Cellular receptors for species B adenoviruses

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Front cover: Cartoon representation of the Ad11 fiberknob trimer. The knob is viewed down the threefold symmetry axis towards the virion surface. The Ad11 knob was crystallized by [1]. The monomers are shown in red, blue, and yellow. The model was built by using COOT after data indexing and scaling in Denzo/Scalepack. All the refinement was performed using Refmac5 (CCP4 suite). The figure was done in Pymol [2]. Figure kindly provided by David Persson, Interfaculty Institute for Biochemistry, University of Tübingen, Tübingen, Germany.
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ABSTRACT

Cellular receptors for species B adenoviruses

by Marko Marttila

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Adenoviruses belong to the most common human pathogens. The severity of infection varies greatly, from subclinical to lethal, depending on the virus type and immune status of the infected host. The 51 known human adenovirus serotypes are divided into six species (A-F) based on characteristics such as tropism. Species B adenoviruses, which are the subjects of this thesis, are further divided into subspecies B:1 that contains Ad3, Ad7, Ad16, Ad21 and Ad50 and subspecies B:2 that contains Ad11, Ad14, Ad34 and Ad35. Species B adenoviruses primarily cause ocular and respiratory tract infections, but certain serotypes (Ad11, Ad34 and Ad35) are also associated with renal disease.

The main aim of this thesis was to identify and characterize cellular receptors for species B adenoviruses. This will ultimately help to understand the diverse tropism shown by different adenoviruses and perhaps contribute to development of antivirals. Also, since adenoviruses are among the most commonly used vector for gene therapy it is of importance to characterize the initial steps of adenovirus life cycle.

Members of species B adenoviruses have been shown to utilize both the complement regulating membrane cofactor protein (MCP), i.e. CD46, and a still unknown receptor. CD80 and CD86, usually found on antigen-presenting cells, have also been suggested as receptors.

We found first that Ad11 used CD46 as a cellular receptor on respiratory A549 cells, and subsequently that CD46 is a cellular receptor for all species B adenovirus serotypes, except for adenovirus types 3 and 7, using cells that represent the tropism of species B adenoviruses, i.e. respiratory, conjunctival and renal epithelial cells.

We further compared the relative roles of CD46 with CD80 and CD86 using cells that represent species B adenovirus tropism. Using soluble candidate receptors and antibodies against corresponding receptors to challenge virus binding to and infection of cells, we found that on these cells, CD46 is a cellular receptor for all species B adenovirus serotypes, except for adenovirus types 3 and 7, and that CD80 and CD86 do not play an important role.

We have further pinpointed the interaction site for Ad11 on CD46 by X-ray crystallography. The extracellular region of CD46 contains four short consensus repeats (SCR1-4) of which the outermost N-terminal SCR1 and SCR2 mediate binding to Ad11. This interaction was confirmed by inhibiting infection and binding of Ad11 to A549 cells using soluble SCR1-2 fragments. Surprisingly the conformation of bound CD46 differs profoundly from its unbound state, with the bent surface structure straightened into an elongated rod. Viral proteins can sometimes undergo large conformational changes upon receptor binding, but this is, to the best of our knowledge, the first example of a virus protein dramatically changing the overall structure of its receptor. CD46 serves as a receptor for a large number of viral and bacterial pathogens and it is structurally and functionally related to other viral receptors such as CD21 and CD55. The mode of interaction presented here may serve as a conceptual framework for studies of many other receptors that are constructed from SCR domains.
**POPULÄRVETENSKAPLIG SAMMANFATTNING**

Cellulära receptorer för species B adenovirus

Adenovirus är en av de vanligaste sjukdomsorsakerna hos människan. Adenovirusinfektioner behöver inte märkas av alls, dvs infektionen kan vara asymptomatic, men den kan också vara dödlig, som hos människor med kraftigt nedsatt immunförsvar. Adenovirus orsakar ofta infektioner i luftvägar inklusive tonsillar, ögon, mage/tarm och urinvägar. Vissa adenovirus orsakar infektioner i både ögon och luftvägar.

De flesta virus påbörjar sin livscykel genom att binda till en receptormolekyl på cellytan och detta gäller även adenovirus. Totalt har 51 olika adenovirus identifierats som angriper människa. Dessa delas in i sex olika grupper (A-F). Det huvudsakliga målsättningen med detta projektet har varit att identifiera och karaktärisera cellulära receptorer för de adenovirus som tillhör grupp B. Dessa virus orsakar infektioner i framförallt ögon, luftvägar och i urinvägar. Vi fann att sju av nio grupp B adenovirus använder en receptor som heter CD46 (även kallad membrane cofactor protein), vars normala funktion är att se till att infekterade celler dödas av immunförsvaret.


Då man nu vet var på CD46 grupp B adenoviruset binder till så kan man tänka sig att man i framtiden kan framställa antivirala medel som baserats på de bitar på CD46 som virus binder till. Läkemedel innehållande små CD46-liknande bitar kan då konkurrera ut virus bindning till cellytan så att celler inte kan infekteras längre. På så sätt kan kroppens eget immunförsvar hinna ikapp och ta hand om infektionen på kortare tid vilket påskyndar tillfrisknandet.

Virus i allmänhet och adenovirus i synnerhet används mer och mer vid sk. genterapi, dvs ”behandling med hjälp av gener”, då dessa fungerar utmärkt som leverantörer av gener in i celler som man av olika anledningar vill behandla. En utmaning är dock att se till att virus när rätt typ av celler. I detta sammanhang är det således av stor vikt att känna till vilka receptorer som dessa virus binder till och på vilka celler dessa receptorer finns. På så vis kan man ”skräddarsy” virus och styra dessa mot ”rätt” typ av celler, så att terapin blir så effektiv och skonsam som möjligt. De virus som använder CD46 som receptor verkar vara ovanligt väl lämpade för genterapi, just tack vare att människor ofta inte är immuna mot flertalet av dessa virusstyper.

Avslutningsvis så kan vi konstatera att det finns flera cellulära receptorer/infektionsvägar som utnyttjas av grupp B adenovirus och åtminstone en är fortfarande okänd, dvs den/de som används för adenovirus typ 3 och 7. Våra studier visar dock att sju av totalt nio grupp B adenovirus kan använda sig av CD46 som cellulär receptor vid infektion av celltyper som representerar de celler/organ som dessa virus infekterar i människor, dvs luftvägar, urinvägar och ögon.
LIST OF PAPERS

**Paper I**  
Adenovirus type 11 uses CD46 as a cellular receptor.  
Segerman A, Atkinson JP, Marttila M, Dennerquist V, Wadell G, Arnberg N.  

**Paper II**  
CD46 is a cellular receptor for all species B adenoviruses except types 3 and 7.  

**Paper III** [Manuscript]  
CD46 but not CD80 or CD86 serves as a cellular receptor for selected species B adenoviruses on human epithelial cells.  
Marttila MM, Ackelind P, Persson BD, Stehle T, Arnberg N.

**Paper IV**  
Adenovirus type 11 binding alters the conformation of its receptor CD46.  
Persson BD, Reiter DM, Marttila M, Mei YF, Casasnovas JM, Arnberg N, Stehle T.  
# ABBREVIATIONS

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>aa</td>
<td>Aminoacid</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>A.D.</td>
<td>Adenoid degenerating</td>
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<tr>
<td>ADP</td>
<td>Adenoviral death protein</td>
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<tr>
<td>AdPol</td>
<td>Ad DNA polymerase</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>APC.</td>
<td>Adenoid-pharyngeal conjunctival</td>
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<tr>
<td>ARD</td>
<td>Acute respiratory disease</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie-adenovirus receptor</td>
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<tr>
<td>CBV</td>
<td>Coxsackie B viruses</td>
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<tr>
<td>CRAd</td>
<td>Conditionally replicating adenovirus</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating factor</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EKC</td>
<td>Epidemic kerato-conjunctivitis</td>
</tr>
<tr>
<td>f</td>
<td>Fiber</td>
</tr>
<tr>
<td>fk</td>
<td>Fiber knob</td>
</tr>
<tr>
<td>GON</td>
<td>Group of nine</td>
</tr>
<tr>
<td>HHV-6</td>
<td>Human herpesvirus 6</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MCP</td>
<td>Membrane cofactor protein</td>
</tr>
<tr>
<td>MLP</td>
<td>Major late promotor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLTU</td>
<td>Major late transcriptional unit</td>
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<tr>
<td>mRNA</td>
<td>Messenger-RNA</td>
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<tr>
<td>ND10</td>
<td>Nuclear domain 10</td>
</tr>
<tr>
<td>NFI-III</td>
<td>Nuclear factor I-III</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PFC</td>
<td>Pharyngoconjunctival fever</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-OH kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMBC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>RCA</td>
<td>Replication competent adenovirus</td>
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<tr>
<td>RGD</td>
<td>Arginine-Lysine-Aspartic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>sBAR</td>
<td>Species B adenovirus receptor</td>
</tr>
<tr>
<td>sB2AR</td>
<td>Species B:2 adenovirus receptor</td>
</tr>
<tr>
<td>SCRs</td>
<td>Short consensus repeats</td>
</tr>
<tr>
<td>STP</td>
<td>Serine-Threonine-Proline</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>TP</td>
<td>Terminal protein</td>
</tr>
<tr>
<td>TVD</td>
<td>Threonine-valine-aspartic acid</td>
</tr>
<tr>
<td>U.S.</td>
<td>United states</td>
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<tr>
<td>VA-RNA</td>
<td>Virus associated RNA</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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</table>
AIMS OF THIS THESIS

The human species B adenoviruses cause significant morbidity in both children and adults and are associated with severe ocular, respiratory and renal infections. In immunocompromized individuals, these viruses may even be lethal. The cellular receptors used by species B adenoviruses have for a long time remained unidentified and consequently the main aim of this thesis was to study the initial steps of species B adenoviral infections by identifying and characterizing cellular receptors used by species B adenoviruses.

Specifically, we aimed to use cell lines that represent the original tropism (respiratory, conjunctival and renal) of species B adenoviruses in order mimic the environment faced by species B adenoviruses when they encounter their target cells.

Using such cells, we then wanted to identify the adenovirus serotypes that used CD46 as a cellular receptor, and compare the relative role of CD46 with that of CD80 and CD86. Unlike CD46, which is expressed on all human, nucleated cells, CD80 and CD86 are expressed mainly by antigen-presenting cells, such as dendritic cells.

Another aim of this thesis was to identify the regions and specific amino acids on CD46 and on the adenovirus fiber knobs that interact with each other. This would then provide a potential target for antiviral treatment, and also make it possible to detarget gene therapy vectors based on species B adenovirus away from CD46, which would enhance efficiency and specificity of subsequently retargeted vectors.

We also aimed to accumulate results that would make it more easy to understand the species B adenovirus entry process and find clues to the specific tropism of these adenoviruses.
INTRODUCTION

HISTORY

Adenoviruses (Ads) were first identified and cultured in 1953 from surgically removed adenoids and tonsils from children [3]. During this time period similar viral agents were isolated in febrile U.S. military personnel that were suffering from a variety of respiratory syndromes [4, 5]. This transmissible viral agent causing degeneration of the epithelial-like cells was at that time called adenoid degenerating (A.D.) [4], adenoid-pharyngeal conjunctival (APC.) [5] and acute respiratory disease (ARD) agents. In 1956, these respiratory tract viruses were named adenoviruses. In retrospective one can see that acute respiratory disease syndromes caused by adenoviruses had been documented perhaps for 100 years before association with this virus (Dingle 1968). Up to 80% of new recruits in the U.S. army acquired lower respiratory tract illness during their first 3 weeks of training, and 20% of them were hospitalized. Adenovirus type 4 (Ad4) and Ad7 consistently accounted for the majority of these cases, causing 60% of the hospitalized cases [6].

However, the prevalent outbreaks of adenovirus infections amongst the US military recruits lead to the need and development of safe and effective vaccines, primarily against Ad4 and subsequently against Ad7 also [7]. Since 1971 live, encapsulated, enteric vaccine against Ad4 and Ad7 had been used routinely to successfully control adenovirus infections in military recruits [8]. However, due to economical reasons vaccine production ceased in 1996, and available vaccine stores were depleted by early 1999, leading to the return of high levels of adenoviral febrile respiratory illness [6, 9, 10]. Restoration of an effective adenovirus vaccine effort within the US army is thus anticipated by 2008 [10].

Adenoviruses have now been found in virtually every human organ system and have been associated with a variety of clinical symptoms such as epidemic keratoconjunctivitis (EKC), pharyngoconjunctival fever (PCF), infantile gastroenteritis, cystitis and urinary tract infections [11].

In 1962 it was shown that Ad12 can cause tumors when injected into hamsters [12] and later in mice [13], which was the first description of a pathogenic human virus that could cause malignant tumors in animals. This resulted in a sudden interest in adenoviruses as tumor viruses, but up to this day no convincing evidence has shown them to be malignant in humans [14-19]. However it has been suggested that even though tumors lack detectable adenovirus-specific molecules they can be of viral origin [20]. Low levels of Ad DNA has indeed been found in tumors [21, 22]. It has also been shown that parts of Ad5 DNA can transform human primary embryonal kidney cells resulting in the HEK293 cell line [23] and human embryonic retinoblasts resulting in HER 911 [24] so the possibility of adenoviruses transforming human cells also in vivo should not be ruled out definitely.

