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CD46 Isoforms and Viral Receptor for Adenovirus Type 64D

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CD46 Isoforms and Viral Receptor for Adenovirus Type 64D

By

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CD46 Isoforms and Viral Receptor for Adenovirus Type 64D

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Abstract

Adenovirus Type 64 (Ad.64) belongs to the adenovirus subgroup D, which causes epidemic keratoconjunctivitis (EKC), otherwise known as viral pink eye. There is currently no known effective treatment for EKC. Membrane Cofactor Protein (CD46) is an integral membrane glycoprotein that, in previous studies, has been identified as a protein receptor for the closely related Ad.37. It has been determined that Ad.64 uses CD46 as a receptor on the cell surface in HeLa cells. CD46 is alternatively spliced when expressed to have different isoforms of interest, including the BC and C isoforms. Certain cell types, like A549 lung carcinoma cells, express only BC isoforms while other cells, like HeLa cells, express both BC and C isoforms. Ad.64 does not seem to use CD46 on A549 cells, but instead likely uses sialic acid as a receptor. Thus, we know that different forms of CD46 are made by different cells, and there may be some functional difference. It is unclear which isoform is being used when Ad.64 infects HeLa cells. Additionally, there is evidence that other proteins may work in partnership with CD46-C. Through electron microscopy, we show that Ad.64 does use CD46 as a receptor for viral infection. Furthermore, the goal of this project is to characterize which proteins work in partnership with CD46-C to further enhance our understanding of the binding mechanism of Ad.64 to human cells.

Introduction

CD46, or membrane cofactor protein, is an integral membrane glycoprotein that serves multifaceted roles in the complement immune system, one of the human body's primary defenses against invading pathogens (Liszewski et al., 1996). CD46 belongs to a class of proteins known as complement regulatory protein and serves as a cofactor for serine protease factor I to cleave and inactivate C3b and C4b deposited on host cells. C3b is a protein that opsonizes pathogens, facilitating their recognition and elimination by immune cells, while C4b initiates the formation of the membrane attack complex (Liszewski et al., 2005). C3b and C4b coat the surface of pathogens, enhancing their recognition and ingestion by phagocytic cells. Additionally, they contribute to the formation of the membrane attack complex (MAC), a structure that can directly lyse pathogens by creating pores in their cell membranes (Liszewski et al., 2005). C3b and C4b are crucial for optimizing the effectiveness of the complement system, and increasing the adhesion of C3b and Cb4 to host cell membranes could lead to the nonspecific binding of these complement proteins (Liszewski et al., 1996). Therefore, CD46 is necessary to prevent autoimmune attacks by serving as a cofactor for serine protease factor I.

CD46 is approximately 45 to 70 kDa in length, depending on the isoform. The structural components consist of four short consensus repeat (SCR) domains, each with distinct ligand-binding properties (Seya et al.,1999). Adjacent to this region are one to three serine/threonine (ST)-rich domains. These domains serve as sites for O-glycosylation by carbohydrate chains, terminating in sialic acid, which attaches to the hydroxyl groups of serine and threonine residues. Additionally, CD46 contains a transmembrane domain and a cytoplasmic tail. The gene encoding this protein consists of fourteen exons, facilitating the production of eight different isoforms through alternative splicing. Among these isoforms, the C and BC isoforms are the most

prevalent (Seya et al.,1999). The C isoform of CD46, lacking the "B" exon that encodes a heavily glycosylated region of the ST-rich domain, measures approximately 50 kDa in length. The C-isoform of CD46 is missing the 15 amino acids from the “B” exon and exhibits significantly lower glycosylation with fewer negatively charged sialic acid residues compared to the BC isoform. The structural differences between the BC and C isoforms are shown in Figure 1.

