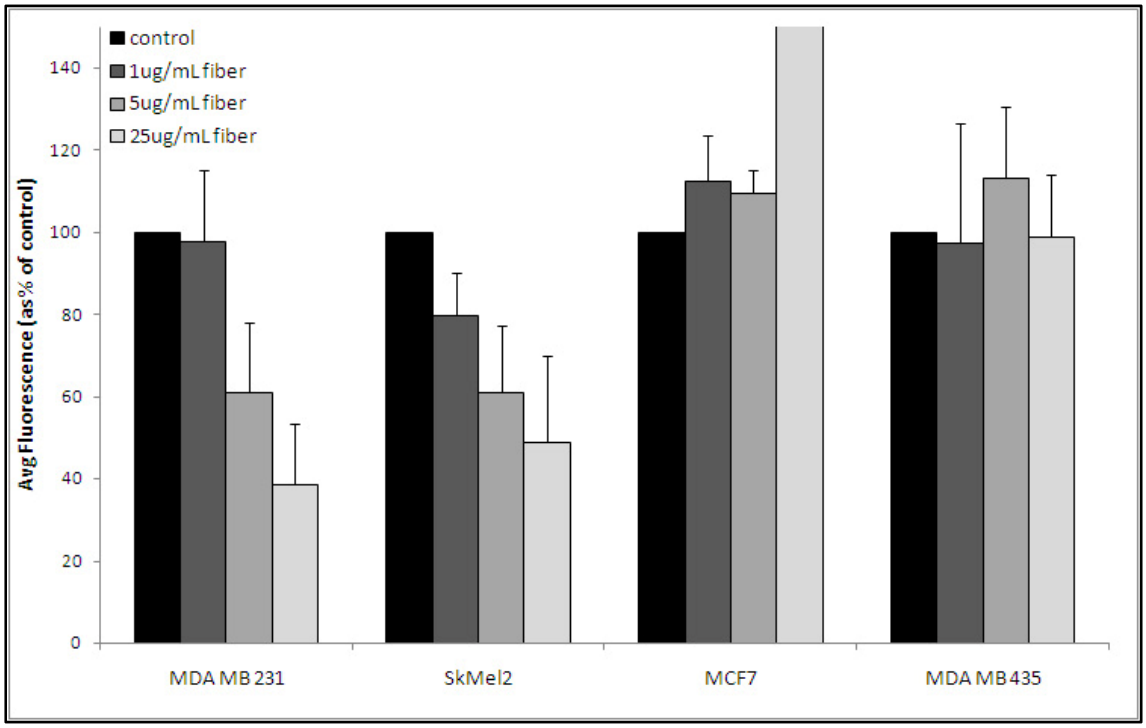
A. Surface CAR levels were detected using flow cytometry. Cells were stained either with the monoclonal antibody RmcB which recognizes CAR (black line) or for control, only the secondary antibody (grey line). The percentage of cells which are positive for CAR is quantified. Data shown is representative of at least two independent experiments

Infection of CAR-negative cells is fiber-independent.

Previous reports have indicated that the fiber-CAR interaction mediates binding of Ad5 to the cell surface [1, 6, 28]. Because we found Ad5 infection of cancer cells can occur in a CAR-independent fashion, we next examined whether Ad5 infection is still dependent on fiber, perhaps by binding to a different cellular receptor. To address this question, we tested the dependence of infection in CAR-negative cells on fiber. Cells

were preincubated with soluble fiber prior to adding Ad5-GFP to the cells and measuring infection, as determined by GFP expression. Infection of MDA MB 231 and SkMel2 cells, both of which express CAR (Figure 2), could be blocked in a dose-dependent manner by preincubation with soluble fiber (Figure 3). In contrast, infection of CAR-negative cells MCF7 and MDA MB 435 was not blocked. Ad5 infection of MCF7 and MDA MB 435 cells is therefore not only CAR-independent but also fiber-independent.

Figure 3. Infection of CAR-negative cells is fiber-independent.



Cells were preincubated for 1 hr with increasing concentrations of soluble fiber followed by addition of Ad5-GFP and futher incubation overnight. Flow cytometry was used to quantify fluorescence intensity. Data shown is the average of at least three independent experiments and error bars represent standard deviation.

Binding to CAR-negative cells is integrin-dependent.

Our fiber blocking studies ruled out the possibility that fiber binds an alternate receptor in CAR-negative cells. A second well-characterized interaction between Ad5 and the cell surface is the binding of the RGD (Arg-Gly-Asp) domain in the penton base of Adenovirus to integrin αvβ3 and integrin αvβ5 [6]. Integrins are heterodimeric cell surface molecules that mediate cell-extracellular matrix and cell-cell interactions and are therefore involved in a number of cellular processes [29]. Additionally, several viruses and bacteria have been reported to use integrins to enter host cells. Integrin-mediated processes are often regulated by both ligand binding and integrin clustering; therefore, many integrin ligands are multivalent, able to bind multiple integrins simultaneously [30]. The crystal structure of the RGD domain of Adenovirus penton binding integrin αvβ5 has been solved, revealing that one penton complex of the virus binds approximately four integrin molecules [31]. Blocking integrin binding prevents Adenovirus from being internalized but does not impact binding of Adenovirus to the cell surface [6]. Although these studies predate the discovery of CAR, the cells used in them expressed a fiber receptor, in retrospect most likely CAR, as infection of the cells could be blocked by soluble fiber [6]. However, the role of integrins in Adenovirus infection in cells lacking CAR is unclear. To distinguish between two possible roles of integrins, binding and internalization, we used an assay to directly measure binding. Cells were plated in 96-well plates and incubated overnight at 37°. Cells were chilled to 4°, a temperature which allows binding but does not permit internalization, and then preincubated with increasing concentrations of an integrin-blocking peptide, RGD, or a control peptide, RGE. Ad5 was added to the cells at 4°. Cells were washed and fixed

and virus bound determined using an antibody directed against Ad5 capsid proteins. Figure 4a shows that in SkMel2 cells, which express CAR, integrin blocking peptide RGD does not block Ad5 binding, consistent with previous reports. However, in both MDA MB 435 and MCF7 cells, which are CAR-negative, Ad5 binding to cells is blocked by RGD, showing that the initial binding event in these cells is integrin-dependent.

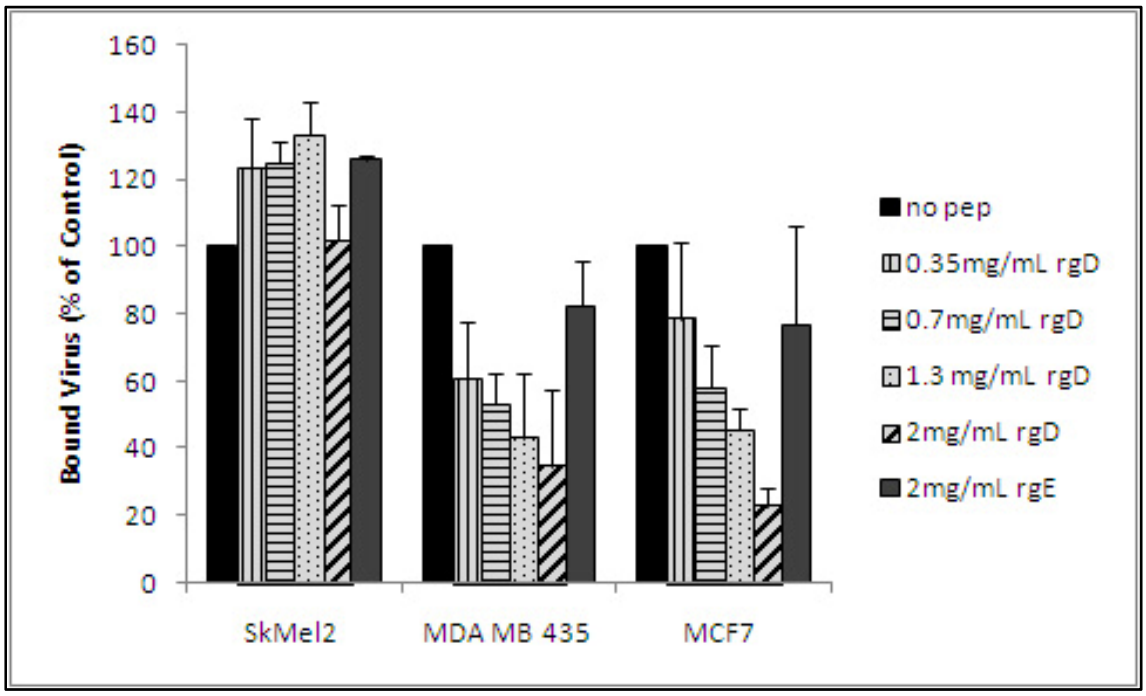
Although eight integrin dimers are reported to bind to ligands that contain RGD, integrins αvβ3, αvβ5, and αvβ1 have been specifically implicated in Adenovirus infection [6, 32, 33]. Therefore, we measured the levels of these integrins on the surface of MDA MB 435 and MCF7 cells, both of which lack CAR expression. We found that MDA MB 435 expresses all three integrins, expressing the highest levels of αvβ5 and the lowest levels of αvβ3 (Figure 4b). We found MCF7 cells only express αvβ5 and β1 (Figure 4b).

Next, we investigated whether one of these integrins is responsible for the binding of Ad5 to CAR-negative cells. Again at 4° to prevent virus internalization, cells were preincubated with antibodies that block ligand binding to integrins and then Ad5 was added to cells and the amount of virus bound measured. Preincubation with blocking antibodies to β1 or αvβ3, in either MDA MB 435 or MCF7 cells, did not inhibit infection. However, blocking αvβ5 dramatically reduced Ad5 binding in both cells. In MDA MB 435 cells, blocking αvβ5 reduced binding to only 5% of control and in MCF7 cells, binding is reduced to 19% of control (Figure 4c). From this result, we conclude that

binding of Ad5 to CAR-negative cells is occurring via the RGD domain of penton binding to integrin αvβ5.

Figure 4. The role of integrins in infection of CAR-negative cells.

