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Glycomics and Proteomics Approaches to Investigate Early Adenovirus–Host Cell Interactions

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Abstract

Adenoviruses as most viruses rely on glycan and protein interactions to attach to and enter susceptible host cells. The *Adenoviridae* family comprises more than 80 human types and they differ in their attachment factor and receptor usage, which likely contributes to the diverse tropism of the different types. In the past years, methods to systematically identify glycan and protein interactions have advanced. In particular sensitivity, speed and coverage of mass spectrometric analyses allow for high-throughput identification of glycans and peptides separated by liquid chromatography. Also, developments in glycan microarray technologies have led to targeted, high-throughput screening and identification of glycan-based receptors. The mapping of cell surface interactions of the diverse adenovirus types has implications for cell, tissue, and species tropism as well as drug development. Here we review known adenovirus interactions with glycan- and protein-based receptors, as well as glycomics and proteomics strategies to identify yet elusive virus receptors and attachment factors. We finally discuss challenges, bottlenecks, and future research directions in the field of non-enveloped virus entry into host cells.

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Introduction to virus–host cell surface interactions

Virus infection of host cells is in most cases initiated by one or more specific interactions between capsid proteins of non-enveloped viruses or glycoproteins of enveloped viruses with host cell receptors/co-receptors [1]. In addition, lipids such as phosphatidylserine embedded in the viral envelope can interact with cell surface receptors in a process termed “apoptotic mimicry” (reviewed in Ref. [2]). Knowledge about these molecules and their interactions is on the one hand of importance to understand virus life cycle, tissue tropism, species tropism, and pathogenesis. On the other hand, it can pave the way toward the development of antiviral drugs that interfere with virus binding and entry into cells, and toward the generation of

efficient viral vectors for targeted gene or cancer therapies.

Most viruses use glycolipids, glycoproteins, or proteins as receptors and/or attachment factors. High-affinity interactions are common for binding to proteinaceous receptors that belong, for example, to the protein (super)families of immunoglobulins (Ig), cadherins, integrins, regulators of complement activation, exo-peptidases, ion channels, LDL receptors, chemokine receptors, TNF receptors, and tetraspanins [3]. Interactions with abundant glycans that contain histo blood group antigens (HBGAs), glycosaminoglycans (GAGs), or sialic acids (SAs) are usually of lower affinity, which can be compensated for by avidity-dependent mechanisms, that is, simultaneous virus binding to multiple glycans.

Receptor interactions serve multiple purposes during the virus life cycle ultimately leading to the

delivery of the viral genome to its intracellular site of replication. Specifically, overcoming the cellular barrier of a lipid bilayer and underlying cortical actin is critical for replication of viruses, which are obligate intracellular parasites. In contrast to attachment factors, receptors therefore not only mediate cell attachment but also dictate uptake of virions into the cell, including intracellular trafficking of incoming virions and ultimately—either directly at the plasma membrane or in intracellular vesicles—the penetration into the cytosol [4]. For viruses replicating in the nucleus, capsids need to additionally traffic to the nuclear pore. Generally, the entry process must solve the assembly/disassembly paradox, that is, a virus particle, which is highly stable in the extracellular milieu, must disassemble at the correct intracellular site. Therefore, viruses reside in a metastable state, which is sensitive to cellular triggering mechanisms such as receptor interactions, pH drop, changes in redox state and ion concentrations, and proteolytic processing of viral surface proteins by cellular proteases [4]. One or a combination of these triggering factors then exposes a membrane-perturbing protein or protein domain leading to penetration into the cytosol.

The family of *Adenoviridae* contains more than 80 different human types that are divided into seven species (A–G) (Fig. 1). They have been isolated from secretions such as stool, bronchoalveolar lavage, and tear fluid, and from surgically removed tissues such as adenoids and tonsils. Thus, human adenoviruses (HAdVs) display a wide cell and tissue tropism resulting in infections of the eyes, airways, gastrointestinal tract, lymphoid tissue, urinary tract, and/or liver. In immunocompromized patients, these viruses can cause severe and even lethal, systemic infections [7]. *Adenoviridae* are furthermore of interest as specific types are associated with obesity [8]. Finally, they frequently serve as viral vectors *in vitro* and *in vivo*. We refer the reader elsewhere for a detailed

discussion of adenovirus-based gene therapy vectors [9]. Thus, HAdVs are not only clinically relevant but also serve as a template illustrating the diversity, complexity, and challenges faced by scientists who study virus–receptor interactions. Animal models such as the Syrian hamster model have only been developed to study species C AdV infections; however, other AdV receptor studies are based on cell culture models.

The linear double-stranded DNA genome of AdVs is surrounded by an icosahedral capsid of 90- to 100-nm diameter that contains three major capsid proteins (hexon, penton base, and fiber) and at least eight core and cement proteins [10]. The hexon protein is the largest (*ca.* 100 kDa) and most abundant (240 trimers) capsomer and interacts with the pentameric, coreceptor-binding penton base in each of the 12 corners, from where the trimeric fibers protrude. The fiber length varies significantly between the seven species and terminates with a receptor-binding fiber knob domain (Fig. 2A). According to their length, long-shafted fibers (LSFs) and short-shafted fibers (SSFs) influence their interactions with receptors. A few AdVs carry both LSFs and SSFs, which contain different knobs (See Box 1).