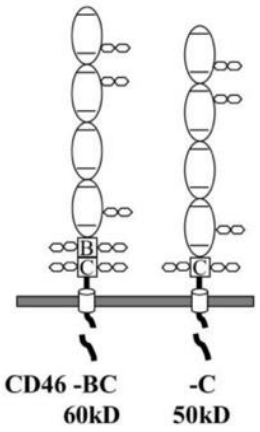

Figure 1. CD46 BC and C isoforms. (Wu et al., 2004) SCR domains (ovals); O-linked sialic acid glycosylation (hexagons); ST-rich domains (squares); cytoplasmic tails (solid black lines).

Previous studies have shown that CD46 associates directly with b1 integrins and indirectly with tetraspans (Lozahic et al., 2000). Integrins are cell surface molecules composed of heterodimers, serving as connectors between the internal signaling components of the cytoskeleton and the extracellular environment. Importantly, the integrin heterodimers that primarily bind to the extracellular matrix (ECM) proteins, which are often upregulated in tumors, feature the β1 subunit (Howe & Addison, 2012). CD46 was found to associate with multiple b1 integrins, including α2b1 and α5b1, even though they did not form complexes with tetraspan molecules (Lozahic et al., 2000). There is little information about why CD46 associates with b1 integrins and how this might contribute to viral infection.

Human adenoviruses are nonenveloped with an icosahedral capsid and contain a double-stranded DNA genome. Human adenoviruses have a hexon, which consists of three copies of a two-domain polypeptide sequence, contributing to the hexagonal shape. They also possess fibers that extend from the vertices of the capsid and are involved in the initial attachment of the virus to host cells. The fiber protein is responsible for binding to specific cellular receptors, facilitating viral entry. The distal end of the fiber protein contains a knob-like structure, known as the fiber knob, which interacts with cellular receptors on the host cell surface (Zhou et al., 2012).

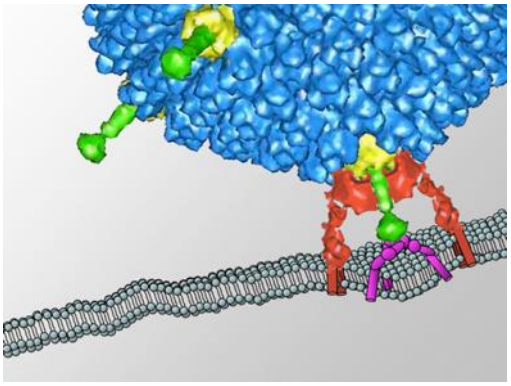

Figure 2. Visual model of adenovirus binding to CD46. The virus (shown in blue) and the fiber knobs (shown in green) attach to CD46 on a host cell.

Epidemic keratoconjunctivitis (EKC) represents a rapidly developing and highly contagious eye infection primarily attributed to human adenoviruses within species D (HAdV-D), notably serotypes HAdV-D8, D64, and D37 (Zhou et al., 2012). EKC is commonly known as viral pink eye, and there is currently no known effective treatment for it. Previous studies have shown that CD46 is a receptor for Adenovirus Type 64-D (Ad.64) host cell attachment and subsequent internalization (Wu et al., 2004). The specific interactions between Ad.64 and CD46 remain to be fully investigated.

Elucidating the role of CD46 in mediating Ad.64 infection holds promise for the identification of novel therapeutic targets and the development of innovative approaches to

combat adenovirus-associated diseases. Thus, comprehensive investigations into the Ad.64-CD46 binding mechanism are essential for advancing our understanding of viral-host interactions and for the development of effective interventions against adenovirus infections.

The current study aims to answer how CD46 and Ad.64 bind to further understand Ad.64 viral infection. This research also investigates how CD46 isoforms contribute to different binding mechanisms and show structural differences between the isoforms. We have shown that CD46 is a viral receptor for Ad.64 through transmission electron microscope (TEM) imaging. Additionally, we have begun to show that CD46-C may work in conjunction with neighboring proteins to be a receptor for Ad.64 in HeLa cells, unlike CD46-BC in A.549 cells.

