Adenovirus-host interactions: implications for tropism and therapy

Annasara Lenman

Department of Clinical Microbiology
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Till min älskade familj och mina underbara vänner

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Abstract

Human adenoviruses (HAdVs) are common viruses often associated with gastrointestinal, ocular and respiratory infections. They can infect a wide variety of cells, both dividing and non-dividing. HAdVs attach to and infect target cells through interactions with cellular receptors. It has also been shown that HAdVs can use soluble host components in body fluids for indirect binding to target cells, a feature that enables the usage of new types of receptors resulting in a more efficient HAdV infection. We therefore evaluated the influence of soluble components from four different body fluids on HAdV infection of epithelial cells, representing the respiratory and ocular tropism of most HAdVs. We found that plasma, saliva, and tear fluid promote binding and infection of HAdV-5 (species C) and that plasma promotes infection of HAdV-31 (species A). Further binding and infection experiments identified coagulation factor IX (FIX) and X (FX) as the components of plasma responsible for increase of HAdV-5 infection while FIX alone mediates increase of HAdV-31 infection. We found that as little as 1% of the physiological concentration of these factors is required to facilitate maximum binding.

The effect of coagulation factors on HAdV infection was thereafter extended to include all species A HAdVs: HAdV-12, -18 and -31. Species A HAdVs normally cause infections involving the airways and/or the intestine. These infections are often mild but species A HAdVs in general, and HAdV-31 in particular, have been shown to cause severe and life-threatening infections in immunocompromised patients. We show here that FIX efficiently increase HAdV-18 and -31 (but not HAdV-12) binding and infection of human epithelial cells, representing the respiratory and gastrointestinal tropism. FIX was shown to interact with the hexon protein of HAdV-31 and surface plasmon resonance analysis revealed that the HAdV-31:FIX interaction is slightly stronger than that of the HAdV-5:FIX/FX interactions, but more interestingly, the half-lives of these interactions are profoundly different. By performing binding and infection experiments using cells expressing specific glycosaminoglycans (GAGs) and

GAG-cleaving enzymes we found that the HAdV-31:FIX and HAdV-5:FIX/FX complexes bind to heparan sulfate-containing GAGs on target cells, but we could also see a difference in GAG dependence and specificity between these complexes.

We conclude that the use of coagulation factors might be of more importance than previously recognized and that this may affect not only the liver tropism seen when administering adenovirus vectors into the circulation but also regulate primary infections by wild-type viruses of their natural target cells. We also believe that our findings may contribute to better design of HAdV-based vectors for gene and cancer therapy and that the interaction between the HAdV-31 hexon and FIX may serve as a target for antiviral treatment.

HAdV vectors are mainly based on HAdV-5 and several problems have recently become evident when using these vectors. Major challenges with HAdV-5 based vectors include pre-existing neutralizing antibodies, poor access to the receptor CAR (coxsackie and adenovirus receptor), and off target effects to the liver due to interactions with coagulation factors. The need for new HAdV vectors devoid of these problems is evident.

HAdV-52 is one of only three HAdVs that are equipped with two different fiber proteins, one long and one short. We show here, by means of binding and infection experiments, that HAdV-52 can use CAR as a cellular receptor, but that most of the binding is dependent on sialic acid-containing glycoproteins. Flow cytometry, ELISA and surface plasmon resonance analyses revealed that the terminal knob domain of the long fiber (52LFK) binds to CAR, and the knob domain of the short fiber (52SFK) binds to sialylated glycoproteins. X-ray crystallographic analysis of 52SFK in complex with sialic acid revealed a new sialic acid binding site compared to other known adenovirus:glycan interactions. Moreover, glycan array analysis identified α2,8-linked oligosialic acid, mimicking the naturally occurring polysialic acid (PSia), as a potential sialic acid-containing glycan receptor for 52SFK. ELISA and surface plasmon resonance confirmed the ability of 52SFK to interact with PSia. Flow cytometry analysis also showed a fivefold

increase in binding of 52SFK to PSia-expressing cells compared to control cells. X-ray crystallographic analysis of 52SFK in complex with oligo-PSia revealed engagement at the non-reducing end of oligo-PSia to the canonical sialic acid-binding site, but also suggested the presence of a 'steering rim' consisting of positively charged amino acids contributing to the contact by long-range electrostatic interactions.

PSia is nearly absent on cells in healthy adults but can be expressed in high amounts on several types of cancers including: glioma, neuroblastoma and lung cancer. We show here that the short fiber of HAdV-52 binds specifically to PSia. Taking into account that HAdV-52 has a supposedly low seroprevalence and is incapable of interacting with coagulation factors we believe that HAdV-52 based vectors can be useful for treatment of cancer types with elevated PSia expression.

Summary in Swedish - Populärvetenskaplig sammanfattning på svenska

Adenovirus är väldigt vanliga virus som bl. a. orsakar förkylning, ögoninfektion och diarré. Dessa infektioner är vanligtvis milda och övergående men hos personer med redan nedsatt immunförsvar, som AIDS patienter och personer som genomgår transplantationer, så kan dessa infektioner vara livshotande. För att adenovirus ska kunna göra oss sjuka så måste dom först ta sig in i vår kropp, mer specifikt, in i våra celler. Det första steget i den processen är att viruset binder till speciella ytstrukturer på cellen, så kallade receptorer. När viruset binder till dessa receptorer så luras cellen att ta upp viruset. Väl inne i cellen börjar viruset att föröka sig och sprider sig vidare till andra celler, vilket gör oss sjuka. Adenovirus har också lärt sig att utnyttja kroppens egna proteiner som dom använder som en förlängningsarm för att kunna binda till nya receptorer. Det gör att viruset kan binda till fler receptorer vilket i sin tur gör att fler celler blir infekterade.

Målet med den här avhandlingen har varit att försöka hitta vilka proteiner som adenovirus kan utnyttja för att lättare ta sig in i våra celler, samt att identifiera vilka olika cellulära receptorer som adenovirus typ 52 (AdV-52) binder till.

Vi började med att undersöka om komponenter i fyra olika kroppsvätskor kunde påverka adenovirus infektion av humana lung- och ögonceller. Adenovirus är uppdelade i sju olika grupper (A-G) beroende på hur lika dom är varandra och vilken typ av infektion som dom orsakar. En representant från varje grupp valdes ut för detta försök. Det visade sig att saliv, tårvätska och blodplasma ökade infektionen av AdV-5 (grupp C) och blodplasma ökade infektionen av AdV-31 (grupp A). Ingen effekt sågs för något annat adenovirus. Vi identifierade därefter koagulationsfaktorer som de proteiner i blodplasma ansvariga för den ökade infektionen av AdV-5 och -31. Genom fortsatta försök kunde vi i mer detalj visa hur AdV-5 och -31 binder till

koagulationsfaktorerna och till vilka receptorer på cellen som adenovirus/koagulationsfaktor-komplexet binder till.

I den andra delen av avhandlingen ville vi identifiera vilka receptorer som AdV-52 binder till. För att binda till receptorer på cellens yta så använder adenovirus sig av sina tolv armar, s.k. fibrer, som sticker ut från viruspartikeln. I vanliga fall har adenovirus bara en sorts fiber, men AdV-52 tillsammans med AdV-40 och -41 skiljer sig från de övriga adenovirus då dessa virus har två sorters fibrer: långa och korta. Vi visar här att AdV-52 använder långa fibrer för bindning till en proteinreceptor som kallas CAR (Coxsackie och Adenovirus Receptor) och att korta fibrer binder till kolhydraten sialinsyra. Fortsatta studier visade att den korta fibern binder extra starkt till långa kedjor av sialinsyra, s.k. polysialinsyra.

Adenovirus kan manipuleras för att bl. a. bekämpa cancer. Det här är ett lovande alternativ för behandling av de cancertyper som visat sig vara väldigt motståndskraftiga mot vanliga behandlingsmetoder såsom strålning och cellgifter. Polysialinsyra är väldigt ovanlig på cellerna i en frisk vuxen person, men kan finnas i stora mängder på vissa cancertyper. Vi tror därför att AdV-52 skulle kunna modifieras och användas som behandling mot de cancerformer vars celler uttrycker polysialinsyra.

Abbreviations

A.D. agent	adenoid degeneration agent
AdV	adenovirus
CAdV	canine adenovirus
CAR	coxsackie and adenovirus receptor
CHO	Chinese hamster ovary
CRAd	conditionally replicating adenovirus
DBP	DNA binding protein
DPPC	dipalmitoyl phosphatidylcholine
DSG-2	desmoglein-2
ECM	extracellular matrix
EGF	epidermal growth factor
FGV	first generation vectors
FIX	coagulation factor IX
FVII	coagulation factor VII
FX	coagulation factor X
GAG	glycosaminoglycan
Gal	galactose
GalNac	N-acetyl galactosamine
GBM	glioblastoma multiforme
GCV	ganciclovir
GLA	γ-carboxyl glutamic acid
GlcNac	N-acetyl glucosamine
GON	group of nine
HAdV	human adenovirus
HD-AdV	helper-dependent adenovirus
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
HV	helper virus
HVR	hyper variable region
ICTV	International Committee of Taxonomy of Viruses
ITR	inverted terminal repeat
LFK	long fiber knob
LRP	low density lipoprotein receptor-related protein
MCP	membrane cofactor protein
MHC	major histocompatibility complex
MLP	major late promotor
mRNA	messenger RNA
NCAM	neural cell adhesion molecule
Neu5Ac	N-acetyl neuraminic acid
NPC	nuclear pore complex

PEG	polyethylene glycol
PSia	polysialic acid
Rb	retinoblastoma protein
RCA	regulators of complement activation
RGD	amino acid motif of Arginine-Glycine-Asparagine
SAdV	simian adenovirus
SCT	stem cell transplants
SFK	short fiber knob
SGV	second generation vectors
SP	serine protease
SR	scavenger receptor
ssDNA	single stranded DNA
TGV	third generation vector
TK	thymidine kinase
TP	terminal protein

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*These authors contributed equally to this work.

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Manuscript

*These authors contributed equally to this work.

Introduction

History

Human adenoviruses were first discovered in 1953 by Rowe and his colleagues (*1*). In their attempt to isolate and identify viruses responsible for upper respiratory infections they found an agent in human adenoids undergoing spontaneous degeneration in tissue culture. This was the first isolation of human adenovirus and the name designated was “adenoid degeneration agent” (A.D. agent). Almost at the same time Hilleman and Werner isolated a similar agent from throat washings of a patient during an epidemic of acute respiratory illness in a military facility. This agent multiplied in human cell tissue cultures, producing obvious cytopathogenic changes (*2*). In 1956 the name adenovirus was proposed (*3*) and since then adenoviruses have been isolated from most human organs and from virtually all vertebrates (*4*).

Due to their easiness to work with and to culture, HAdVs have been of great importance for many discoveries in eukaryotic molecular biology. They have helped scientists to better understand viral and cellular gene expression and regulation, DNA replication, cell cycle control and cellular growth regulation. One of the greatest contributions of HAdV research was the discovery of messenger RNA (mRNA) splicing, for which Sharp & Roberts were awarded the Nobel Prize in physiology/medicine 1993 (*5*).