Members of the *Adenoviridae* family as most viruses use either glycans or proteinaceous receptors, or a combination of both for infection of host cells. In addition, they also bind to secretory extracellular molecules, such as coagulation factors and lactoferrin [5]. After engagement of primary receptors, some AdV types enter the cell by clathrin- and dynamin-dependent endocytosis often involving integrins, or by macropinocytosis [11–14]. In the endocytic compartment, the membrane lytic AdV cement protein VI then penetrates the endosomal membrane, thereby inducing endosomal escape [15–18]. Exposure of cement protein VI, which resides underneath the capsid wall prior to cell attachment, is in some cell types induced by drifting

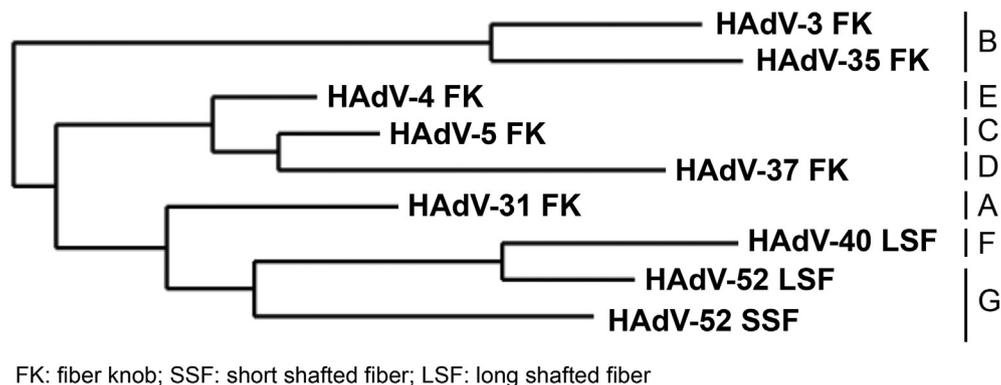


Fig. 1. Selected adenovirus types and their genetic grouping. Phylogenetic tree showing the relationship of selected HAdV fiber knob (FK) sequences. HAdV-31, -3 and -35, -5, -37, -4, -40, and -52 are representing HAdV species A to G, respectively. HAdV FK sequences were obtained from NCBI. The phylogenetic tree was generated by using phylogeny.fr (<http://www.phylogeny.fr/index.cgi>) [6].

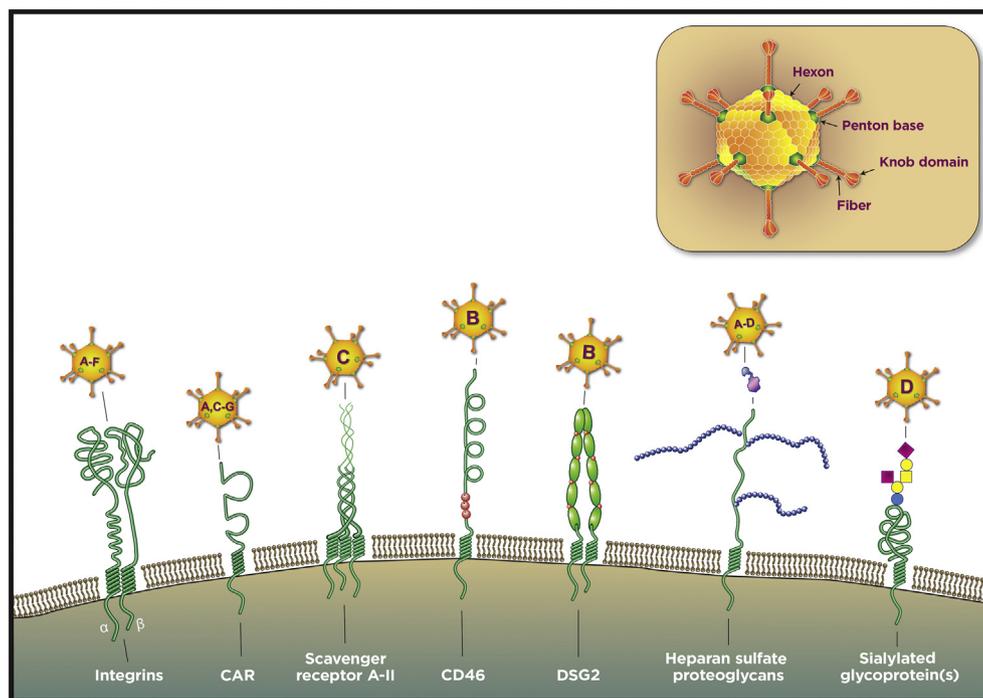


Fig. 2. Adenovirus structure and important adenovirus attachment factors/receptors. (A) The adenovirus particle is 90 to 100 nm in diameter and characterized by icosahedral symmetry with a triangulation number of 25. The capsid proteins critical for cell surface binding are shown. Each of the 240 hexons comprises three identical capsid proteins. Twelve penton bases are located at the vertices. From each vertex a fiber extends and ends in a knob domain. (B) Selection of human adenovirus attachment and entry factors on the cell surface. Structures should be considered as models. The capsid component interacting with the attachment factor is indicated by the connecting line. CAR, coxsackie and adenovirus receptor; DSG2, desmoglein-2; FIX/FX, coagulation factor IX and X. Figure adapted from Arberg [5].

motions of AdV receptors [19]. Interestingly, efficient AdV endocytosis and endosomal membrane piercing requires cellular lipid signaling and membrane repair mechanisms, which the virus itself triggers during cell attachment [15]. In epithelial-like cells, escape of AdVs from endosomes occurs rapidly, that is, 10 min after surface binding, and was thought to be associated with a drop in endosomal pH. However, recent data suggest that escape may be pH independent [20]. In the cytoplasm, AdVs are transported to the nucleus along microtubules [21]. Finally, the nuclear pore disassembles the already partially disassembled particle and shuttles the AdV DNA into the nucleus [22]. In this review, we will primarily discuss early AdV–host cell interactions as well as approaches to identify host determinants of AdV attachment and early entry.