A



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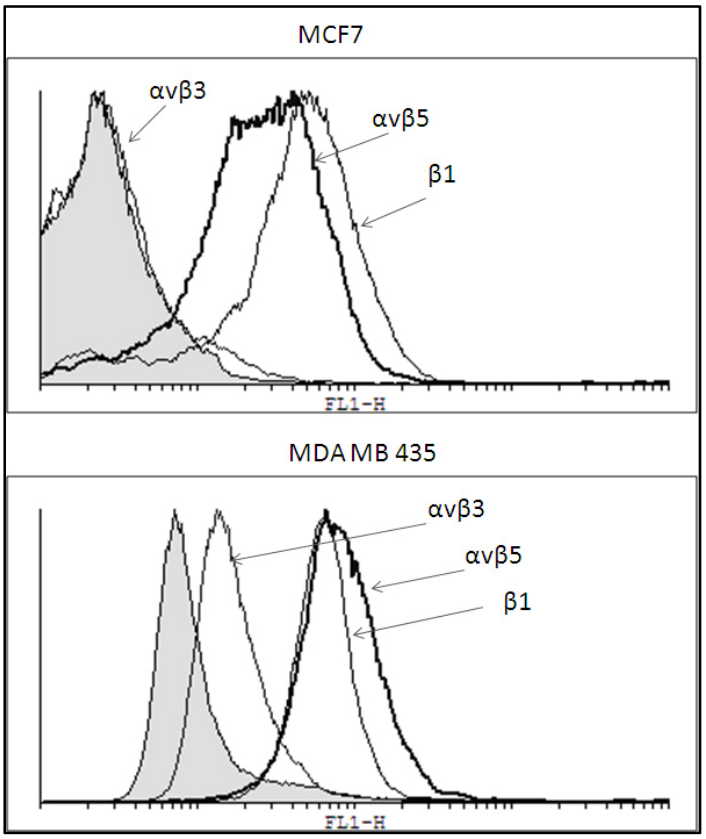
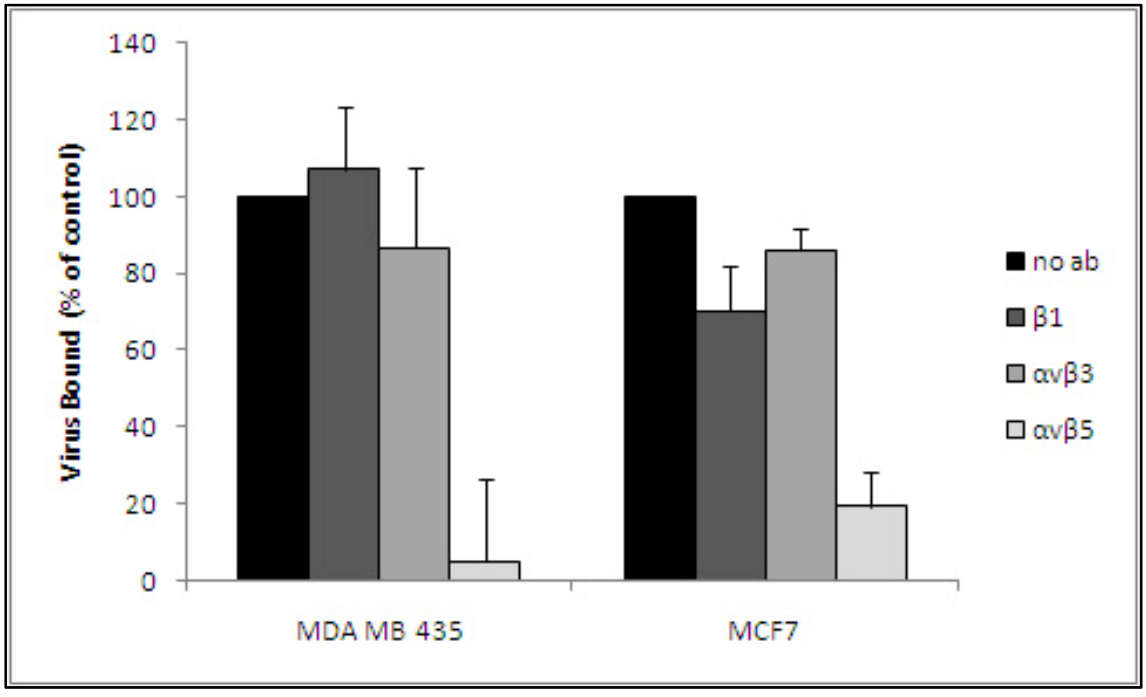

MCF7
αvβ3
αvβ5
β1
FL1-H
MDA MB 435
αvβ3
αvβ5
β1
FL1-H

C


Virus Bound (% of control)
140
120
100
80
60
40
20
0
MDA MB 435
MCF7
no ab
β1
αvβ3
αvβ5

C. Cells were preincubated with media alone, increasing concentrations of synthetic peptide GRGDSP, or control peptide GRGESP for 1 hr followed by the addition of Ad5, all at 4^0. Samples were then washed and fixed and virus bound was detected using an antibody to Ad5 as described in materials and methods. Data shown is the average of at least three independent experiments and error bars represent standard deviation.

D. Surface integrin levels were determined using flow cytometry. Cells were stained with LM609 (Integrin αvβ3), P1F6 (Integrin αvβ5), JB1A

Binding of Ad5 via integrin αvβ5 to CAR-negative cells is high affinity.

To further characterize the interaction between Ad5 and the surface of cells in which binding occurs via integrin αvβ5, we measured the binding affinity of whole Ad5 and the surface of MDA MB 435 cells, which bind Ad5 through integrin αvβ5 (Fig 4c). Figure 5a shows binding to MDA MB 435 cells is specific and saturable and represents a typical binding isotherm. To determine the dissociation constant (K_d), a measure of the strength of an interaction, we fit the data to the Langmuir binding isotherm (Eqn 1) [34].

$$Y = \frac{[L]}{(K_d + [L])}$$

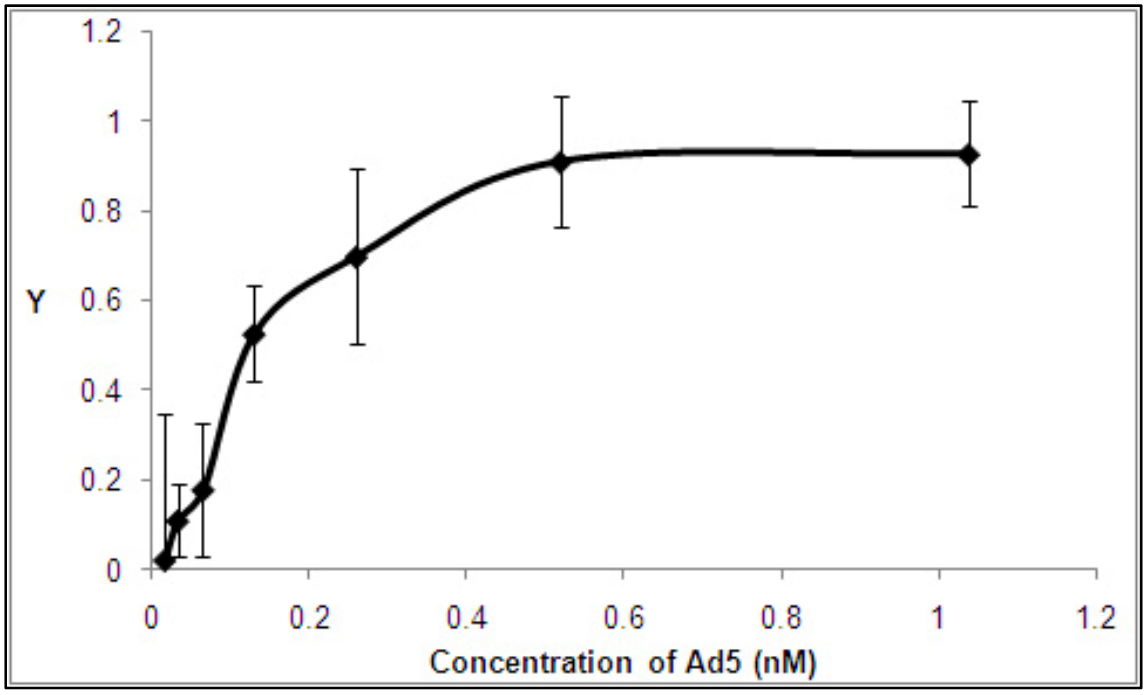
(Eqn 1)

Here, Y is the fractional occupancy of the receptor and [L] is the ligand concentration. We performed a non-linear least-squared analysis using MS Excels's Solver function to

calculate the K_d. Figure 5b shows observed versus calculated values of Y, demonstrating the observed values fit this equation. We calculate a K_D of 1.4 x 10^{-10} M. Therefore, we conclude that Ad5 can initiate infection by binding to cells via integrin αvβ5 and this interaction is high affinity, in the picomolar range.

Figure 5. Ad5 binding to cells via Integrin αvβ5 is high affinity.

A



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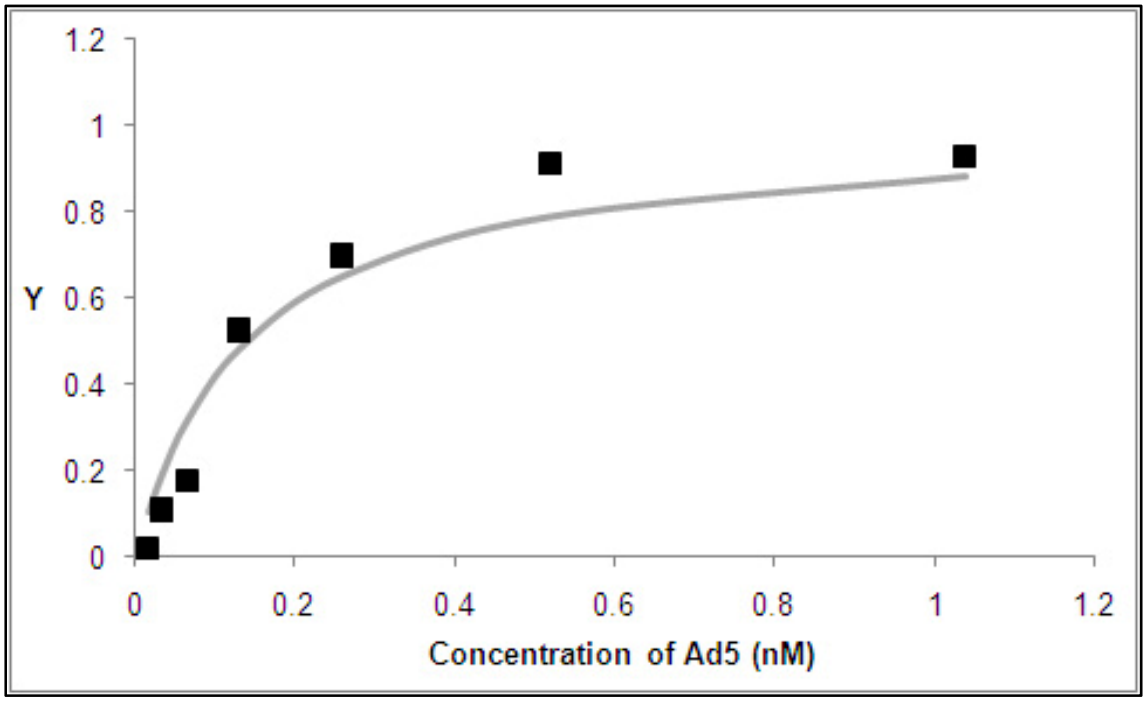


F. Cells were incubated with various concentrations of Ad5 at 4^0 for sufficient time for virus bound to reach equilibrium. Virus bound was measured as described in Figure 4. Y is the fractional occupancy, which is a ratio of virus bound to maximum virus bound. Data shown is the average of at least three independent experiments and error bars represent standard deviation.