Taxonomy

Human adenoviruses belong to the family *Adenoviridae*. According to the International Committee of Taxonomy of Viruses (ICTV) (*6*) the *Adenoviridae* family contains five different genera: *Atadenovirus* containing Ads with a high A+T content, mainly isolated from reptiles and birds; *Aviaadenovirus* containing Ads isolated from birds; *Ichadenovirus* containing Ads isolated from fish; *Mastadenovirus* containing Ads isolated from mammals including HAdVs; and *Siaadenovius* containing Ads isolated from reptiles and birds.

Today 56 different HAdVs have been identified and they are further divided into seven different species, A-G (*7*) (**Table 1**). However, approximately 10-15 additional candidate HAdV types have been described but not yet classified. Historically, HAdVs have been classified upon their ability to agglutinate erythrocytes, their oncogenicity in rodents, genetic sequence similarity and tropism. Serology has also been used for classification of HAdV-1 – 51, where the different serotypes were separated based on their resistance to neutralization by antisera from already-known serotypes. The most recently identified HAdVs (52-56) have not been classified using serology but instead characterized based on whole genome sequencing and bioinformatic analysis. It has been agreed to replace the term “serotype” by “type” and new criteria for assignment and classification of new types have been suggested (*8, 9*). However, no consensus has been reached yet. Due to the power of novel genetic and bioinformatics tools and since recombination is an accepted feature of HAdV evolution (*10*), recombinants will be classified as new types provided that they are sufficiently different (genomic, biological and pathological) from related types, potentially resulting in a large increase of HAdV types in the future. More than 30 simian adenoviruses (SAdVs) have sequence similarities to their human counterparts to such a high degree that they have also been included in the taxonomy of HAdVs, within species B, C, F and G (*6*).

Table 1. Classification and tropism of human adenoviruses.

Species	Type	Tropism
A	12, 18, 31	Intestine
B:1	3, 7, 16, 21	Respiratory tract, eye
B:2	11, 14, 34, 35, 50, 55	Respiratory tract, eye, urinary tract
C	1, 2, 5, 6	Respiratory tract
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 33, 36-39, 42-49, 51, 53, 54, 56	Eye, intestine
E	4	Respiratory tract, eye
F	40, 41	Intestine
G	52	Intestine?

Clinical and pathological aspects

Human adenoviruses are commonly associated with infections in the respiratory tract (species A-C, E), intestine (mainly F, but also A, C, D and G) and eyes (B-E). Specific types may also cause infections in the urinary tract (B), liver (C), tonsils and adenoids (C). The majority of HAdV infections occurs during the first five years of life due to lack of protecting antibodies. In healthy individuals HAdV infections are normally mild and self-limiting, but severe and even fatal cases can occur sporadically (*11-13*).

HAdVs can infect a broad range of cells and there are reports indicating that they are able to persist in a latent state after primary infection. This type of latent HAdV infections occur in tonsillar lymphocytes, lung epithelial cells, the central nervous system and the entire gastrointestinal tract (*14*).

The transmission of HAdVs is typically from person to person, either by inhalation of aerosolized droplets, tears from infected persons, fecal-oral spread or contact with contaminated environmental surfaces. HAdVs are very stable and can remain infectious even after several weeks in dry

environments, and since they are non-enveloped they are also resistant to several disinfectants (*15*). Extensive decontamination is of extreme importance especially in hospital settings for transplant patients and intensive care units as HAdV infections in these patients are life-threatening.

Adenovirus infections in immunocompromised patients

HAdV infections pose a great threat in immunocompromised patients, including transplant patients, congenital immunodeficiency patients and patients undergoing chemotherapy. The risk is highest in patients with depletion of T-cells, such as allogenic stem cell transplants (SCT), since T-cells are extremely important for the immune defense protection against HAdV infections. On the positive side, HAdV infections in HIV/AIDS patients are no longer severe and life-threatening due to the highly effective antiviral treatments used by these patients, given that the patient is actually undergoing HIV therapy (*14*).

In transplant patients HAdV complications can occur from new infections derived either from the graft or the environment, or from reactivation of persistent endogenous HAdVs (*16, 17*). The reactivation of endogenous HAdVs seem to be the most important cause for infections in severely immunocompromised patients. Even though this reactivation can occur at different sites, a long term study of SCT patients showed that HAdV proliferation preceding invasive infection almost exclusively occurred in the gastrointestinal tract (*18, 19*). Monitoring viral load in stool samples can therefore be of importance for early signs of an approaching disseminated disease (*19*).

HAdVs causing infections in immunocompromised patients belong mainly to species C HAdVs (-1, -2 and -5) but also to species A HAdVs (-12 and -31) and to species B HAdVs (-3, -11, -16, -34 and -35) (*14*). HAdV-associated disease in immunocompromised patients can range from mild gastroenteric or respiratory syndromes, to severe manifestations including hemorrhagic enteritis or cystitis, pneumonia, hepatitis, nephritis, encephalitis,

myocarditis and disseminated infection associated with high mortality due to multiorgan failure (*14*). Disseminated disease is mainly caused by species C HAdVs, however, in recent decades HAdV-31 infections have been reported as an increasing threat, especially in children following allogenic SCT (*20-23*). HAdV-31, along with species C HAdVs, have also been shown to be strongly overrepresented in immunocompromised patients as compared to immunocompetent patients (*24*). This was suggested to be due to reactivation rather than to new infections. Simultaneous infection of more than one HAdV type in SCT patients is common and HAdV-31 is often included (*25, 26*). High amounts of HAdV-31 are shed with feces from infected patients and HAdV-31 has caused severe problems in nosocomial settings where it has been transmitted between immunocompromised patients (*27, 28*).

Allogenic SCT patients are believed to have the highest number of HAdV infections with an overall frequency of 3-47% depending on the study, and there the mortality rate can be as high as 60%. Children are also more prone to infections than adults (reviewed in (*29*)). Kampmann et al reported species A HAdVs to be the most commonly detected species in these patients and among them only HAdV-31 was found in patients with viremia (*23*) .

The earlier antiviral treatment is started, the better the chances are for successful control of HAdV infections, even if the availability of effective therapeutic strategies is limited. This urges the importance of fast and reliable HAdV diagnostics. The presence of HAdVs in blood is a sign of disseminated infection and viral load is usually equivalent to the severity of organ damage. Studies have shown that rapidly increasing viral load in the blood can be monitored before onset of clinical symptoms, suggesting that viral load in blood together with the viral load in stool samples are parameters to use for detection of potentially severe and invasive HAdV infections (*14*).

Treatments

Treatments against HAdV infections are mostly used for immunocompromised patients, particularly allogenic transplant patients since they are at most risk of severe and life-threatening disease. Several studies of HAdV infections in SCT patients show that patients developing viremia were exclusively those who received T-cell-depleted grafts. Lymphocyte recovery was also shown to be the most important factor for survival in these viremic SCT patients and mortality was closely related to absence of lymphocytes (*23, 30, 31*). The treatment regimens for HAdV infections in immunocompromised patients are therefore based on methods to increase the lymphocyte count or buy time for lymphocyte recovery. Treatments include: i) reduction or complete withdrawal of immunosuppressive therapy whenever possible, ii) use of antiviral drugs, and iii) immunotherapy with donor-derived HAdV-specific T-cells for patients that do not respond to antiviral therapy. It should be noted that administration of HAdV-specific T-cells is still to be considered as experimental and can only be used in the context of clinical trials in specialized centers (*14*).

Cidofovir is an antiviral drug commonly used for HAdV infections. It is a nucleotide analog of cysteine that preferentially inhibits the viral DNA polymerase, and is also incorporated into the DNA chain, both of which prevents viral replication (*14*). Cidofovir has been shown to have antiviral effects on all HAdVs species, but the efficacy seem to be best in preemptive settings (*32-34*). It is therefore currently the primary anti-HAdV drug used for preemptive therapy (*35*). An important side effect of cidofovir is nephrotoxicity and monitoring of renal and tubular functions are required (*35*).

Ribavirin is a nucleoside analog of guanosine and have effect on both RNA and DNA viruses. It inhibits viral polymerases, viral RNA capping and increases mutation rates in newly synthesized DNA (*36*). It has a known effect against species A-D HAdVs (*34*), however the concentrations needed

to reach IC_{50} *in vitro* cannot be achieved in plasma or tissue and ribavirin is not generally recommended for treatment of HAdV infections (*34, 35*).

Brincidofovir is a recently introduced drug that is a prodrug of cidofovir. It is conjugated to a lipid and designed to release cidofovir intracellularly. This increases the intracellular concentration of cidofovir and decreases the extracellular concentration, thereby reducing the risk for nephrotoxicity. It has shown promising results against HAdV infections with EC_{50} values 5-333 fold better than cidofovir, but the clinical development of the drug is still ongoing (*37-39*).

Adenovirus structure

Human adenoviruses are non-enveloped viruses containing one linear double stranded 34-37 kb DNA genome within an icosahedral capsid. The capsid is about 90-100nm in diameter and has a mass of 150MDa. The capsid has 20 triangular facets with 12 vertices and a fiber protein protruding from each vertice. Eleven structural proteins have been identified with SDS-page and named II-XII, in order of their decreasing molecular weight (*40*). They are divided into major and minor capsid proteins as well as core proteins (**Figure 1**).

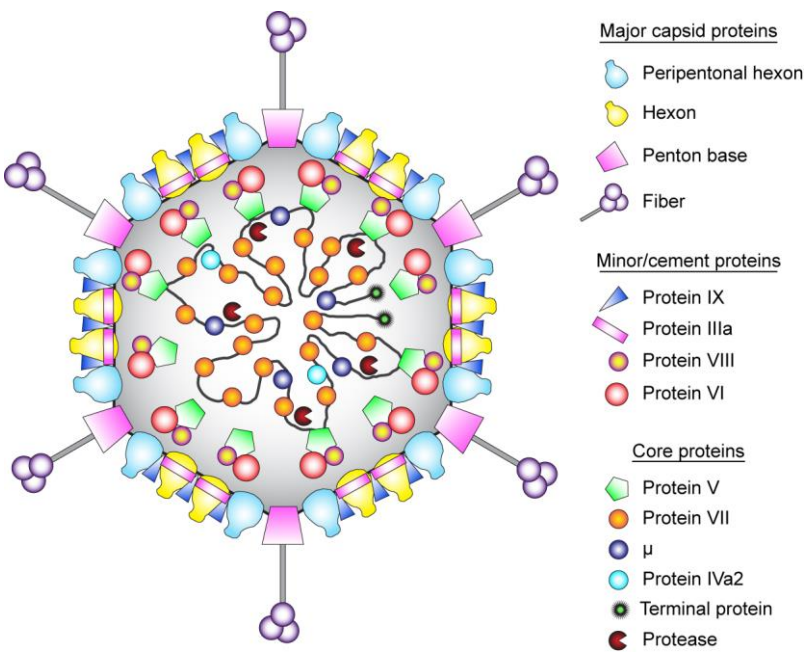

Figure 1. Schematic picture of a HAdV particle showing the major and minor structural proteins and the core proteins.

Hexon (pII)

The hexon protein is the major component of the icosahedral capsid; 720 hexon monomers form 240 homotrimers that constitute the 20 facets of the capsid (*41, 42*). Each monomer consists of two antiparallel β-barrels stabilized by an internal loop and three protruding loops on the external side (*43*). These external loops contain up to nine hyper variable regions (HVR) and generate type-specific epitopes for neutralizing antibodies (*43-45*) (**Figure 2A**). This structure contains extensive intermolecular contacts within the hexon trimer, which render it extremely stable and explain the stability of the hexon towards proteolysis (*40, 46*). Depending on their interactions with neighboring proteins, the hexons can be classified into four groups, H1-H4. There are 60 H1 which interact with the penton base at the capsid vertices and these hexons are referred to as peripentonal hexons. H2-H4 are clustered together and form a unit referred to as "group of nine" (GON) and make up the center of the capsid facets (*42, 47*). The hexon has been shown to mediate coagulation factor-dependent binding of HAdV-5 to heparan sulfate proteoglycans on target cells (*48*).