Materials and Methods

Ad.64 and CD46 Binding Solution Preparation. A combination of purified Ad.64 virus expressing an EGFP transgene, sterile DPBS, 5 nm Ni-NTA-Nanogold® beads, and soluble human CD46 extracellular domain with C-terminal 6His-tag (1.81 mg/ml) were used to create four different treatment groups. The first treatment group served as a control, containing sterile DPBS and gold beads. The second and third treatment groups also served as controls, containing sterile DPBS and gold beads with only CD46 protein or only purified Ad.64, respectively. Finally, the fourth treatment group contained sterile DPBS, gold beads, CD46 protein, and Ad.64 virus, as well as $CaCl_2$ to facilitate the binding of Ad.64 and CD46. Each solution was centrifuged twice at 6,000 rpm for 30 seconds using Ultrafree®-MC Centrifugal Filter Devices with a 0.1 μm pore size to filter out unbound beads and CD46, replacing any lost volume with sterile DPBS.

Transmission Electron Microscope Imaging. Treatment solutions were placed on a formvar-coated copper grid and allowed to incubate for 10 minutes before placing glutaraldehyde and paraformaldehyde onto the grid to covalently crosslink the capsid components. Grids were stained with 4% $H_4O_{40}Na_4SiW_{12}$ and air dried before imaging. Images were taken on JEM-2100 Plus Transmission Electron Microscope at 60,000x magnification.

Maintenance of Cells and Lysate Harvesting. HeLa cervical carcinoma cells were maintained in Advanced Dulbecco's Modified Eagle's Medium (DMEM) with additives including 3% bovine serum (HyClone® FetalClone®), Glutamax (Gibco® GlutaMAXTM), and antibiotics/antimycotics (Amresco® - Penicillin/Streptomycin). HeLa cervical carcinoma cells were grown to at least 75% confluence before being harvested. The old media was removed from the flasks, and they were then washed with DPBS. A detergent solution of 1.0% CHAPS hydrate in PBS was added to flasks and cells were scraped and collected. This sample was incubated on ice for 30 minutes before being centrifuged for 10 minutes at 10,000 rpm at 4 °C.

Immunoprecipitation of CD46 Complex. Pierce™ Protein A/G Magnetic Beads were washed with 0.1% CHAPS detergent solution twice. CD46 antibody was combined with HeLa cell lysate and incubated for one hour at room temperature on a rocker. This solution was then incubated with the magnetic beads for one hour at room temperature on a rocker. The supernatant was removed, and the beads were washed with 0.1% lysis buffer three times. The beads were washed in purified water before finally being eluted with a small volume of SDS loading buffer. The sample was then heated for 10 minutes at 95°C in a heating block. The sample was run on an any kD SDS page gel at 150 volts for 45 minutes. The SDS-Page gel was transferred to a piece of Immobilon®-P PVDF Membrane and hydrated with ethanol. A Western Blot was performed at 100 volts for 30 minutes. The membrane was then blocked with Bio Rad

EveryBlot Blocking Buffer for one hour at room temperature. The blocking buffer was removed and replaced with 1 μg/mL of CD46 antibody and rocked overnight at 4 °C. The membrane was washed five times with TBST. The membrane was imaged with Alexa Fluro 488 fluorescence. To prepare the sample to be digested and characterized, the same protocol was applied to the cell lysate solution as stated above for Western Blotting except the elution step. The beads were washed with of pre-urea buffer (Tris 8.0 pH 50 mM, ECTA 1 mM, KCl 75mM) once before finally being eluted in 8M urea. Beads were in urea solution for 20 minutes at room temperature on a rocker before elution.