Glycan-based interactions

Interactions with cellular glycans alone are rarely sufficient for entry and productive infection, and are mostly followed by interactions with proteinaceous receptors. However, in many cases, virus–glycan interactions are required to concentrate viruses on the cell surface and bring them into contact with their

proteinaceous receptors. In these cases, both glycan-based attachment factors and proteinaceous receptors are needed for infection. The most commonly used glycosylated attachment factors include SA-containing glycans, GAGs, and HBGAs.

SA-containing glycans

SA-containing glycans are used as attachment factors by human viruses of more than 10 families [23], of which influenza A viruses (IAVs) are the best studied. SAs differ from most other monosaccharides in that they are built by a nine-carbon backbone instead of the common five or six carbons [24]. They usually occupy the non-reducing end of the glycan chain where they are typically linked to galactose units via α 2,3- or α 2,6-glycosidic bonds. Alternatively, they are linked to a neighboring SA via α 2,8-glycosidic bonds. Via glycan chains, SA is conjugated to lipids or to proteins via serines or threonines (*O*-linkages) or to asparagines in the sequon Asn-X-Ser/Thr (*N*-linkages). 5-*N*-acetylneuraminic acid (Neu5Ac) is usually depicted as a prototype SA and is the most abundant SA in humans, but more than 60 different types of SA have been identified in nature. The different SA types are modified mainly by acetyl and glycolyl groups but

Box 1

Definitions and abbreviations in adenovirus entry, proteomics, and glycomics.

Viral attachment protein (VAP): viral surface protein that binds to cellular receptors. VAPs of non-enveloped viruses such as adenoviruses are viral capsid proteins such as penton bases and fibers.

Attachment factor: Enables virus attachment to host cells, but is usually not sufficient for entry and infection.

Receptor: Enables both attachment and entry of viruses into host cells.

Entry factor: Does not mediate attachment, but is needed for entry/infection.

Co-/auxiliary receptor: Facilitates attachment/entry, but is not essential for infection.

Hexon: The main capsomer of adenovirus. Two hundred forty trimers constitute the main surface area of adenovirus.

Penton base: Adenovirus capsid component. Each particle contains 12 of these pentameric proteins, which anchor fiber proteins to the capsid. Needed for entry and endosomal release, but not for attachment.

Fiber: Protein protruding from each of the 12 corners of the icosahedral capsid. Binds to receptors through the terminal knob domain. The length of the fibers varies significantly between adenovirus species.

Mass spectrometry (MS): Spectrometric method used to determine the molecular weight of an organic compound such as a protein-derived peptide. MS is based on the specific mass and charge of a given analyte, which is determined by trapping it in a dynamic electric field.

Proteomics: Large-scale analysis of the sum of all proteins in a biological sample. The term was coined in 1994. Often the term is specifically used for the MS-based analysis of proteins.

Glycomics: Large-scale analysis of glycomes, that is, the complete repertoire of glycans and glycoconjugates on or in a cell, tissue, or other biological sample.

JAM: Junctional adhesion molecule. JAMs belong to the immunoglobulin superfamily. The family of JAM proteins comprises three classical family members and four non-classical members. JAMs are expressed at tight junctions of epithelial and endothelial cells and on the surface of leukocytes, platelets and erythrocytes.

CAR: Coxsackie and adenovirus receptor. A non-classical JAM, which serves as a receptor for Coxsackieviruses and adenoviruses.

also by sulfation, phosphorylation, and so on, which affect the affinities of viral ligands and thus also cell, tissue and host specificity of viruses. Other viruses that have been well studied with respect to their SA-containing receptors include reovirus, polyomavirus, paramyxovirus, and adenovirus [25].

A small subset of species D HAdV types (here represented by HAdV-37) with a pronounced ocular tropism uses SA-containing receptors for adhesion to and entry into human corneal epithelial cells [28] (Fig. 2B). In this case, receptor identification was guided simply by the calculated, unusually high isoelectrical point of the fiber knobs of HAdV-37, and the hypothesis that the receptor would feature a negative charge. SA-cleaving neuraminidase, SA-blocking lectins, and soluble glycans were used to validate SA as a HAdV-37 receptor in human corneal epithelium cell binding and infection. X-ray crystallography analysis of the HAdV-37 fiber knob in complex with a selection of SA-containing glycans revealed that there are three SA-binding sites on the top of the knob, in a highly positively charged cavity. Precisely,

the carboxylic acid group of each SA interacts with a positively charged lysine in the knob cavity [27]. Subsequent glycan array analysis pointed out a preference for a glycan corresponding to that of the SA-containing ganglioside GD1a [28]. This ganglioside carries a hexasaccharide that branches into two arms that both terminate with SA linked via α 2,3-linked glycosidic bonds to neighboring galactose units. Structural analysis revealed that in this context, both SAs bind to the canonical SA-binding site of HAdV-37. Surface plasmon resonance analysis of the interaction revealed an affinity that was higher (μ M level) than for many other viruses, which typically bind to SA with low nM affinity [29,30]. Strikingly, the GD1a ganglioside itself is not used as a receptor. Instead HAdV-37 knobs use SA-containing glycoproteins, as revealed by cell-based assays using, for example, metabolic inhibitors of ganglioside biosynthesis and specific enzymes. Combined with the abundance of SA in secretions where they contribute to lubrication but also as a barrier to pathogens, and the ocular tropism of HAdV-37, these results suggested that SA-containing

compounds may be used as a topical treatment of ocular HAdV infections. Indeed, two types of compounds have been developed and evaluated *in vitro*. The first type comprises larger, protein- or lipid-based compounds with the capacity to aggregate HAdV-37 progeny and thereby limit the number of infectious units in the eye [31,32], and the other type is a smaller, trivalent compound where each SA is designed to fit into the three SA-binding sites of the knob [33,34]. The latter molecules cannot aggregate virions, but can bind virions with high-affinity, block virus attachment and thereby prevent infection at low concentrations (IC₅₀, i.e., the concentration that reduces infection rates to 50% of controls, in low nM range).

