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CD46 interaction with adenovirus type 64, a causative agent for viral pink eye

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Abstract

Human adenovirus type 64 (Ad64) is a causative agent of contagious viral pink eye. We do not understand why Ad64 causes eye infections. One likely reason is the presence of a receptor molecule on the exposed surface of eye cells, but not other cells. Ad64 binds to a protein called CD46 on the cell surface. We aim to visualize how CD46 binds to the surface of Ad64. I mixed the extracellular portion of CD46 with purified Ad64 virus, then visualized it using negative stain transmission electron microscopy. In order to gain insight into how the virus binds CD46, we used cryo-electron tomography to increase the signal-to-noise ratio. In addition, we performed a binding experiment using only the Ad64 fiber knob with CD46 to determine which location the binding is occurring using Surface Plasmon Resonance analysis (SPR). This knowledge may reveal why Ad64 binds to eye cells to cause disease and lead to the future design of drugs that block Ad64 from entering eye cells.

Introduction

Adenoviruses are non-enveloped pathogens characterized by their icosahedral capsid structure and double-stranded DNA genome. These viruses are responsible for a range of clinical conditions, including respiratory illnesses, gastrointestinal disorders, and conjunctivitis, affecting individuals of all ages worldwide[1]. Among over 70 various strains, Human adenovirus types 64 (Ad64; formerly Ad19c), 37, and 19, homologous members of species D adenoviruses, have been identified as a prominent causative agents of epidemic keratoconjunctivitis, commonly

[1]Wu E, Fernandez J, Fleck SK, Von Seggern DJ, Huang S, Nemerow GR. A 50-kDa membrane protein mediates sialic acid-independent binding and infection of conjunctival cells by adenovirus type 37. Virology. 2001 Jan 5;279(1):78-89. doi: 10.1006/viro.2000.0703. PMID: 11145891.

known as pink eye disease[2]. This type of conjunctivitis is highly contagious and can lead to severe inflammation and even corneal damage, resulting in prolonged discomfort and visual impairment.

The interaction between Ad37 and human cells is mediated through the cellular receptor CD46[3], which is expressed throughout the body including eye cells[4], thus I hypothesized that Ad64 also uses CD46 as a receptor. CD46 serves multiple roles in immune regulation and cellular infection processes, making it a critical focal point for understanding viral entry mechanisms[5]. However, despite its clinical significance, the specific interactions between Ad64 and CD46—particularly how the virus exploits this receptor for cellular entry—are not fully understood. Previous studies have suggested that the fiber knob of adenovirus may play a crucial role in receptor engagement, but the exact mechanisms and efficiency of this interaction remain unclear.

This study aims to delve deeper into the mechanism of the binding between Ad64 and CD46. By employing advanced imaging techniques and biochemical assays, we intend to map out the precise binding sites and configurations that facilitate viral attachment and entry. Understanding these interactions at a molecular level is crucial, as it could lead to the development of targeted therapies that inhibit the initial steps of viral infection, thereby preventing the onset and spread of pink eye. This research not only has the potential to impact public health by mitigating the

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[2] Zhou X, Robinson CM, Rajaiya J, Dehghan S, Seto D, Jones MS, Dyer DW, Chodosh J. Analysis of human adenovirus type 19 associated with epidemic keratoconjunctivitis and its reclassification as adenovirus type 64. Invest Ophthalmol Vis Sci. 2012 May 14;53(6):2804-11. doi: 10.1167/iovs.12-9656. PMID: 22467570; PMCID: PMC3367469.

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[4] Wu E, Fernandez J, Fleck SK, Von Seggern DJ, Huang S, Nemerow GR. A 50-kDa membrane protein mediates sialic acid-independent binding and infection of conjunctival cells by adenovirus type 37. Virology. 2001 Jan 5;279(1):78-89. doi: 10.1006/viro.2000.0703. PMID: 11145891.