Adenoviruses have also been used as a model system by Philip A Sharp and Richard J Roberts whom in 1977 discovered how genes could be split (i.e. splicing) and could be present several well-separated segments in the genome. This discovery was awarded the Nobel Prize in physiology and medicine in 1993.
TAXONOMY

Adenoviruses have so far only been isolated from vertebrates. Adenoviruses belong to the virus family *Adenoviridae* that can further be subdivided into four genera (and a fifth suggested genus) according to the International Committee on Taxonomy of Viruses (ICTV). These genera are the *Atadenovirus* (named after the bias of their genomes containing high A+T content infecting a broad range of hosts including ruminants, as well as avian, reptilian and marsupial hosts), *Aviadenovirus* (infecting birds), *Mastadenovirus* (infecting mammals) and *Siadenovirus* (infects birds and frogs). The fifth suggested genus is called *Ichadenovivirus* (isolated from fish; [25, 26]). Within each genus adenoviruses are grouped into species, named from the host and supplemented with letters of alphabet [27, 28]. Species designation of adenovirus depend on several of the following characteristics [27]:

- Calculated phylogenetic distance (more than 5-10% based mainly on the distance matrix analysis of the protease, protein VIII (pVIII), hexon, and/or DNA polymerase amino acid sequence in comparisons)
- DNA hybridization
- Restriction fragment length polymorphism analysis
- Percentage of GC in the genome
- Oncogenicity in rodents
- Growth characteristics
- Host range
- Tropism
- Cross-neutralizing neutralizing antibodies
- Possibility of genetic recombination
- Number of virus associated (VA) RNA genes
- Agglutination of red blood cells
- Genetic organization of the early 3 (E3) region

Human adenoviruses, which belong to the *Mastadenovirus* genus, includes 51 different serotypes that are divided into six species (A-F; Table 1) [27]. The classification of human adenoviruses have evolved and changed along with the emergence of new analyzing methods. Human adenoviruses were originally divided into different species (formerly called subgenera) based on their ability to agglutination human and animal red blood cells [29, 30], followed by their oncogenicity in newborn hamsters [31], virion polypeptide profile [32], DNA restriction endonuclease patterns [33, 34], DNA hybridization analysis [35, 36] and tropism/symptoms [33].

The term *serotype* was previously defined by neutralization of the infectivity of the virus by hyperimmune sera and the ratio of homologous to heterologous neutralization titers had to be grater than 16:1 [11]. Even today, the most numerous serotypes can clearly be separated into six species according to this classification system, supported by DNAs equence distance matrix analysis [27].

Today, lack of cross neutralization together with a calculated phylogenetic distance of more than 10% separates two serotypes into different species. If the phylogenetic distance is less than 5%, any additional common grouping criteria from the above may classify separate serotypes into the same species even if they have been isolated from different hosts [27]. For example, adenoviruses isolated from chimpanzee resemble certain human adenoviruses so
extensively so that they are classified into “human” adenovirus species. Simian Ad21 belong to the species Human adenovirus B, while simian Ad22-25 belong to Human adenovirus E [27].

Table 1. Properties of human adenovirus serotypes of species A to F

<table>
<thead>
<tr>
<th>Species</th>
<th>Ad serotype</th>
<th>Tropism</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
<td>Intestine</td>
</tr>
<tr>
<td>B:1</td>
<td>3, 7, 16, 21, 50</td>
<td>Respiratory tract, eye</td>
</tr>
<tr>
<td>B:2</td>
<td>11, 14, 34, 35</td>
<td>Respiratory and/or urinary tract, eye</td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6</td>
<td>Respiratory tract</td>
</tr>
<tr>
<td>D</td>
<td>8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36, 37-39, 42-51</td>
<td>Eye, intestine</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>Respiratory tract, eye</td>
</tr>
<tr>
<td>F</td>
<td>40, 41</td>
<td>Intestine</td>
</tr>
</tbody>
</table>

Modified from[37] and kindly provided by Niklas Arnberg.

Other terms used is prototype, which is the first isolate in each serotype found (for example Ad7p; Table 2). Strains that differ from the prototype at a genome level are called genome types “a, b, c, d” etc. (for example Ad7a, Ad7b etc.). Furthermore, each isolate of any prototype or serotype will receive a specific strain name (for example Ad7p: Gomen; Ad7a: S-1958).

Species A

Species A includes Ad12, Ad18, and Ad31, which are all highly oncogenic in hamsters [31]. All have been found in stools from healthy humans [11]. These serotypes agglutinate rat erythrocytes incompletely [11].

Species B

Species B is subdivided into subspecies B:1 (Ad3, Ad7, Ad16, Ad21, and Ad50) and B:2 (Ad11, Ad14, Ad34, and Ad35) [27, 38]. These have different tropism. Subspecies B:1 viruses primarily cause respiratory disease (but also ocular infections and systemic infections, including diarrhea) while B:2 viruses mainly cause persistent infections of the urinary tract, and to a lesser extent ocular and respiratory infections [38] [11]. In addition to this, three species B:2 viruses (Ad11 mainly, but also Ad34 and Ad35) have been shown to cause renal infections and are potentially lethal in immunocompromized patients [11, 39-42]. Ad3 and Ad7 cause disease in humans more frequently than other species B serotypes and not surprisingly the seroprevalence of these two viruses is higher than for the other species B adenoviruses [43]. Species B adenoviruses are weakly oncogenic in newborn hamsters and specifically agglutinate monkey erythrocytes [31] [30] [11].
Species C

Species C contains Ad1, Ad2, Ad5, and Ad6. They infect more than 80% of the human population in early life [44]. These were also the first adenoviruses identified [3]. Although the primary infections are respiratory, species C adenoviruses can persist with non-symptomatic periods for years in immunocompetent hosts while viruses are intermittently shed in faeces [11, 44-47]. The probable source of persistent infection is mucosa-associated lymphoid tissue, and more precisely human mucosal T lymphocytes [44]. Species C adenoviruses incompletely agglutinate erythrocytes from rat [30] and are non-oncogenic in humans, even though they transform rodents cells \textit{in vitro} [48].

Species D

Species D consists of 32 different serotypes making it the largest human Ad species. Some of these serotypes show a tropism for the eye, such as Ad8, Ad19, and Ad37 which all cause EKC as well as sporadic keratoconjunctivitis [11]. Many of the species D adenoviruses are rarely encountered, therefore poorly characterized. Several serotypes are found in AIDS patients [49, 50]. Typical AIDS-associated serotypes are Ad9, Ad17, Ad20, Ad22, Ad23, Ad26, Ad27, Ad42-49, and Ad51 [51]. Species D adenoviruses completely agglutinate erythrocytes from rat and human [37]. They are non-oncogenic in humans, even though they transform rodents cells \textit{in vitro} [52].

Species E

Species E contains only one serotype, Ad4, which has been associated with epidemic follicular conjunctivitis and acute respiratory disease [53-55]. Several new Ad4 genome types have been found during the years [9, 33, 56-59]. The pattern of evolution appears to resemble that of influenza virus where a small but constant genetic drift is punctuated by the periodic appearance of a new strain that replaces former strains [57]. Species E adenoviruses incompletely agglutinate rat erythrocytes [37] and transform rodent cells \textit{in vitro} [11].

Species F

Species F consists of the enteric Ad40 and Ad41, which are associated with infantile diarrhea with long duration [60, 61], that can sometimes become fatal [61, 62]. Species F adenoviruses are difficult to culture \textit{in vitro} in established epithelial cell lines [11]. They show hardly discernable agglutination of rat erythrocytes and transform rodents cells \textit{in vitro} [11, 37].
<table>
<thead>
<tr>
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<th>Subgenus</th>
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<th>Source</th>
<th>Diagnosis</th>
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*Modified from [37] and kindly provided by Niklas Arnberg.*
BIOLOGY

VIRION STRUCTURE

Adenoviruses have been extensively studied and a substantial amount of data has been collected and presented, primarily from Ad2 and Ad5 which are the most studied adenoviruses. Adenoviruses are non-enveloped particles approximately 90nm in diameter [63] with fibers (9-33nm, depending on the serotype) projecting from the vertices of the icosahedron (Figure 1) [11]. Virions are composed of a protein shell (the icosahedral capsid) encapsulating the DNA-protein containing core-complex. The virion has a mass of about 150 x 10^6 Da and 13% of the mass is DNA and 87% is protein [64, 65] Trace amounts of carbohydrate is found on fiber (of Ad 2 and Ad5) because of the addition of O-linked N-acetyl-glucosamine [66]. Comparison of electrophoretic results with genomic open reading frames (ORF) suggests that the virion contains about 11 proteins, which are numbered by convention with roman numerals II-IX, IIIa, µ, terminal protein, and the p53 viral protease [67]. Protein I is excluded since it was later found to be composed of aggregated proteins [67].

Structural proteins

The non-enveloped capsid is composed principally of two types of building blocks: 240 trimeric hexon capsomers (also known as protein II or polypeptide II; pII) occupying the 20 triangular faces of the virus, and 12 penton capsomers, one at each of the 12 vertices of the icosahedron. As the names imply the pentons and hexons are surrounded by five and six neighboring subunits respectively [68]. Each penton consists of a pentameric base (pIII), which has a rod-like protruding trimeric fiber (pIV) ending with a terminal knob domain attached to it. The fiber is non-covalently attached to the penton to form the penton capsomer [69]. Most human adenoviruses encode only a single type of fiber with a specific length, which is characteristic to the species. This is not the case for Ad40 and Ad41 however, as they encode two fiber proteins of different lengths which are both incorporated in the virion [70-72].

Major structural proteins

The hexon protein

The hexon is the most common of the structural proteins, accounting for 63% of the total protein mass [64]. The 720 hexon monomers in each virion form 240 hexon homotrimers, which in turn form 20 triangular capsid facets, each consisting of 12 hexon homotrimers [67, 73, 74]. The pseudo-hexagonal shape seen on electron micrographs of isolated hexons is created by the 3-fold repetition of two similar β-barrel “double barrel” domains in the base of each subunit [64]. This characteristic shape has been evolutionally conserved and provided insights into viral evolution, thus recognizing bacteriophage PRD1 (which infects gram-negative bacteria) as a distant relative to adenovirus [75] [76]. On top of the hexon trimer three towers are formed, one from each subunit. These towers are composed of three intertwined hypervariable loops originating from each hexon subunit. The three towerlike structures seem to generate the type specific epitopes where the antigenic determinants are located [64, 77]. Hexons can be subdivided into group of nine (GONs) and peripentonal hexons. The GONs form the outer facets and the peripentonal hexons link the pentons with the facets [78].
Figure 1. Schematic structure of adenovirus. The locations of the capsid and cement components are reasonably accurate. However, the disposition of the core components and the virus DNA is largely conjectural. Adapted from [79], with permission from the publisher, Society for General Microbiology.
The penton: penton base protein
The penton base is a pentamer of pIII and seals the capsid at each of the 12 vertices. Determination of the molecular structure of the penton base for Ad2 has revealed that it has a roughly pentagonal cross section with five lobes circling a ~30Å central cavity that will harbor the trimeric fiber [73, 74, 80]. On top of the lobes loops are located containing the Arg-Gly-Asp (RGD) motif (Figure 2), which allows binding to cell-surface $\alpha_v$-integrins during adenovirus internalization [64, 81]. However, loss of the RGD motif slows down internalization, but it does not abolish adenovirus infectivity completely [82] [83]. It appears that for Ad5 the fiber-CAR interaction alone is sufficient for Ad entry and expression, although it is far less efficient than if the the RGD-integrin interaction is available [83]. Species F Ad penton bases are the only ones that do not contain RGD motifs at all. [82, 84, 85].

Figure 2. Structure of the Ad2 penton base, without attached fiber. The pentamer, the functional unit of the penton base protein is shown as a surface representation (left) and as a ribbon diagram (right). Each monomer is colored uniquely. The variable loop, RGD loop and N- and C termini are marked. Adapted from [80], with permission from the authors.

The penton base amino acid sequence is exceptionally highly conserved, even between human and animal adenoviruses, with a typical 70% homology between any two penton bases. The differences are almost exclusively localized to the flexible hypervariable loop regions that are of variable length, and except for Ad40 and Ad41 contain the RGD motif [80]. It has been observed for some serotypes that pentons can assemble without other virion components to form dodecahedral particles that can efficiently enter cells by endocytosis and accumulate at the nuclear membrane [86] [87]. Naturally occurring penton deodecahedra have been observed in the extracts of Ad3-, Ad4-, Ad7-, Ad9-, and Ad15-infected cells [86]. The physiological role of the penton is still a bit unclear, although it has been proven that the RGD motif of the penton base is causing cell rounding in infected adherent cells [81] and pentons are involved in haemagglutination [88] [38].

The penton: fiber protein
The trimeric fiber protein (pIV) can be divided into three structural parts; the N-terminal tail, a central shaft with repeating motifs, and a C-terminal globular knob [89] [74] [90] [91] [67]. A crystal structure of the Ad2 fiber/penton base-complex has shown that a conserved amino acid sequence in the N-terminal fiber tail, FNPVYPY, is responsible for the penton base/fiber
interaction via hydrogen bonds and salt bridges [80]. The average diameter of the fiber shaft of Ad2 is 15Å, excluding the surface loops [91]. The fiber also shows distant evolutionary relationship with the reovirus attachment protein σ1 [92]. This has led to the possibility to create pseudotyped adenovirus vectors with σ1 fibers to target cells expressing the reovirus receptor junctional adhesion molecule 1 (JAM-1) [93, 94].

The variation in fiber length between different serotypes is caused by the difference in the number of sequence repetitions in the shaft. The number of sequence repetitions range from six in Ad3 with the shortest shaft [95] to 22 in Ad2 and Ad5 with the longest shafts [89, 91, 96]. The structure of the shaft was first revealed in a crystallographic analysis of the Ad2 knob and nearby repeats [91], which showed that the shaft repeat has a novel topology. This topology is described as a triple β-spiral fold, which is characterized by intertwining of three polypeptide chains similar to a spiral staircase in which each step is defined by a β-repeat. The repeating motifs typically consist of 15-20 amino acids [96].