Another AdV that also binds to SA is HAdV-52, which is associated with gastroenteritis in humans [35]. HAdV-52 is equipped with two different fibers, SSFs and LSFs. The SSF binds to polysialic acid-containing glycans, but through a different pocket than that in the knob of HAdV-37 [36,37]. In addition, several non-human AdV types can also bind to SA-containing glycans [38–40].

GAGs

GAGs are linear polysaccharides with disaccharide units containing *N*-modified glucosamine or galactosamine and uronic acids or galactose [41]. GAGs have a substantially longer polymer chain than most other glycans. The monosaccharides are sulfated in their 2, 4, 6, and/or *N* positions, which contributes to negative charge in addition to the carboxylic acid groups of the uronic acids. The organization and modifications of the disaccharide units determines the GAG type [heparan sulfate (HS), chondroitin sulfate, keratan sulfate]. HS is the most common GAG-containing attachment factor and is used, for example, by herpesviruses, flaviviruses, parvoviruses, papillomaviruses, HIV, multiple hepatitis viruses, bunyaviruses, and a few AdV types [42,43].

Some members of species B HAdVs, that is, HAdV-3 and -35, which infect the eye, respiratory and/or urinary tract, use GAGs as low-affinity cellular attachment factors [44] in addition to proteinaceous receptors (see below). Proteomics and glycan array-based approaches pointed out HS proteoglycan (HSPG) as a candidate attachment factor, which was validated by cellular assays using enzymatic degradation and GAG-deficient cells. Here, a striking conclusion was that two independent mechanisms of interaction with HSPG exist: Whereas HAdV-3 binds in a conventional mechanism to HSPG via the fiber knob, HAdV-35 binds to HSPG independent of the knob. The relative importance and mechanism of these GAG interactions have not yet been characterized in detail. Species C HAdV-2 and -5 have also been suggested to use HS as cellular receptors [45,46]. In these studies, the viral GAG-binding protein was not identified. However, the shaft domain of the

HAdV-5 fiber contains a putative HS-binding domain, and point mutations of this domain altered HAdV-5 gene transfer [47]. HS also influences HAdV-based gene transfer indirectly since many HAdVs bind via the hexon to blood factors, which in turn mediate binding and gene transfer to hepatocytes via HS [48]. Generally, HS usage may be the consequence of cell culture adaptation of viruses as cell lines typically express high levels of HS [49,50]. Thus, HS dependency needs to be analyzed cautiously including testing of primary virus isolates and HS dependency *in vivo*.

HBGAs and other virus:glycan-based interactions

HBGAs are based on precursor disaccharides that contain Gal, GalNAc, Glc, or GlcNAc linked by different types of glycosidic bonds [51]. The precursors are further decorated by additional monosaccharides, including fucose or SA, resulting in chains that commonly contain three to five monosaccharides. These antigens are expressed on multiple proteins on cells and in secretions, and are well-characterized receptors for gastrointestinal calicivirus and rotavirus [52].

Glycosphingolipids (GSLs) are glycosylated lipids present in the outer leaflet of the plasma membrane [53]. The glycan chain faces the extracellular space and contains up to seven monosaccharide units, which can include HBGAs and SAs. SA-containing GSLs are also referred to as gangliosides. The latter are associated with cholesterol rich membrane microdomains. Generally, GSLs are enriched on the apical side of polarized cells and contribute to, for example, endocytosis. Since SAs and HBGAs are building blocks of these molecules, viruses such as IAV, rotaviruses, and caliciviruses, but also polyomaviruses, parvoviruses, and others can use GSLs as receptors [54].

Interestingly, many enveloped viruses attach to cells using glycosylated molecules on the virus surface, thereby flipping the coin. Specifically, these viruses use transmembrane viral spikes, which are glycosylated by viral or host cell-encoded glycosyltransferases. This results in escape from neutralizing antibodies but also allows binding to host cell lectins, with extended viral tropism as a consequence. Examples of lectins are C-type lectins such as DC-SIGN, which is expressed on dendritic cells and contributes to cell–cell adhesion [55]. DC-SIGN and other C-type lectins bind to mannose-containing glycans that commonly decorate viral spikes [56]. While several viruses have been reported to interact with C-type lectins, a clear role in cell entry has mainly been demonstrated for phleboviruses, dengue virus, and West Nile virus [57].

Protein-based interactions

In addition to glycan-based cell surface interactions, AdVs bind to several proteinaceous receptors including

coxsackie and adenovirus receptor (CAR), integrins, CD46, desmoglein-2, and scavenger receptors (SRs) (Fig. 2B). Here, we will review the most prominent protein receptors for AdVs.