[5] Wu E, Trauger SA, Pache LMullen T, Von Seggern DJ, Siuzdak G, Nemerow GR2004.Membrane Cofactor Protein Is a Receptor for Adenoviruses Associated with Epidemic Keratoconjunctivitis. J Virol78:.https://doi.org/10.1128/jvi.78.8.3897-3905.2004

effects of this common eye disease but also enhances our broader understanding of adenovirus biology and pathogenesis.

Methods

1. Viral Cultivation and Purification

The overall experiments consist of two parts. In the first part of the experiment, our goal was to make a large quantity of the Adenovirus type 64 and bind it with CD46 receptor protein, and visualize the binding through negative stain transmission electron microscopy (TEM) and Cryo-electron tomography. The second part of the experiment was to make the viral fiber knob portion of the virus, which is located in the outermost part of the virus that is traditionally considered as the binding location for adenovirus. After the production of the fiber knob, we then performed a direct binding experiment and analyzed using SDS-page gel to verify if the binding was successful, and the goal was to determine the binding location of the virus to the receptor cell. The result was then further confirmed with Surface Plasmon Resonance analysis, which is an advanced technique for any ligand-analyte binding experiment.

Cultivation of Ad64 in cell cultures

The research began with the cultivation of Human adenovirus type 64 (Ad64) in A549 cell cultures. A549 cells, a human alveolar basal epithelial cell line, are commonly used for adenovirus propagation due to their high susceptibility to infection and efficient viral production. These cells were cultured in Advanced Dulbecco's Modified Eagle Medium (Advanced DMEM),

and were maintained in a warm temperature incubator (37°C), ensuring optimal conditions for cellular growth and maintenance.

Upon reaching 80% confluence, the cells were infected with Ad64 through the direct addition of the virus into the cell flask. After the addition of the virus Ad64, the cells were further incubated to allow virus adsorption. The infected cultures were then incubated for 7 days until most A549 cells fluoresce, which is indicative of successful infection. The goal of this step is to produce a large quantity of virus.

For virus extraction, the infected cells were subjected to three freeze-thaw cycles to lyse the cells and release the viral particles into the supernatant. This crude lysate was clarified by low-speed (1000x*g* for 10 minutes) centrifugation to remove cellular debris. The supernatant containing the virus was further purified using a cesium chloride (CsCl) density gradient through a twelve-hour long ultracentrifugation at 120,000x*g*, a technique that separates particles based on their buoyant density. The virus forms a distinct band in the middle of the gradient, which was extracted using a fine needle and syringe by directly poking through the test tube.

CD46-affinity chromatography

Following density gradient purification, Ad64 virus binding to CD46 was tested using nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography. This technique exploits the affinity of 6 histidine residues (His-tag) engineered into the CD46 proteins (Sino Biologicals) for nickel ions attached to the agarose beads. The virus preparation was mixed with soluble CD46 with a C-terminal His-tag and applied to a column containing Ni-NTA agarose (Qiagen). aAfter binding, the column was washed extensively with a wash buffer containing 25 mM

imidazole to remove non-specifically bound proteins. The bound virus was then eluted with 250 mM imidazole, and the end product of this purification step is the Ad64 plus CD46 mixture, and the results were verified through the SDS-page analysis.

Genetic Transformation and Cloning

The transformation process was mainly used for generating recombinant plasmids containing Ad64 fiber knob. Plasmids containing the modified and wild-type adenovirus fiber knob genes were introduced into competent *E. coli* cells through restriction enzymes Ndel and BamHI, which opens up the bacterial pET28a-TEV plasmid and allows the viral plasmid DNA to be inserted just after 6 histidine codons. After, ligase was used to seal off the plasmid and a quick heat shock step was used to allow bacteria to take up the plasmid. The cells were allowed to recover in rich media and then plated on LB agar plates containing an antibiotic (ampicillin) to select for bacteria containing the plasmids. We then picked several healthy colonies and transferred them to bigger culture tubes for further growth. 0.5 mM IPTG was used to induce over-expression, and B-PER was used to break open the cells and release either viral fiber knob. Finally, Pierce nuclease (ThermoFisher) was added to remove unwanted nucleic acids (such as DNA and RNA) from the bacteria. Ad64 fiber knob was purified using Ni-NTA Agarose in the same way as previously described above. The end product of this experiment is to produce pure fiber knobs.