Penton base (pIII)

The penton base is a homopentameric protein approximately 340 kDa in size (depending on the HAdV type). The monomeric structure is similar to the hexon with a β-barrel domain at the base and two loops extensions forming the distal end. The pentamer has a large central cavity containing five equivalent fiber-binding sites whereas only three are used to anchor the N-terminal domains of the trimeric fiber (*49*).

The penton base together with the fiber form the penton complex that protrudes from the 12 vertices of the icosahedral capsid. The fiber initiates attachment to the host cell and the penton base mediates internalization (*7*). The penton base causes internalization by interactions with cell surface αvβ3/5 integrins (*50, 51*). This interaction is enabled by an amino acid sequence, Arg-Gly-Asp (RGD), located on a long, variable and flexible loop at

the surface of the penton base (**Figure 2B**). The RGD motif is conserved in all HAdV species, except species F (HAdV-40 and HAdV-41) (*52-55*). Instead HAdV-40 has a RGAD (Arg-Gly-Ala-Asp) motif and HAdV-41 has an IGDD (Ile-Gly-Asp-Asp), which may result in the usage of other internalization pathways not normally used by RGD-containing HAdVs (*56*).

The penton base also has the ability to assemble without other virion components to form dodecahedral particles that can enter cells by endocytosis (*57*). Cryo-EM studies of HAdV-3 dodecahedra, with and without fiber, revealed that the penton base undergoes structural rearrangements upon fiber binding (*58*).

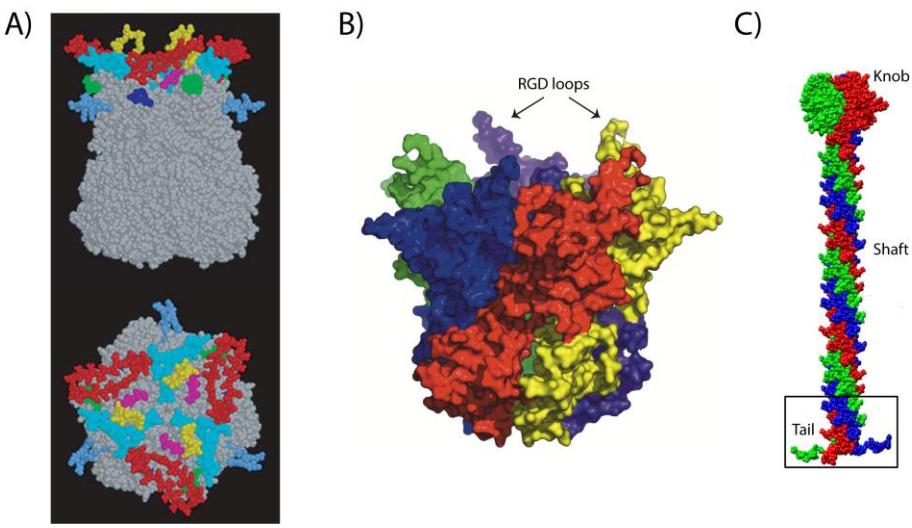

Figure 2. Structures of the three major capsid proteins. A) Model of the HAdV-5 hexon (sideview followed by a topview) with highlighted HVRs (HVR1, red; HVR2, green; HVR3, pink; HVR4, light blue; HVR5, yellow; HVR6, blue; HVR7, cyan). The remainder of the hexon trimer is shown in grey. Reprinted with permission from publisher (*59*). B) Side view of a crystallised HAdV-2 penton base. Each subunit is shown in a different color. Reprinted with permission from publisher (*49*). C) Model of the HAdV-5 fiber with each subunit in a different color. Reprinted with permission from publisher (*60*).

Fiber (pIV)

The fiber is a trimeric protein that can be divided into three distinct parts: an N-terminal tail that attaches the fiber to the penton base, a shaft, and a knob responsible for interactions with cellular receptors (**Figure 2C**). The length of the fiber is determined by a variable number of 15-20 amino acids long pseudorepeats in the shaft, ranging from 6 repeats in HAdV-3 (*61*) to 22 in HAdV-2 and -5 (*62*). The three monomers are intertwined to form a stable shaft (*63*). Both the length and flexibility of the fiber has been shown to be of importance for cellular attachment and uptake (*64, 65*). The trimeric structure of the C-terminal knob creates a globular structure with a central cavity on the top. A number of loops extending from the knob (designated DG, HI and AB) are of importance for receptor interactions (*66*).

HAdVs normally have one type of fiber, except for HAdV-40, -41 and -52, which all contain two different types: one long and one short (*67-69*). As for all other HAdVs only one fiber is attached to each penton base. In HAdV-52 the two different fibers are incorporated into the capsid in equal amounts (*70*), while in HAdV-40 and -41 short fibers are in majority (*71*).

Minor capsid proteins

The HAdV minor capsid proteins are not as well characterized as the major capsid proteins but their main functions are to act as cement proteins to stabilize the capsid and to facilitate its assembly/disassembly.

Protein IIIa (pIIIa) is located on the inside of the capsid where it interacts with penton base and peripentoneal hexons presumably acting as a stabilizer (*72*). It has also been suggested to be important for viral DNA packaging into the capsid (*73, 74*).

Protein VI (pVI) is located on the inside of the capsid and interacts with peripentoneal hexons. pVI is important in the infectious cycle where it enables endosomal escape by disrupting the endosomal membrane (*75*). It is

also important for nuclear import of hexons, virion assembly and activation of the HAdV protease (*76, 77*).

Protein VIII (pVIII) is also located on the inside of the capsid where it is believed to stabilize the interactions between GONs and their neighboring hexons (*78, 79*).

Protein IX (pIX) is the smallest of the minor capsids and is unique for the mastadenovirus genus (*80*). It is located externally in the cavities between the peaks formed by hexons (*46, 81*). Besides being a major stabilizer of GONs, pIX has also been suggested to be important for viral entry, microtubule-dependent transport to the nucleus as well as transcriptional activation of the major late genes (*82-86*). pIX has also been used as a basis for anchoring other polypeptides to the capsid, a feature useful in constructing gene therapy vectors (*87*) and for tagging viruses with a fluorescent marker that enables tracking of viruses in infected cells (*88*).

The adenovirus core and non-structural proteins

The HAdV core consists of a ~35 kb long double stranded DNA molecule and five different core proteins. **The terminal protein (TP)** binds to the 5´ends of the DNA molecule and is important for initiation of replication (*89, 90*). **Protein VII (pVII)** is together with **μ** responsible for packaging of the viral genome into nucleosome-like particles (*91, 92*). It is also believed to be involved in import of the viral DNA into the nucleus in the initial steps of infection (*93*). **Protein V (pV)** connects the core to the capsid by interacting with the penton base, pVI and DNA (*94, 95*). **Protein IVa2 (pIVa2)** binds to the DNA in a sequence-specific manner and is involved in regulation of the major late genes as well as DNA packaging (*96, 97*).

There are also about 30 non-structural HAdV proteins identified so far and they have mainly been shown to have catalytic or regulatory functions (*7*). The functions of some of them are better established than others and will be described here shortly. The DNA polymerase is essential for viral genome replication and DNA proof reading (*98*). The DNA binding protein (DBP)

binds to DNA, and protects ssDNA from degradation as well as destabilize the DNA helix in order to facilitate replication (*99, 100*). The viral protease cleaves several viral proteins, which is an essential step for virion maturation (*101*).

Adenovirus life cycle

Human adenoviruses are non-enveloped viruses that must be stable enough to withstand the harsh environments that they may encounter outside the host, but also have the ability to disassemble and release its genome at the right time and place once inside a target cell. The HAdV particle is therefore said to be "metastable" and disassembly is triggered by various signals, the first one being attachment to cellular receptors. The life cycle is divided into two phases that are separated by the onset of viral replication: the early phase includes cellular attachment, internalization, endosomal release, transport of the viral DNA to the nucleus and expression of early genes. The late phase includes expression of late viral genes and assembly of progeny viruses. The whole replication cycle takes around 24-36 h and can yield 10^5 new virions from one infected cell (*7*).

Attachment and cellular receptors

Attachment of HAdVs to cellular receptors is the initial step of infection and most HAdVs bind to the receptor with the knob domain of the fiber protein. HAdVs can interact with one or several receptors, which mediate internalization into the host cell. Several HAdV receptors have been identified so far including membrane-bound proteins, glycans and soluble proteins. The receptor usage depends on the HAdV type and species (**Figure 3**).

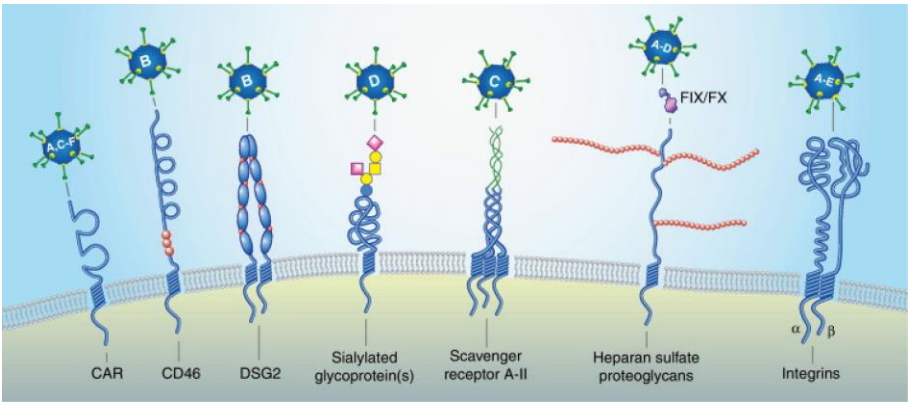

Figure 3. Cellular receptors used by human adenoviruses.
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The coxsackie and adenovirus receptor

The coxsackie and adenovirus receptor (CAR) was identified in 1997 as a common receptor for coxsackie B viruses and human adenoviruses (HAdV-2 and -5) (*103*). It has since then been shown to be the major receptor used *in vitro* by members of all HAdV species, except species B HAdVs (*103, 104*). CAR is a transmembrane protein that belongs to the immunoglobulin (Ig) superfamily. It has an intracellular domain, a transmembrane domain and an extracellular domain that is composed of two Ig-like domains, designated D1 and D2 (D1 being the more distal domain). CAR has been identified as a component of the tight junctions where it enables cell-cell interactions by forming intercellular homodimers (*105*). The predominant isoform of CAR is mainly expressed on the basolateral side and in tight junctions on polarized epithelial cells and is therefore not readily available for a virus approaching from the exposed apical side of the cell. This brings the role of CAR as a cellular receptor *in vivo* into question. However, a minor isoform of CAR (CAR^{Ex8}) has been found to be exposed on the apical side of some airway epithelial cells and was shown to support viral attachment (*106*).