CAR

CAR is a 46-kDa transmembrane protein, which belongs to the junction adhesion molecule (JAM) family within the immunoglobulin (Ig) superfamily [58–60]. CAR consists of an extracellular domain with two Ig-like domains (D1 and D2), a transmembrane region and a cytoplasmic tail [59,61]. The physiological function of CAR is to promote cell–cell adhesion by forming homodimers through the Ig-like domains [60]. The receptor is expressed in many organs including the brain, heart, lung, intestine, pancreas, liver, and kidney, which indicates that CAR possesses an important physiological role in several organs *in vivo* [62,63]. CAR was first identified as a receptor for coxsackie B viruses and species C (serotypes 2 and 5) AdVs [59,61] and subsequently shown to play a role as an attachment protein for species A, D–G AdVs, but not for species B AdVs [36]. On the virus side, the knob domain of the viral fiber protein is mediating binding to CAR [64]. The crystal structure of the AdV knob domain and CAR revealed that surface-exposed loops of the knob and the Ig-like domain D1 of CAR form a high-affinity complex [65]. In fact, the soluble D1 domain of CAR alone is sufficient for knob binding and leads to an inhibition of AdV infection *in vitro* [66]. In dilated cardiomyopathy patients, a positive correlation between AdV load and CAR expression was observed [67]. Another function of the fiber–CAR interaction, in addition to cell attachment, is to promote escape of newly assembled and released virions from the site of infection. This is achieved by production and release of a large excess of free fiber protein from AdV-infected airway epithelial cells. These free fiber proteins bind to intercellular CAR homodimers, thereby interrupting cell–cell adhesion and facilitating AdV escape from the apical surface [68].

Integrins

Integrins are cell adhesion molecules that—similar to CAR—mediate heterodimeric cell–cell interactions with other cell adhesion molecules, but they interact also with extracellular matrix components such as collagens, laminin, and fibronectin. All integrins form heterodimers of α and β subunits. In vertebrates 18 α and 8 β subunits, which form 24 different known heterodimers, are characterized. They are found in a variety of cells with most cells expressing distinct sets of integrins (reviewed in Refs. [69–71]). Under rare conditions, integrins serve as attachment factors, but primarily, they mediate internalization and membrane

penetration [14]. The HAdV-2 penton base interacts with $\alpha\text{M}\beta\text{2}$ integrin in hematopoietic cells *in vitro* [72]. The interaction is mostly mediated by the conserved Arg–Gly–Asp (RGD) motif on the penton base protein that is expressed in all HAdV members except those belonging to species F, which undergoes a delayed uptake in epithelial cells [73]. Mutations in the RGD motif of HAdV-2 virus penton base lead to a delayed virus reproduction in flat adherent cells [74]. Other integrins including $\alpha\text{V}\beta\text{1}$ [75], $\alpha\text{V}\beta\text{3}$, $\alpha\text{V}\beta\text{5}$ [76,77], and $\alpha\text{3}\beta\text{1}$ [78] are also used as AdV co-receptors. The integrins $\alpha\text{V}\beta\text{3}$ and $\alpha\text{V}\beta\text{5}$ both promote virus internalization, while $\alpha\text{V}\beta\text{5}$ is also involved in membrane penetration [77].

CD46 and desmoglein 2

CD46, also known as membrane cofactor protein (MCP), is expressed on all nucleated cells and belongs to the family of regulators of complement activation. The main function is to protect uninfected cells from complement attack [79]. CD46 consists of four extracellular short consensus repeats (SCRs), followed by a serine, threonine, proline-rich domain (STP), a small domain of unknown function, a transmembrane domain and a cytoplasmic tail [80]. For most species B HAdVs, which do not bind CAR, CD46 was shown to function as a cellular receptor [81,82]. Of note, other viruses including measles virus use CD46 as receptor [83,84]. For AdVs, mutational analysis and infection assays demonstrate that the N-terminal SRC domains 1 and 2 comprise the AdV fiber binding site. In addition, the crystal structure of HAdV-11 fiber knob in complex with SRC domains 1 and 2 of CD46 confirmed this interaction and revealed that the binding leads to a conformational change of CD46 [85,86]. Moreover, surface expression of CD46 is downregulated early in AdV infection through interactions with the AdV fiber knob [87,88]. Several studies showed that CD46 functions as a cellular receptor for all species B AdVs except HAdV-3 and -7 *in vitro* [81,82,89]. In contrast, Sirena *et al.* [90] reported that CD46 serves as a receptor for HAdV-3 in CD46-expressing human and rodent cells. In line with this, Trinh *et al.* [91] demonstrated that HAdV-3 and -7 use CD46 for avidity dependent attachment, and that increasing levels of CD46 lead to enhanced HAdV-3 and -7 infection. While some species B AdVs use CD46 as their main receptor [92], desmoglein-2 (DSG2), a protein that belongs to the cadherin superfamily, was identified as main receptor for HAdV-3, -7, -11, and -14 [93]. Unlike for CD46 interactions, high-affinity binding to DSG2 requires both penton base and fiber protein [93,94].