Protein Interaction Assays

The interaction between Ad64 and the CD46 receptor was assessed using two primary techniques: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Surface Plasmon Resonance (SPR).

SDS-PAGE was performed to analyze the protein composition of the samples and verify the purity and integrity of the virus and receptor protein. This method separates proteins based on their molecular weight through a polyacrylamide gel matrix under the influence of an electric field. Samples were prepared by mixing with SDS sample buffer and boiling for 5 minutes to denature the protein to break up any trimers that are common for adenovirus fiber knobs. The denatured proteins were then loaded onto a polyacrylamide gel and subjected to electrophoresis, and the gel was analyzed under a stain-free, protein gel imager.

Surface Plasmon Resonance (SPR):

SPR is a powerful technique for analyzing real-time biomolecular interactions without the need for labeling. In our setup, CD46 was immobilized on a gold sensor chip via amine coupling, which involves the covalent attachment of the amino groups on the protein to the functional groups on the chip surface. Purified Ad64 was then flown over the immobilized CD46 at various concentrations to measure the binding kinetics (performed by Ichor Life Sciences, Potsdam, NY). The interaction was monitored in real time, providing data on the association and dissociation rates, from which the affinity constants were calculated. This quantitative data was effective in characterizing the strength and specificity of the interaction between viral fiber knobs and CD46.

Imaging Techniques

Advanced imaging techniques were employed to visualize the interaction between Ad64 and CD46, providing insights into the structural aspects of the binding interface.

Negative Stain Transmission Electron Microscopy (TEM):

Negative staining is a rapid method for examining the morphology and aggregation state of viruses and their complexes with receptors. For TEM analysis, a small aliquot of the Ad64-CD46 complex was placed on a carbon-coated grid and stained with an aqueous solution of uranyl acetate. This stain fills the background but leaves the virus and protein complex unstained, enhancing contrast in the electron microscope. Images were captured at various magnifications using a JEOL 1010 transmission electron microscope with a CCD camera to observe the overall shape and structural details of the virus-receptor complex.

Cryo-Electron Tomography:

For high-resolution three-dimensional imaging, cryo-electron tomography was conducted. This technique involved plunging the sample grid into liquid ethane to vitrify the sample, preserving it in a near-native state within a thin layer of ice. The frozen-hydrated specimen was then transferred to a cryo-electron microscope equipped with a tilting stage. Images were collected over a range of angles as the sample was tilted (performed by Dr. K. Dryden, University of Virginia). This approach allowed us to visualize the spatial arrangement of Ad64 and CD46 at molecular resolution, providing potential insights into the binding sites and the conformational changes that occur upon interaction.

Results

Part 1: Visualization of Viral Binding

The first part of our results focused on the characterization of the interaction between Human adenovirus type 64 (Ad64) and the cellular receptor CD46 using affinity chromatography. If Ad64 binds directly to soluble CD46 containing a 6-His tag, the complex can be precipitated

using Ni-NTA Agarose beads. SDS-page analysis was first used to verify the successful binding of CD46 and virus Ad64 (Figure 1). Soluble CD46 (lane 1) was depleted from solution by Ni-NTA Agarose (lane 3), but it appears in the high-imidazole elution fractions (lanes 6-9). Importantly, the hexon protein, the largest and most abundant viral protein, also appears in the elution fractions, indicating that CD46 pulled the Ad64 virus with it. Other adenovirus proteins, seen in lane 2, are not visible in the elution fractions, likely due to low relative abundance. The SDS-page gel verifies the successful binding between Ad64 and CD46.