HAdVs use the fiber knob to interact with the D1 domain. However, both the length and flexibility of the fiber shaft have been shown to be of

importance for efficient binding to CAR (*64*). HAdV fiber knob binding to CAR is usually of high affinity and the site of interaction on D1 is the same site involved in CAR-CAR dimerization (*107, 108*). It has been shown that soluble fiber interactions with CAR can disrupt the CAR-CAR interaction which has a relatively lower affinity. Excess production of fiber protein during an infection can interfere with CAR homodimerization and thereby disrupt tight junctions and facilitate virus spread from infected tissue (*109*).

CD46

Human CD46 is also known as membrane cofactor protein (MCP) and is expressed on all human nucleated cells (*110*). It belongs to the family of proteins known as regulators of complement activation (RCA) and its function is to protect healthy host cells from complement-mediated destruction by facilitating factor I-dependent inactivation of complement factors C3b and C4b (*111*).

The extracellular domain of CD46 consist of four short consensus repeats (SCR1-SCR4) that form an elongated structure. Domains SCR1-3 are arranged in a nearly linear fashion, while there is a bend between domains SCR3 and SCR4 (*112*). As for CAR, CD46 also contains a transmembrane and an intracellular domain. CD46 has been shown in a couple of studies to be located on the apical side polarized epithelial cells (*113, 114*), while others report CD46 to be localized within the intercellular junctions (*115*).

CD46 is used as a cellular receptor by most species B HAdVs (*116, 117*) and it has also been suggested to be a receptor for HAdV-37 (species D) (*118*). HAdVs use their fiber knob to interact with SCR1 and SCR2 domains on CD46 (*119*). The affinity between the fiber knob and CD46 range from nM to mM, depending on HAdV type (*120, 121*). CD46 can also be used as a receptor by many other viruses as well as bacteria, including measles virus, human herpes virus 6, group A *Streptococci* and some *Neisseria* strains (*122-125*).

Desmoglein-2

Desmoglein-2 (DSG-2) is a cell adhesion molecule that belongs to the cadherin protein family. It is a transmembrane glycoprotein component of desmosomes that adhere adjacent cells together in polarized epithelia (*126*). DSG-2 is found in most human tissues but due to the intercellular localization, DSG-2 is not easily accessible for HAdV infection on the apical side of the cell. HAdVs that have been shown to use DSG-2 as a cellular receptor belong to species B, including HAdV-3, -7, -11 and -14. The interaction of these HAdVs with DSG-2 is mediated by the fiber, but only in the spatial constellation that is formed in the viral particle (*127*).

Species B HAdVs are known to overexpress and secrete incomplete particles called dodecahedral particles that consist only of penton base and fiber proteins. These can bind to DSG-2, which triggers intracellular signaling that results in the cleavage of the extracellular domain of DSG-2, thereby disrupting DSG-2 homodimers between adjacent cells. This result in opening of intercellular junctions which may facilitate subsequent virion spread from or within the epithelial layer (*127-129*).

Sialic acid

Sialic acids are a family of negatively charged monosaccharides with a shared nine-carbon carboxylated backbone. They are found ubiquitously at the cell surface of higher vertebrates and the most common sialic acid in humans is N-acetyl neuraminic acid (Neu5Ac) (**Figure 4A**). However, modifications of the sialic acid backbone can give rise to more than 50 different variants (*130*). Sialic acids are mostly found in the terminal position of different glycan structures located on *N*-linked (asparagine-linked) or *O*-linked (serine- or threonine-linked) glycoproteins as well as on glycolipids. In the glycan chain, sialic acids are usually linked to galactose (Gal) or N-acetylgalactosamine (GalNAc) in an α2,3 or α2,6 manner (i.e. from carbon 2 on sialic acid to carbon 3 or 6 on Gal/GalNAc). Sialic acids can also be linked

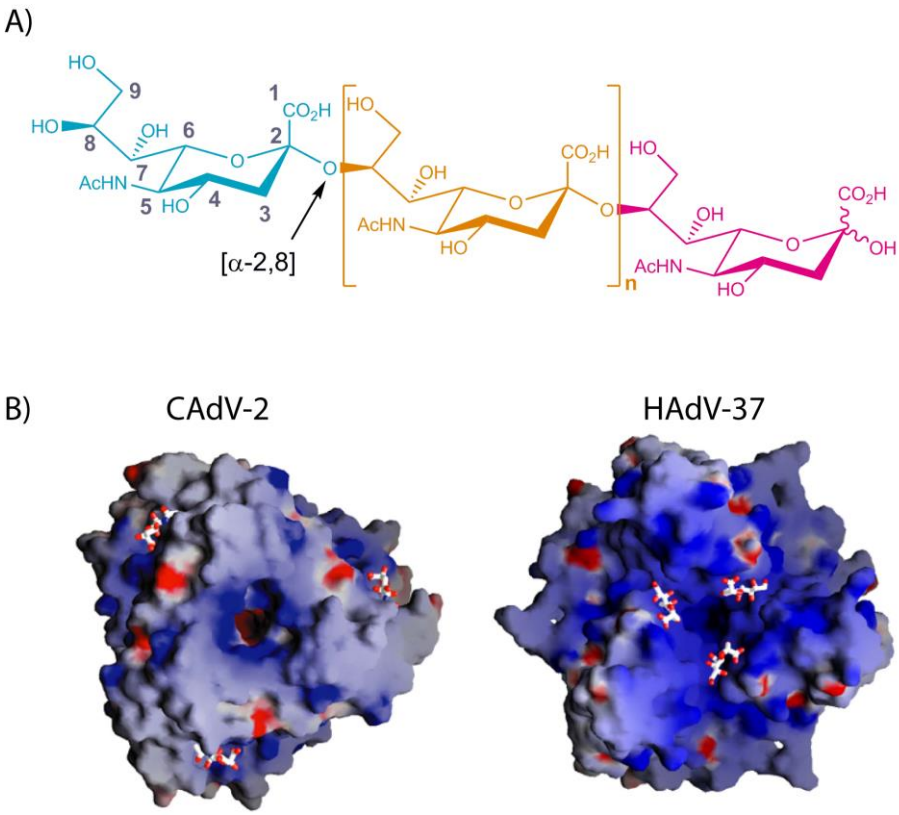

Figure 4. Sialic acid structure and sialic acid binding sites in HAdVs. A) General structure of polysialic acid. Each color represent one sialic acid residue. Up to ~100 sialic acid residues can be linearly connected via α2,8-linkages. B) Surface charge representation of CAV-2 and HAdV-37 fiber heads and cartoon representation of sialic acids as found in the complexes. Reprinted from the publisher which permits unrestricted use (*131*).

to each other with α2,8- or α2,9-linkages, which enables internal positioning in glycans as well as formation of long sialic acid chains, called polysialic acid (PSia) (**Figure 4A**) (*132-134*). Neu5Ac has four protruding functional groups (carboxylate, N-acetyl, glycerol and hydroxyl groups) that enable formation of a large number of hydrogen bonds, salt bridges and non-polar

interactions, which in addition to its abundance on the apical side of the cells makes it an attractive target for viral attachment (*135*). Sialic acid is used as a receptor by several human pathogens besides HAdVs, including rotavirus (*136*), influenza virus (*137*), JC and BK polyoma-viruses (*138, 139*), coxsackievirus (*140*) , *Helicobater pylori* (*141*) etc.

HAdV-8, -19, -37 (and most likely other species D HAdVs) have been shown to use sialic acid-containing glycans as cellular receptors (*142*). HAdVs engage sialic acid with the fiber knob domain where each monomer contains a sialic acid binding site, however, the localization of these binding sites are fundamentally different between AdV types. In HAdV-37, sialic acid binds to the top of the fiber knob in a central cavity formed between the three fiber monomers (*143*), while the binding to canine adenovirus (CAdV-2) takes place on the side of the fiber (*131*) (**Figure 4B**).

Because of the small contact surface, interactions between sialic acid and individual fiber knobs are often of low affinity (in the millimolar range). Stronger attachment to target cells can be achieved by avidity effects where multiple low affinity interactions together increase the functional affinity by receptor clustering (*135*). Other ways of achieving higher affinities to sialic acid-containing glycans is to: i) identify the oligosaccharide structure that enable sialic acid engagement in more than one binding site, which is the case for HAdV-37 and the GD1 glycan that utilizes two out of three sialic acid binding sides increasing the affinity 250-folds (*144*) or ii) identify the glycan structures where sialic acid confer the initial binding and the remaining glycan structure form additional interactions, thereby increasing the affinity (*135*). Glycan arrays have been very useful for identification of sialic acid-containing glycans that can contribute to these relatively high affinity interactions with viral attachment proteins (*145, 146*).

Sialic acids play essential roles in several biological systems including cellular and molecular recognition and protection. The long glycan chains with terminating negatively charged sialic acids can function as a shield/coat that protects proteins from proteases or cells from degradation (*147*). The immune system uses sialic acid patterns to distinguish from self and nonself structures and the negative charge of sialic acid also affects the attraction

and repulsion phenomena between cells (*133, 147*). Induction of sialic acid expression is common in cancer cells, which enables immune escape and repulsion between cells, thereby increasing their malignancy (*148, 149*).

Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPGs) are glycoproteins composed of a core protein to which one or more heparan sulfate (HS) chains are covalently linked. They are found at the cell surface and in the extra cellular matrix where they interact with a large number of ligands. HSPGs have numerous biological activities and functions, including cell adhesion, developmental processes, regulation of cell growth and proliferation, inhibition of blood coagulation, cell surface binding of lipoprotein lipase and other proteins, angiogenesis, and tumor metastasis (*150*).

HS is a linear polysaccharide belonging to the family of glycos-aminoglycans (GAGs). It is built from repeating disaccharides that form long chains between 40-300 residues in length. The most common disaccharide within HS is composed of a glucuronic acid linked to *N*-acetylglucosamine (GlcNAc), making up around 50% of the HS chain. The GlcNAc units can become *N*-deacetylated followed by *N*-sulfation and this type of modification creates a pattern along each HS chain with clusters of unmodified *N*-acetylated disaccharide units (denoted NA domains) and consecutive sequences of *N*-sulfated disaccharide units (NS domains). These domains are separated by transition segments with alternating *N*-sulfated and *N*-acetylated disaccharides (NA/NS domains). The degree of sulfation of HS chains vary depending on growth conditions and in response to growth factors. The arrangement of the negatively charged sulfate groups and the orientation of the carboxyl groups specify the location of ligand-binding sites (*151*).

HSPGs have been shown to act as a primary receptor for HAdV-2 and -5 and as a potential co-receptor for HAdV-3 and -35 (*152, 153*). The interaction of HAdV-5 with HSPG has been shown to involve a KKTK motif in the fiber

shaft (*152*) while HAdV-3 interacts with HSPGs through the fiber knob (*153*). Other pathogens have also been shown to interact with HSPGs, including herpes simplex virus (*154*), dengue virus (*155*), cytomegalovirus (*156*), *Borrelia burgdorferi* (*157*) etc.

Coagulation factors and other soluble components

Vitamin K-dependent coagulation factors are important players in the coagulation cascade and fibrinolysis pathway that maintain homeostasis in the vascular system. They are produced in the liver and circulate in the blood in their inactive form. The group of vitamin K-dependent coagulation factors include factor II, VII, IX, X and protein C. They are all serine proteases that cleave peptide bonds in proteins and they require Ca^{2+}, phospholipids and cofactors for their biological activity (*158*). All except FII are structurally very similar, with an N-terminal γ-carboxyl glutamic acid (GLA) domain, two epidermal growth factor (EGF) like domains and a serine protease (SP) catalytic domain at the C-terminal end (*159*).