SRs

SRs belong to a large group of membrane-bound receptors and have been classified, according to their sequence, into 10 families (A–J) [95]. The first SR was

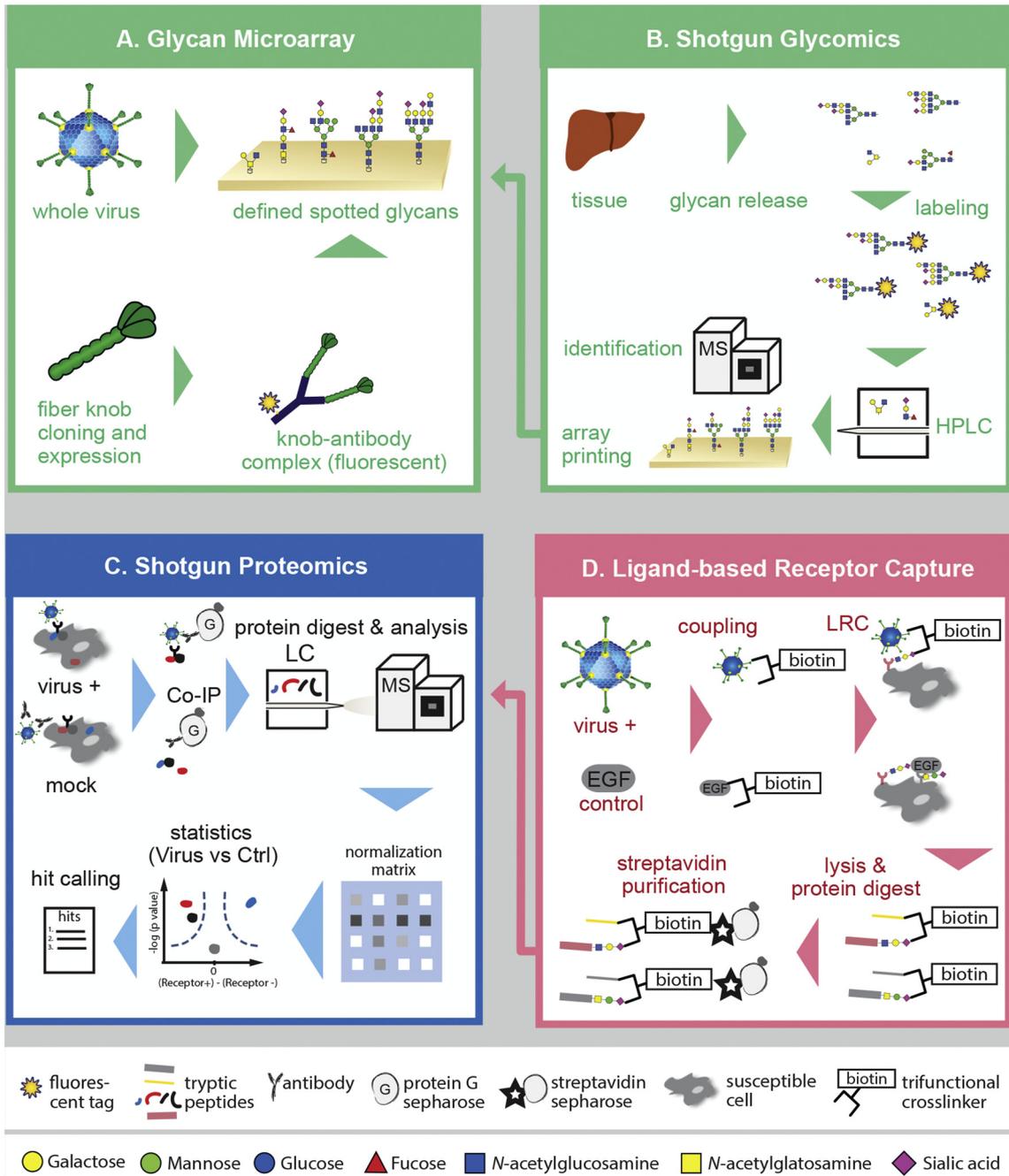


Fig. 3. Methods to identify early virus–host cell interactions. (A) Classical glycan array with synthetical glycans printed on a microarray plate. The array is probed either with purified whole virus particles, virus like particles or VAPs, such as the fiber knob domain of adenoviruses, and binding determined by fluorochromes conjugated to the probe or a probe-binding antibody. (B) Shotgun glycomics. Tissue homogenates are used to extract glycans, fluorescently label them and separate them by liquid chromatography (LC) before spotting the glycans on a microarray. The nature of each glycan can be determined by mass spectrometry (MS) while virus binding experiments are performed as in panel A. (C) Shotgun proteomics of co-immunoprecipitated virus–receptor complexes. Virus is cold-bound to cells and virus attachment protein–receptor complexes purified from cell lysates by affinity enrichment. Purified proteins are then identified and quantified by peptide fingerprinting and label-free interaction proteomics. (D) Ligand-based receptor capture uses specifically designed trifunctional organic compounds such as TRICEPS. These first react with free amines of virus surface proteins such as the fiber knob of adenoviruses and then crosslink glycosylated cell surface receptors by a hydrazine reactive group. Finally, a biotin group allows for efficient capture of receptor peptides after tryptic digest of cell lysates using streptavidin (SA) resins. Captured peptides are identified and quantified by MS as in panel C.

described by Goldstein *et al* [96] and named after its ability to bind modified low-density lipoproteins. In addition to lipoproteins, SRs also recognize ligands such as proteoglycans, ferritin, carbohydrates, cholesterol ester, and a variety of pathogens [95]. In AdV-based vector applications, SRs mediate virus uptake and accumulation in the liver. Specifically, liver-resident Kupffer cells clear AdV-based vectors after systemical administration. The interaction with scavenger receptor A (SR-A) was shown to be responsible for liver uptake [97,98]. Mutational analysis of AdV capsid proteins and *in vivo* administration in mice revealed that the SR-A interaction is mediated by the hypervariable regions of the AdV hexon protein [99]. In addition, Piccolo *et al.* [99,100] demonstrated that helper-dependent adenoviral vectors interact with SR-A and SR expressed on endothelial cells *in vitro* and *in vivo*. Moreover, the SR MARCO was shown to facilitate AdV infection of murine alveolar macrophages [101].