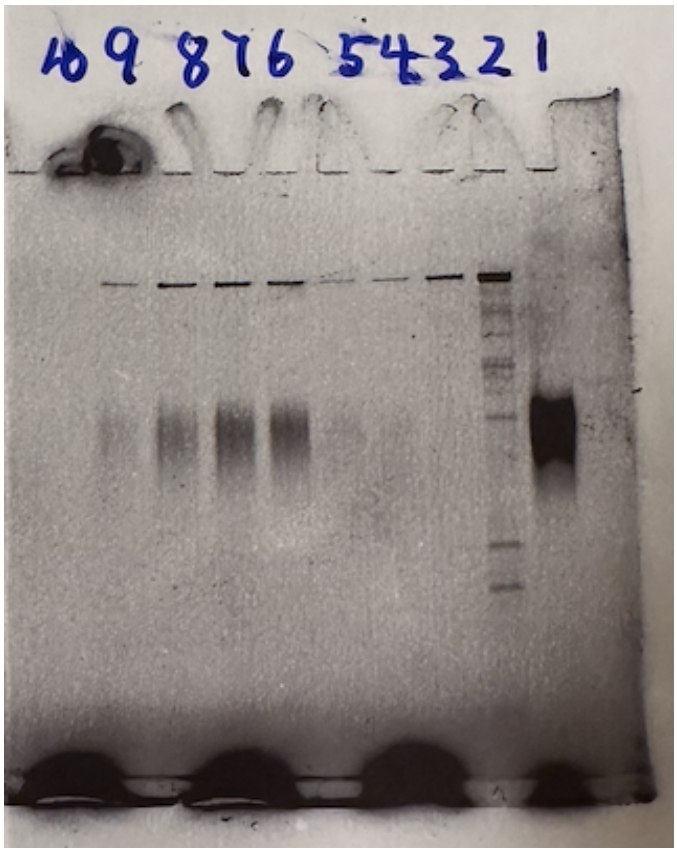

Figure 1. SDS-PAGE gel of fractions from Ad64-CD46 affinity chromatography. (1) soluble CD46 with 6-His tag, (2) Purified Ad64, (3) Flowthrough, (4-5) Low imidazole Wash , (6-9)

High imidazole Elution .

Negative Stain TEM:

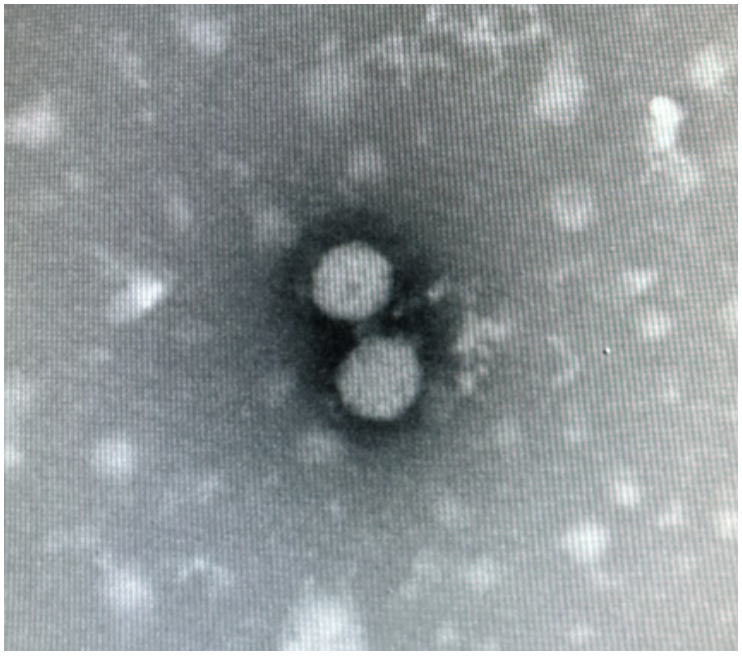

Figure 2: Transmission electron microscopy. Ad64 detected under electron microscope. The dark color represents the stain, and the two white particles in the middle represent two Adenovirus type 64. The CD46 protein can not be visualized.