The knob structure involves three monomers, which each form an eight stranded antiparallel β-sandwich structure (Figure 3) [97] [98] [99] [100] [101] [1]. The knob, which is responsible for binding to cellular receptors, has a somewhat propeller-like structure with a central depression and tree symmetry-related valleys on top of the knob, which were first thought to be the binding site for the coxackie-adenovirus receptor (CAR). The structure of Ad12 knob, which was solved in complex with CAR (CAR D1), showed that the interacting site was in fact composed of surface loops at the monomer interfaces [99]. The Ad11 knob also interacts with its receptor CD46, by binding to the side of the knob monomer interface regions [1]. Ad19 and Ad37 on the other hand attach to the sialic acid residues at the top of the knob trimer [100].

Despite the claim that all species D adenoviruses use CAR as a cellular fiber receptor [102], Ad19 and Ad37 (species D) have been shown to use sialic acid as a cellular receptor also [100, 103-105]. These contradicting facts might be explained by the difference in shaft length between species B and D adenoviruses. Adenoviruses with relatively short fibers may not use CAR but adenoviruses with longer fibers (more than 11,5 β-repeats) may do so [96]. It has been shown that chimeric Ad5 adenovirus vectors expressing short Ad9 fibers (from species D) show ~40-65% decrease in virus attachment and internalization into CAR-expressing cells compared to wt Ad5 virus with long fibers [106]. Shaykhmetov et al. propose that the strikingly negatively charged hexons on the Ad5 capsid surface might cause an electrostatic repulsion between the capsid and the acidic cell surface proteins. This could be more pronounced in the Ad5 chimera with short-shafted Ad9 fibers than in the wildtype Ad5, which uses CAR for primary binding to the host cell. Neutralization of the negative cell surface charges abrogated the differences in attachment and infection between the fiber length chimeras [106]. On the other hand, the attachment of a short Ad9 fiber to an Ad5 penton might disturb the correct spatial arrangement of CAR binding knob and penton base RGD motifs required for efficient binding and infection. Also, Ad5 chimeras with short shafted Ad35 fibers infected cells efficiently which might be explained by an interaction with a primary cell surface receptor high above the acidic cellular glycocalyx which rises only 10nm above the cell surface [106]. The length of the fiber is not the only characteristic suggested to be of importance in receptor binding and there are observations of a bend in the fiber shaft and in the transition between the shaft region and the knob thus making the fiber more flexible in its interactions with cellular receptors [91, 96, 107]. The bend in the fiber-shaft is localized to the third β-repeat (close to the penton Figure 4, about 60Å from the capsid surface) [107]. This repeat contains two to four additional amino acids inserted into the β-
repeat, which are thought to be involved in bending of the fiber shaft [96, 107]. Interestingly the non-consensus sequence motif is not present in the fiber of species D serotypes, such as Ad37, making the shaft rigid and straight [107-109]. Most adenoviruses, again with the exception of species D viruses, have a KLGXGLXFD/N consensus motif in the last complete β-repeat (repeat 21 in Ad2). This sequence may allow for the bending of the fiber at the knob [107].

Both fiber-CAR and penton base-integrin interactions are necessary for efficient Ad5 infection which is proven by that the infectivity of both fiberless [110, 111] and RGD deficient [81, 83] vectors was greatly diminished.

**Figure 3.** Cartoon representation of the Ad11 fiberknob trimer [1], monomers shown in blue, red, and yellow. The model was built using COOT after data indexing and scaling in Denzo/Scalepack. All the refinement was performed using Refmac5 (CCP4 suite). The figure was done in Pymol [2]. Figure kindly provided by David Persson, Interfaculty Institute for Biochemistry, University of Tübingen, Tübingen, Germany.

**Figure 4.** Schematic model of the Species C Ad surface receptor interactions. The sterical hindrance restricting secondary integrin interactions may be overcome by the the flexibility of the fiber. This figure was adopted from [107] by permission from the authors.
Minor capsid proteins
The minor capsid protein structure and functions are much less studied as compared to the major capsid components since the major capsid proteins fiber, penton base and the hexons have all been exploited for virus retargeting as scientists have been trying to understand the initial interactions between virus and host cell. The four minor capsid proteins IIIa, VI, VIII and IX (pIIIa, pVI, pVIII and pIX, respectively) are thought to act as cementing proteins in the capsid, thus being important for the virion structure stability [64].

pIIIa
The precursor of pIIIa has a mass of 67kDa and is cleaved at the N-terminus during the maturation of the virion to create the 63.5kDa final version of pIIIa [112]. pIIIa is a monomeric protein extending from the exterior surface of the capsid through to the interior of the capsid [73, 112]. It is situated at the point of the edge between facets and is thought to act like a “rivet” to stabilize the interface between two capsid facets [64]. It resides at each vertex close to the penton base where it interacts with the hexamers of neighboring facets [67, 113]. It is also involved in viral assembly [114, 115]. It is believed that pIIIa interacts with pVII because they can be co-immunoprecipitated [114]. At least one part of pIIIa is phosphorylated in the capsid [116, 117]. The physiological impact of the phosphorylation is unknown [112]. It has also recently been shown that an artificial expression of pIIIa during the early phase of adenoviral infection results in a significant reduction in late viral protein synthesis and a moderate block to viral DNA replication, thus indicating a role for pIIIa in adenovirus late gene expression, however the mechanism is still unclear [118].

pVI
The 22kDa pVI is generated by the cleavage from a larger precursor. pVI is situated on the inner capsid surface and anchors the peripentonal hexons together and connects the capsid to the core [73, 112, 119]. The N-terminus is basic and may interact directly with the DNA [65, 73]. During the infection pVI helps the virion to escape from the endosome as pH-dependent capsid disassembly liberates pVI, which in turn promotes endosomal membrane disruption. This membrane disruption is dependent of the exposure of a predicted N-terminal α-helix in pVI that is buried within the hexon-protein interface in the intact capsid [120]. pVI is also involved as a shuttle in the nuclear import of hexons from the cytoplasm in infected cells as it links hexons to the importin α/β-dependent nuclear import partway. The key nuclear import signals of pVI are located in a short C-terminal segment, which is proteolytically removed during virus maturation. The removal of these transport signals appears to convert the precursor of pVI from the role of supporting nuclear import of the hexons to a structural role of pVI in the capsid [78, 112, 121, 122]. The C-terminus of pVI, together with viral DNA, are both cofactors to adenovirus protease thus increasing the protease activity [121]. Actin is also a cofactor for the adenovirus protease which may help adenoviruses to destroy the cytoskeleton, leading to cellular lysis and subsequent viral exit [64, 121].

pVIII
The 15.3 kDa pVIII is located at the inner surface of the capsid as dimers and interacts with hexons between neighboring facets. It is processed from a large precursor protein just like pVI and pIIIa [73]. It is noteworthy that the precursor of protein VIII is only found in empty capsids but is undetectable in complete particles. This feature has been used to identify empty capsids in adenoviral vector preparations [123-125].
**pIX**
The 14.3 kDa pIX is the smallest of the minor capsid proteins and is unique to the *Mastadenovirus* genus [112]. It is a minor component of the planar group-of-nine hexons (GONs) which form the central part of each of the 20 facets of the icosahedral capsid [112, 126]. GONs are released upon disruption of the adenoviruses under mild conditions [127-129] suggesting a difference from the peripental hexons that dissociate separately [74]. pIX is localized between the hexons and is slightly exposed from the capsid and is probably stabilizing the capsid [73, 130-132]. pIX has been traditionally placed in the cavities residing between the hexon tops where four trimers of pIX cement the central nine hexamers together [67, 74, 113, 133] although alternative and complementary positions of pIX have been proposed [112, 133].
pIX have been found to act as a transcriptional activator of adenoviral late genes in transient transfection experiments [134] although the effect during wild type infection is uncertain [135]. Furthermore, in adenovirus-infected cells pIX is involved in the formation of nuclear inclusion bodies, to which the cellular promyelocytic leukaemia (PML) protein is relocated late in infection thus forming the PML (also known as ND10)-inclusion bodies. PML protein is involved in regulating the cellular antiviral response, which might be the beneficial reason for the adenoviruses to sequester it [136]. PML nuclear inclusion bodies have also been shown to be involved in the regulation of the infection process for many other viruses [137].
pIX has also been studied as a tool for adenoviral gene therapy, and tropism alteration by capsid modification in particular, due to the ability to anchor heterologous ligands on it [138] [139] [140] [112].

**The core proteins**
The adenoviral core contains the linear double stranded viral DNA of about 36kb with a molecular weight of about 22,2x10^6 Da and five known proteins [38, 67, 74]. Polypeptides V, (pV) VII (pVII) and μ (mu, also called protein X; pX) are basic, arginine rich proteins that are in contact with the viral DNA [141-143] and probably condense the DNA in the core [67]. IVa2 and the terminal protein (TP) are associated with the core.

**pV**
Each virion contains about 157 copies of pV [74], and appear non-specifically associated with the viral DNA [144]. pV appears to form a layer around the core providing a structural bridge to the capsid via interactions with pVI and pVII and/or the DNA [78, 145]. Deletion of pV gene in Ad5 causes a thermostability/infectivity defect, which is rescued by thermo-selectable mutants in core protein μ precursor, thus suggesting that pV and μ precursors might have a role in viral assembly [146]. Protein V localizes to both the nucleolus and the nucleus and redistributes nucleolin (also called C23) and nucleophosmin (also known as B23) from the nucleolus to the cytoplasm. [147]. Nucleolus is the center of ribosome biogenesis whereby rRNA is synthesised, processed and incorporated into ribosomes [148, 149]. B23 is implicated in RNA processing [150] and directing of many proteins to the nucleolus [151-153]. Nucleolin has been associated with rRNA processing, ribosomal assembly, transcriptional repression and the transport of ribosomes to the cytoplasm [154-157].

**μ**
The core also contains the small, basic L2 encoded 19 amino acid DNA-binding peptide μ (mu) which have been associated with adenovirus DNA condensation and charge
neutralization [141, 158]. During the import of the adenoviral genome at the nuclear pore complex protein VII and μ have been reported to be remain associated with the DNA, while pV may dissociate from it before or immediately after DNA translocation into the nucleus [144].

pVII
The most common core protein is pVII with more than 800 copies [64, 65] and has functional features similar to the cellular histones condensing the viral DNA [159-165] The precursor of pVII has an additional 23 amino acids at the N terminus [166] which are cleaved off by a viral protease inside the virion capsid to form pVII in the late steps of viral maturation. During the early infection process the complex of protein VII-DNA is transported and imported to the nucleus and may serve as a template for early viral gene transcription [165, 167-169].

During the late phase of viral infection pVII associates with unpacked viral DNA [159, 170], which is later packaged into the viral capsid, the exact mechanism is not known at the moment, where it is organized in an viral chromatin-like manner [169].

pVII interacts with the viral pIVa2 and L1 52/55 kDa proteins, which are known viral DNA packaging molecules. It is possible that the interaction between pVII or VII with L1 52/53 kDa protein might be mediated by pIVa2 [169]. pIVa2 mediates the specific binding with the packaging sequence in adenoviral DNA [171], while pVII and L1 encoded 52/55 kDa proteins bind to DNA non-specifically [169]. pIVa2 also mediates the serotype specific packaging of DNA into capsids [171], condenses DNA, represses transcription and furthermore associates with the adenoviral transcriptional activator E1A [172].

pTP
In the nuclear core, the E2 encoded terminal protein (TP) is attached covalently to each 5’ termini of the viral genome and is involved in the initiation of replication [173, 174].

Non-structural proteins
About 30 adenoviral non-structural proteins have been described to date. With a few exceptions, they function in the adenoviral life cycle by having catalytic and regulatory functions [65]. The DNA binding protein (DBP) is an E2-encoded phosphoprotein which size varies with serotypes (473-529 amino acids, aa) [65]. It has multiple functions and is involved mainly in DNA-replication and other viral DNA metabolic processes [65, 175, 176]. Other E2 encoded proteins involved in DNA replication are the viral DNA polymerase and the terminal protein (TP) [65]. The L3 region encoded 23kDa cysteine protease is a non-structural protein that is crucial for viral capsid assembly as well as for the uncoating of the viral particle at the nuclear membrane during the viral entry process [65, 177]. It has been estimated that and adenovirion contains about 10-30 protease molecules [178, 179]. The activity of the protease is increase by presence of adenoviral DNA and by the cleavage of pVI, specifically by the 11 amino acid peptide cleavage product pVIc [64, 65, 121, 180].
ADENOVIRUS LIFE CYCLE

Adenovirus receptors

Attachment of adenoviruses into host cells is generally preceded by fiber-mediated viral binding to primary cellular receptors. Most studies on adenoviral life cycle has been done on the closely related Ad2 and Ad5, mainly because they were amongst the first to be isolated and because they are easily produced in large amounts [67]. It was early discovered that the adenovirus structure responsible for cellular attachment was the fiber protein [181]. Later, several studies have showed that soluble fibers of different serotypes inhibit adenovirus attachment and/or infection of homotypic virions to cells in vitro [182-186]. The importance of the high affinity fiber knob-receptor interaction is illustrated by the fact that ectopic attachment receptor expression [187] or exchange of the serotype specific fiber to another fiber enabled infection/transfection of previously non-permissible cells [94, 188-193], a feature that has been widely exploited in adenovirus gene-therapy vector design. Swapping of fibers (or parts of fibers, such as the knob domain) between serotypes is one of the most common naturally ocuring recombination events, suggesting that it can be beneficial in the emergence of new adenoviral strains [60, 194-196].