Discussion

In this study we report that cells which do not express CAR can be efficiently infected by Ad5. This infection is not dependent on fiber binding to cells but rather can be blocked by synthetic peptides that block the RGD/integrin interaction, indicating binding is occurring between the RGD motif in the viral penton base and cellular integrins. Further, we find that binding to CAR-negative cells is inhibited specifically by a blocking antibody to integrin αvβ5, demonstrating that integrin αvβ5 alone is sufficient for Ad5 attachment to cells. The binding mediated by integrin αvβ5 is extremely high affinity, in the picomolar range. Our data challenges the current model of Adenovirus infection, in which binding to a primary receptor, CAR, is required in order for subsequent interactions between Adenovirus and integrins to initiate viral entry.

Our results suggest that Ad5 does not require a primary receptor to dock it to the cell before it can interact with internalization receptors. Other viruses are also reported to use both primary and internalization receptors. HIV-1 first binds to CD4 followed by binding to the chemokine receptors CCR5 or CXCR4, which trigger membrane fusion [16]. Binding to CD4 induces conformational changes in the HIV protein gp120, revealing the previously hidden binding site for its coreceptors [35]. Variants with mutations in gp120 allowing for direct interaction with coreceptors have been isolated *in vitro*; however, these variants are sensitive to neutralizing antibodies and therefore selected against *in vivo* [36]. Therefore, the role of CD4 binding in HIV-1 infection may be particularly critical in evading the immune system of the host.

Unlike HIV-1 binding to CD4, Ad5 binding to CAR does not induce conformational changes in viral proteins, thus facilitating subsequent entry steps. It also does not induce cell signaling events which might prepare the cell for viral entry as only the extracellular domain of CAR is required for Adenovirus infection [37]. Rather, CAR is thought only to hold the virus close to the cell surface allowing the penton base to engage integrins and initiate internalization. Our results suggest this step is not required for infection, but rather Integrin αvβ5 is sufficient to bind to Ad5. However, CAR binding is conserved in a number of Adenovirus serotypes and the fiber-CAR interaction is one that is well characterized and of high affinity [11, 38]. Therefore, CAR binding likely plays an important role somewhere in the Adenovirus life cycle. One possibility is that the major role of CAR in Adenovirus infection is as an exit receptor, which it has been proposed to be [39]. When Ad5 lyses a cell, excess fiber is released and through binding to CAR, disrupts neighboring cell-cell junctions, allowing for release of the virus back to the apical surface where it may continue infecting cells [39]. Supporting this hypothesis, at least two serotypes of Adenovirus, Ad9 and Ad37 have fibers which bind CAR but do not use CAR as an attachment receptor [11, 40].

Both HIV, as evidenced by CD4-independent variants isolated *in vitro*, and Ad5, as evidenced by the results of this paper, can infect cells without binding to their so-called primary receptors. Binding to these receptors, instead of being strictly required for infection, may contribute to other necessary parts of the virus life cycle, such as evading

the host immune system or facilitating virus escape. Many other viruses with less characterized receptors seem to also use multiple receptors, some classified as binding receptors [14]. For example, rotaviruses are thought to first bind to a sialic acid (SA)-containing molecule, which anchors the virus to the cell, and then bind to coreceptors to initiate viral entry [14]. Mutant variants of rotaviruses that are SA-independent and interact directly with coreceptors have been isolated *in vitro*, suggesting that similarly to HIV and Adenovirus, binding to the primary receptor is not strictly required for infection [41, 42]. Therefore, the interaction between rotaviruses and SA-containing molecules may facilitate an as yet unidentified aspect of rotavirus infection.

In addition to being used as a model system for viral entry, much effort has been put into developing adenoviruses, especially subgroup C Adenoviruses including Ad5, as vectors for gene therapy. In fact, Adenovirus vectors have been used in more than one quarter of gene therapy trials worldwide [43]. Cancer is one of the most common targets of Adenovirus -mediated gene therapy. As mentioned previously, CAR expression is often lost as cancers progress and this loss has been viewed as a major hurdle to using Adenovirus -based therapies in cancer [23-27]. However, integrin αvβ5 has been reported to often be overexpressed in cancers. Therefore, our conclusion that Ad5 can use integrin αvβ5 to bind to and infect cells lacking CAR suggests that cancer cells having lost CAR expression may still be good targets for Adenovirus -based therapies. We also observed what may be an as yet unidentified obstacle to these therapies, however. T47D cells, which express CAR (Fig 2a) and integrin αvβ5 (data not shown)

are still resistant to Ad5 infection (Fig 1). Therefore, future studies to determine why these cells are not infected, even in the presence of the required receptors, are needed.

Interestingly, cells which do express CAR are dependent on CAR, as indicated by the fiber blocking experiments, despite expressing Integrin αvβ5 (data not shown). One possible explanation is that fiber binding to CAR sterically hinders the virus from accessing Integrin αvβ5. In support of this idea, Einfeld et al showed that preincubating cells with fiber blocked infection of AE25 cells an order of magnitude more than using a mutant virus ablated for CAR binding [18]. Another possibility is that cells which have lost CAR, a cell-cell adhesion molecule, likely have very different cell-cell contacts. This difference could lead to a very different integrin profile, potentially altering accessibility to integrins or activation status of integrins.

Finally, we further characterized the binding of Ad5 to cells via integrin αvβ5 by measuring the affinity of the virus for the cell surface. We find Ad5 has an affinity for CAR-negative cells of 1.4×10^{-10} M. An affinity of 140 picomolar is an extremely high affinity interaction. Previous studies have reported an affinity of the penton complex for cells to be 55 nM [6]. However, in the context of the intact virus, more than one complex is likely to engage the cell surface, thus explaining the increased affinity. Generally, studying virus-receptor interaction using purified viral proteins or purified receptors likely underestimates the strength of binding since virtually all viruses have multiple copies of attachment proteins available to bind the cell surface.

In conclusion, we have found that cells which do not express CAR can still be efficiently infected by Ad5. In these cells, binding and subsequent infection occurs directly through Integrin αvβ5, previously thought to be insufficient for Adenovirus infection.

Experimental Procedures

Cell Lines and Viruses

SkMel2 cells were cultured in MEM supplemented with sodium pyruvate, non-essential amino acids, and 10% FBS. WM278 were cultured in DME-H16 and supplemented with 10% FBS. All other cancer cell lines were cultured in DMEM supplemented with 10% FBS. Virus used was replication incompetent E1A deleted and expressed GFP (Ad5-GFP). Virus was propogated in 293/E4/pIX cells and harvested by CsCl gradient ultracentrifugation as previously described [44, 45]. Virus titer was determined as previously described [46].

Antibodies and Peptides

The MAb RmcB was used to detect CAR expression [1]. The Mabs LM609, P1F6, and JB1A directed against Integrins αvβ3, αvβ5, and β1 respectively were purchased from Chemicon. The secondary antibody Alexa 488 was purchased from Molecular Probes. The synthetic peptides GRGDSP and GRGESP were purchased from Sigma.

Recombinant Fiber

Full length Ad5 fiber cloned into a Gateway entry vector was a kind gift of Demetris Iacovides. Fiber was then cloned into a his-tagged destination vector using the Gateway system per manufacturer's instructions (Invitrogen). Fiber was transformed into and grown up in BL21 Star (DE3) E.coli (Invitrogen). Overnight starter culture was diluted 1:100 in LB/amp and grown until bacteria reached log phase. 50uM IPTG was added and bacteria were grown at room temperature overnight. Pellets were disrupted using

Bugbuster (Novagen) per manufacturer's instructions. Fiber was purified via its his-tag by incubation with Probond resin (Invitrogen), several washes with 20mM Imidizole, and elution using Poly-Prep Chromatography Columns (BioRad) with 0.2 M Imidizole. Purified recombinant fiber was then dialyzed into PBS for use in experiments.

Cell Infection Assay

Cells were plated in 6-well plates and incubated overnight at 37°. Cells were infected with Ad5-GFP at MOI 25 in DMEM with 2% FBS. After overnight incubation at 37°, cells were trypsinized, washed with PBS, and GFP expression quantitated using flow cytometry. For fiber blocking experiment, prior to addition of Ad5-GFP, different quantities of soluble fiber (1ug/mL, 5ug/mL, or 25 ug/mL) were added to cells, incubated at room temperature for 1 hr. Then Ad5-GFP was added to cells at MOI 25 and cells were incubated overnight at 37° before flow cytometry analysis.

Surface expression levels

Cells were trypsinized, washed with PBS, and $1x10^6$ cells were incubated with primary antibody for 30 minutes on ice. Cells were washed, incubated with secondary for 30 minutes on ice, and analyzed by flow cytometry. Dilutions were as follows: RmcB (1-50), LM609 (1-100), P1F6 (1-100), JB1A (1-100), Alexa 488 (1-100).