Despite the successful detection of the virus using this technique, the results were inconclusive with respect to the binding of CD46 to the virus. The images obtained showed the typical icosahedral structure of Ad64 clearly, but there was no visible evidence indicating the presence of CD46. The lack of visual evidence for CD46 binding could be attributed to the orientation of

the complexes on the grid, the stain obscuring, not adequately contrasting the bound receptor, or the size of CD46 being too small to visualize.

Cryo-Electron Tomography:

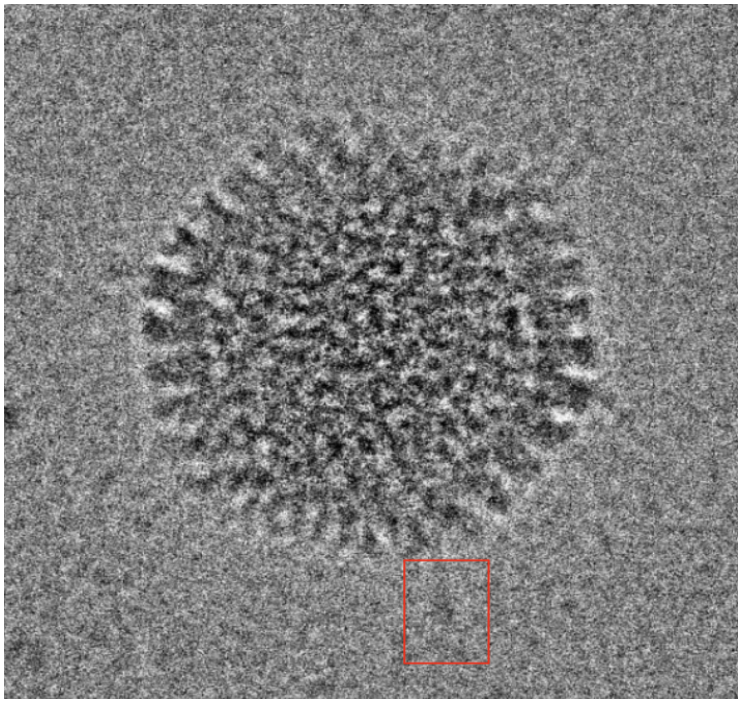

Figure 3: Cryo-electron tomography. Highlighted region indicates viral fiber knob. No evidence of CD46 protein detected anywhere on the Ad64 structure.

To overcome the limitations observed in negative stain TEM, cryo-electron tomography was employed. This technique provided three-dimensional reconstructions of the virus at near-atomic resolution, ideal for observing the spatial arrangement and potential receptor binding sites. The tomograms were meticulously analyzed for any signs of CD46 interaction with the viral capsid. Similar to the TEM results, the cryo-electron tomography did not show the expected binding of

CD46 on the viral surface. The high-resolution images confirmed the integrity and detailed structural features of Ad64 such as the hexons and the fiber knobs, but did not provide evidence of the receptor, suggesting that CD46 may be simply too small to visualize.

Part 2: Binding Experiments with the Fiber Knob

The second part of the results involved binding experiments using the fiber knob of Ad64, which was produced via genetic transformation and then purified for these tests. The primary objective was to determine if the fiber knob directly interacts with CD46, as hypothesized based on the known biology of adenoviruses. These experiments utilized both SDS-PAGE and Surface Plasmon Resonance (SPR) to assess the interaction.