CAR

Although it has been known for a long time that species C adenoviruses and group B coxsackieviruses (CBV) share the same attachment receptor [197] it was not until 1997 the coxsackie adenovirus receptor (CAR) was identified as a receptor for Ad2 and Ad5 [198]. It was later discovered that selected serotypes from five out of six species (A, C, D, E, and F but not species B) bind to soluble CAR which lead to the conclusion that all serotypes from these five species use CAR as a functional cellular fiber receptor [102]. This generalization has later proven to be somewhat optimistic as several serotypes from at least Species D have been shown to bind and utilize another receptor [100, 103, 105].

CAR: Structure, function and localization

Species C adenoviruses have been shown to use CAR in vitro [198, 199] and probably in vivo too [200-203]. CAR has a predicted molecular weight of about 38kDa but it migrates on SDS polyacrylamide gels as 46kDa, which is probably due to glycosylation. CAR contains a single membrane-spanning domain connecting the intracellular 107 amino acid domain from the extracellular adenovirus-binding 216 amino acid domain. This domain is composed of two immunoglobulin (Ig)-like domains, D1 and D2 [204]. Both CBV and adenoviruses bind to the D1 domain, however targeting different epitopes [99, 205]. Neither the cytoplasmic nor the transmembrane part of CAR is required for adenovirus infection, strongly implicating that CAR merely act as an cellular anchoring point for attaching adenoviruses [206]. CAR is a transmembrane component of the epithelial cell tight junctions [207] and is abundantly expressed in heart, pancreas, the central and peripheral nervous system, prostate, testis, lung, liver and intestine, but little or no CAR is found on B and T cells, adult muscle cells and many malignant cells [208]. The expression of CAR seem to be developmentally regulated [209] and is induced by inflammation regulators [210]. Studies with cell lines of different origin show that the expression of the CAR correlate well the ability of species C adenovirus ability to infect these [178, 211, 212]. Nonetheless, CAR-binding adenoviral vectors are relatively inefficient in infecting differentiated airway epithelia [213-216], which has a well developed extracellular matrix composed of various proteins and sugars which could possibly reduce, or sometimes enhance infection [208]. However, wildtype adenoviruses regularly initiate their
infections by aerosols and are found in these very areas, suggesting that delivery to airways is feasible [217, 218].

Since CAR is mainly localized in tight junctions and along the basolateral membranes [207, 219], hidden below the exposed apical surface of polarized epithelial cells (for example in the respiratory tract epithelium; Figure 5) it has for some time been a mystery how adenoviruses can use CAR as a functional cellular receptor when CAR appears to be so inaccessible from the adenovirus point of view. One theory is that the airway infections starts by the infection of specialized nonpolarized (M) cells that express CAR on the luminal side of the airway, followed by spread to neighboring polarized cells, or that lesions in the epithelium exposes basolateral membranes so that CAR is exposed to adenoviruses [208, 220]

**Figure 5.** CAR-mediated adenovirus entry and escape. Hypothetical scheme describing events leading to epithelial infection with the CAR-interacting Ad2 or Ad5. Ads are thought to access the epithelial cells by infecting cells displaying CAR receptors in the apical membrane. This first leads to a local infection and the spreading of the particles to basolateral membranes of epithelial cells where CAR is commonly located. Viral spreading is aided by the release of fiber-penton base complexes which have been suggested to disrupt the tight junctions of epithelial cells prior to virus release by binding and competing out the CAR-CAR protein interactions that maintain the cell-cell adhesion. With permission from the authors [221] and the publisher. Copyright John Wiley & Sons Limited.

**CAR: Mediator of adenovirus entry or escape**

It has also been suggested that adenoviruses might also use CAR as an escape mechanism as well as an entry mechanism. Following replication, in the late phase of the adenoviral infection and prior to cell lysis, virions and viral structural components, in particularly fibers are released from the infected cells and affect the surrounding tissue integrity [222]. The export of fiber components interrupts adherent junction cell-cell contacts and increases epithelial permeability by outcompeting homodimeric, intercellular CAR-CAR interactions due to the higher CAR-binding affinity of the fiber [216, 223] aided by the likelihood of excess molar ratio of fiber compared to CAR [220]. This mechanism may provide the means
for adenoviruses to cross tissue barriers so it can spread to new body compartments (bloodstream, other body organs) and to the environment [220].

**CAR: Interactions with the adenovirus fiber knob**

The crystal structure of Ad12 fiber knob domain in complex with CAR D1 has been solved [99]. The regions of the knob that are responsible for CAR interaction were localized to the AB-, DE-loops and the F-strand of one knob monomer and the FG-loop of the neighboring knob monomer, all of which are located on the side of the knob [99]. The AB-loop mediates over 50% of the interfacial protein-protein interactions of the binding [99], however the binding also seem to a great extent be water mediated as interface cavities are filled up and bridged by water molecules [99] [224]. Mutational studies have confirmed the importance of the AB loop in Ad5 CAR binding [225, 226] despite the divergence in knob amino acid sequence between the CAR binding adenoviruses, thus implicating the importance of patches of conserved amino acid sequences within the knob [226]. From mutational studies of the Ad5 knob AB-loop Ser408, Pro409 DG-loop Tyr477 and β-strand F Leu485 are direct CAR contact residues, while Ala406, Arg412 in the AB-loop, Arg481 in the E-loop [225] and Tyr491 in the DG-loop [227] are peripherally or indirectly involved in CAR binding. The binding sites for CAR are thus mostly positioned in flexible loops structures in the fiber knob.

**Sialic Acid**

A small group of species D adenoviruses (Ad8, Ad19 and Ad37) that cause EKC have been found to use α2,3-linked sialic acid as a cellular receptor rather than CAR [103, 105 Burmeister, 2004 #110, 228, 229]. The knobs of these adenoviruses have relatively high theoretical isoelectric points (pI:s), compared to the adenoviruses that mainly are associated with respiratory and/or mild eye infections (Species B, C and E types) that seem to contain knobs with intermediate pI values [230]. Adenoviruses associated with enteric infections have intermediate pI values (A and F adenoviruses) [230]. Homology modeling has predicted that the top surface of the fiber knobs of the sialic acid-interacting adenoviruses is also positively charged and consequently Ad8, Ad19, and Ad37 seem to interact with sialic acid in an charge dependent manner [104].

The crystal structures of the species D Ad37 in complex with sialyl-lactose have been solved [100] and the sialic acid interacting site is indeed located on the top of the knob. The two key residues of the Ad37 sialic acid binding site, Tyr 312 and Lys345 are conserved within the investigated species D knobs (Ad8, Ad15, Ad17, Ad28, Ad30 and, Ad37) and the other residues demarcating the binding site (Tyr308, Pro317 and Val322) are also highly conserved [100]. Furthermore, additional interactions with secondary molecules/receptors on the cell surface or simply increased avidity due to multiple interactions between virions and cells moreover other post-binding events may be of importance for the tropism and EKC causing ability [100]. Compared to many protein-protein interactions, the knob-siaclic acid interaction may appear to be of low affinity. However, the dissociation constants are in the same range as observed for other sialic acid binding proteins, like for the influenzavirus hemagglutinin (Kd ~3mM) [231] and rotavirus VP8 core protein (Kd ~1,2mM) [232].

**CD46**

For a long time it was known that species B adenoviruses use a receptor different from other adenoviruses [233]. In 2003 CD46, also known as membrane cofactor protein (MCP), was identified as a cellular receptor for the species B Ad11 [186]. Subsequent reports have shown that many, but not all, species B adenoviruses bind to, or use, CD46 as a cellular receptor for
internalization into host cells [234-236]. Additionally, Ad37 from species D has been reported to bind to CD46 [237].

CD46 as a pathogen “magnet”
CD46 (Figure 6) has been reported to serve as a receptor for a number of different human pathogens; human herpesvirus 6 (HHV-6) [238], Measles Virus [239, 240], Streptococcus pyogenes [241] [242], and Neisseria spp. [243]. These pathogens interact with different sites on CD46.

Figure 6. Schematical structure of human CD46. Most human tissues express the four major CD46 isoforms depicted here. The isoforms derive from alternative splicing so additional splice variants exist. The cellsurface part of CD46 is N-glycosylated in the SCR-regions and O-glycosylated in the STP region.

HHV-6 binds to SCR2 and 3 [244], measles virus (mainly the laboratory strains) bind to the SCR1 and 2 region [245]. Streptococcus pyogenes bind to SCR3 and 4 [242], and Neisseria gonorrhoeae uses SCR3 and the STP domain [246]. Finally, Ad11 from the species B adenoviruses have been crystallized in complex with CD46 and was shown to bind to and alter the conformation of CD46 SCR1-2 [1], which is the same regions to which another adenovirus species B Ad35 have been shown to use [247, 248]. Another report by Gaggar et al. [249] demonstrated that chimeric adenoviruses based on the species C serotype 5 capsid but possessing species B Ad11 or Ad35 fibers, bounds to conformationally sensitive domains located in SCR2. However, the transduction of the Ad5/35 chimera was dependent on both SCR1 and 2. It was furthermore stated that N-glycosylations did not critically contribute to the transduction. In partial support of this, Fleischli et al. showed that removal of the N-glycan attached to SCR1 had no significant effect on Ad35 binding [247]. However, removal of the SCR2-attached N-glycan decreased both Ad35 binding and luciferase transgene expression [247]. Transgene expression from Ad5/35 varied greatly depending on receptor length. This might be explained by electrostatic repulsion that may be obtained between short-shafted
Ad35), negatively charged Ad5-based capsids and the acidic cell surface proteins [106]. The deletion of the cytoplasmic tail rather increased the transduction by Ad5/35, however downregulation of CD46 was not observed in transgenic Chinese hamster ovary (CHO) cells expressing human CD46 in this experimental setup. In a recent report however, Sakurai et al. Nonetheless found cell surface downregulation of CD46 in several human cell lines e.g. human peripheral blood mononuclear cells (PBMCs), but not in HeLa cells. This was caused by Ad35 vectors, however the total cellular levels of CD46 were not reduced [248]. This suggests that CD46 can be internalized without degradation following infection of Ad35, similar to the mechanisms seen during measles virus infection [248]. However, measles virus haemagglutinin, which binds to CD46, also downregulated CD46 expression in several human cell lines including HeLa [250] and CHO-CD46 [251]. This suggests that subsequent cellular events following the Ad35 attachment to CD46 differ from those induced by measles virus [248].

The type of ligand binding to CD46 may determine if CD46 is internalized by clathrin-coated pits or by macropinocytosis [252]. More specifically, cross-linking of CD46 on the cell surface either by multivalent antibodies or by measles virus lead to internalization via a macropinocytic-like pathway that ultimately leads to CD46 degradation. However, another internalization pathway using monovalent antibodies, that do not crosslink cell surface CD46, resulted in constitutive internalization via clathrin mediated endocytosis, followed by transport to multivesicular bodies and recycling back to the cell surface.

CD80 (B7-1) and CD86 (B7-2)

Short et al. recently described that the knob of Ad3 binds to CD80 and CD86, and Ad5 vectors pseudotyped with Ad3 fiber knobs (Ad5/3), transduce CHO cells that express human CD80 or CD86 more efficiently than ordinary CHO cells [253]. CD80 and CD86 also appeared to serve as cellular receptors for Ad3 pseudotyped vectors transducing human dendritic cells. Furthermore, preincubating HeLa cells with antibodies against CD80 and/or CD86 inhibit transduction with up to 55% [253], which suggests the existence of a second receptor on these cells. In another recent study by Short et al. it was shown that all species B adenoviruses bind to CHO cells that express human CD80 or CD86 more efficiently than ordinary CHO cells [254].

Infection of CD80 and CD86 expressing CHO-cells was further demonstrated by analyzing the accumulation of the Ad hexon capsid protein by immunofluorescence after 36 hours post infection. Following addition of wildtype Ad3, Ad7, Ad11 and Ad35 the cells were examined and found to contain hexon protein. However, productive viral infection is not believed to occur in the non-human CHO-cells so the method in this case just shows accumulation of hexon proteins and can not be proven to be accumulation of newly synthesized functional viral particles [254]. From these studies it seems that species B adenoviruses can use more than one pathway to enter host cells, but more efforts are required in order to elucidate the roles of these molecules in adenovirus life cycle.

Additional Receptors

There have been reports of additional receptors for species C serotypes Ad2 and Ad5, but these are much less studied. Receptors that have been reported include MHC class I α2 domain [255], heparan sulfate glycosaminoglycans [256, 257] and vascular cell adhesion molecule-1 (VCAM-1) [258].
Internalization

Role of the penton base and integrins
After initial attachment of species C Ad2 to cells, the adenoviral penton base is introduced in the entry process [259-261]. This viral protein acts as a co-receptor and binds to cellular integrins. Clustering integrins initiates a multitude of signals, which facilitate virus internalization by clathrin-mediated endocytosis [80, 208, 262]. Most adenovirus penton bases bind integrins via the protruding, flexible RGD. Examples of integrins that are known to mediate adenovirus entry via the RGD-motif are αvβ3, αvβ5 [261], αvβ1 [263], α5β1 [264], αMβ2 and αLβ1 [265]. Although both αvβ3 and αvβ5 can mediate endocytosis, αvβ5 promotes better membrane permeability and subsequent release from endosomes [260, 266]. Specifically, the threonine-valine-aspartic acid (TVD) amino acid motif found in the cytoplasmic tail of the β5 integrin subunit regulates gene delivery and endosomal escape of Ad2 to the cytosol [260, 266].