Quantitative PCR

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen). PCR was performed by the Genome Analysis Core Facility, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco. PCR was conducted in triplicate

with 20 uL reaction volumes of 1X Taqman buffer (1X Applied Biosystems PCR buffer, 20% glycerol, 2.5% gelatin, 60nM Rox as a passive reference), 5.5 mM $MgCl_2$, 0.5 mM each primer, 0.2 uM each deoxynucleotide triphosphate (dNTP), 200 nM probe, and 0.025 unit/uL AmpliTaq Gold (Applied Biosystems) with 5 ng cDNA. A large master mix of the above-mentioned components (minus the primers, probe, and cDNA) was made for each experiment and aliquoted into individual tubes, one for each cDNA sample. cDNA was then added to the aliquoted master mix. The master mix with cDNA was aliquoted into a 384-well plate. The primers and probes were mixed together and added to the master mix and cDNA in the 384-well plate. PCR was conducted on the ABI 7900HT (Applied Biosystems) using the following cycle parameters: 1 cycle of 95° for 10 minutes and 40 cycles of 95° for 15 seconds, 60° for 1 minute. Analysis was carried out using the SDS software (version 2.3) supplied with the ABI 7900HT to determine the Ct values of each reaction. Ct values were determined for three test and three reference reactions in each sample, averaged, and subtracted to obtain the ΔCt [ΔCt = Ct (test locus) – Ct (control locus)]. PCR efficiencies were measured for all custom assays and were greater than or equal to 90%. Therefore, relative fold difference was calculated for each primer/probe combination as $2^{-\Delta Ct}$ x 100. PCR primer and TaqMan probe sequences were synthesized by Integrated DNA Technologies (Coralville, IA) [or purchased from Applied Biosystems]. The sequences were as follows

Human CAR

Amplicon:

GGCGCTCCTGCTGTGCTTCGTGCTCCTGTGCGGAGTAGTGGATTTCGCCAGAA
GTTTGAGTATCACTACTCCTGAAGAGATGATTGAAAAAGCCAAAG

Forward: GGCGCTCCTGCTGTGC

Reverse: CTTTGGCTTTTTCAATCATCTCTTC

Probe: TGCGGAGTAGTGGATTTCGCCAGAAG

Human GapDH:

Amplicon:

ATTCCACCCATGGCAAATTCCATGGCACCGTCAAGGCTGAGAACGGGAAGCT

TGTCATCAATGGAAATCCCA

Forward: ATTCCACCCATGGCAAATTC

Reverse: TGGGATTTCCATTGATGACAAG

Probe: ATGGCACCGTCAAGGCTGAGAACG

Ad5 Binding Assay

Cells were plated in 96-well SigmaScreen poly-D-lysine coated plates (Sigma) and incubated overnight at 37°. For peptide and antibody blocking experiments, cells were prechilled at 4° for 30 minutes followed by addition of either peptide at indicated concentration or antibody (500ug/mL) for 1 hr. Ad5-GFP (0.04 ug/uL) diluted in DMEM with 50% FBS was added to cells and incubated for 6 hrs at 4°. Cells were washed several times and fixed with ice cold solution of 95% EtOH/5% Acetic Acid. Cells were washed 1x TBST (0.05M Tris, 0.15 M NaCl, 0.5% Tween-20, pH 7.5) and incubated in Superblock (Pierce) for 1 hr at RT. Cells were washed 2x Superblock followed by incubation with a non-related control IgG antibody to block any non-specific interactions for 30 min, RT. Cells were washed 1x TBST and incubated with polyclonal rabbit anti-

Ad5 ab (Access Biomedical) for 30 min, RT, followed by washing 4xTBST. Cells were next incubated with Goat-anti-Rabbit-AP (Pierce) for 30 min, RT, followed by washing 4xTBST. Signal was then amplified and detected using an Elisa Amplification System per manufacturer's instructions (Invitrogen). For determining K_D of Ad5 binding to cells, the above protocol was used except cells were incubated with Ad5 at varying concentrations for 18 hrs at 4° prior to fixing.

Chapter 2:

Measuring Avidity: Intact Adenovirus binds to the cell surface 1000 fold stronger than individual viral attachment proteins

Introduction

Attachment between a virus particle and a cell is one of the first steps in viral infection. The attachment step is often a critical factor in determining cellular tropism, host range, and the pathogenesis of a virus [47]. Understanding the exact nature of the interaction between viruses and cell surfaces is therefore essential to understanding the early events in viral infections.

The initial attachment step occurs via binding of the viral attachment protein (VAP) to a specific cellular receptor. Viruses present multiple copies of their VAP on the viral surface and the ability of multiple VAPs to interact with multiple receptors likely increases the strength of the virus/cell interaction. A complete understanding of this interaction requires understanding not only how an isolated VAP binds to its receptor, but also how multiple VAPs on an intact virus particle engage the cell surface. Often, a quantitative comparison between the strength of the purified VAP/receptor interaction and that of the intact virus/receptor interaction is technically challenging due to complications growing intact virus, isolating VAP, or identifying and isolating the cellular receptor. One virus system in which all of these challenges can be met is adenovirus. Both whole adenovirus and the VAP can be easily grown and purified and its cellular receptor has been identified and well characterized.

Adenovirus is a non-enveloped double-stranded DNA virus. Infections of the six subgroups (A-F) and approximately 50 serotypes of Adenovirus most commonly cause

upper respiratory tract infections, gastroenteritis, and conjunctivitis in children [48]. Adenovirus infection is also well-documented in outbreaks in the military and in the immunocompromised; severe adenovirus pneumonia in immunocompetent adults has also been reported [10, 49, 50]. In addition to its natural role as a pathogen, Adenovirus has played a prominent role in gene therapy, being used as the vector in more than one quarter of all gene therapy clinical trials [43].

The structure of Adenovirus has been extensively studied. Adenoviruses have an icosohedral shape and measure approximately 100nm in diameter. Hexon, penton, and fiber are the primary components of the Adenovirus capsid. Hexon is present in 240 copies and makes up most of the icosohedral capsid. Penton is present at each of the 12 vertices and forms the base out of which fiber protrudes. Fiber is a homotrimeric protein consisting of an N-terminal domain that interacts with penton at the vertices of Adenovirus, a long flexible shaft, and a C-terminal globular knob domain that interacts with specific cellular receptors [51]. Coxsackie and Adenovirus Receptor (CAR) has been identified as the primary receptor with which the fibers of most subgroups of Adenovirus, including subgroup C, interacts [1]. The crystal structure of Adenovirus fiber knob binding to the D1 domain of CAR has been solved and the specific residues required for this binding event identified [52]. In addition to the fiber-CAR interaction, our lab has recently reported that in cells which are infectible but lack CAR, the initial attachment event is Adenovirus binding via its penton base to Integrin αvβ5. CD46 has been identified as the receptor for subgroub B and a number of other less characterized receptors have been reported to be involved in Adenovirus infection [11, 53].

Adenovirus serotype 5 (Ad5), a member of subgroup C, is one of the best characterized adenoviruses. In this study, we measured the binding affinity of purified Ad5 fiber for the cell surface and compared it to the binding affinity of whole Ad5. We found that the intact virus has a binding affinity for the cell surface 1000-fold stronger than that of purified fiber.

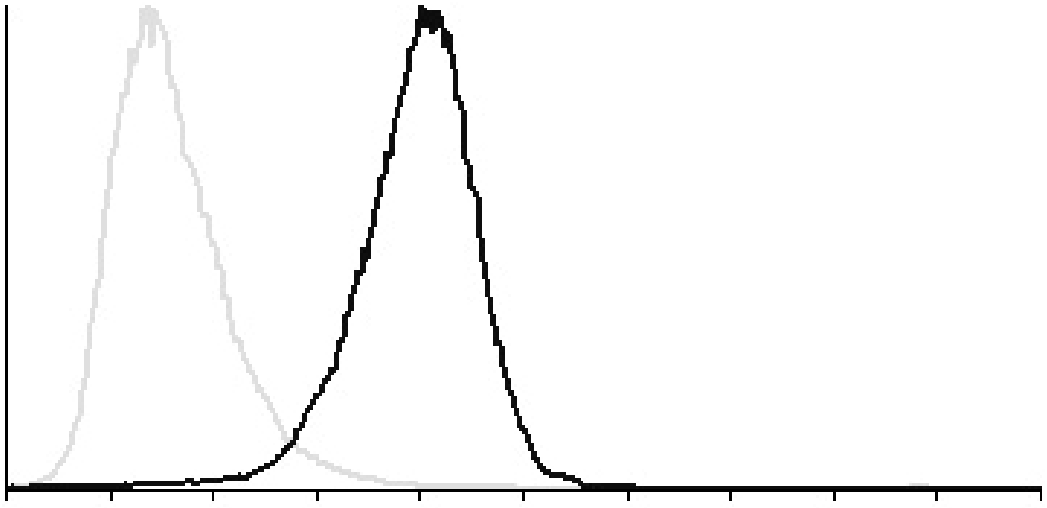
Results

Analysis of soluble fiber binding to CHO-CAR cells.

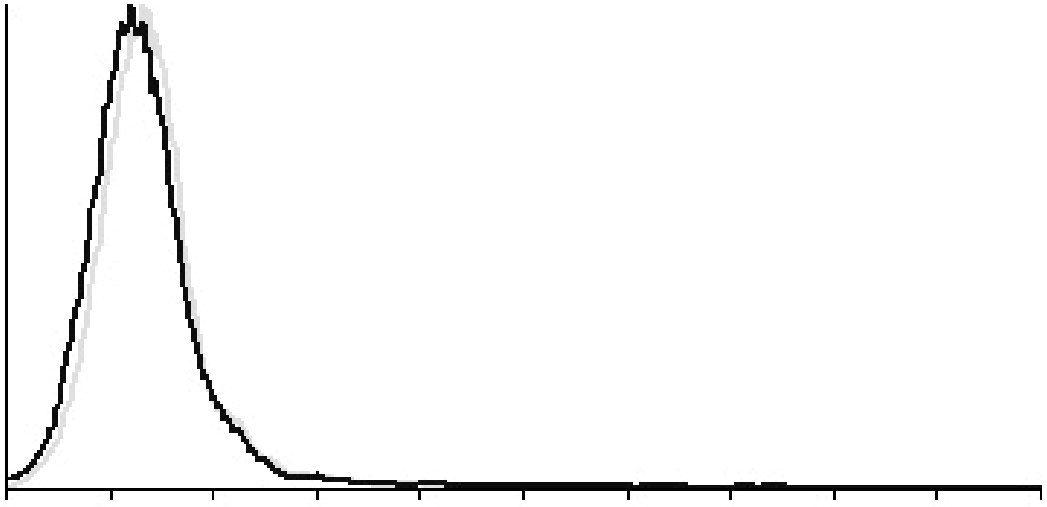
We first sought to determine the equilibrium binding constant, a measure of the strength of the interaction, between purified fiber and CAR expressed at the cell surface. Previous studies have shown that recombinant fiber grown in *E.coli* forms the appropriate trimer and that its C-terminal knob domain maintains its ability to bind to CAR [1, 51]. The cell line we used for these experiments were CHO cells, either stably expressing human CAR (CHO-CAR) or the parental CHO cells which do not express any CAR (Fig 1a). CHO-CAR cells are much more infectible than CHO cells (Fig 1b) and 90% of this infection is blocked by preincubating with soluble fiber (Fig 1c). This matched pair of cell lines ensures we can determine if binding is CAR-specific while, unlike studies using purified receptor, allows binding to be studied in the context of the cell surface.