SDS-PAGE Analysis:

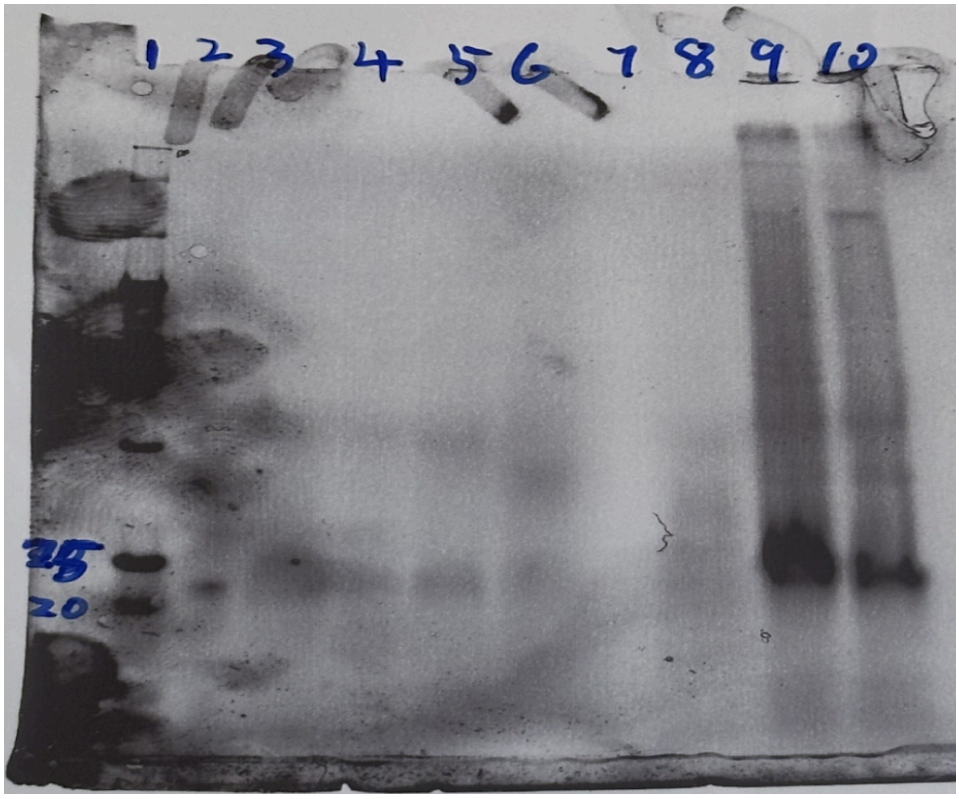

Figure 4: SDS-gel analysis. (1) Ladder, (2) Flowthrough, (3) Wash , (4,5,6) Elution, (8) Pure CD46, (9-10) purified Adenovirus fiber knob .

Following incubation of the fiber knob with CD46, the mixture was again precipitated with Ni-NTA Agarose and then subjected to SDS-PAGE to detect possible complexes. Purified fiber knobs and CD46 proteins were run on SDS-PAGE individually along with the Ad-CD46 mixture to verify their purity and identity. The individual run (9,10) on the gel showed distinct bands corresponding to pure CD46 and pure fiber knob alone, confirming their successful expression and purification. Unfortunately, in the elutions, the bands were able to match the viral fiber knob, but there seems to be no evidence of CD46. This result challenged the assumption that the fiber knob of Ad64 directly engages CD46 necessary for viral attachment and entry. However, the evidence to support such a conclusion is weak, because the band for CD46 seems to be very

smeared and diluted due to its glycosylation, which means it is possible that the binding did occur but it is simply hard to visualize due to its smeared and broad band.

Surface Plasmon Resonance (SPR):

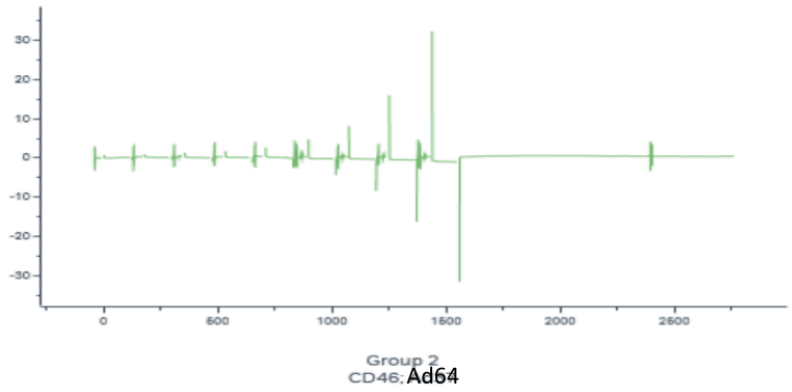

Figure 5: Surface Plasmon Resonance analysis (SPR). The quickly disappearing peaks show very weak to no binding for Ad64 fiber knob.