Integrin and clathrin mediated internalization of Ad2 and Ad5 is a major entry route but there is evidence of at least a second endocytic process, macropinocytosis, that is induced simultaneously [267]. Macropinocytosis requires integrins, F-actin, protein kinase C (PKC), Na+/H+ exchanger, and small G-proteins of the Rho family, but not dynamin. This has been reviewed recently by Meier et al. [208].

Intracellular dismantling of virions
The integrin mediated internalization of adenoviruses is followed by release of fiber from the capsid [268, 269]. Viral escape from the acidified endosome have been associated primarily with the penton base, fiber, protease and recently with the internal capsid pVI, although the exact details are still uncertain [86, 120, 267, 270-276]. The internalization process appears to be rapid, 80% of cell bound Ad2 viruses are internalized into KB cells (human epidermoid carcinoma) within 20 minutes and in A549 cells the half life of the endosomal escape into the cytosol is five minutes [268]. The incoming Ad2 particles thus continue to undergo a stepwise dismantling, and as the early endosome containing the virus matures, the vesicular pH drops, due to the activity of proton pumps, and at ~pH5.5-pH6.0 the virus penetrates the endosomal membrane and is released into the cytosol, thus evading further degradation by lysosomal enzymes (Figure 7). [268, 275, 277].

Integrins are involved in subsequent internalization events
An tyrosine-based internalization and endocytic sorting signal, NXPY (X representing any amino acid) [278], is found in the cytoplasmic tails of β3 and β5 integrin subunits [279] and has been associated with localization of certain receptors into coated pits [280]. Ad2 penton attachment to the cells via integrins activate phosphatidylinositol-3-OH kinase (PI3K) which initiates an activation of Rac and CDC42 GTPases, which leads to actin polymerization and viral endocytosis into clathrin coated vesicles [281, 282]. It has also been suggested that protein kinase C (PKC) can be targeted by this activation [208]. PKC has been suggested to be required for membrane trafficking and endosomal escape of Ad2, but not required for endocytosis per se [267, 269]. The precise function of PKC is still unclear. The clathrin mediated uptake is also dependent on dynamin, a 100-kDa cytosolic GTPase, which enables the constriction and budding of the clathrin coated pits into coated vesicles [283]. Dynamin is also a signal transducing molecule, further activating downstream effectors [208].
Intracellular transport and nuclear docking

After the endosomal escape, partially dismantled capsids are transported rapidly, but intermittently, with bidirectional movement at μm/s velocities along the microtubule, towards the microtubule organizing center near the perinuclear envelope, and thus accumulate at the nuclear periphery within ~30-60 minutes of infection (Figure 7) [284, 285]. Upon arrival to the perinuclear membrane the capsids dock at the nuclear pore complex (NPC) where the subsequent dismantling occurs [286]. The viral protease L3/p23 degrades the internal capsid stabilizing pVI and finishes the disassembly [177]. It has also been suggested that the NPC itself has dismantling capabilities [286]. The majority of the capsid proteins remain at the perinuclear membrane after dismantling [177, 268, 272, 286].

Figure 7. Summary of intracellular trafficking of species B and species C Ads. Species C Ad (e.g. Ad5) is internalized via interaction with CAR and a secondary receptor, integrin, and then enters the cell via receptor-mediated endocytosis. When the endocytic compartment containing Ad5 fuses with a sorting endosome (pH 6.2), Ad5 breaks out the early endosome, escapes to the cytosol, and translocates to the nucleus along microtubules within 1 h. Species B Ads (e.g., Ad7) binds to cells via an unidentified receptor (R) and is internalized via receptor-mediated endocytosis. The endocytic compartment containing Ad7 fuses with sorting endosomes, but unlike Ad5, Ad7 does not disrupt the sorting-endosome membrane, remaining inside that organelle as it matures to become a late endosome (pH 5.5) and finally a lysosome (pH 5.0). Ad7 escapes from late endosomes and/or lysosomes and translocates to the nucleus. The internalization of the two species is equally rapid (t½ = 2 to 3 min), however, Ad5 reaches the nucleus rapidly (t½ = 40 min) while Ad7 trafficking progresses more slowly (t½ = 220 min). Adapted from [287], with permission from the authors.
The NPC represents a physical barrier that small molecules can pass through via passive diffusion while molecules >45 kDa must be actively transported and possess a specific targeting signal, the nuclear localization signal (NLS) [288]. Furthermore it seems that the NPC cannot import macromolecules bigger than ~40nm [289] [288] and thus physically restrict the import of the whole, intact adenovirus capsids. Proteins containing the nuclear localization signal (NLS) are imported to the nucleus and since the adenoviral terminal protein contains a NLS it is possible that it plays a role in threading the adenoviral DNA and DNA associated proteins, such as pVII, through the NPC [286]. The adenoviral terminal protein, covalently bound to each 5’ end of adenovirus DNA strand, also serves to initiate viral DNA replication and binds the adenoviral DNA to the nuclear matrix [286] [290]. Although it is known that the adenovirus genome is tightly associated with the adenoviral core proteins pV, pVII and μ their role in the nuclear entry is still somewhat unclear [288]. The nuclear import can be inhibited by the lack of intracellular calcium, possibly due to calcium dependent conformational changes plugging the NPC [286, 291]. About 40% of the bound Ad2 viruses succeed to release their DNA into the nucleus [268].

Internalization of non-species C adenoviruses
Studies of entry and intracellular trafficking of species B have been done mainly with Ad3 [292] and Ad7 [287, 292, 293] and to some extent using pseudotyped Ad-vectors expressing predominantly the species C capsid and species B fiber, or parts of the fiber, such as Ad5/Ad7f [294] and Ad5/Ad35fk [295]. Ad3 infection of malignant melanoma M21-L4 cells expressing v3 and v5 has been shown to be inhibited by a combination of antibodies directed at the v integrins, thus indicating that at least Ad3 of the species B adenoviruses depend on integrin co-receptors for internalization [259]. Another study confirmed this by showing direct association between Ad3 and soluble v5 integrins [296]. It was earlier reported that species B (Ad3 and Ad7) and species C (Ad2 and Ad5) used different internalization routes [292, 293]. Ad5 is released early from endosomes at ~pH6.0, and Ad7 is released late from endosomes or endolysosomes at ~pH5.5 [287]. The Ad7 fiber seems to be the pH-dependent modulator of endosomal escape, since a pseudotyped Ad5 capsid expressing Ad7 fiber showed a similar endocytic transportation pattern as Ad7 [287, 294]. In A549 cells Ad5 virions are rapidly translocated to the nucleus, while Ad7 virions remain widely distributed in the cytoplasm, and thus within 1h ~72% of Ad5 but only ~32% of Ad7 were localized to the nucleus [294]. Despite the seemingly dramatic difference in initial kintetics of nuclear targeting between Ad5 and Ad7, the genomic delivery of Ad7 8h p.i. appear to reach almost similar levels as that for Ad5 [287, 294].

The adenoviral uptake and intracellular transportation is a very complicated process involving many steps and signaling procedures. Many of the processes used by adenoviruses are in fact naturally occurring cellular processes that are exploited by viruses to infect the cell. However, as our knowledge about the adenoviral infectious processes grows so does our understanding of the cellular mechanisms, and vice versa.

Adenovirus genome
The human adenoviruses are medium sized DNA viruses with a linear two-stranded genome, which is 34-36kbp in size and large enough to encode up to ~40 proteins [28]. The genome organization of adenoviruses is widely conserved between the adenoviral genera (Figure8) [28]. It has been proposed that the ends of the genome (the E1- and E4-regions) are
transcribed first, leading to an opening and subsequent transcription, of the central core of the genome structure [297, 298]. The genome is characterized by several RNA polymerase II dependent transcription units, the five early (E1A, E1B, E2, E3, E4), the delayed early units (IX, IVa2, and E2 late) and the major late transcriptional unit (MLTU) [67]. The large MLTU transcript is further processed by polyadenylation and alternative splicing, creating 5 (or 6) different mRNAs (L1-L5/L6) [299, 300]. The genome also encodes one or two small virus-associated RNAs (VA RNA I and II) transcribed by RNA polymerase III [67, 301].

**Mastadenovirus: SAdV-25 (HAdV-E)**

**Atadenovirus: OAdV-7 (OAdV-D)**

**Aviadenvirus: FAdV-1 (FAdV-A)**

**Siadenovirus: TAdV-3 (TAdV-A)**

**Figure 8.** Overview of the gene layout showing similarities in representatives of four adenovirus genera. Figure adapted from: [28], with permission from the authors.

The different transcriptional units often express proteins with related functions (Figure 9) [302]. In general, E1A encodes two proteins that are responsible for transcriptional activation and inducing the cell into S-phase. E1B encodes two proteins that inhibit apoptosis. E2 encodes three proteins involved directly in viral DNA replication: the DNA polymerase, TP and DBP. The E3 encoded proteins are involved in evasion of immune response, and the E4 encoded proteins are involved in various functions such as modulating DNA replication, mRNA transcription, transport, translation and apoptosis, [67, 302, 303]. The late genes encode structural components that build up the capsid [67, 302, 303].

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Figure 9. Schematic view describing the transcription of the human adenovirus genome. The early transcripts are outlined in green, the late in blue. Arrows indicate the direction of transcription. The gene locations of the VA RNAs are denoted in brown. MLP is the Major late promoter. Adapted from [79], with permission from the publisher, Society for General Microbiology.

DNA replication

Ad2 and Ad5 DNA replication in HeLa-cells begins 5-8 h post infection and lasts until the host cell dies [67]. The inverted terminal repeats (ITRs) at both ends of the viral DNA act as origins of replication (Figure 10). DNA replication is further divided into two stages. Type I replication begins at either end of the DNA duplex and forms a new duplex with the template parental and a newly synthesized daughter strand, thus displacing the other, now single stranded, parental DNA strand. Furthermore, several daughter strands can be queued up and synthesized simultaneously. In type II replication the single strand circularizes by annealing the two self-complementary ITRs at each end resulting in a “panhandle” which has the same terminal structure as the original DNA duplex. This allows the single stranded “circularized panhandle” to be recognized by the same replication initiation machinery that facilitated the type I replication and thus leads to synthesis of another DNA duplex [304] [67].

The adenoviral preterminal protein (pTP) and DNA polymerase (AdPol) bind to ITR. The pTP, precursor of the adenoviral terminal protein, TP. AdPol functions as both 5’ to 3’ polymerase and 3’ to 5’ exonuclease, thus having as a proofreading function during DNA replication [305, 306]. The ITR contain regions that enhance replication by allowing cellular transcription factors, such as nuclear factor I (NFI) [307-309] and Oct-1 (also known as NFIII) [309, 310] to bind. NFI binding is furthermore promoted by DBP [311, 312]. DNA binding of NFI and Oct-1 allows recruitment, proper positioning and stabilization of AdPol-pTP complex at the origin of replication [67]. The binding of the AdPol-pTP complex to the DNA is facilitated by NFI interaction with AdPol [308, 313] and Oct-1 binding to pTP [314, 315].
The priming reaction starts with the AdPol catalyzed binding of pTP to the 5’ deoxycytidine monophosphate (dCMP) at the end of the DNA chain [316-318]. Attachment of pTP appears to open up the end of the DNA duplex, allowing the end of the template strand to enter the active site of AdPol [67, 319]. The 3’OH-group of pTP-dCMP then serves as a primer for continuous synthesis of viral DNA in the 5’ to 3’ direction by AdPol [67]. During this process pTP is released from the AdPol-pTP complex shortly after initiation [306]. The DNA replication is also promoted by DBP, which unwinds and coats the displaced single, non-template strand of DNA [175, 311, 312]. NFII, a type I cellular topoisomerase, is also required for efficient replication [320].

**Figure 10.** DNA replication model of human adenoviruses.

**Procession and transport of structural proteins**

Transport of mRNA:s that encode the structural proteins to the cytoplasm initiates the translation process. Monomeric hexon assemble to trimeric hexon capsomers shortly after synthesis [321]. Hexon trimerization is facilitated by L4 encoded 100kDa protein, [322, 323]. Hexon import into nucleus is facilitated by a precursor of pV1 [122]. Pentons, built up by penton base and fibers assemble slower in the cytoplasm, but are eventually transported into nucleus [321].

**Virion assembly**

Replication of viral DNA and massive production of capsid components preceeds the viral assembly step, which occurs in the nucleus. Several details of this is process are still not known. In general, adenovirus DNA is packaged in a polar fashion with the left end of the genome first [324-327]. This packaging is facilitated by a domain situated somewhere within ~600bp from the terminus [328]. In Ad5, packaging is dependent on seven “packaging sequence repeats”, also known as A-repeats due to their AT-rich nature [328]. The packaging
repeats for Ad5 are situated within regions where transcriptional enhancers for viral genes act [328].

Proteins that have been suggested to have a functional role in packaging and assembly are pIVa2, L1 coded 53/55KDa and a 22kDa protein encoded by L4, which all bind to the packaging sequence in the viral DNA [329-331]. They further interact with nonstructural and structural adenoviral proteins and are thought to contribute to the assembly of a procapsid, and subsequently promote DNA packaging into this[328, 329]. Insertion of DNA has been suggested to be mediated by an, yet to be identified, ATP-driven “packaging motor”, in a similar way as in the packaging procedure in the distantly related bacterial phage PRD1 [75, 328, 332]. After encapsidation the viral particle matures as the viral proteases cleave many structural precursor proteins, resulting in the mature, infectious form of adenovirus [87, 328, 333].