Results

CD46 binding capability to Ad.64. A collection of images of Ad.64 particles in the presence or absence of CD46 and 5 nm Ni-NTA-Nanogold beads was taken of each treatment group using a transmission electron microscope (Figure 3). The average of the four treatment groups is shown in Figure 4. Our first control group established that the 5 nm gold beads mostly spun through the 0.1 μm filter when no other species are present in the solution, leading to a low number of beads in the images. The first treatment group only including gold beads had an average of 7 ± 2.6 beads. Our second treatment group was used to confirm that the beads would not be retained if they were only bound to CD46 because the pores in the membrane would allow the complex to pass through during centrifugation. The second treatment group of gold beads and CD46 had an average of 8.6 ±1.5 beads. Our third treatment group was used to confirm that the beads would not bind to Ad.64 without CD46 present. The third treatment group of gold beads and Ad.64 virus had an average of 38 ± 8.7 beads. The fourth treatment group was used to investigate the binding capability of CD46 to Ad.64 in solution. The fourth treatment group of gold beads, Ad.64 virus, and CD46 protein had an average of 179.6 ± 26.7 beads. There is a

statistically significant difference between the control groups and the experimental group, confirming that CD46 is binding to Ad.64 in some capacity. The fourth group was statistically significant from all other treatment groups using a one-way ANOVA.

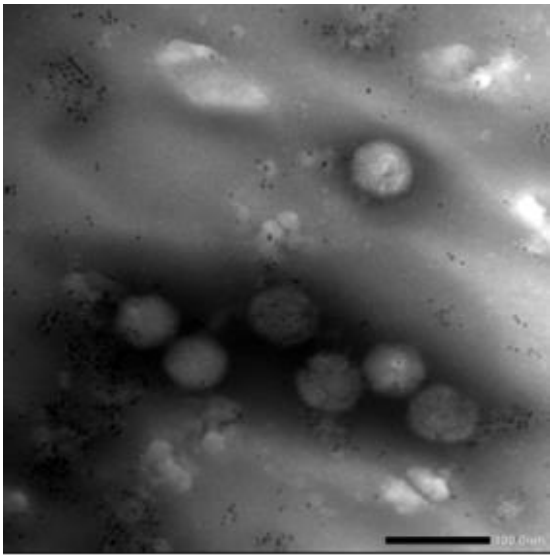

Figure 3. Imaging of Ad.64 Virus and CD46-Bead Solution. Image as taken on 60,000x magnification on a transmission electron microscope. The scale bar indicates 100.0 nm.