To further quantify the binding interactions, SPR analysis was conducted. Similar to the SDS-page analysis, the SPR data revealed minimal to no significant interaction between either fiber knob and CD46. The response curves did not show the characteristic binding and dissociation phases typically observed with specific interactions, instead indicating negligible affinity between the two proteins. This lack of detectable binding suggests that the interaction does not involve the fiber knob binding directly to CD46 or is below the detection limits of these assays.

The combined findings from both parts of the study suggest that the interaction between Ad64 and CD46 does not occur via the fiber knob in a manner that is detectable by the methods used.

Discussion

The results of this study challenge some of the existing assumptions about the interaction between Human adenovirus type 64 (Ad64) and the cellular receptor CD46, particularly regarding the role of the viral fiber knob in mediating this interaction. Despite employing advanced imaging techniques and detailed biochemical assays, our study did not provide evidence to support the hypothesis that CD46 binds directly to the fiber knob of Ad64 in a stable or detectable manner. This finding suggests that the binding dynamics between Ad64 and CD46 disobeys the traditional mechanism of adenovirus and is more complex than previously understood, potentially involving other viral or cellular components not yet identified.

Reevaluation of Viral Entry Mechanisms

The absence of detectable CD46 on the viral capsid in both negative stain TEM and cryo-electron tomography images suggests that if CD46 is involved in the entry process, its interaction with Ad64 might be that the CD46 binds in a location on the virus that was not captured under the experimental conditions used (the fiber knob). This could imply that other regions of the virus or additional cofactors are involved in stabilizing this interaction, which were not considered in this study. One recent study published in 2021 claims that adenovirus type D can also bind to CD46 through the hexon protein, which is the membrane layer of the virus structure. Therefore, future experiments to test this hypothesis could potentially reveal the actual binding locations between Ad64 and CD46 .

Implications for Therapeutic Development

The findings of this research have important implications for the development of therapeutic interventions aimed at preventing Ad64 infections. The traditional approach of designing inhibitors that block the fiber knob-CD46 interaction may need to be reconsidered. Instead, a broader strategy that targets multiple potential binding interactions or pathways might be more effective. This could involve the development of broad-spectrum antivirals that inhibit several steps of the viral entry process or the use of combination therapies that target different aspects of the viral life cycle.

Future Directions

Identification of Alternative Receptors and Co-factors:

Future studies should focus on identifying other receptors or co-receptors involved in the Ad64 entry process. This could include a systematic screening of cellular proteins that interact with different components of the viral capsid or the use of genetic approaches to identify host factors that are critical for infection. Such studies could uncover new targets for antiviral drug development.

Advanced Imaging Studies:

Further imaging studies using techniques such as single-particle cryo-electron microscopy or super-resolution microscopy could provide more detailed insights into the interaction dynamics between Ad64 and host cell receptors. These techniques could help visualize the interactions in

real time and in the context of intact cells, potentially revealing the transient or subtle interactions that were not detectable with the methods used in this study.

In Vivo Models:

To validate the relevance of the findings and to better understand the pathophysiology of Ad64-induced keratoconjunctivitis, *in vivo* models should be utilized. Animal models that closely mimic human eye infections could be used to study the efficacy of potential therapeutics and to observe the natural course of infection and immune response.

The unexpected results of this study highlight the complexity of viral-host interactions and underscore the need for a multifaceted approach to understanding and combating viral infections. By expanding the scope of research and employing diverse methodologies, future studies can build on these findings to develop more effective antiviral strategies against Ad64 and other pathogenic adenoviruses.

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