Figure 1. Characterization of CHO and CHO-CAR cells

A

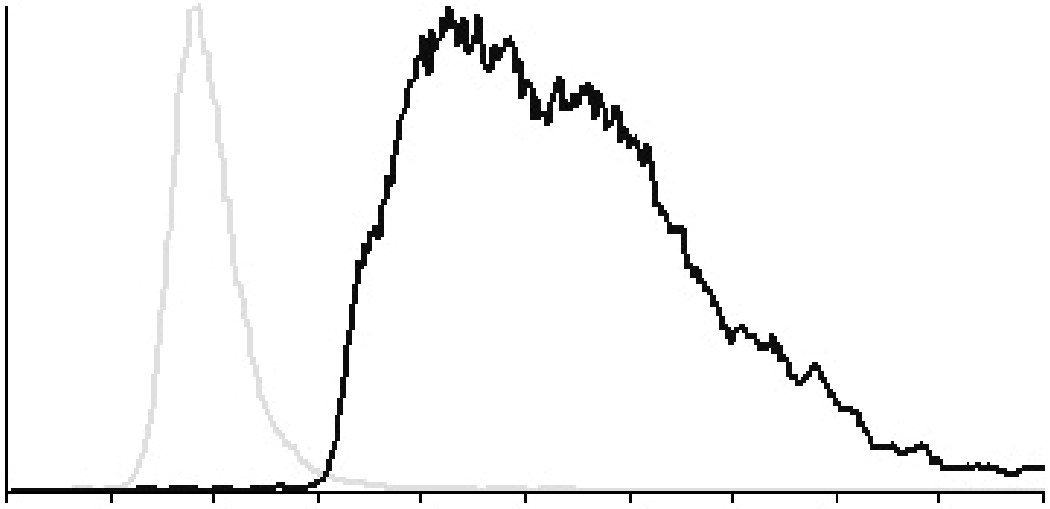


CHO-CAR

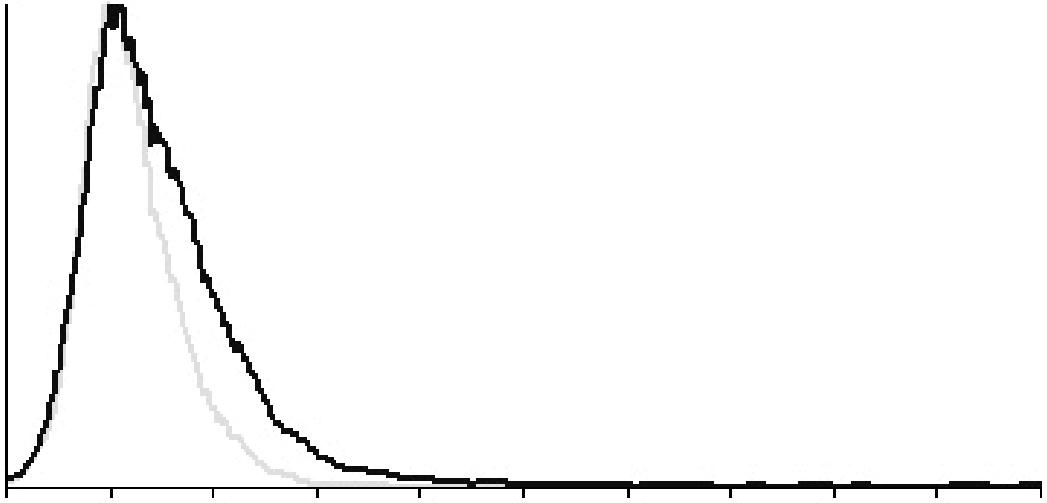


CHO

B

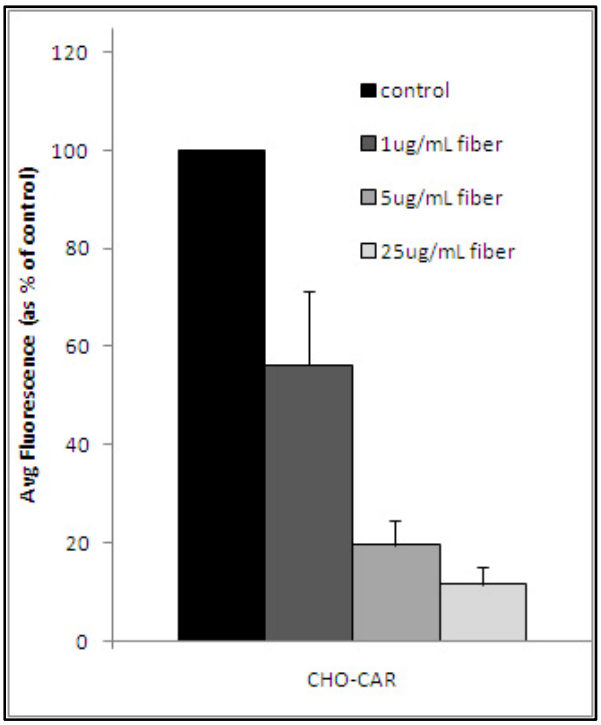


CHO-CAR



CHO

C



H. Surface CAR expression measured by FACS. Surface levels of CAR expression were measured in CHO-CAR cells (top panel) and CHO cells (bottom panel). Grey line is secondary only control, Black line is CAR measured with Rmcb antibody.

I. Adenovirus infection of CHO-CAR and CHO cells. CHO-CAR (top panel) or CHO (bottom panel) were infected with Ad5-GFP at MOI 25 and GFP expression was measured. Infected cells (black line) are

Although fiber has been shown only to bind to and not to be internalized by cells, in order to make direct comparisons between fiber binding and whole Ad5 binding, all experiments were done at 4°C, a temperature which allows binding but does not allow internalization of the virus [6]. A time course was performed and a 12 hour incubation is required to achieve equilibrium across the entire range of concentrations (0.03-300 nM) used in the binding analysis (data not shown).

To measure binding, cells were plated on 96-well plates and incubated overnight at 37°. Cells were chilled to 4° followed by addition of varying concentrations of soluble fiber. After fiber incubation, cells were washed and fixed, and fiber bound was detected using a fiber-specific antibody. Fig 2a shows the binding of increasing concentrations of fiber to CHO-CAR and CHO cells at equilibrium. The binding observed saturates CAR and is specific. Non-specific binding is minimal as demonstrated by the flatness of the plateau on the binding isotherm. No specific binding was observed in CHO cells as was expected since they do not express CAR (Fig 2a). To determine the dissociation constant (K_d), we fit the data to the Langmuir binding isotherm (Eqn 1) [34].

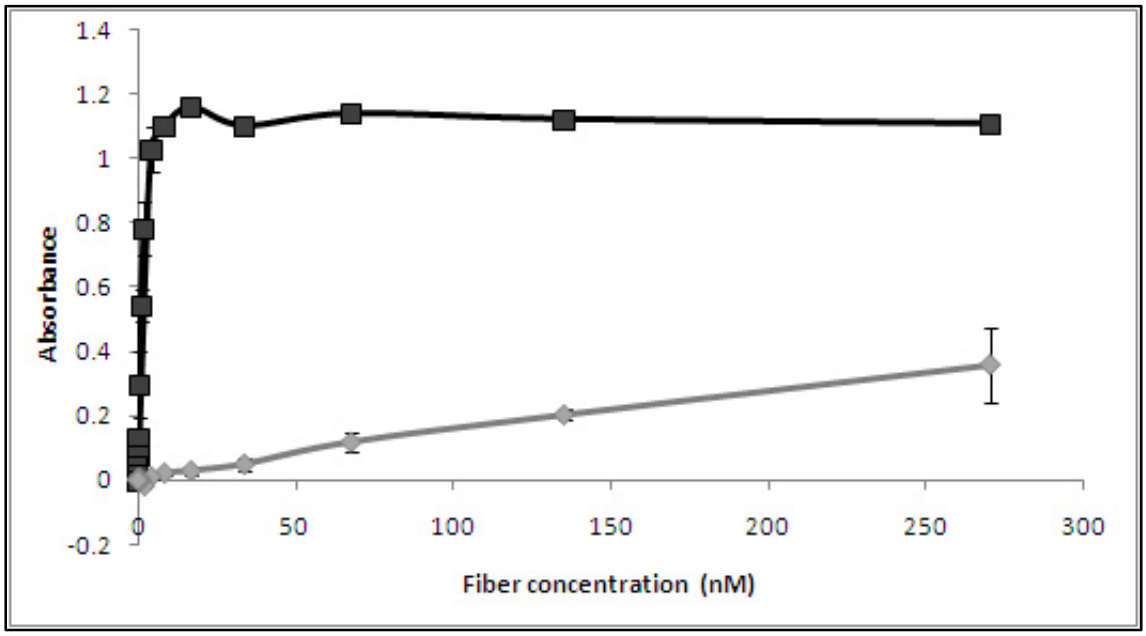
$$Y = \frac{[L]}{(K_d + [L])} \qquad \text{(Eqn 1)}$$

Here, Y is the fractional occupancy of the receptor and [L] is the ligand concentration. We performed a non-linear least-squared analysis using MS Excels's Solver function to

determine the K_d. Fig 2b shows the observed versus calculated values of Y, demonstrating that the data fit this equation. Using this method, we calculated a K_d of $1.3x10^{-9}$ M.