Coagulation factors were found to mediate HAdV transduction of hepatocytes in an attempt to explain the accumulation of HAdV-5 based vectors in the liver after intravascular administration. It was first shown that coagulation factor IX (FIX) and the complement component C4-binding protein increased hepatocyte transduction *in vitro*, although the *in vivo* situation could not be explained by these two components alone (*160*). Further studies showed that all structurally alike vitamin K-dependent coagulation factors, FVII, FIX, FX and protein C could enhance transduction of hepatocytes *in vitro* (*161*). Coagulation factor X was, however, later shown to be the main contributor of liver HAdV-5 transduction *in vivo* (*48*). FX interacts with HAdV-5 though the GLA domain on FX and the HPVR5 or 7 on the hexon protein of HAdV-5. FX then in turn binds to HSPGs through the heparin-binding exosite in the SP domain, thereby bridging HAdV-5 to the cell (*48*). HSPGs have a core protein to which one or several heparan sulfate glycosaminoglycans are linked. HAdV-5:FX-mediated binding was

shown to occur through HSPGs with *N*-sulfated N-acetylglucomsamine and *O*-sulfated D-glucuronic acid residues in the heparan sulfate (*162*).

Several other HAdV types have also been assessed for their ability to interact with FX and classified in three categories; relatively strong binders (e.g. HAdV-5, -2, -16, -50), relatively weak (e.g. HAdV-35, -3, -7, -11, -18) and non-binders (e.g. HAdV-26, -48, -20, -44) (*48*).

HAdVs have also been shown to use other soluble components for more efficient binding and infection of host cell. **Lactoferrin** is an iron transport protein that mediates HAdV-5 binding and infection of human epithelial cells (*163*), however, the exact mode of action is still not known. **Dipalmitoyl phosphatidylcholin** (DPPC) is a phospholipid and a major component of pulmonary surfactant that has been shown to bind to the hexon protein on HAdV-5 and increase the infection of human lung epithelial cells. These cells constantly produce and reincomporate DPPC and it was shown that reincomporated DPPC liposomes delivered the attached virus (*164*).

Integrins

Integrins are adhesion molecules that mediate cell-cell contacts and connect cells to the extracellular matrix (ECM). They are heterodimeric transmembrane glycoproteins, consisting of one α and one β subunit. Humans express 18 different α subunits and 8 different β subunits, which combine into 24 unique integrins. Each subunit consist of an extracellular domain, a transmembrane domain and a short cytoplasmic tail that interacts with cytoskeletal components and signal molecules inside the cell (*165*). Integrins play an important role in cell signaling where they transduce signals from the ECM to the cell interior and vice versa, as well as in the immune system where they enable leukocyte trafficking and migration within tissues (*165, 166*).

HAdVs bind to their primary receptor on the cell surface, which enables subsequent interaction with integrins through the RGD motif in the penton base. Integrins thereby act as secondary receptors or coreceptors, which facilitate HAdV internalization (*51, 167*). It has also been suggested that initial capsid disassembly is induced at the cell surface in a manner dependent on the dual engagement of mobile CAR and the less mobile α_v integrins (*168*). HAdVs interact with several different integrins including αVβ1/3/5, αMβ2 and α3β1 (*50, 51, 169-171*).

Other adenovirus receptors

Scavenger receptor A (SR-A) has been suggested as a receptor for HAdV-5 and is responsible for uptake of the virus in macrophages both *in vitro* and *in vivo*. HAdV-5 was suggested to bind to SR-A via the fiber knob (*172*).

CD80 & CD86 are cell surface markers expressed mainly on dendritic cells and have been suggested to function as receptors for species B HAdVs on these cells *in vitro* (*173, 174*). However, several receptors have been suggested to be used by species B HAdVs and more investigations are needed to elucidate their relative importance during natural infections *in vivo*.

Major histocompatibility complex 1 (MHC-1) are antigen-presenting molecules found on the cell surface of all nucleated cells. They have been suggested to serve as cellular receptors for HAdV-2 and -5 on HeLa cells *in vitro*, through interaction with the fiber knob (*175*). However, other studies have not been able to confirm the role of MHC as a HAdV receptor (*176, 177*).

Internalization and trafficking

Studies of the HAdV replication cycle have mainly been conducted with HAdV-2 and HAdV-5 and, if not otherwise stated, the following sections are based on research performed with these two types, summarized in **Figure 5**.

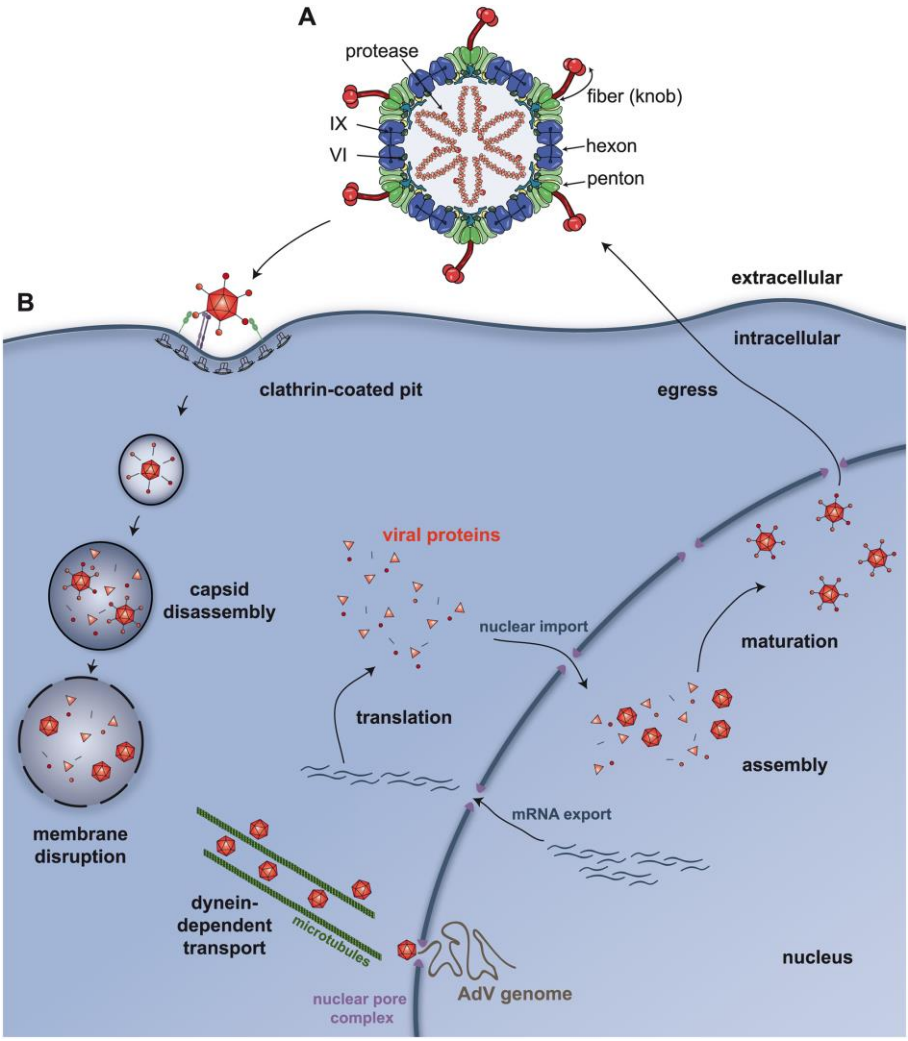

Figure 5. HAdV life cycle. Illustration showing essential steps of the HAdV life cycle in epithelial cells. Reprinted from a publisher which permits unrestricted use (*178*).

HAdVs attach to primary receptors on the cell surface, which in turn enable binding to αVβ3 and αVβ5 integrins through the RGD loop on the penton base (*50, 51, 53*). Interactions with integrins require a flexible fiber shaft to allow simultaneous binding to both the primary receptor and penton base (*64*). Approximately four integrins can bind to one penton base, and the clustering of integrins triggers transmembrane signaling. This results in activation of phosphatidylinositol-3-OH and Rho GTPases that induce rearrangement of the actin cytoskeleton, which in turn facilitate endocytosis of the HAdV particle via clathrin-coated vehicles (*179-182*). It is also possible that integrin interactions trigger signals that increase HAdV entry via micropinocytosis (*183*). Physical movements of CAR and integrins following dual engagement of HAdV initiate disassembly of the HAdV particle and leads to detachment of the fiber so that a fiberless particle is endocytosed (*168*). Once inside the cell, the endosome acidifies as it matures and the lowering of pH facilitates further uncoating of the viral particle in an ordered fashion. Removal of the vertex region, which includes penton base, fiber protein and possibly also peripentonal hexons, allows release of the membrane lytic protein VI from the inner surface of the virus capsid. Protein VI disrupts the endosomal membrane and enables endosomal escape of the virus particle approximately 15 minutes after binding to the cell surface (*7, 75, 184*). In the cytoplasm the partially-dismantled HAdV particle associates with the dynein motor protein complex and is transported along the microtubules to the nucleus where it docks at the nuclear pore complex (NPC) (*185*). Association with the NPC probably initiates final disassembly of the virus and release of DNA together with bound core proteins into the nucleus (*7*).

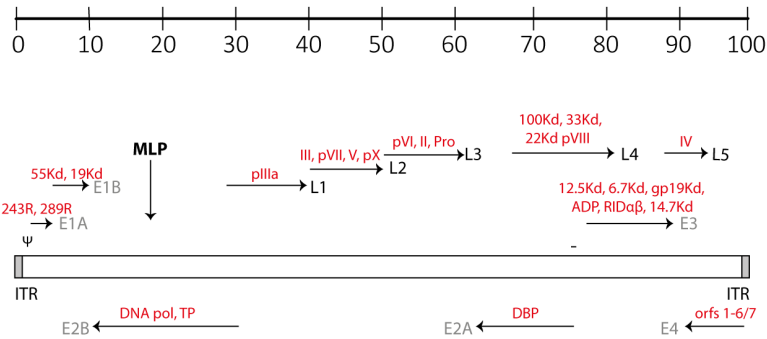

Figure 6. Transcription of the HAdV genome. Arrows indicate the direction of transcription. MLP= major late promotor.

Genome organization and replication

The HAdV genome consist of a single, double-stranded DNA molecule, approximately 35 kb long. Both ends are flanked by inverted terminal repeats (ITRs) ranging from 36 to more than 200 bp and a terminal protein (TP) associated with the 5´ends (*95*). The genes on the HAdV genome have been divided in groups based on their time of transcription during the replication cycle; early genes include five transcription units (E1A, E1B, E2, E3 and E4), intermediate genes include four transcription units (IX, IVa2, L4 intermediate and E2 late) and the late genes with one transcription unit under the regulation of the major late promotor (MLP) (**Figure 6**). This single mRNA is spliced to generate at least 14 distinct mRNAs grouped into five families of late mRNAs (L1-L5). This is, however, a convenient but simplified version of transcription events since early genes continue to be expressed at late times and late genes have a low level of transcription already early after infection. In general, gene products from the early genes function to mediate further viral gene expression, enable viral DNA replication, induce cell cycle progression to provide an optimal environment for viral replication, block apoptosis and antagonize host antiviral

mechanisms. The late genes encode structural proteins that build up the capsids of progeny virus (*7*).