**Viral escape**

Late in the infectious cycle the L3 coded 23kDa viral protease also cleaves and rearranges cellular cytokeratins, blocking their ability to form filaments and thus weakening the cytoskeletal mechanical stability of the cell, which precedes cellular lysis [334]. Another process that aid the adenoviral escape from the host cell is the E3 encoded 11.6 kDa protein, also known as adenovirus death protein (ADP), which after sufficient accumulation kill the host cell in the late stages of infection [335, 336]. As mentioned earlier, soluble fibers may be released from infected cells and, in the case of CAR interacting adenoviruses, these open up epithelial tight junctions which allows free virions to escape from the site of infection and to invade non-infected cells [220].
ADENOVIRUSES AS GENE THERAPY VECTORS

Gene therapy can briefly be described as delivery of a genes into cells/tissue in order to achieve a therapeutic effect. Gene therapy is a vague term comprising many possible methods and therapeutic fields. These include, among many others, several genetic diseases and acquired genetic diseases, such as cancer [337]. Currently, more than 1300 clinical trials with gene therapy have been initiated worldwide. Viral vectors are used in ~70% of the trials and adenoviruses specifically in ~25% of all trials, making it the most commonly used viral vector today. Retroviruses are the second (23%) most common gene therapy vector. The clinical trials are mainly focused on cancer diseases with ~66 % of the trials, far beyond is the numbers for the second most common cardiovascular disease with ~9% of the clinical trials [338]. Non-viral methods of gene transfer include naked DNA injection, lipofection, gene gun and in vivo electroporation [337].

Adenovirus vectors, commonly based on species C Ad2 and Ad5, do not integrate their genome into the host genome, thus the transgene will remain episomal. This does not only increases safety but also implies a relatively transient expression of the delivered genes and that the transgenes will not be transferred to progeny cells[337]. However, a major drawback in using viruses as gene therapy vectors is that the human immune system reacts and efficiently neutralizes viruses. To overcome this, larger adenoviral doses have been used, with limited success. To minimize uncontrolled spread of the vector they have been made replication incompetent through removal of either the replication initiating genes or the viral genes altogether. However, conditionally replicating vectors are sometimes used for tumor targeting.

Viruses are natural, evolutionarily perfected gene delivery vehicles, which seem ideally suited as gene therapy vectors. Adenoviruses are easy to propagate in high titers, can infect many cell types regardless of their growth state and can accommodate large (close to ~37kb) DNA inserts [339-341].

REPLICATION INCOMPETENT VECTORS

Three generation of replication incompetent adenoviral vectors have been developed [337, 339]. The first generation has a deletion in the E1 region, allowing for an <7kb insert. The deletion of E1 creates space for foreign DNA and disrupts the control and activation of DNA synthesis and late viral protein expression, thus restricting the viral spread to adjacent cells. Some first generation vectors combine the deletion of E1 and E3 to create more space. Traditionally, the first generation E1 vectors are generated in E1 trans-complementary packaging cell lines. However, E1-deleted vectors can be complemented by co-infection of other adenoviruses or even papillomavirus or cytomegalovirus and there thus exists a risk for reversion to replication-competent adenoviruses (RCA). The first generation vectors are also to a large extent immunogenic due to the impaired, but not abolished, production of viral gene products which leads to an immunologic response [337, 339].

The second generation vectors have additional viral regions deleted, such as E2, E3 and/or E4. This leads to, besides additional space for genomic inserts, reduced expression of viral proteins and subsequent reduction in cytopathic effects and immune response, however long term expression of the transgene is still not achieved. However, the second generation vectors are less prone to generate RCA,[337] [339]. The E1 and E4 deleted vector have been reported

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induce less apoptosis and appear more long lasting in vivo [342, 343]. However, the contribution of E4 deletions is controversial. One study showed no significant difference between E1 and E1/E4 deleted vectors considering immune response and in vivo persistence [344]. Other reports indicate that deletion of E4 leads to the loss of transgene expression [345, 346].

The third generation vectors are also known as “high capacity”, “gutless” or “helper dependent” vectors. These vectors have had all viral genes deleted with only the viral ITRs and a packaging signal remaining. They can accommodate large transgenes close to the maximum physical capacity of the adenoviral capsid and are characterized by highly reduced immunogenicity. Production of gutless vectors is consequently dependent on a system that expresses all viral proteins that are required for vector production in trans. Several approaches have been used to achieve this, with varying success [339]. The high capacity vectors are superior to the earlier generations, showing much improved safety, toxicity and long term transgene expression [347]. Indeed, in two studies using rodents treated with helper-dependent adenovirus vectors lifelong (i.e. 2-3 year) corrections of the disease phenotypes have been observed after single vector administrations [347-349].

The major drawbacks with adenoviral vectors so far have been acute toxicity/inflammatory reactions, especially after delivery of very high vector doses, which caused the death of 18 year old Jerry Gelsinger who died due to fatal acute respiratory distress syndrome (ARDS) and multiorgan failure [350-353].

CONDITIONALLY REPLICATING ADENOVIRUSES (CRAdS)

The early generations of replication deficient vectors have been used for tumor therapy but the results have not been very successful. In general, vectors transduce cancer cells poorly but the therapeutic effects have been modest [354, 355]. For obvious reasons, replication incompetent vectors do not spread far away from the actual injection site if administered locally [356]. In advanced disease with large tumors, tumor penetration is of the essence [355]. One approach to achieve this is to use replication competent oncolytic agents, such as CRAdS. The majority of the approaches to create CRAdS have aimed at modifications of the E1 genes for sustained gene expression. Two main strategies have been used; the first being the genetic complementation-type (type-1) CRAdS. These CRAdS contain small modifications in the E1A or E1B regions, which are complemented in tumor cell lines but not in healthy cells [355, 357, 358]. One approach to do this has been to incorporate mutations in the viral E1B55k protein. E1B55k normally binds and inactivates the tumor suppressor protein p53, thus allowing viral replication in the infected host cell causing it to lyse, thus facilitating further viral spread. However, E1B55k mutated viruses lack this p53 regulating activity, so upon infection p53 can block viral infection, initiate cell cycle arrest and the virus is unable to spread. In cancer cells, which lack p53, the virus is able to replicate and undergo lysis facilitating further viral spread to cancer cells while healthy cells should be safe [355]. The type-2 CRAdS are of the transcomplementation-type where viral replication is controlled by a tumor/tissue specific promoter [359] [360] [361] [355]. The main concern for the development of type-2 CRAdS is the poor availability of promoter fragments with specific activity. Today the interrelationships between structure and function of promoter regions are not understood well enough so the construction of tissue specific promoters is based on empirical data [355].
Furthermore, the approach to combine adenoviral gene therapy treatment with traditional anti-cancer treatments, such as chemotherapy or radiation therapy has shown some promising results [362-365].

VECTOR RETARGETING

The commonly used adenovirus vectors have several limitations built in, such as the high seroprevalence against Ad2 and Ad5 in the human population. Another problem is the poor transduction capacity in vivo. Some tissues that express or expose little or no CAR are refractory to transduction by these serotypes. Thus, the efficiency of adenoviral vector delivery can potentially be improved by more efficient targeting. A common way to do this is to modify the capsid to recognize a new target. This can be achieved in several ways, one of them is through usage of bispecific antibody conjugates which bind to both to the virus and to target cells, thus eliminating the tropism of wildtype virus and retargeting the vector to specific, suitable cells/tissues [366-369]. Another method is through construction of pseudotyped vectors which contain fibers or parts of fibers from other serotypes [188, 192]. Fiber exchange between serotypes is a naturally occurring phenomena and intermediate strains are frequently found [49, 370], these serotype modifications have recently been adapted to fit the man-made vector design concept. Furthermore, there have been several recent reports of adenoviral vectors based on Ad5 that have been fiber modified to target several new receptors, amongst them chimeras with parts originating from reoviruses [93, 94]. Another approach to retarget vectors is through transcriptional retargeting, which utilizes tissue specific promoters that only allow transgene expression in the targeted tissue, such as cancer cells. This concept has already briefly been discussed when addressing the type 2 CRAds and has been recently reviewed by Sadeghi et al. [371].
THE COMPLEMENT SYSTEM

The immune system fights pathogen in general by two means: initially by the non-specific, innate immune response followed by the adaptive antibody mediated immune response which is acquired some time after exposure to the pathogen (or vaccine) [372]. The complement system is an important component of the innate immune response. It initiates a complex cascade of proteolytic events that, amongst other things, produce chemotactic factors that direct phagocytic and inflammatory cells to the scene, increases vascular permeability, opsonize the infecting agent and may also directly eliminate the target by lysis [303] [373] [374] [372]. The complement system can be activated in three ways (Figure 11).

THE CLASSICAL PATHWAY

The classical pathway is mediated by binding of the complement protein C1q to antibody-antigen complexes, or sometimes directly to the surface of some pathogens and is thus mainly dependent on the adaptive immune response [303, 372, 374]. C1q has six globular heads and when it is associated with C1r and C1s it forms the C1 complex. Binding of more than one C1q to the antibody-antigen complex or to the surface of the pathogen causes a conformational change in the C1 complex that activates C1r, which in turn cleaves C1s, thus generating an active serine protease. This protease cleaves C2 (to C2a and C2b) and C4 (to C4b and C4a) and initiates further activation of the complement cascade via the common pathway junction point, involving degradation of C3 [303, 372, 374].

THE LECTIN PATHWAY

The second complement activating pathway is the mannan-binding lectin (MBL) pathway, more commonly known as the lectin pathway. MBL shows structural similarities to C1q and this pathway is conseqently similar to the classical pathway. MBL is associated with two serine proteases MASP 1 and-2, which are in turn related to C1s and C1r. MBL binds to several monosacharides, particularly mannose and N-acetyl glucosamine, which are found on some pathogen surfaces. The active MBL complex also acts by cleaving C2 and C4 [372, 374].

THE ALTERNATIVE PATHWAY

The third complement activation pathway is the alternative pathway. It is in fact a default process, mediated by the spontaneous deposition of complement factor C3b on the surface of host cells and present pathogens. After surface deposition C3b will, unless removed by specific mechanisms, mediate complement activation and subsequent target elimination. C3b is produced by spontaneous hydrolysis of the C3, which is abundantly present in plasma. C3b can also act as an opsinin that promotes phagocytic uptake by leukocytes [372]. The cleavage of C3b results in a cell surface bound C3bi, which cannot support further complement activations while the other cleavage product C3f remains soluble. The cleavage of C4b present in the classical pathway results in soluble, extracellular C4c and cellbound C4d, which are unable to support further complement activation [375].
Figure 11. Schematic view of the complement system. Activation of the complement cascade via the classical (A), lectin (B) or alternative (C) pathway results in the initiation of the terminal complement pathway (D), leading to the formation of membrane attack complexes, MACs. Also shown schematically is the physiological regulation of the complement cascade. Host proteins that interfere with different steps of the complement cascade are indicated with blue text. Figure adapted from [372], with permission from authors and publisher, Society for General Microbiology.
Activation of any of the three pathways will eventually lead to the cleavage of C3 to C3b and subsequently cleavage of C5, which initiates the terminal pathway and subsequent formation of the membrane attack complex (MAC) that can form pores in the lipid bilayers, resulting in osmotic disruption followed by lysis. Since unregulated complement activation is potentially a self-hazardous system it needs to be tightly regulated by the host. Normal mammalian cells are protected by several complement regulating systems/proteins [372].

THE ROLE OF CD46
One of components is CD46, the cellular receptor for the majority of species B adenoviruses. CD46 is considered to be more effective in controlling the alternative complement pathway amplification loop [376-379]. CD46 serve as a cofactor for the factor-I-mediated cleavage of cellbound C3b and C4b present on the very same cell as CD46 is present on. Factor-I is a plasma protein, which is constitutively active and critical for the control of the fluid phase and the cellular complement reactions. Factor-I requires a substrate binding co-factor protein, such as CD46, that promotes binding of factor-I to the substrate [379]. Thus, CD46 is rapidly downregulated from the cell surfaces after infection [238, 240, 248, 380, 381], thus allowing the complement system to form MAC and kill the infected cells.
RESULTS AND DISCUSSION

Adenoviruses cause 10% of all respiratory infections that require hospitalization of children younger than four years [38]. Specifically, severe and possibly fatal pneumonia is primarily associated with Ad and Ad7 from species B1. Ad3 and Ad7 are also frequently associated with pharyngoconjunctival fever, manifesting as the name implies as conjunctivitis, fever and respiratory disease. The other members of species B1 (Ad16, Ad21, and Ad50) are also, however less frequently, associated with respiratory and/or ocular infections. Ad3 and Ad7 are clearly the most seroprevalent of the species B adenoviruses [43]. Respiratory and ocular infections may however also be caused by the species B2 adenoviruses they, particularly Ad11, Ad34 and Ad35, are however most commonly associated with renal infections, which can sometimes become fatal in immunocompromised individuals [38, 39]. However, in healthy individual adenoviral infections tend to be self-limiting and cause type specific immunity following recovery [38]. When studying species B adenoviruses one should strive to use cells originating from, and representing, the natural tropism areas of these viruses.