Other receptors

In addition to the above-outlined receptor interactions, AdVs have also been described to interact with CD80 and CD86, vascular cell adhesion molecule-1 (VCAM-1), and the $\alpha 2$ domain of the major histocompatibility complex 1 (MHC-I). CD80 and CD86 are expressed on the cell surface of human dendritic cells and mature B lymphocytes [102]. Species B AdVs use CD80 and CD86 as receptors and the virus knob domain is required for the interaction [103]. VCAM-1, like CAR, belongs to the immunoglobulin superfamily and consists of an extracellular domain with seven Ig-like domains, a transmembrane region and a cytoplasmic domain. The main function of VCAM-1 is to act as an endothelial receptor for leukocytes [104]. Homology analysis showed that domain 7 of VCAM-1 and domain 1 of CAR, which are critical for AdV attachment, have significant homology. These findings are in line with more efficient AdV-mediated gene transfer in VCAM-1 expressing cells compared to VCAM-1-negative cells [105]. Finally, MHC class-I molecules, which are broadly expressed on all nucleated cells, act as receptors for HAdV-5 through their $\alpha 2$ domain. HAdV-5 fiber knob binds to MHC-I $\alpha 2$

and MHC-I $\alpha 2$ expression increases susceptibility to HAdV-5.

Taken together, a wide range of cellular protein receptors for AdVs have been described, most of them for species B and C AdVs. Some of these, for instance CAR, function as receptors for AdV members of multiple species, while others are rather specific. Given the vast number of AdV species and types, several as-of-yet unknown protein receptors likely exist. In the following paragraphs, we will discuss possible methodological advancements, which may aid in discovery of current orphan AdV receptors.

Large-scale approaches to study early virus–host cell interactions

Glycan arrays and shotgun glycomics

Glycan arrays are high-throughput assays that allow for rapid identification of protein binding glycans (Fig. 3A). These arrays have been used to identify virus attachment factors and receptors and to determine the specificity of virus–glycan interactions (Table 1). The first glycan-based arrays were based on immobilization of microbe-derived polysaccharides on a nitrocellulose-coated glass slide [112]. Alternatively, smaller glycans can be chemically linked to amino-lipids resulting in neoglycolipids [113] and printed [114]. Importantly, clustered neoglycolipid printing allows for the analysis of low affinity, avidity-dependent interactions [115,116]. Another variation is the immobilization of cyclopentadiene-conjugated monosaccharides on gold-coated glass slides containing benzoquinone and pentaethyleneglycol groups [117]. The different variants of printed arrays were used to examine specificities of a broad spectrum of glycan-recognizing antibodies and glycan binding proteins. Moreover, this early work laid the foundation for the development of more stable glycan arrays with increased sensitivity.

A more complex glycan array comprising 200 synthetic and natural glycans with defined structures was developed by the US-based Consortium for

Table 1. List of virus attachment factors/receptors identified by glycan array

Virus	Receptor	Glycan array platform	References
HAdV-3	HSPGs	CFG	[44]
HAdV-35	HSPGs	CFG	[44]
HAdV-37	GD1a-glycan	CFG	[28]
HAdV-52	$\alpha 2,8$ -linked Neu5Ac	ICL	[36]
Human polyomavirus 9	Neu5Gc	ICL	[106]
B-lymphotropic polyomavirus	$\alpha 2,3$ -linked Neu5Ac	ICL	[107]
JC polyomavirus	LSTc pentasaccharide	ICL	[108]
Simian virus 40 (SV40)	GM1-gangliosides	CFG and ICL	[109,110]
Rotavirus	A-type histo-blood group antigen	CFG	[111]
Reovirus	GM2-glycan	ICL	[30]
Middle East respiratory syndrome coronavirus (MERS-CoV)	$\alpha 2,3$ -linked Sias	other	[29]

Functional Glycomics (CFG). Here, amino group derivatized glycans are printed on amino-reactive (*N*-hydroxysuccinimide) glass slides. In a proof of concept study, the CFG array determined the binding specificities of various glycan binding proteins, including galectins, antibodies, hemagglutinin protein of influenza virus, and whole virions [118]. Other examples of glycan arrays that contain limited, or more specific types of glycans include arrays with glycolipids, *N*-glycans, *O*-glycopeptides, and GAGs. A glycolipid array with 50 glycolipids non-covalently printed on a polyvinylidene difluoride membrane [119] could determine the presence of autoantibodies in human serum and cerebrospinal fluid. For GSL interactions, an array with covalently immobilized GSLs on *N*-hydroxysuccinimide- or epoxide-activated glass array slides is available [120]. A mucin 1 (MUC1) *O*-glycopeptide array could identify glycopeptide epitopes of cancer-specific antibodies [121], and an *N*-glycan-derived array successfully determined the glycan-binding specificity of anti-HIV antibodies. In the latter case, enzymatically produced *N*-glycans including high-mannose, hybrid, and other complex-type *N*-linked oligosaccharides were displayed on an aluminiumoxide-coated glass slide [122]. Due to their complex and heterogeneous nature, the development of GAG-based arrays has been hampered. However, a synthetic approach was recently employed to generate a HS-based glycan library. This array displays 47 HS-based oligosaccharides and revealed specificities of various HS-binding proteins [123].