HAdV DNA replication begins ~6h after infection and takes place in two stages. The ITRs at each end of the genome function as replication origins and in the first stage viral replication can start at either terminus and proceed continuously to the other end giving rise to one duplex genome and one single strand of DNA (ssDNA). In the second stage of replication a complement to the ssDNA is synthesized. The ssDNA template circularizes and anneals with itself in the self-complementary ITRs, thereby restoring the functional replication origin. This can then be recognized by the same replication machinery that functioned in the first stage, resulting in synthesis of a complementary strand. The newly-synthesized genomes can either enter subsequent replication rounds or be processed and encapsidated into progeny virus (*7, 186*).

Three viral proteins play major roles in the HAdV genome replication, i) the terminal protein which function as a primer for initiation of DNA synthesis, ii) the adenovirus DNA polymerase which has a 5´-3´ polymerase activity along with a 3´-5´ exonuclease activity that enables replication and proofreading and iii) the DNA binding protein (DBP) that stabilizes ssDNA from degradation, helps with stabilization of the replication machinery and facilitate unwinding of the DNA helix. These viral proteins are able to initiate replication of the viral DNA on their own, but with help from the host cell transcription factors NFI and OCT1 the rate of initiation is substantially enhanced (*187, 188*). After initiation of replication the adenovirus polymerase separates from TP and chain elongation continues with the polymerase, DBP and cellular topoisomerase I (*7, 186*).

Adenovirus assembly and release

Replication of the viral DNA and production of large amounts of adenoviral structural proteins is the starting point for virus assembly. Capsid proteins are produced in the cytoplasm and transported into the nucleus where assembly of the virus particle occur. Hexon folding and trimerization in the cytoplasm is a rapid process assisted by the viral L4-encoded 100K protein (*189*) and nuclear import of the hexon trimers is facilitated by pVI (*76*). Penton base and fiber protein assemble individually but join to form a penton complex before nuclear import (*190*). The viral genome together with its associated core proteins is packaged into the capsid in a process requiring viral IVa2, L4-22K and L1 52/55K proteins (*7, 191, 192*). Final maturation of the provirions into infectious virions require cleavage of the structural precursors pVI, pVII, pVII, TP and μ by the adenovirus protease (*7*). Release of mature virions from the cell occurs upon lysis of the cellular membrane. This process is facilitated by several systems including viral protease cleavage of cellular cytokeratin, which destabilizes the intermediate filaments rendering the cell more susceptible to lysis (*193*), and the E3 11.6 kD protein, also referred to as the "adenovirus death protein", which kills the cells as it accumulates and promote cell lysis (*194*). As mentioned before, viral spread from the tissue can be enabled by excess production and secretion of fiber proteins that disrupt intercellular CAR homodimers and thereby interfere with tight junctions (*109*).

Clinical applications

HAdVs have been used extensively as vectors for multiple applications, including vaccination (*195-197*), cardiovascular applications (*198*) and treatment of cancer (*199, 200*). The advantages of using vectors based on HAdVs include the ability of the virus to infect a wide variety of cell types, both dividing and non-dividing, and the possible incorporation of large transgenes into their genome without risk of subsequent integration into the host genome. HAdVs are considered to be safe vectors due to the relatively mild diseases caused by these viruses and their well characterized genome. They are also easily produced in high titers, making them suitable for industrial scale productions. Most HAdV vectors are based on HAdV-5 and they comprise the majority of vectors used in gene therapy trials worldwide (*201*). However, decades of research have identified certain challenges when using HAdV-5 based vectors, which have limited their clinical applications (**Table 2**). The pronounced liver accumulation of vectors after intravascular administration due to interactions with coagulation factors causes severe off-target effects and reduced bioavailability (*48*). Another drawback is the global, high prevalence of neutralizing antibodies against HAdV-5 (and many other common HAdV types) in the human population, which rapidly eliminate systemically-delivered vectors and thereby severely hamper the therapeutic efficacy (*202*). Poor access to the HAdV-5 receptor CAR also limits transduction of target cells/organs (*203*). However, these problems cannot simply be solved by increasing the vector dose for intravenous administration since it was shown to overwhelm the innate immunity, resulting in systemic cytokine shock and death of a patient (*204*). Therefore other strategies have been developed to overcome the problems with HAdV-5 based vectors. Coating of the capsid with polymers, such as polyethylene glycol (PEG), reduces recognition by host neutralizing antibodies resulting in increased half-life in the blood and decreased toxicity (*205, 206*). The majority of these neutralizing antibodies have been shown to be directed against the HVRs on hexons, leading to development of chimeric HAdV-5 vectors with HVRs from a different AdV type, which was sufficient to bypass

neutralization *in vivo* (*59, 207, 208*). Pseudotyping of the whole hexon of HAdV-5 vectors with the hexon of HAdV-3 was also shown to diminish interactions with coagulation factors, thereby reducing liver accumulation and increasing bioavailability of the vector (*209*). To increase transduction efficiency in target cells with low or no CAR expression HAdV-5 vectors have been pseudotyped with fibers from other AdVs which use different cellular receptors (*210-212*). A simpler way of overcoming the problems with HAdV-5 based vectors is construction of vectors based on less common HAdVs or even non-human AdVs. The advantages with this approach include lack of pre-existing neutralizing antibodies, use of other cellular receptors than CAR and hopefully inability to interact with coagulation factors. Therefore the research community has started to exploit the whole *Adenoviridea* family, which has resulted in a number of novel vectors based on AdV types with different human and non-human origins (reviewed in (*213*)).

Table 2. Summary of research performed with HAdV based vectors.

Advantages

+ Efficiently transduce both dividing and non-dividing cells

+ Large packaging size

+ Easy to produce in high titers

+ No risk of transgene integration into the host genome

Drawbacks (with HAdV-5 based vectors)

- High prevalence of neutralizing antibodies in the population

- Off-target effects on the liver due to interactions with coagulation factors

- Poor access to CAR on target cells

Strategies to overcome drawbacks (with HAdV-5 based vectors)

**** Coating the viral surface with polymers to escape neutralizing antibodies***

**** Capsid pseudotyping***

**** Ablation of motifs involved in unintended binding***

**** Use of vectors based on less common HAdV types/ non-human AdV***

Adenovirus vector development

The first vectors based on HAdVs, known as first generation vectors (FGV), have foreign DNA inserted to replace the whole E1 region and sometimes also the E3 region to increase the transgene insertion capacity (*214*) (**Figure 7**). Deletion of the E1 region render the vectors replication incompetent and production must be carried out in E1-complementing cell lines (*215*). Although these vectors cannot replicate *in vivo*, the FGVs still contain all remaining viral genes and residual expression of these genes target the transduced cells for elimination by the adaptive cellular immune response, resulting in loss of therapeutic transgene expression (*216*).

In an attempt to decrease the immune response and increase insertion capacity, a second generation of HAdV vectors (SGV) were constructed. SGVs lack all the early transcript regions, E1-E4 (*214, 216*). However, the strong induction of anti-vector immune response and thereby early clearance of transduced cells remained a problem with SGVs (*214*). The additional failure to develop a suitable cell line for large-scale production hampered further development of SGVs (*217*).

The third (and latest) generation HAdV vectors (TGV) are depleted of the entire viral genome except for elements needed for genome replication (ITRs) and encapsidation (Ψ). These vectors are known as "gut-less", "high capacity vectors" or "helper-dependent HAdV vectors" (HD-AdVs) (*214, 216*). Removal of all viral genes enable tremendous insertions of transgenes up to 36 kb. However, these vectors cannot be produced on their own and is dependent on a helper virus (HV) for propagation, thus the term "helper-dependent HAdV vectors". The HV must replicate normally and express all of the viral proteins needed to replicate and package the HD-AdV genome. A major issues with this procedure is contamination of HV in the final preparations of HD-AdVs, therefore several strategies have been developed to reduce this contamination (*217*). HD-AdVs lack all viral genes and thereby do not produce immunogens in the host, i.e. they will not activate the adaptive cellular immune response. Thus, HD-AdVs have been shown to enable long term transgene expression in target cells. However, the capsid is

still identical to the wild type virus and HD-AdVs can therefore still face problems with neutralizing antibodies (*216*).

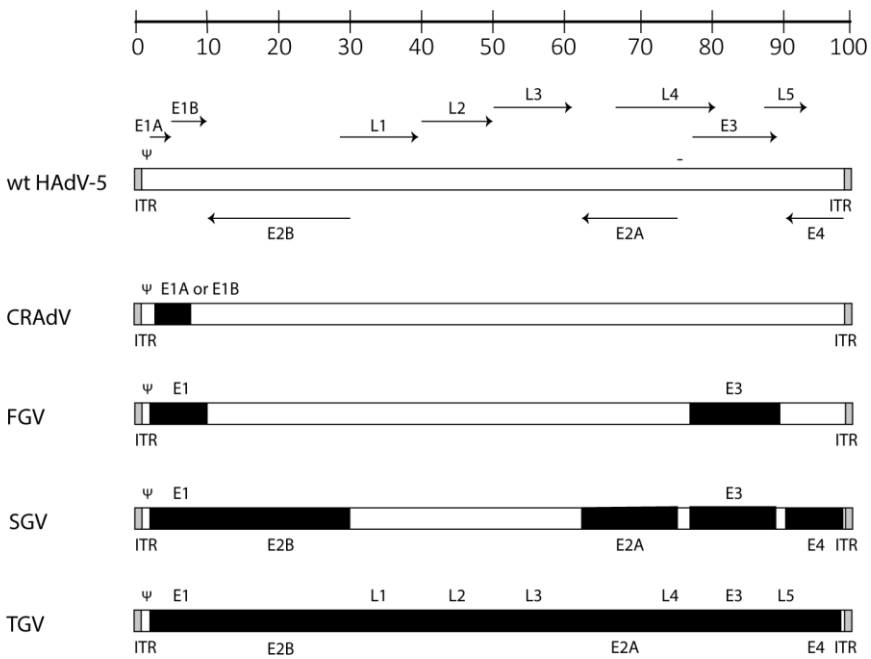

Figure 7. Schematic representation of wild-type HAdV genome and different generations of HAdV based vectors. Genomes are divided into 100 map units and deletions in the different generations of HAdV vectors are shown in black.

CRAd=conditionally replicating adenovirus, FGV=first generation vector, SGV=second generation vector, TGV=third generation vector, ITR=inverted terminal repeat, Ψ=encapsidation signal

A different type of HAdV-based vectors are the so-called "conditionally replicating adenoviruses" (CRAds), which have been used as an alternative therapeutic strategy for cancer. CRAds are recombinant adenoviruses that can selectively replicate within and kill cancer cells. The selectivity for cancer cells can be achieved in different ways. One way is by making a deletion in the E1a region, which abolish interactions between the E1A protein and the retinoblastoma protein (Rb). This restricts viral replication to tumour cells with abrogated Rb levels without affecting normal cells (*218*). Similarly, deletion of the E1B-55K gene prevents the vector from replicating in cells with an active p53, but when it infects cancer cells with an abnormal p53 function, it replicates and causes cellular lysis. The E1B-55K deletion has been used in ONYX-015, a clinically approved CRAd for treatment of head and neck squamous cell carcinoma in China (*199, 219*). Another way to achieve selectivity is by encoding the E1 gene under the control of tissue/tumour-specific promotors or enhancers (*220*).