PAPER I

ADENOVIRUS TYPE 11 USES CD46 AS A CELLULAR RECEPTOR


The cellular receptors used by species B adenoviruses have for a long time remained unidentified. In this paper we identified CD46 as a cellular receptor for Ad11. Ad11 and Ad7 clearly bound to CHO-CD46 cells more efficiently (i.e. up to 10 times better) than CHO-cells expressing CAR, DAF (decay accelerating factor)/CD55 or ordinary CHO cells (figure 1, paper I). Furthermore, ordinary non-permissive CHO-cells were rendered permissive for Ad11, but not for Ad7, by the expression of human CD46 (figure 4, paper I). Moreover, polyclonal antibodies against CD46 abolished binding of Ad11 and infection of CHO-CD46 cells (figure 3, paper I). In addition, soluble Ad11 fiber knob blocked binding of Ad11 to A549 cells and CHO-CD46 cells, whereas knobs of Ad7 (and Ad5) did not (figure 7, paper I). This clearly indicated that Ad11 and Ad7 use different cellular receptors for binding.

The obtained result that Ad7 did not use CD46 to infect cells was somewhat surprising since in the same study, Ad7 was found to bind CD46. We hypothesize that Ad7 is able to bind weakly to CHO-CD46 cells, but not efficient enough for subsequent cellular entry. Other studies have since then confirmed that Ad7 does not use CD46 to infect host cells, nor does the fiber knob bind to CHO-CD46 expressing cells [235, 382].

Binding of both Ad7 and Ad11 to CHO-CD46 cells was reduced by anti-CD46 antibodies, however binding of Ad7 and Ad11 to A549 cells was only slightly affected (figure 3, paper I). This suggested that on A549 cells both Ad7 and Ad11 may use additional receptors besides CD46. The incomplete inhibitory effect of anti-CD46 antibodies during Ad11 binding to and infection of A549 cells might be explained by the use of non-sufficient amounts of antibody or usage of antibodies that bind to domains of CD46 to which Ad11 do not interact. In subsequent work (paper II) we have been able to efficiently inhibit Ad11 infection in A549 cells using rabbit anti-CD46 serum [235].
It was also noted that Ad7 binding to CHO-CD46 cells was efficiently improved by divalent cations, Mg\(^{2+}\), Ca\(^{2+}\) and Mn\(^{2+}\), while Ad11 binding was only slightly improved by Mn\(^{2+}\) only (figure 5, paper I). These findings are supported by an earlier study where the binding of Ad3 and Ad7 to J82 and A549 cells was dependent on divalent cations, primarily Ca\(^{2+}\), while Ad11 and Ad35 binding was less dependent on divalent cations [383]. Thus, it appears that Ad7 requires divalent cations for binding while Ad11 can bind to CD46 in the absence of cations. In accordance to this observation it has recently been reported that Ad7 depends on the presence of extracellular Ca\(^{2+}\) for infection of A549 cells [384]. Since our later studies have shown that all species B adenoviruses, except Ad3 and Ad7, use CD46 as a functional cellular receptor on A549 cells [235] it may be that species B adenovirus interaction with CD46 in general, is independent of divalent cations.

The binding of Ad7 to CHO cells in the presence of Ca\(^{2+}\), Mn\(^{2+}\), and Mg\(^{2+}\) was only slightly lower as compared to the levels on CHO-CD46 cells (figure 5, paper I). One might speculate that there exists a hamster cell-surface protein present on CHO-cells that Ad7 can bind to which is structurally somehow similar to the human Ad7 receptor. This putative hamster receptor homolog/analog should in that case be upregulated/congregate upon the expression of human CD46 on the CHO-cell surface allowing Ad7 binding (as seen in figure 1 and 5, paper I). If this hypothetical hamster cell-surface receptor is located in close proximity to CD46 it might be structurally camouflaged by sterical hindrance by the anti-CD46 antibodies, thus also inhibiting the effect of the divalent cations on Ad7 binding. On A549 cells however, the structural arrangement of CD46 and the Ad7 receptor appears to be different since Ad7 binding was not hampered by the different antibodies tested.

Several proteins on the cell surface have been known to be associated with CD46, amongst them \(\alpha_1\beta_1\), \(\alpha_3\beta_1\), \(\alpha_5\beta_1\), and \(\alpha_6\beta_1\)-integrins [385]. Furthermore, integrin ligand interaction is known to be dependent on divalent cations [386]. It has been reported that transgenic expression of cell-surface human \(\alpha\)-integrin subunits in CHO-cells leads to the association with hamster \(\beta\)-integrin subunits on the cell surface forming a biologically active cellsurface \(\alpha\beta\)-integrin, such as \(\alpha_3\beta_1\) [387-389]. It has also been reported that Ad3 fiber binds to a 130kDa, and the virion itself binds a 100kDa membrane protein present on HeLa cells in a divalent cation dependant manner [182]. Hamster \(\beta_1\)-integrin subunit has been reported to be a 130 kDa protein [389] and several \(\beta_1\)-integrins have been reported to serve as secondary receptors for Ad5 [390] [391]. In addition, Ad3 have been reported to infect M21-L4 cells expressing \(\alpha\beta_3\) and \(\alpha\beta_5\) about three-fold better than M21-L12 cells that do not express these integrins [259]. This interaction was moreover inhibited by soluble RGD peptides by ~90% and incubation of cells with a function blocking antibody against \(\alpha\beta_3\) or \(\alpha\beta_5\) inhibited the infection by ~50%. In another study soluble \(\alpha\beta_5\) integrin exhibited significant cation dependant binding to Ad3 [296]. Consequently, we hypothesized that integrins could be receptors for specific species B adenoviruses. However, transfection of CHO cells with human \(\alpha_2\), \(\alpha_2\beta_1\), \(\alpha_3\), \(\alpha_5\) or \(\alpha\) did not increase the binding of Ad7 or Ad11 [186]. In addition it would have been interesting to test Ad3 as well.

Removal of sialic acid from the surface of A549 cells using neuraminidase resulted in increased binding of Ad3 and Ad7, which might be explained by increased accessibility to the receptor [383].

Trypsination of CHO-CD46 cells prior to binding inhibited binding of Ad7 while Ad11 binding is enhanced (figure 6, paper I) [186]. This result was in agreement with an earlier study [383] where the binding of Ad3 and Ad7 but not Ad11 and Ad35 were sensitive to
trypsinization of the host cells (A549 and J82) prior to binding. Binding was further reduced for all tested adenoviruses when the cells were pretreated with broad specificity proteases such as subtilisin and proteinase K and it was thus concluded that the adenoviruses bound to a protein [383]. It was also reported that the Ad11 and Ad35 blocked binding of Ad3 and Ad7 to host cells (A549). However the reverse inhibition was not possible, i.e. Ad3 and Ad7 could not inhibit binding of the Ad11 and Ad35. Thus, based on these experiments it was suggested that species B:1 and B:2 adenoviruses both bound to a cellular receptor designated sBAR (species B adenovirus receptor), and that species B:2 adenoviruses bound to an additional receptor designated sB2AR (species B:2 adenovirus receptor) [383]. Taken together, CD46 was the first species B receptor to be identified and was at this time thought to be identical with sB2AR.

**PAPER II**

**CD46 IS A CELLULAR RECEPTOR FOR ALL SPECIES B ADENOVIRUSES EXCEPT TYPES 3 AND 7.**


Following the discovery that Ad11 uses CD46 as a cellular receptor we set out to investigate if/which of the remaining species B adenoviruses that use CD46 as a functional cellular receptor.

We found that the poorly permissive CHO-cells were rendered more permissive to infection by all species B adenoviruses, except types 3 and 7, when transduced with CD46 cDNA (BC1 isoform; **figure 1, paper II**). Accordingly, we also found that pre-incubation of CHO-CD46 cells with rabbit antiserum against human CD46 prior to addition of virions efficiently inhibited or even blocked infection of all species B adenoviruses, except types 3 and 7 on A549 cells (**figure 2, paper II**). Based on these results we concluded that all species B adenoviruses, except Ad3 and Ad7 used CD46 as a functional cellular receptor on A549 cells, a cell line that represents the respiratory tropism of species B adenoviruses.

We also investigated selected serotypes from each of the remaining species: Ad31 (species A), Ad5 (species C), Ad37 (species D), Ad4 (species E), Ad41 (species F) using the same setup. Under the conditions used, the expression of human-CD46 did not render CHO cells more permissive to infection by any of the selected non-species B adenoviruses. When infecting A549 cells in the presence of antisera against CD46 none of the selected non-species B adenoviruses showed reduced infectivity. However, much to our surprise Ad31 (species A) infected A549 cells more efficiently in the presence of CD46 antiserum. The reason behind the observation that Ad31 infection in A549 cells is more efficient in the presence of CD46 antiserum is unknown at the moment.

Taken together none of the serotypes belonging to species A (Ad31), C (Ad5), D (Ad37), E (Ad4) or F (Ad41) used CD46 for infection of target cells. This is of interest since it has been suggested that Ad37 uses CD46 as a cellular receptor [237]. However, several reports from different groups state that Ad37 does indeed use sialic acid containing oligosaccharides as cellular receptors [100, 103, 105].

It is noteworthy that Ad7 have been shown to be able to bind to CHO-CD46 cells, more efficiently than to CD46-negative CHO-cells [186, 247], and despite this however not being
able to infect the CHO-CD46 cells more efficiently [186, 235]. Both Sirena et al. and we in this paper used the CHO-CD46 BC1 isoform (BHK-CD46-c154 and CHO-CD46-BC1 cells respectively) so the resulting differences should not be due to different splice variants of CD46 used. However, the data presented by Gaggar et al. showing that Ad3 does hardly bind CHO-CD46 better than ordinary CHO cells is consistent with our results that Ad3 does not use CD46 as a cellular receptor on CHO-CD46 or A549 cells [234]. We believe that even if Ad3 and Ad7 might interact with CD46, and perhaps use CD46 as a co-receptor, these viruses are likely to use other cell surface components as major cellular receptors in our model system.

It had previously been suggested that all species B adenoviruses use CD46 as cellular receptor [234]. Gaggar et al. reported that several species B:2 adenoviruses (Ad11, Ad14 and Ad34) attached to CHO-CD46 cells better than to CHO cells not expressing CD46, as did Ad16, Ad21 and Ad50 of species B:1. This was done in spite of the facts that a) one serotype (Ad3) did not bind CHO-CD46 cells more efficiently than the CHO cells not expressing CD46, and b) two species B serotypes (Ad7 and Ad34) were not tested at all. In this work we set out to clarify if all species B adenoviruses used CD46 for binding and subsequent infection. Our results showed that all species B adenoviruses, except Ad3 and Ad7 used CD46 as a functional cellular receptor on A549 cells [234].

We also wanted to compare the amino acid sequences of species B fiber knobs to see if we could see any patterns between the different serotypes that were evident of their different receptor usage. However, since the Ad50 fiber knob sequence was not previously published and available we set out to sequence it. From this work we found it evident that the fiber knob of Ad50 is very similar to that of Ad21 (figure 3 and table 1, paper II). Upon alignment of the fiber knob amino acid sequences (figure 4, paper II) we tried to identify potential areas of interest to the receptor interaction. The knobs of Ad3 and Ad7, that do not use CD46 in our hands, seem to be sequence-wise not very closely related (figure 3, paper II). Furthermore, the phylogenetic tree built on fiber knob amino acid sequences did not reveal obvious similarities between the species B:1 and B:2 adenoviruses. This appears to underline the importance of small areas in the fiber as being the critical determinants for the function and receptor binding. This is more clearly seen in (figure 4, paper II) where we can see areas and amino acids that have been identified as critical to receptor interaction for different adenoviruses. Many of these amino acids of importance for receptor interaction appear to be located in loop structures extending from the knobs. Species B adenovirus knobs (but not adenoviruses from other species) contain a three amino acid insert in the otherwise conserved AB-loop which has previously been suggested to contain a CAR-binding motif in knobs of non-species B serotypes [99, 226]. We also found that Ad3 and Ad7 differed from the rest of the species B adenoviruses by having hydrophobic amino acid residues at positions 240 and 296 (numbering according to figure 4, paper II) while the other CD46-using adenoviruses contained charged or hydrophilic amino acids at position 240 and a negatively charged amino acids at position 296. These two positions were probably exposed and in close proximity with previously suggested receptor-interacting regions so we hypothesized that these might also be of importance and perhaps explain why Ad3 and Ad7, in our hands, did not use CD46 for infection.
CD46 BUT NOT CD80 OR CD86 SERVES AS A CELLULAR RECEPTOR FOR SELECTED SPECIES B ADENOVIRUSES ON HUMAN EPITHELIAL CELLS.

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During the last few years several candidate receptors have been identified for species B adenoviruses. Segerman et al. [383] suggested that species B adenoviruses use one common receptor called species B Adenovirus Receptor (sBAR), while the species B2 adenoviruses used a species B2 Adenovirus Receptor (sB2AR). Shortly after this CD46 was identified as a receptor for Ad11 [186]. This was the first species B receptor to be identified (paper I) and was at that time thought to be identical with sB2AR. However, later studies showed that CD46 could be used by both B1 and B2 adenoviruses for binding [234] and infection [235], thus abolishing the theory that sB2AR was the sole B:2 adenovirus receptor. We found (paper II) that all species B adenoviruses, except Ad3 and Ad7 used CD46 as a functional cellular receptor on A549 cells, a cell line chosen to represent the respiratory tropism of species B Ads.

However the immunoregulatory proteins CD80 and CD86 and a new, yet to be identified, receptor “X” have also been suggested to serve as cellular receptors for species B adenoviruses [254, 392]. In those reports, the relative roles of CD80 and CD86 were not compared with that of CD46 and the cells used in these studies did not represent the tissue tropism of species B adenoviruses. With this in mind, we thus wanted to investigate the impact of the known candidate receptors using four species B adenoviruses; the non-CD46 using Ad3 and Ad7 (both from B:1) and Ad11 and Ad35 (both from B:2) using two cell lines that represent the conjunctival (normal human conjunctival; NHC) and renal (J-82) tropism of species B adenoviruses.