Figure 2. Soluble fiber binding to CHO-CAR and CHO cells

A



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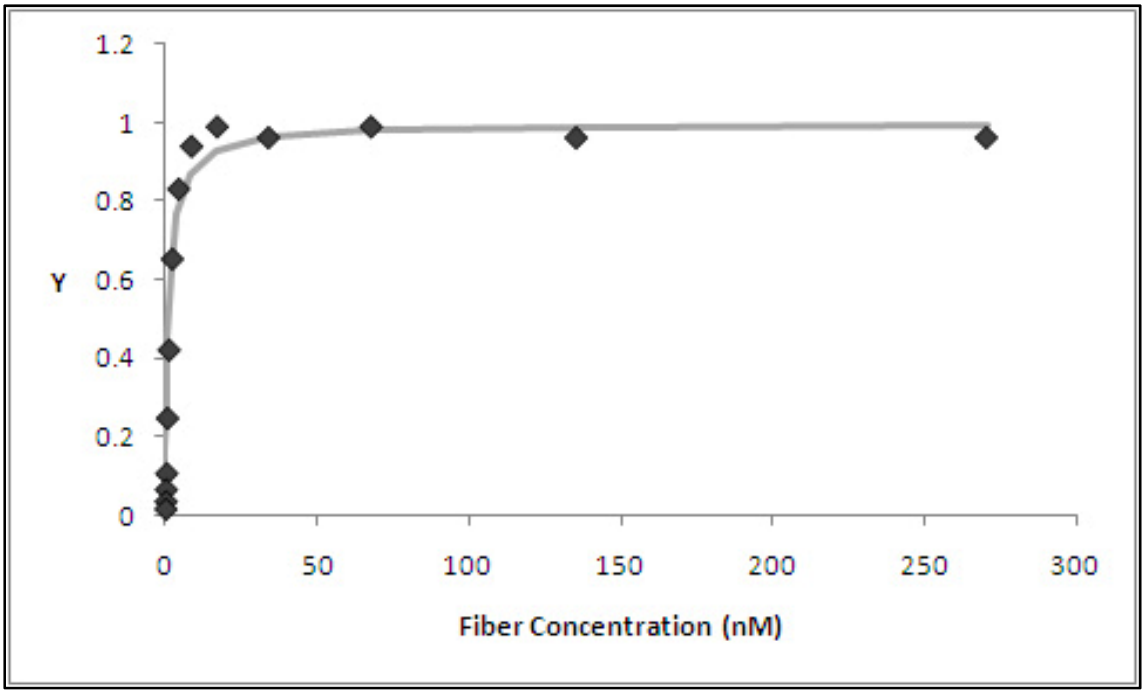


K. Cells were incubated with various concentrations of soluble fiber at 4^0 for sufficient time to reach equilibrium. Fiber bound was measured as described in materials and methods. Binding to CHO-CAR cells (black line) is compared to CHO cells (grey line) Data shown is the average of at least three independent experiments and error bars represent standard deviation.

Analysis of whole adenovirus binding to CHO and CHO-CAR cells.

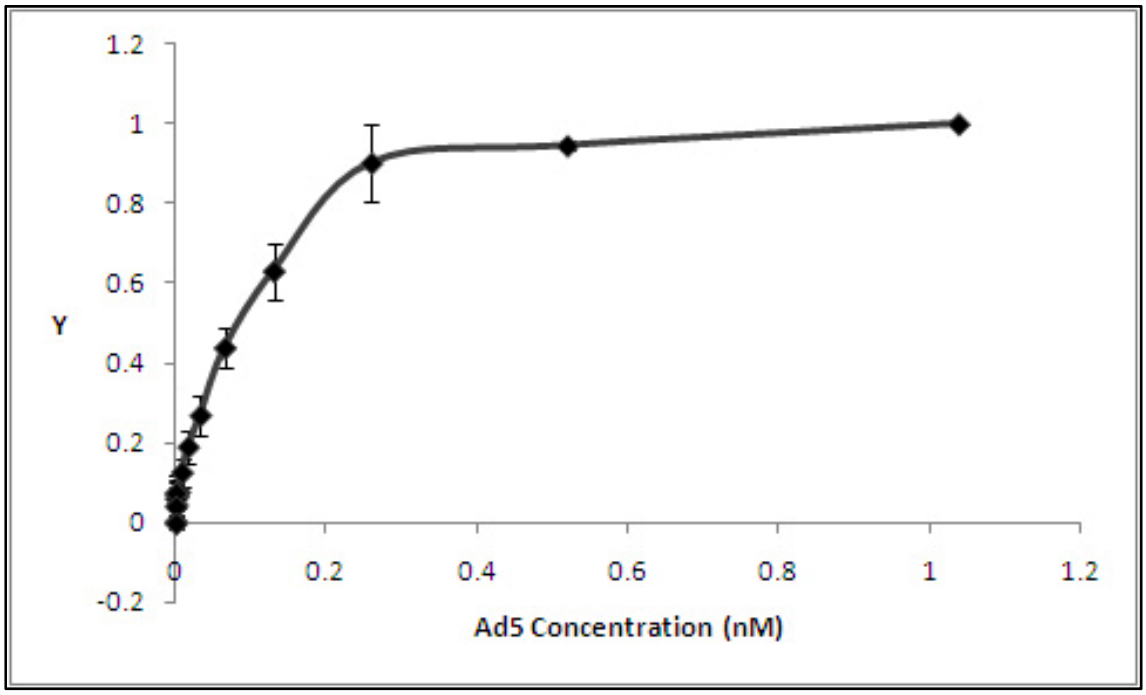
Ad5 has 12 fibers extending from its viral capsid and this multivalency likely allows the virus to bind more tightly to the cell surface than the binding of individual fibers alone. Additionally, other fiber-independent virus-cell interactions may contribute to the overall stability of Ad5 binding. Therefore, we next sought to determine the equilibrium binding constant between whole Ad5 and the cell surface. Again, we used the matched cell lines CHO and CHO-CAR. A time course was performed and an 18 hour incubation of Ad5 with cells was required to achieve equilibrium across the entire range of concentrations ($3.17x10^{-5}$-1.04 nM).

Although CHO cells do not express CAR and therefore do not bind to fiber, CHO cells do express Integrin αvβ5, a receptor sufficient to bind to Ad5 and initiate infection in CAR-negative cells. However, CHO cells are not highly infectible as shown in Fig 1 so we were unsure whether Ad5 binds to these cells. Figure 3a shows the equilibrium binding of increasing concentrations of Ad5 to CHO cells. We observed Ad5 binding to CHO cells is specific and saturable. We fit the data to the Langmuir binding isotherm (Fig 3b). Non-linear least-squared analysis gave a K_d of $6.7x10^{-11}$ M. The binding affinity observed in CHO cells is extremely similar to what we previously observed in cells where the penton base of Ad5, through its RGD domain, binds Integrin αvβ5 to initiate binding and infection. Therefore, we investigated whether Ad5 binding to CHO cells is mediated by integrins. We preincubated CHO cells with integrin blocking RGD

peptide or control RGE peptide and then allowed Ad5 to bind. We found that binding of Ad5 to CHO cells is blocked by integrin-blocking peptides (Fig 3c).

Figure 3. Intact Ad5 binding to CHO cells

A



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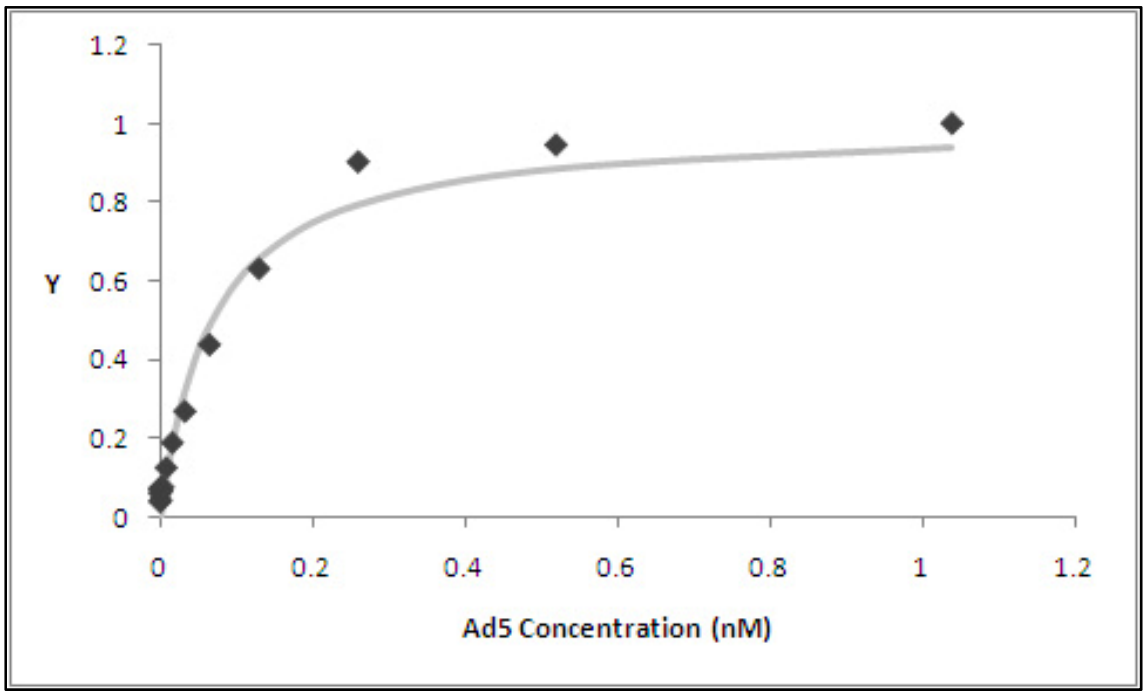
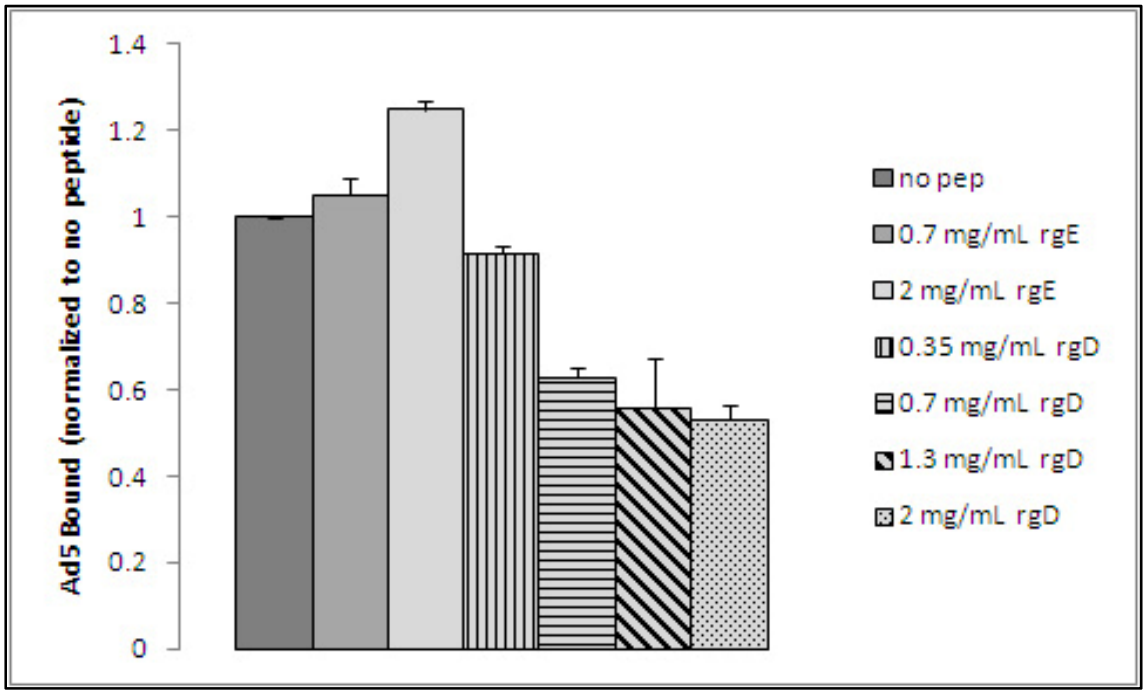