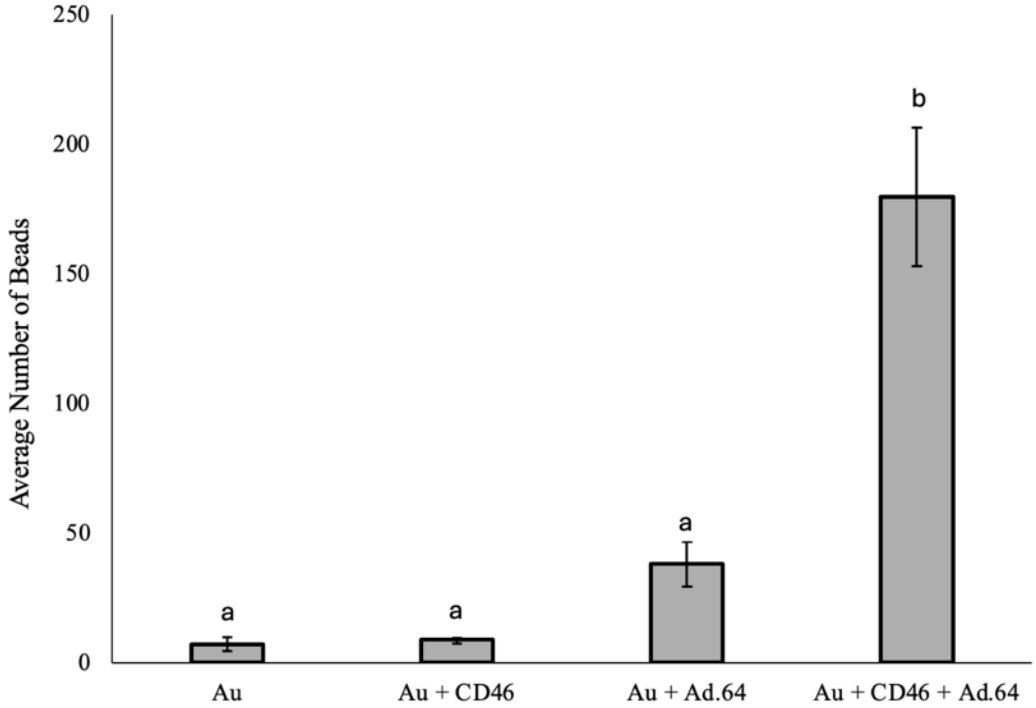

Figure 4. Average Number of Beads in Viral Imaging. Images were taken on 60,000 magnification and had the same area per picture. The number of gold beads were counted in each photo and an average was calculated for each

treatment group. The error bars represent the standard deviation of the samples. Bars with different letters are statistically different.

CD46 Immunoprecipitation Preliminary Results. To determine which, if any, proteins are bound to CD46 on the cell surface, CD46 and associated proteins were immunoprecipitated from HeLa cervical carcinoma cells lysed with CHAPS detergent. Preliminary SDS-PAGE and Western Blots were conducted to determine if the experimental solution contained CD46-C and any neighboring covalently bound proteins. Figure 3 shows an SDS-PAGE gel of an immunoprecipitation elution. A bright band around 150 kD in weight is seen, along with a lighter band around 50-75 kD. The band at 150 kD corresponds to the CD46 antibody. The lighter band at 50-75 kD most likely correlates to CD46 protein in the solution, though it is at a lower concentration. The Western Blot (fig. 6) shows similar evidence of a protein around 50-75 kD.

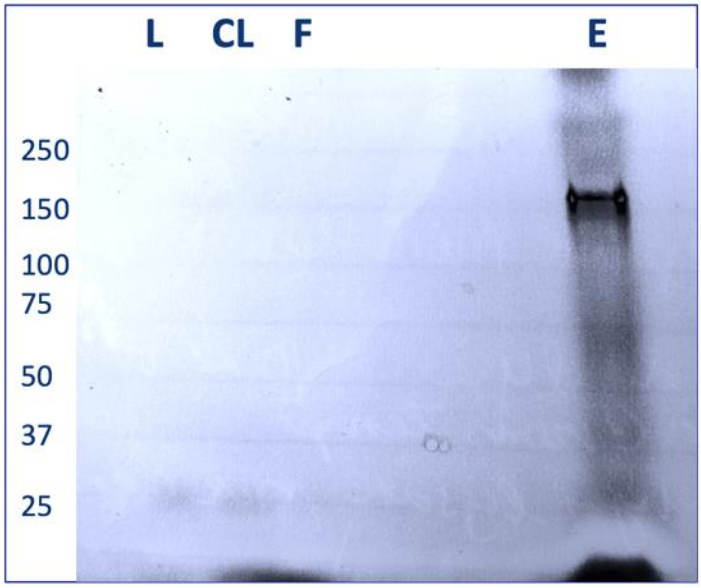

Figure 5. SDS-page gel from HeLa cells under fluorescence. The CD46 antibody is fluorescently labeled and appears under Pro-Q Emerald 488 detection. Lanes are labeled as L (ladder), CL (cell lysate), F (flow through), and E (elution). Unlabeled lanes are various washes in the immunoprecipitation. Markings on the ladder are correlated with kD sizes, labeled next to the ladder.