We found that binding (figure 1, paper III) and infection (figure 3 and 5, paper III) of Ad3 and Ad7 to J82 cells was not inhibited by neither rabbit sera against CD46 nor soluble CD46 protein. However, Ad11 and Ad35 binding and infection to J82 cells was efficiently hindered by both anti-CD46 antiserum and soluble CD46 protein. Similar observations regarding binding and infection were seen for all tested adenoviruses on NHC cells (figure 2, paper III) and (figure 4 and 5, paper III). However, soluble CD80 or CD86 proteins did not inhibit neither binding nor infection of any of the tested serotypes (figure 1-5, paper III).

In a recent report using selected human stem- and tumor cell lines Ad16, Ad21, Ad35, and Ad50 were suggested to use CD46 almost exclusively, whereas Ad11 were suggested to use both CD46 and receptor “X” and the rest of the species B adenoviruses would use receptor “X” exclusively [392]. This is in agreement with our earlier results [235] (figure 2, paper II) where Ad11 infection was only partially blocked by rabbit anti-CD46 sera [186] (figure 3, paper I). In this manuscript we found that soluble CD46 and anti-CD46 serum almost completely blocked infection of both Ad11 and Ad35 in J82 cells. Ad11 and Ad35 infection in NHC cells was strongly inhibited, but not completely blocked, by soluble CD46 or anti-CD46 serum. These results suggested that on J82 cells CD46 is probably the sole cellular receptor for both Ad11 and Ad35 while on NHC cells, Ad11 and also Ad35 might use both CD46 and another receptor, such as receptor “X”. We found that CD46 is expressed in higher amounts on NHC cells than on J82 cells (figure 6, paper III) and we thus thought that CD46 would be an important receptor for Ad11 and Ad35 also on this cell line. Surprisingly we saw
less effect on Ad11 and Ad35 infection using soluble CD46 or anti-CD46 sera. The reason why we observed a less obvious inhibition of Ad11 and Ad35 on J82 cells than on NHC cells by the anti-CD46 serum and soluble CD46 might thus be explained by a) that we did not use sufficient amounts of anti-CD46 serum/soluble CD46 protein or b) more abundant expression of the other receptor “X” on J-82 cells than on NHC cells.

It has also been suggested that Ad3 uses CD46 as a cellular receptor [236]. The data provided by Sirena et al. indicate that the Ad3 knob interacted with CD46 on erythroleukemia K562 cells and on genital epithelial HeLa cells. Furthermore, a mixture of antibodies against CD46 only inhibited the Ad3 binding by only ~50% on K562 cells. None of these cells do however, to the best of our knowledge, represent the tropism of Ad3 in humans.

It has also been reported first that Ad3 [253], and in an recent report [254] that all species B adenoviruses, use CD80 and CD86, costimulatory molecules present on mature dendritic cells and B lymphocytes, as cellular attachment receptors. As far as we know these cells do not represent the major targets for Ad3, or the other species B adenoviruses as Short et al. suggest [253] [254]. However, the species B adenoviruses might have evolved to be able to use CD80/CD86 as a mean to infect antigen-presenting cells and thus modulate the immune responses. It may be that the distribution of CD80/CD86 is more widely spread than previously thought, even in tissues representing the major tropism areas of the species B adenoviruses, but that remains to be seen. However, inhibition of HeLa cell infection using an Ad5 vector expressing Ad3 fiber was only inhibited by ~55% by the simultaneous use of anti-CD80 and anti-CD86 antibodies clearly indicating that there exists, at least, another entry pathway [253].

Since soluble CD80 and CD86 did not inhibit infection mediated through CD46 or receptor “X” as seen in our experiments (figure 1-4, paper III), we concluded that the binding sites for CD80 and CD86, if present on the fiber knobs of the tested viruses, are not in close proximity to that of CD46.

Based on these findings we suggested that on these cell lines (J82 and NHC), chosen to represent the renal- and ocular tropism of species B adenoviruses a) CD46 is the major cellular receptor for Ad11 and Ad35, but not for Ad3 and Ad7 and b) CD80 and CD86 are of insignificant importance as cellular receptors for the tested adenoviruses. However, additional experiments are still needed to further understand the role and importance of CD80 and CD86 in species B adenoviral infections. Following that line of thought, it would be interesting to evaluate the presence of CD80/CD86 and repeat the blocking and infection experiments using primary cells originating from species B adenoviral tropism tissues, however the availability and culture of these might pose problems. Since CD80 and CD86 are mainly found on antigen presenting cells, such as dendritic cells and macrophages, it would also be interesting to study the immunomodulating aspects of species B adenoviruses upon infection. However, these experiments are beyond the scope of this project.

Altogether, it appears that there are multiple cellular receptors for species B adenoviruses and it seems likely that they play different roles in different tissues. It remains to be seen if they share common epitopes, which could explain the multiple receptor usage reported. We have also considered that Ad3 and Ad7 may use a splice isoform [393-395] of CD46 different from the ones used by the other species B adenoviruses. However, this track remains also to be followed up. One could also speculate that species B adenoviruses use different receptors during different timepoints in their journey in the human body and when they encounter
different cell types. If this is the case we are bound to see a lot of contradicting reports until the whole scheme is apparent.

Finally, at least one major receptor/uptake mechanism is yet to be identified and characterized - the one(s) used by Ad3 and Ad7, the most seroprevalent of the species B adenoviruses. However, the majority of the species B adenoviruses appear to use CD46 as a functional cellular receptor when infecting cells chosen to represent the tropism of the corresponding adenovirus.

**PAPER IV**

**ADENOVIRUS TYPE 11 BINDING ALTERS THE CONFORMATION OF ITS RECEPTOR CD46.**


Since we first identified CD46 as a functional cellular receptor for Ad11 (paper I) [186] and later (paper II) [235] for all species B adenoviruses, except Ad3 and Ad7 on A549 cells we consequently wanted to identify and characterize the binding sites on CD46. The approach chosen to do this was to crystallize the Ad11 knob in complex with CD46 and thus pinpoint the interacting amino acids. The extracellular region of CD46 constitutes of four short consensus repeats (SCR1-4) and a serine-threonine-proline (STP-) domain. Previous studies by others, utilizing mutational approaches, suggested that either SCR1 and SCR-2 [247, 396] or SCR-2 only [249] constitute the receptor binding domain. We found here that the Ad11 fiber knob interacts with the outermost N-terminal SCR1-2 regions (figure 1, paper IV). The complex of Ad11 knob and SCR1-2 was solved at a resolution of 2.85Å using the Swiss Synchotron Light Source (SLS, Villigen, Switzerland), which allowed us to define the interactions between CD46 and Ad11 fiber knob at atomic level. Three Ad11 trimer subunits, or protomers, assembled into a compact fiber knob that can bind three CD46 molecules. The Ad11 knob was homologous to those from other serotypes [64, 65] but differences were seen primarily in the loop regions that define the receptor interactions. Each CD46 unit rests diagonally across the interface between two Ad11 monomers as seen in (figure 1, paper IV). The SCR1-SCR2 domains of CD46 obtain an almost linear conformation upon binding to the fiber knob and the fiber knob covers almost the entire length of the glycan free side of SCR1-2. Unlike the Ad12-CAR interface [99] the Ad11-CD46 interface appears to be very shape complementary, with many protein-protein interactions. Consequently, the affinity of the Ad11 knob and CD46 SCR1-2 is high (~2 nM).

In order to better understand the interacting surfaces of the Ad11 knob and CD46 SCR1-2, three different contact regions have been described: A, B and C (figure 2a, paper IV). Contact region A is situated centered at the Ad11 HI loop and residues at the side of SCR1 (figure 2b, paper IV). Van der Waal contacts helps to maintain shape complementarity. Additional contacts include some hydrogen bonds and a salt bridge between Aps284 of the Ad11 knob and His43 on CD46. The second region (figure 2c, paper IV) involves both HI and DG region of the fiber knob. A key residue here is Arg280 that, besides forming a salt bridge with Glu63 on CD46, also aligns the hydrophobic portion of Arg280 with the CD46 Phe35 side-chain. Several other contacts strengthen the interaction in this region. The third contact region (figure 2d, paper IV) is formed by the IJ loop of the neighboring Ad11 monomer and the lower portion of SCR2 and consist of two hydrogen bonds involving the main chain of CD46. Considering the relative position of the Ad11 knob and the complex...
bound SCR1-2 molecules we think that the N-terminal shaft of the Ad11 fiber and the CD46 SCR3-4 would face in the opposite directions. The area of CD46 that is recognized by the Ad11 fiber knob is most likely the most exposed and outermost part of the cell surface bound CD46 molecule. The fact that recombinant soluble CD46 SCR1-2 and SCR1-4 protein inhibit Ad11 binding and infection in respiratory epithelial cells (A549) at nearly identical doses and with very similar kinetics proves that the SCR3-4 domains do not contact the Ad11 knob (Supplementary figure 1, paper IV).

A fascinating feature of the Ad11 knob-CD46 interaction is that the relative orientation between SCR1 and SCR2 is changed drastically upon Ad11 knob interaction. Ligand free SCR1-2 appears with a distinct ~60° bend which was straightened out upon ligand binding. Ad11 knob thus alters the conformation of its receptor, by realigning the two SCR-repeats, converting them to a rod like structure, thus exposing structures that were previously hidden in the unliganded CD46. This, to the best of our knowledge, the first time a viral ligand alter the conformation of its receptor.

It was recently reported that the cation independent CD46-binding phenotype of Ad11p fiber knob could be converted to a phenotype that mimicks Ad7p (CD46-independent) by one amino acid substitution: Ad11p Arg279Gln Ad7p [382]. Furthermore, the reverse mutation in the Ad7 fiber knob created a phenotype resembling that of Ad11p. This fascinating discovery underlines the fact that seemingly small amino acid changes can have profound effect on an adenovirus phenotype. However, by looking at the crystal structure we see that although Ad11 Arg279 did not directly interact with CD46, its proximity with neighboring residues Asn247 and Arg280 (figure 2c, paper IV) probably mediates HI- and DG-loop stability. Mutations in Arg279 may consequently lead to an improper folding that do not allow the knob to interact with CD46 efficiently. Since the protein tertiary structure is dependent on so many amino acid interactions, mutational studies, although providing very interesting and insightful data, have a built in weakness. In addition, our speculations in our earlier study (figure 4, paper II) about which amino acids were of importance to CD46 binding proved to be wrong.

CD46 is used as a receptor for a multitude of pathogens, from viruses to bacteria [397] and is structurally similar to other viral receptors such as DAF/CD55 and CD21 [398] that might also interact with their respective pathogen in a similar way. The interaction model presented also provides new information that will aid development of antiviral drugs as well as development of new adenoviral vectors. In time, the knowledge of the details in the Ad11 receptor-knob interaction may be the base for designing drugs that prevent Ad11 binding and thus prevent or terminate Ad11 and related infections. Small molecules carrying the “wedge residue” (Phe35) found between SCR1-2 could potentially be the base for this anti-viral therapy. The majority of the gene-therapy vectors used today are based on the species C Ads that bind CAR, because of the broad expression of CD46, high affinity of Ad11 to CD46, and the lower seroprevalence against the species B Ads in general the species B based Ads have been described as potential, attractive alternatives for to expand the cellular targets for adenoviral vectors [43] [399] [400] [401].

The viral receptors should no be looked upon as non-moving stiff structures just sitting statically on the cell surface but rather as flexible, moving “receptionists” capable of transferring messages, distributing material and mediating entry and exit, among many other things. And, as with buildings, cells might just have many “doors”, so just because you have found one, it does not mean that there is not another one waiting to be discovered.
CONCLUSIONS

The human species B adenoviruses cause severe respiratory, ocular and renal infections in humans and may even be lethal in immunocompromised patients. Despite this, the critical initial steps in the life cycle of these adenoviruses have been poorly characterized.

However, our studies first concluded that CD46 is a functional cellular receptor for species B Ad11, and later on we found that this molecule is a cellular receptor for all species B adenoviruses, except Ad3 and Ad7, using cells (A549, NHC, and J82) that represent the respiratory, conjunctival and renal tropism, respectively of these adenoviruses.

From our work we can also exclude CD80 and CD86 cellular receptors for species B adenoviruses on NHC and J82 cells. However, it is still possible that these molecules may serve as cellular receptors for species B adenoviruses, if and when antigen presenting cells such as dendritic cells are infected by these viruses. Bearing in mind that species B adenoviruses cause latent/persistent infections in immunocompromised patients, a role of these molecules appears likely upon species B adenovirus infections of dendritic cells in vivo.

Since yet another receptor is used by species B adenoviruses, receptor "X", further research is necessary in order to elucidate the relative roles of these molecules during species B adenovirus infection in human cells. At least one major receptor/uptake mechanism is yet to be identified and characterized - the one(s) used by Ad3 and Ad7, which are the most prevalent among the species B adenoviruses.

Finally we have pinpointed the interaction site for Ad11 on CD46 using X-ray crystallography. The Ad11 fiber knob have three possible binding sites for CD46 and these sites are located across the DG- and HI loops, thus binding between two knob monomers of the Ad11 knob. The Ad11 binding to CD46 is mediated by the two outermost regions on CD46 SCR1-2. Surprisingly, the conformation of bound CD46 differs significantly from its unbound state, with the bent surface structure straightened into an elongated rod upon binding. Since CD46 serves as a receptor for a large number of viral and bacterial pathogens and it is structurally and functionally related to other viral receptors such as CD21 and CD55, it might be that the conformational change of CD46 during adenovirus binding also takes place in other related ligand-receptor interactions.
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REFERENCES