1.2
1
0.8
Y 0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
Ad5 Concentration (nM)

C


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ad5 Bound (normalized to no peptide)
no pep
0.7 mg/mL rgE
2 mg/mL rgE
0.35 mg/mL rgD
0.7 mg/mL rgD
1.3 mg/mL rgD
2 mg/mL rgD

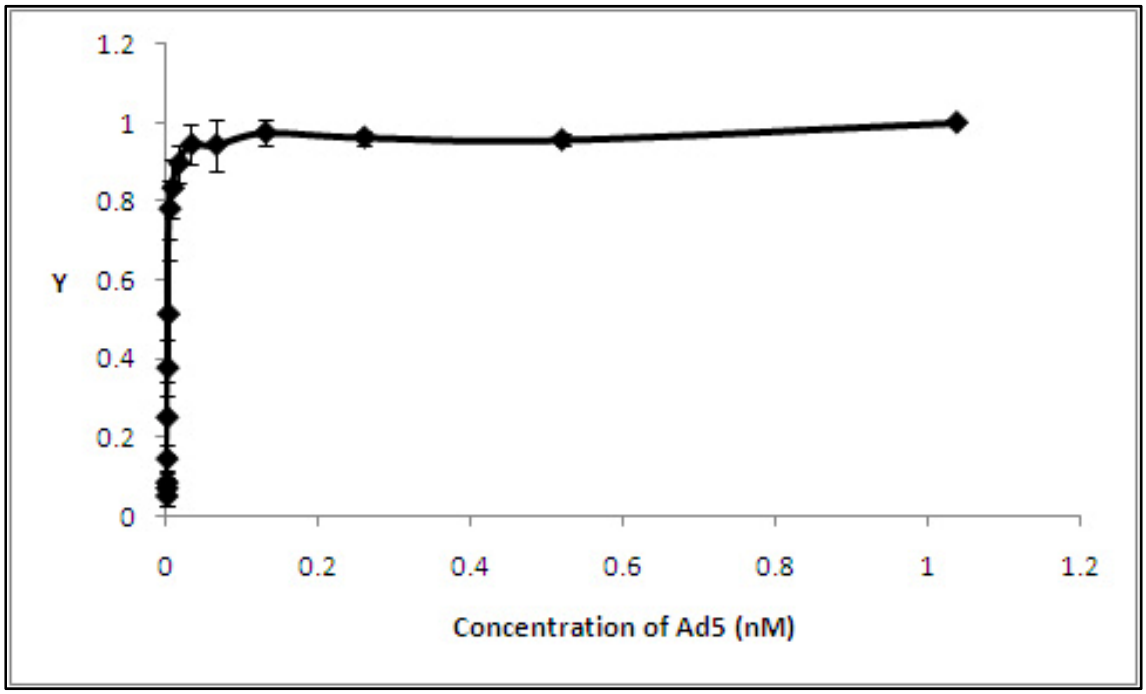
M. Cells were incubated with various concentrations of Ad5 at 4^0 for sufficient time for virus bound to reach equilibrium. Virus bound was measured as described in materials and methods. Y is the fractional occupancy, which is a ratio of virus bound to maximum virus bound. Data shown is the average of at least three independent experiments and error bars represent standard deviation.

N. Observed data (diamonds) was fit to calculated values (line) using the Langmuir Binding Isotherm (Eqn 1). K_d was determined by Microsoft Excel's Solver function using non-linear regression analysis to solve Eqn

Next we measured the binding of Ad5 to CHO-CAR cells. Figure 4a shows the equilibrium binding of increasing concentrations of Ad5 to CHO-CAR cells. Ad5 binding to CHO-CAR cells shows a typical binding isotherm with specific and saturable binding. As above, we fit the data to the Langmuir binding isotherm (Fig 4b). Non-linear least-squared analysis gave a K_d of $1.6x10^{-12}$ M. The affinity of whole Ad5 binding CHO-CAR cells represents the stability introduced by allowing the virus to interact with both integrin receptors, through its RGD motif, and CAR receptors, potentially through multiple fibers. Ad5 has an affinity for CHO-CAR cells three orders of magnitude greater than soluble fiber. Additionally, Ad5 binds CHO-CAR cells 40-fold stronger than CHO cells.

Figure 4. Ad5 binding to CHO-CAR cells

A



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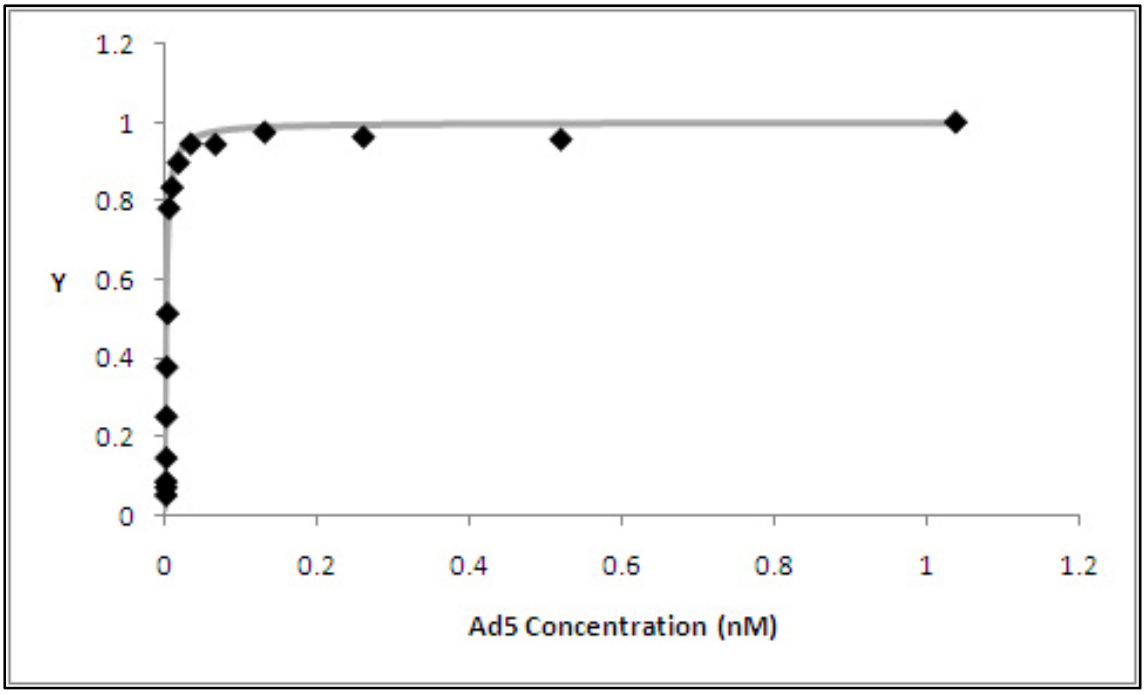


P. Cells were incubated with various concentrations of Ad5 at 4^0 for sufficient time for virus bound to reach equilibrium. Virus bound was measured as described in materials and methods. Y is the fractional occupancy, which is a ratio of virus bound to maximum virus bound. Data shown is the average of at least three independent experiments and error bars represent standard deviation.

Discussion

Binding of a virus to the cell surface is the initial step in viral infection and thus understanding the complexities of this step is critical to understanding infection. In this study we have further elucidated the contribution of different capsid proteins and two different cellular receptors to the resulting strength of binding between an intact virus particle and live cells. We found that the intact virus has an affinity for cells expressing both CAR and Integrin αvβ5 (CHO-CAR) three orders of magnitude greater than isolated fiber and approximately 40 fold stronger than the intact virus binding cells expressing only one of the cellular receptors, Integrin αvβ5 (CHO).

The binding of purified fiber to the bacterially produced D1 domain of CAR has been studied by surface plasmon resonance (SPR) [38]. In this study, they compared binding affinities of fiber-CAR when fiber was immobilized on the surface and CAR flowed across it or vice versa. They found that when immobilizing CAR a K_D of $1x10^{-9}$ M was observed, a value 25-fold lower than when the experiment was done in the reverse orientation. Lortat-Jacob et al argue that this increased affinity is due to an avidity mechanism whereby trimeric fiber can bind to three CAR molecules; further, that immobilizing the receptor better mimics the cell surface and studies in which VAP is immobilized instead likely greatly underestimate the binding affinity of the VAP for the cell surface. In the current study, we measured the affinity of purified fiber for CAR expressed on the surface of live cells. We obtained a very similar value of $1.3x10^{-9}$ M for the binding of fiber. These results confirm the hypothesis of Lortat-Jacob et al that

immobilizing receptor, rather than ligand, is a more physiologically relevant model system.

Although the CAR-fiber interaction has typically been credited with mediating Ad5 binding to cells, we found that Ad5 binds to CHO cells, which do not express CAR or bind soluble fiber, with a high affinity of $6.7x10^{-11}$ M [1, 6, 28]. We found this binding to be integrin-dependent. This data is consistent with our recent report that Ad5 uses Integrin αvβ5 as a binding receptor in CAR-negative cells. Interestingly, although binding of Ad5 to CHO cells is observed, these cells are not very infectible. This observation implies that CHO cells may be deficient in another step of viral infection, though this deficiency can be overcome with the overexpression of an additional Ad5 receptor, CAR, as seen in CHO-CAR cells.