The ability of CRAds to selectively replicate in cancer cells provides several advantages including increase of the input dose of the virus, high expression levels of therapeutic transgenes, and transmission of vectors to adjacent tumour cells. Oncolytic Ads can kill the cancer cell by direct lysis as a natural step of the replication cycle, but they can also express cytotoxic proteins (*221, 222*), activate anti-tumor immune responses (*223, 224*), and sensitize tumour cells to chemotherapy (*225*).

Treatment of glioma - an example of HAdV-based cancer therapy

Malignant glioma is a type of cancer with tumours formed in the brain or spine and encompasses several types of glial cell-based tumours, including anaplastic astrocytoma, glioblastoma multiform (GBM), mixed oligoastrocytoma and anaplastic oligodendroglioma. They are all high grade tumours and classified by the World Health Organization as either grade III (all but GBM) or grade IV (GBM).

GBM is the most common primary malignant brain tumour and the current standard of care includes maximal surgical removal, radiotherapy

and simultaneous chemotherapy with temozolomide (*226*). However, even with treatment the median survival is only between 12-15 months (*227*). There is therefore an urgent need for novel therapeutic approaches to combat this devastating disease. In recent years, a number of adenovirus-based therapies have been developed, including adenoviral vectors for gene delivery and the use of modified oncolytic adenoviruses (reviewed in (*228*)).

An approach exploited for treatment of GBM is construction of Ad vectors carrying a conditional "suicide gene". These gene encodes enzymes that convert nontoxic compounds into cytotoxic molecules. In the case of GBM treatment, the "suicide gene" most commonly used is the herpes simplex virus type 1-thymidine kinase (TK) (*229*). TK phosphorylates the prodrug ganciclovir (GCV) to GCV-monophosphate, which is further phosphorylated by cellular kinases to yield GCV-triphosphate. GCV-triphosphate becomes incorporated in the duplicating DNA leading to DNA chain termination and inhibition of the DNA polymerase (*221*). GCV can diffuse freely over the cell membrane, but will only be converted to the cytotoxic GCV-triphosphate in the transduced cancer cells expressing TK. GCV-triphosphate is highly charged and cannot diffuse over membranes but can access neighbouring cells trough gap junctions thereby inducing cell death in non-transduced proliferating cells surrounding the infected cell, while leaving healthy non-dividing neurons unaffected (*228*). TK and GCV have also been shown to make GMB tumour cells more sensitive to radiotherapy and chemotherapy (*225*).

Result and discussion

Papers I and II

Paper I

Coagulation factors IX and X enhance binding and infection of adenovirus types 5 and 31 in human epithelial cells.
Jonsson M, Lenman A, Frangsmyr L, Nyberg C, Abdullahi M, Arnberg N.
J Virol. 2009;83(8):3816-25.

Paper II

Coagulation factor IX mediates serotype-specific binding of species A adenoviruses to host cells.
Lenman A, Muller S, Nygren MI, Frangsmyr L, Stehle T, Arnberg N.
J Virol. 2011;85(24):13420-31.

HAdVs can bind directly to several different cellular receptors in a type- and species-specific manner. They have also been shown to utilize soluble host components in body fluids for indirect binding to cells. Coagulation factors, lactoferrin and DPPC are all secreted host components that are known to interact with HAdV particles and mediate cellular attachment and internalization (*48, 163, 164*). This lead us to investigate the possibility that additional soluble components in various body fluids may influence cellular attachment and effect HAdV tropism more than previously recognized.

In paper 1 we therefore evaluated the effect of four different body fluids: plasma, tear fluid, breast milk and saliva, on HAdV infection of human epithelial cells representing the ocular and respiratory tropism of multiple HAdVs. A representative HAdV member from each species was included in the study. We found that saliva, tear fluid and plasma increased infection of HAdV-5 (species C) and that plasma increased infection of HAdV-31 (species A).

We further showed that the plasma-mediated increase in binding and infection was due to interactions between virions and coagulation factors. FIX and FX increased HAdV-5 binding to and infection of human epithelial cells while FIX alone increased binding to and infection of HAdV-31. We also showed that this increase in binding and infection was dependent on divalent cations and that only 1% of the physiological concentrations of these factors were required for maximum binding. FIX- and FX-dependent HAdV-5 binding have previously been shown to be mediated through interactions with cell surface heparan sulfate on hepatocytes (*48, 160*). By performing blocking studies with soluble heparin and treating the cells with the heparan sulfate-cleaving enzyme, heparinase I, we showed that this was also the case on epithelial cells. However, while FIX-mediated HAdV-31 binding and infection was completely reduced with soluble heparin, no effect at all was seen after heparinase I treatment, leaving the role of heparan sulfate for the HAdV-31:FIX complex unclear.

In paper 2 we extended the study of coagulation factor-mediated HAdV binding and infection to include all HAdVs belonging to species A; HAdV-12, -18 and -31 along with HAdV-5 (species C). We also aimed to characterize the HAdV-31:FIX interaction in more detail and determine the cellular receptor used by this complex.

Out of the four structurally similar vitamin K-dependent coagulation factors investigated, only FIX increased HAdV-18 and -31 binding to and infection of human epithelial cells representing the respiratory and gastrointestinal tropism of these HAdVs. FIX promoted HAdV-31 binding and infection more efficiently than HAdV-18 in all experiments, and no effect could be observed for HAdV-12. Using surface plasmon resonance we showed that FIX interacted with the hexon protein of HAdV-31 with an affinity quite similar to that of HAdV-5 and FIX/FX. However, the half-lives of these complexes were profoundly different, with the HAdV-31:FIX complex being 10 times more stable than the HAdV-5:FIX/FX complexes.

FIX- and FX-mediated binding of HAdV-5 to cells have been shown to depend on either cell surface heparan sulfate-containing GAGs or the low

density lipoprotein receptor-related protein (LRP) both *in vitro* and *in vivo* (*48, 160, 161*). Using cells expressing or lacking specific GAGs or LRP we could exclude LRP from serving as a cellular receptor for the HAdV-31:FIX complex. From additional studies using soluble GAGs and GAG-cleaving enzymes we determined that both HAdV-31:FIX and HAdV-5:FIX/FX complexes bound to heparan sulfate-containing GAGs on target cells. However, we could also see a difference in GAG dependence and specificity between these complexes.

In these two studies we observed a significant difference between the abilities of HAdV-31 and HAdV-5 to utilize coagulation factors for binding to target cells. HAdV-5 was able to utilize both FIX and more efficiently FX, for binding and infection, while HAdV-31 could only utilize FIX. The HAdV-31:FIX interaction exhibited an overall higher affinity than the HAdV-5:FIX/FX complexes but more surprisingly, a 10 times longer half-life. Besides a difference in interaction between virus and coagulation factors we also observed a variance in binding to cellular heparan sulfate-containing GAGs. Heparinase I-treatment of cells efficiently decreased the binding of HAdV-5:FIX/FX but did not affect HAdV-31:FIX binding, while heparinase III-treatment of cells efficiently reduced the binding of all complexes. Heparinase I preferentially cleaves glycosidic bonds in regions that are highly sulfated, specifically bonds between N-sulfated glucosamine and 2-O-sulfated iduronic acid, which are less common in heparan sulfate (*230-232*). Heparinase III cleaves less sulfated domains in the linkages between N-sulfated or N-acetylated glucosamine and glucuronic acid (*233, 234*). These motifs are relatively common in heparan sulfate, and therefore, heparinase III cleaves heparan sulfate more efficiently than heparinase I (*234*). Furthermore, FIX/FX-mediated HAdV-5 binding to cells with different GAG expression was completely abolished both in cells specifically lacking heparan sulfate and cells lacking all GAGs, while FIX-mediated HAdV-31 binding to the same cells was significantly reduced but not abolished. Taken together we concluded that heparan sulfate-containing GAGs are of importance for cellular binding of both HAdV-5:FIX/FX and HAdV-31:FIX

complexes. However, the difference in binding between the complexes after heparinase I/III treatment suggest a specificity in heparan sulfate binding where HAdV-31:FIX seem to prefer less sulfated domains whereas HAdV-5:FIX/FX binding is less specific. Another possible explanation for the difference in heparan sulfate binding could be the longer half-life of the HAdV-31:FIX complex compared to the HAdV-5:FIX/FX complexes, which could make the HAdV-31:FIX complex less sensitive to decreased amounts of heparan sulfate on the cell surface. This could explain why heparinase I, which does not cleave heparan sulfate as efficiently as heparinase III, failed to affect the HAdV-31:FIX binding while completely abolishing HAdV-5:FIX/FX binding. It would also explain the ability of HAdV-31:FIX to show a reduced, but still significant, binding to the GAG-lacking cells. These cells still retain approximately 10% expression of GAGs compared to control cell lines, which could in theory be enough to mediate HAdV-31:FIX binding but not HAdV-5:FIX/FX binding.

In paper 2 we also compared the ability of species A HAdVs to interact with coagulation factors. Species A HAdVs normally cause infections involving the airways and/or the intestine. The infections are often mild but species A HAdVs in general, and HAdV-31 in particular, have over the last decades become a rising threat for immunocompromised patients where they cause severe and life threatening infections (*20-23*). Out of the three species A HAdVs, HAdV-31 was the one that showed the strongest increase in FIX-mediated binding and infection. The concentration needed for this increase was also significantly lower than the concentration needed for HAdV-18. Since HAdV-31 is able to cause disseminated disease in immunocompromised patients (*23, 235*) it is tempting to speculate that the ability of HAdV-31 to efficiently utilize FIX could in part explain this phenomenon. Once in the circulation HAdV-31 would have unlimited access to FIX which it could use to infect cells throughout the body thus causing life-threatening multiorgan infections. If this is the case the high affinity interaction between FIX and HAdV-31 could constitute a target for development of novel antiviral drugs.

Coagulation factors were identified as mediators of HAdV-5 infection when trying to explain the pronounced liver accumulation upon systemic administration of HAdV-5 based vectors (*48, 160, 161*). Since then, large attention has been drawn to characterize the specific interactions between HAdV-5, coagulation factors and the cellular receptors used by the complex, in order to understand how coagulation factors affect the native tropism of HAdVs and to optimize vector design (*213*).

HAdV-5 is mainly recognized as a respiratory pathogen while HAdV-31 is known to cause both respiratory- and gastrointestinal infection. Yet both of them have evolved the ability to interact efficiently with coagulation factors, components that are mainly found in blood. This ability is not restricted only to HAdV-5 and -31, since many other HAdVs have been reported to interact with FIX and FX (*48, 236*). Coagulation factors can be found in other tissues besides blood, though at unknown concentrations. Upon inflammation or tissue damage they can be found in the respiratory mucosa due to exudation or direct production by bronchial cells (*237, 238*). We have shown here that as little as 1% of the blood concentration of FX and FIX is enough to mediate HAdV-5 and HAdV-31 infection, indicating that coagulation factors may also be of importance during HAdV primary infections of their natural target cells.

Interestingly, it has recently also been shown that FX binding to hexons can shield HAdV-5 from neutralizing IgM antibodies and complement-mediated destruction, thereby improving virus survival (*239*). Taken together, this shows that HAdV:coagulation factor-mediated increase in infection along with the shielding effect is a complex situation, which needs to be carefully considered when designing adenovirus vectors. Inability to interact with coagulation factors would strongly reduce liver accumulation upon systemic administration, thereby significantly increasing bioavailability of the vector and at the same time avoiding the toxicity associated with liver transduction and the possible detrimental side effects that the therapeutic transgene expression could have in the liver. But at the same time, inability to interact with coagulation factors would leave the viral particle unprotected against neutralizing antibodies and complement-mediated destruction,

which might lead to HAdV neutralization and reduced target transduction. To add to this complex situation, HAdVs can also use other soluble host components, such as lactoferrin and DPPC, which may also influence the tropism and *in vivo* targeting of HAdV-based vectors. Therefore, these molecules and the corresponding HAdV interactions require more intensive studies in order to obtain a complete picture of the attachment mechanisms that regulate the tropism of wild-type HAdVs and HAdV-based vectors.