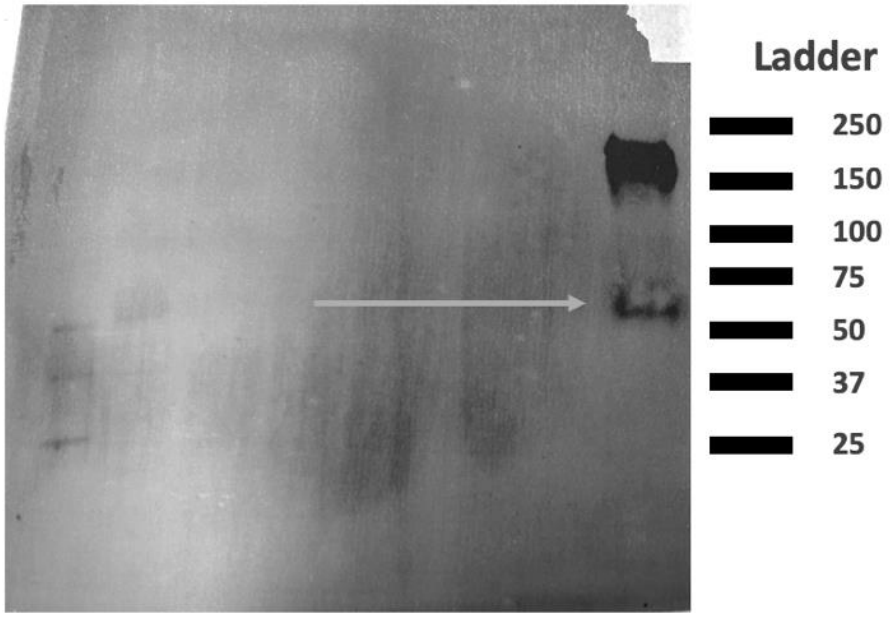

Figure 6. Western Blot of SDS-page gel under fluorescence. The CD46 antibody is fluorescently labeled and appears under Pro-Q Emerald 488 detection The last lane carries the protein from the SDS-page. The arrow points to the protein of interest, presumably CD46. The ladder on the left shows kD markings.

Discussion

We hypothesized that CD46 binds to Ad.64 to act as a receptor for viral binding. The gold beads should correlate to the amount of CD46 bound to Ad.64, we predicted that if CD46 did successfully bind to Ad.64, that there would be an increased number of beads in the images. While it was difficult to determine where and how CD46 is binding to Ad.64, this information solidified that CD46 is in some way a viral receptor for Ad.64. Previously, it was believed that CD46 might bind to the fiber knobs of the virus because of similar results in Ad.37 viruses and CD46 binding **(**Wu et al., 2004). With fiber knob binding, we would expect to see groups of three beads symmetrically around the virus. Alternatively, we see the beads appear mostly on and around the virus, suggesting they might not be bound to the fiber knob. Recent studies have shown that species D adenovirus hexon capsid proteins directly interact with CD46 for cell entry

(Persson et al., 2020). The placement of the beads on top of and around the virus in imaging suggests that CD46 may bind to the capsid instead of the fiber knobs.

It is important to note that many of the CD46-bead complexes did not stay attached to Ad.64 during imaging. This may be due in part to the grid preparation protocol, specifically the glutaraldehyde and paraformaldehyde solution that is used to covalently crosslink the capsid. Despite this, the beads staying in the solution demonstrates that it was initially bound before imaging and still contributes to the overall understanding of CD46 as a viral receptor. Further work is needed to determine why the complex may not stay bound during imaging and how to keep the bound complex together to illustrate where the CD46 complex is binding.

The preliminary SDS-PAGE and Western Blot results indicate that from the HeLa cell lysate, we were successfully able to elute CD46. There are hints of other proteins in the solution in the SDS-PAGE gel, specifically the smear of bands in the elution lane (fig. 5). Further research is needed to sequence any partner proteins in the solution with CD46 using trypsin digestion and mass spectrometry. Once characterization of the partner proteins is complete, future studies can build molecular modeling of the protein complex and Ad.64 viral binding to this complex.

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