Several previous studies have attempted to quantify the affinity of adenovirus for the cell surface. Affinities ranging from approximately $1x10^{-10}$ M to $5x10^{-12}$ M (Table 1) have been reported [54-57]. These studies were done in a variety of cell lines, at several different binding temperatures, and with different times allowed to achieve equilibrium. Each of these parameters could potentially contribute to the large range of K_d's reported. For example, within 15 min of adding virus and warming to 37°, a substantial amount of adenovirus internalization is seen [6]. Persson et al did not consider in their experiment conducted at 37°, internalization of virus and the subsequent potential change in receptor availability [56]. In this study, all binding experiments were carried out at 4° to prevent

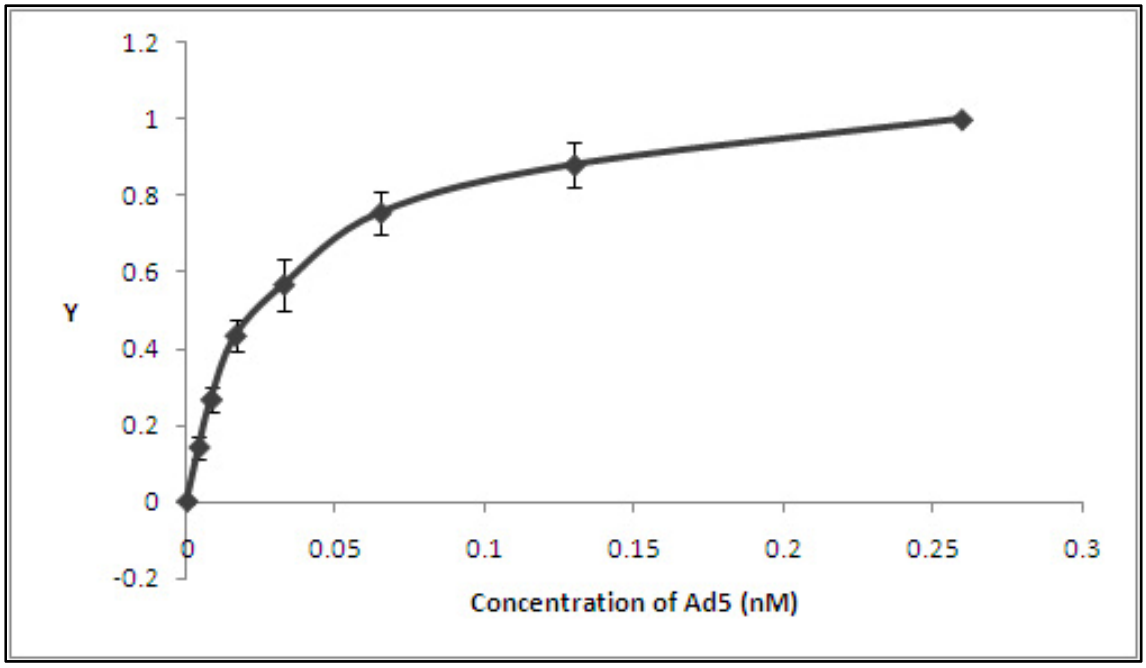
internalization from confounding the results. Additionally, all of the previous studies have allowed only 45 min or 3 hrs for virus binding to cells. In our study, a time course indicated 18hrs was required to achieve equilibrium across the entire range of concentrations we used. From data obtained after only 1.5 hrs, we calculated a K_d of $2.2x10^{-11}$ M for Ad5 binding to CHO-CAR cells (Supplementary Fig 1), a value more than an order of magnitude greater than what we observed once the system had reached equilibrium, thus demonstrating that shorter incubation times can lead to an underestimation of affinity.

Table 1.

Cell Line	Temp (°C)	Incubation time (hours)	K_d (M)	Reference
HeLa	3	3	1.43E-09	Persson et al 1985
HeLa	3	3	6.67E-11	Persson et al 1985
HeLa	37	0.75	6.25E-11	Persson et al 1985
KB	20	0.75	1.09E-10	Defer et al 1990
A549	20	0.75	1.25E-10	Defer et al 1990
293	4	3	6.94E-11	Shayakmetov et al 2000
293S	4	3	5.26E-12	Gilbert et al 2007
CHO-CAR	4	18	1.60E-12	Current study

Supplementary Figure 1. Binding to CHO-CAR cells, 1.5 hrs

A



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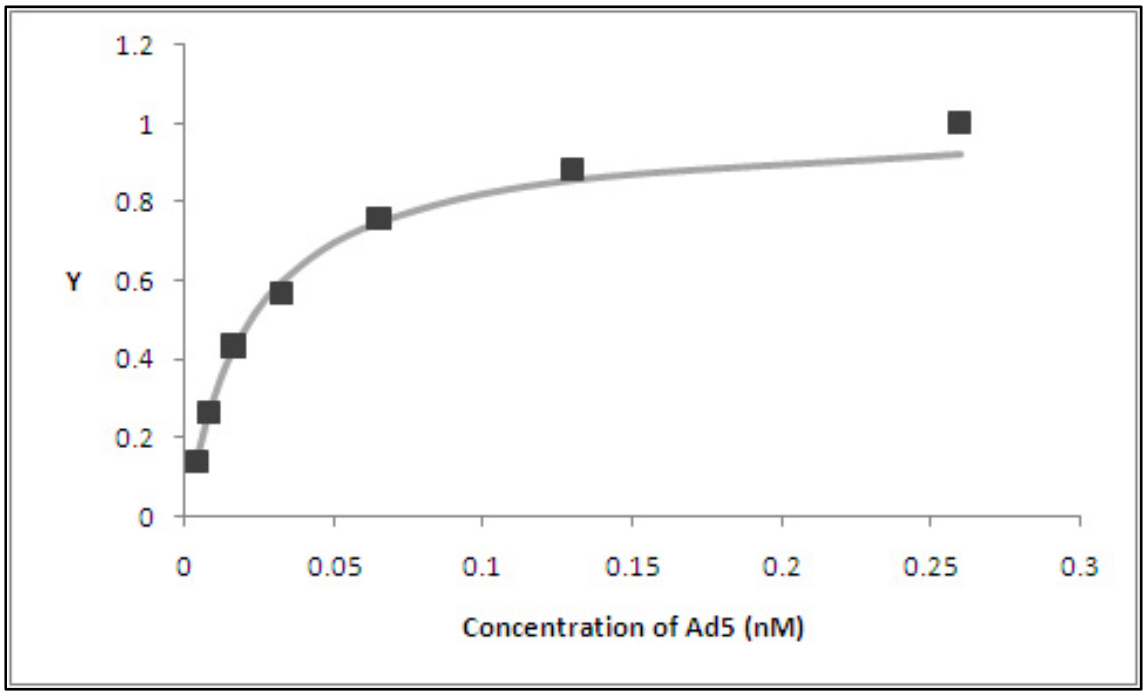

R. Cells were incubated with various concentrations of Ad5 at 4^0 for only 1.5 hours. Virus bound was measured as described in materials and methods. Y is the fractional occupancy, which is a ratio of virus bound to maximum virus bound. Data shown is the average of at least two independent experiments and error bars represent standard deviation.

In many studies attempting to quantify the binding between a virus and its receptor, purified VAP is allowed to bind either to purified receptor or to the cell surface. In our study we found that whole Ad5 binds 1000-fold stronger than purified fiber. This increase in binding affinity is likely due to a number of factors. Intact Ad5 binds to more than one receptor, as demonstrated by the binding of Ad5 to both CHO and CHO-CAR cells. In fact, referring to fiber as the VAP of Ad5 is not entirely correct since penton, through its RGD domain, also contributes to the binding. Additionally, Ad5 has twelve copies of both penton and fiber, and it is highly likely that more than one copy engages the cell surface at a time, adding an avidity mechanism to the binding dynamic. Many other viruses also have been reported to bind to multiple receptors and it is a general trait of viruses to display multiple copies of their VAPs. Thus studying the virus/receptor interaction in the context of any purified viral component likely greatly underestimates the true nature of the virus-cell binding event. Similarly, binding results obtained by immobilizing virus and flowing receptor over it will also miss any contribution of avidity or multiple receptor engagement. For example, the affinity of poliovirus for its cellular receptor was found to be only in the micromolar range; however, this study was done by immobilizing virus and allowing receptor to bind to it [58].

One area in which how strongly a virus binds to the cell surface has practical consequences is in the design of viral inhibitors. For example, the dengue virus VAP has been found to interact with highly sulfated heparan sulfate [59]. Subsequent studies attempted to identify potential dengue inhibitors by interfering with the VAP/receptor interaction [60]. However, inhibitors sufficient to block the individual VAP from binding

to its receptor may not be strong enough to actually block the binding of intact virus where many VAPs may engage many receptors. Alternatively, perhaps inhibitors identified could be themselves linked into multivalent entities, making them potentially much more potent at blocking the multivalent virus.

Understanding the binding dynamic between a virus and cell surface is critical to a complete understanding of what is required to initiate a viral infection and therefore, what is required to prevent that initiation. Another area in which this study has potential implications is in the design of nanoparticles. Currently, the development of nanoparticles that could deliver drugs, genes, or siRNA is of high interest. One major area of research in this field is how to direct these nanoparticles to the desired target cells and often this goal is met by having a targeting ligand displayed on the outer surface of the nanoparticle, much like a virus. Therefore, an accurate estimate of the affinity between a virus, or nanoparticle, and the cell surface required to initiate infection should influence the design of future nanoparticles.

In conclusion, we have quantitatively compared the binding of soluble adenovirus fiber to CAR expressed at cell surfaces to the binding of intact Ad5 to CAR expressed at cell surfaces. We found that the intact virus binds to cells with a 1000-fold greater affinity than individual fibers. This much higher affinity is the type of binding actually experienced by cells during a viral infection.

Experimental Procedures

Cell lines and viruses

Chinese hamster ovary (CHO) cells expressing ectopic human CAR (CHO-CAR) or containing vector without insert (CHO) were kind gifts from Dr. J. Bergelson, Children's Hospital of Philadelphia, Philadelphia, PA. Cell lines were grown in DMEM supplemented with 10% FBS. Virus used was E1A deleted and expressed GFP (Ad5-GFP). Virus was propagated in 293/E4/pIX cells and harvested by CsCl gradient ultracentrifugation as previously described [44, 45]. Virus titer was determined as previously described [46].

Antibodies, surface expression analysis, and recombinant protein

The MAb RmcB was used to detect CAR expression [1]. Surface levels were determined as described in Chapter 1 using flow cytometry. Soluble fiber was grown and purified as described in Chapter 1. Briefly, fiber was his-tagged, grown in E.coli, and purified using nickel beads.

Cell Infection Assay

Infection with Ad5-GFP was determined as described in Chapter 1. Briefly, cells were incubated with Ad5-GFP at MOI 25 overnight and GFP expression was determined using flow cytometry. For fiber blocking experiment, soluble fiber at increasing concentrations was added to cells for 1 hr RT prior to the addition of Ad5-GFP at MOI 25.

Binding Assays

Binding assays for fiber and Ad5 were performed as described in Chapter 1 except for the following changes. Cells were incubated with fiber for 12 hours prior to fixing. To visualize fiber binding, cells were incubated with MAb 4D2 (Abcam) directed against adenovirus fiber followed by Mouse IgG antibody --H+L, Alkaline Phosphatase Conjugated (Abcam). The remaining protocol remains the same as with Ad5. Peptide blocking experiments were also performed as described in Chapter 1.

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