Papers III and IV

Paper III

Human adenovirus 52 uses sialic acid-containing glycoproteins and the coxsackie and adenovirus receptor for binding to target cells.

Lenman A, Liaci AM, Liu Y, Ardahl C, Rajan A, Nilsson E, Bradford W, Kaeshammer L, Jones MS, Frängsmyr L, Feizi T, Stehle T, Arnberg N. *PLoS Pathog*. 2015;11(2):e1004657.

Paper IV

Human adenovirus 52 short fiber binds to polysialic acid.

Lenman A, Liaci AM, Frängsmyr L, Liu Y, Blaum B, Podgorski I, Harrach B, Benkö M, Feizi T, Stehle T, Arnberg N.

Manuscript

HAdVs are commonly used as vectors for many applications, including vaccination and cancer therapy. These vectors are mainly based on HAdV-5 and the main challenges with these vectors include pre-existing neutralizing antibodies, poor access to the HAdV-5 receptor CAR, and off-target effects on the liver due to interactions with coagulation factors. Other HAdVs devoid of these problems have therefore been investigated for vector development.

HAdV-52 was isolated in 2003 from a small outbreak of gastroenteritis in adults and it was found to be so divergent from all other known HAdVs that it was classified into a new species (species G). HAdV-52 differs from most other HAdVs in that it has two different fiber proteins, one long and one short. HAdV-52 has rarely been isolated from humans and the seroprevalence is supposedly very low in the population, making HAdV-52 suitable as a vector candidate. In paper 3 we set out to identify the receptors used by HAdV-52 in order to better understand its tropism and to improve

targeting of future HAdV-52 based vectors. By performing binding studies using Chinese hamster ovary (CHO) cells expressing or lacking previously known HAdV receptors we found that HAdV-52 used both CAR and sialic acid-containing glycans as cellular receptors. This was further verified on a human epithelial lung cell line by pre-incubating HAdV-52 virions with soluble sialic acid or CAR, or by pretreating the cells with sialic acid-cleaving *V. cholerae* neuraminidase or anti-CAR antibodies, all of which reduced HAdV-52 binding to different extents. Reduction of virion binding was more pronounced by interference with sialic acid interaction versus CAR interaction, suggesting that sialic acid-containing glycans serve as major receptors for HAdV-52 on the cells investigated. We could further pinpoint the sialic acid-binding to occur through the HAdV-52 short fiber knob (52SFK) while the long fiber knob (52LFK) bound to CAR. Surface plasmon resonance analysis revealed a strong affinity of 52LFK to CAR (2.6-5 nM), which is comparable to the affinity of several other CAR-binding HAdVs (*240*). By solving the crystal structure of 52SFK in complex with sialic acid we were able to identify the sialic acid binding site. Surprisingly, the location of this binding site was distinct from those of other structurally-characterized sialic acid-binding AdV fiber knobs (*131, 143*).

The predominance for HAdV-52 binding to sialic acid-containing glycans could not be explained by an incorporation of more short fiber knobs in the viral capsid, since long and short fibers were present in a 1:1 ratio. Neither could it be explained by a low expression of CAR on the cells investigated. The 52LFK:CAR interaction is probably of higher affinity than the 52SFK:sialic acid interaction, still sialic acid-containing glycans seemed to be the major receptor used by HAdV-52 on A549 cells. We suggest that the lack of high affinity binding to sialic acid may be compensated by increased avidity due to multiple interactions between the virion and sialic acid. Another explanation could be that sialic acid creates the initial contact and the remaining glycan chain confer additional interactions with the fiber knob, thereby increasing the overall affinity. Receptor usage may also be a question of accessibility. *In vivo*, CAR localizes to the lateral and basolateral

side of polarized epithelial cells, which are target cells for multiple HAdVs, while sialic acid is abundant on the apical surface. Thus it is plausible that virions approaching non-infected cells from the apical side have access to sialylated glycans, but not to CAR. If this is the case, it would question why multiple HAdVs still bind to CAR through high affinity interactions. A possible explanation for this may be found in the observation that excess fibers are secreted basolaterally, prior to virion release from infected cells, and disrupt CAR-CAR homodimers in the tight junctions. As shown for HAdV-2, this results in increased extracellular space and improved intercellular transport of subsequently released virions (*109*). We speculate that HAdV-52 uses the short fiber to mediate virion attachment to non-infected cells whereas excess of long fibers are secreted from infected cells and facilitate transmission of subsequently released virions within a tissue, or between tissues.

Sialic acid is widely distributed in the human body and is mainly found as terminal residues on glycan structures. In paper 4 we set out to identify the exact structure of the sialic acid-containing glycan used by 52SFK for cellular attachment. Glycan array analysis of 52SFK binding to 128 different sialylated glycans pointed out a group of linear α2,8-linked oligosialic acids as candidate receptors. α2,8-linkage enables formation of long chains of sialic acid residues known as polysialic acid (PSia). ELISA experiments using immobilized *E.coli*-derived PSia (colominic acid) confirmed the ability of 52SFK to interact with PSia while none of the CAR-binding fiber knobs showed any binding at all. 37FK, which binds with relatively high affinity to the branched, disialylated GD1a glycan (*144*), bound less efficiently to PSia than 52SFK. We could therefore conclude that HAdV-52 was able to interact specifically with PSia via the knob domain of its short fiber. Furthermore, 52SFK bound more than five times better to PSia-expressing human neuroblastoma cells compared to PSia-deficient control cells. 52SFK binding to PSia-expressing cells was efficiently reduced by pre-incubating 52SFK with soluble pentameric sialic acid.

With X-ray crystallographic analysis of 52SFK in complex with oligosialic acid we found that 52SFK engage the non-reducing end of PSia by means of the same sialic acid-binding site identified in paper 3. Additional, direct interactions could not be identified, however structural analysis suggested that long-range effects accounted for the increased affinity. In line with this, an inspection of the electrostatic potential of the 52SFK identified a positively charged rim, which we termed the 'steering rim', located around the sialic acid binding site that may be able to attract the second, third and fourth sialic acid moiety of PSia, thereby adding to the overall affinity.

The PSia-binding RGN motif along with the amino acids constituting the steering rim are conserved in the short fiber knobs of other species HAdV-G AdVs; SAdV-1, -2, -7 and -11 as well as SAdV-19, hypothetically giving these AdVs the ability to interact with PSia. We therefore evaluated the binding of the SFKs from these HAdVs to PSia-expressing/non-expressing cells. Indeed SAdV-1, -7, -11, and -19 but not SAdV-2, bound more efficiently to PSia-expressing cells compared to control cells. However, 52SFK still displayed the strongest discrepancy between PSia-expressing and PSia-deficient cells, indicating a more specific interaction of 52SFK with PSia rather than a general high binding to both cell lines as seen for S-7SFK.

Polysialylation is an unusual posttranslational modification that occurs mainly on the neuronal cell adhesion molecule (NCAM). In humans, PSia is mainly found on NCAM during embryogenesis, but is close to absent in healthy adults (*241-243*). It is currently not known why HAdV-52 would have evolved the ability to interact with a receptor that is rarely found in its main host. PSia is however present on bacteria in the gut microbiota (*244*), and with the recent indications for an interplay between commensal bacteria and viruses to cause infections in the gut (*245, 246*), it might be so that PSia on bacteria is of importance for the assumed gastroenteric tropism of HAdV-52.

PSia can also be highly expressed in some types of cancer such as glioma (*247-249*), neuroblastoma (*250, 251*) and lung cancer (*252, 253*). PSia expression is often associated with higher tumor invasiveness and poor

prognosis (*249, 254*). There is therefore an urgent need for new treatments to combat this devastating disease. We have shown that HAdV-52 interacts specifically with PSia and taking into account that HAdV-52 most likely has a low seroprevalence in the population and is unable to interact with coagulation factors we believe that HAdV-52-based vectors could be useful for treatment of cancer types with elevated PSia expression.

Concluding remarks

Human adenoviruses have previously been shown to use soluble host components in body fluids, such as lactoferrin and coagulation factors, for indirect binding to target cells, a feature that enables more efficient infection. Here we have added to this knowledge by characterizing coagulation factor IX and X interactions with HAdV-5 (species C) and HAdV-31 (species A) and determined the implications of these interactions for infection of human epithelial cells, which represent the natural tropism of these viruses. We further showed that as little as 1% of the physiological concentration of these factors was enough to reach maximum increase in binding, suggesting that coagulation factors can aid in infection at other locations in the body where their concentration is much lower than in the circulation, and thus play an important role for the natural tropism of these viruses.

Subsequent studies showed that FIX-mediated increase in infections caused by species A HAdVs was most pronounced for HAdV-31 followed by HAdV-18, while no effect was seen for HAdV-12. Species A HAdVs, and especially HAdV-31, have become a problem in the latest decades since they cause life-threatening disseminated disease in immunocompromised patients. The ability of HAdV-31 to utilize FIX for more efficient infection might be a possible reason to why HAdV-31 cause severe infections in these individuals.

In the same study affinity measurements showed a similar affinity for the HAdV-31:FIX and HAdV-5:FIX/FX complexes, however, the HAdV-31:FIX complex harbored a ten times longer half-life, which could potentially explain the difference in binding to cellular heparan sulfate seen with these complexes. We also suggest that the high affinity interaction between HAdV-31:FIX may constitute a target for antiviral development.

In another study we characterized the receptors used by the newly identified HAdV-52 equipped with two fiber proteins: one long and one short. We

showed that HAdV-52 used the long fiber to attach to host cells via CAR, but that most of the binding was dependent on short fiber binding to sialic acid-containing glycans. This lead us to hypothesize an *in vivo* situation where HAdV-52 uses the easily accessible sialic acid-containing glycans as a primary receptor to facilitate infection and that excess production and secretion of long fibers can disrupt hidden CAR-CAR dimers in the tight junctions to facilitate virus escape and spread within and between tissues.

Extended studies to characterize the sialic acid-containing glycans used by 52SFK identified polysialic acid as a potential sialic acid-containing receptor for HAdV-52. PSia is close to absent on cells in healthy adults begging the question why HAdV-52 would have evolved a specificity for this glycan rarely found in its main host. PSia can however be found in the gut microbiota, and given the increasing amount of evidence for an interplay between commensal bacteria and viruses to cause infections in the gut, one could speculate that PSia on commensal bacteria could be of importance for the assumed gastroenteric tropism of HAdV-52.

PSia is also commonly re-expressed on several types of cancers, where it is associated with higher tumor invasiveness and poor prognosis. Taking into account the low seroprevalence of HAdV-52, its inability to interact with coagulation factors and its ability to bind specifically to PSia, we believe that HAdV-52 based vectors could be useful for treatment of cancer types with elevated PSia-expression.

In summary I believe that my results can add to our understanding of the natural tropism of HAdVs and aid in the development of HAdV-based vectors for gene and cancer therapy.

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