A limitation of the above-described glycan libraries is that they do not fully cover the glycan diversity present on cells or in tissues. A solution to this challenge is “shotgun glycomics” (SG), that is, arrays displaying the complexity of cell/tissue-derived glycans (Fig. 3A) [124]. The SG technology was demonstrated with GSLs isolated from human erythrocytes (blood types A and O) and prostate cancer cells. Specifically, chloroform extracted GSLs are tagged with a bifunctional fluorescent molecule, that is, *N*-(aminoethyl)-2-amino benzamide (AEAB). AEAB contains an aryl amine, which allows for conjugation to free glycans, and an alkyl amine, which permits conjugation to reactive surfaces. The additional fluorescent tag in AEAB facilitates quantification and separation of conjugated GSLs. An example of whole tissue SG is the pig lung glycoprotein and glycolipid array. Here, glycans were released from *N*-linked glycoproteins, *O*-linked glycoproteins and glycolipids using enzymes, nonreductive elimination, and ozonolysis, respectively. The released glycans were fluorescently tagged, separated by HPLC, covalently printed on glass slides, and subsequently probed with IAV [118,129].

Glycan arrays developed in particular by the CFG and the Imperial College of London (ICL) have facilitated the identification of several attachment factors and receptors of HAdVs. An updated CFG-

based array containing approximately 260 glycans identified HAdV-37 glycan-containing receptors (Fig. 2A and B). Specifically, the HAdV-37 knob bound to GD1a ganglioside. Subsequent cell-based assays and structural studies confirmed specificity of the knob for GD1a-like glycans [28]. A different version of the CFG array explored binding partners for species B HAdVs. This revealed that unlike species D HAdV-37, which use SA as the main attachment factor, species B HAdV-3 and -35 use HSPG as a low-affinity attachment factor [44]. Finally, CFG arrays demonstrated that HAs of pandemic IAVs interact with α 2,3- or α 2,6-SA in a manner that corresponds to tissue and host tropism of these viruses [127,128]. These observations supported previous findings on IAV specificity and moreover reliability of glycan arrays.

The ICL arrays facilitated the discovery of SA-containing glycans as attachment molecules for HAdV-52. Of note, HAdV-52 shares an unusual feature with the two only human types of species F AdVs, that is, the presence of two fibers, SSF and LSF. Specifically the SSF interacts with SA. Structure function analysis revealed that the SA interface is on the side of the trimeric short fiber knob rather than in the central cavity as is the case for HAdV-37 [36].

Despite the remarkable knowledge about virus-glycan interactions generated by the CFL and ICL glycan arrays, SG opens up unprecedented opportunities to identify glycan-containing viral attachment factors and receptors not represented in conventional glycan arrays. For instance, SG identified possible endogenous glycan attachment factors and receptors for IAV in pig lung [129]. The pig lung glycome was probed since IAV can broadly disseminate in swine, which is postulated as an intermediary host for both human and avian IAVs. IAVs mainly interacted with SA-containing *N*-glycans confirming previous results [130]. Recognition of IAV by some novel endogenous *N*-glycans recognition was also observed [129]. Finally, SG can not only identify attachment factors and receptors for productive virus infection, but also host restriction factors or decoy receptors. For instance, SG identified soluble glycans from human breast milk as soluble decoy receptors for rotavirus [131].

In summary, glycan array-based technologies have undergone tremendous advancements during the last two decades. This has enabled the discovery of glycan-containing attachment factors/receptors for species B, D, and G HAdVs (Table 1). In the future, this technology may be used to uncover additional glycan-containing HAdV attachment factors/receptors.

Shotgun proteomics

In addition to the development of glycan array technologies, the field of virology has in the past decade benefited from advances in mass

spectrometry-based proteomics. In particular shotgun proteomics, which is the unbiased detection of all proteins in a given biological sample, holds great promise for the identification of host factors of viral infection [132–134]. The basic principle of shotgun proteomics is the digestion of all proteins in a mixture with a defined enzyme such as trypsin and the subsequent identification of proteins based on their characteristic peptide fingerprint, for example, tryptic peptides unique to one protein in the whole proteomic space of a given species. Improved sensitivity of mass spectrometry and the recent development of label-free protein quantification methods are collectively driving applications in virus receptor discovery. Here we will highlight how mass spectrometry-based proteomics identified adenovirus attachment factors and receptors in the past and give an outlook, and how this technology can aid to unravel novel receptors in the future.

Historically, virus overlay protein binding assays (VOPBAs) first successfully used mass spectrometry-based proteomics for receptor discovery. VOPBA resolves all cellular proteins or purified plasma membrane protein fractions of virus susceptible cells by gel electrophoresis and transfers the size separated cellular proteins onto a membrane. This membrane is then probed with whole virus or virus attachment proteins (VAPs), that is, the virus surface structures mediating cell attachment. Bands with bound virus or VAP are visualized with antibodies. Corresponding bands in a duplicate protein gel can finally be subjected to mass spectrometry fingerprinting to identify putative receptors. VOPBA successfully identified the Lassa virus and lymphocytic choriomeningitis virus receptor alpha-dystroglycan [135] and the AdV receptor CD46 [81]. In the latter study, HeLa cell membrane proteins separated by gel electrophoresis were probed with the HAdV-35 fiber knob domain. Previously, the authors had shown that fiber knob domains of HAdV-35 can compete for whole virus attachment. The authors confirmed the interaction of CD46 and the HAdV-35 fibers by immunoprecipitation of fiber binding proteins from HeLa cell lysates and immunoblotting for CD46. The role of CD46 as a *bona fide* receptor was further shown by binding of fluorescent HAdV-35 particles, competition with soluble CD46, and siRNA knockdown of CD46 [81]. Subsequent analysis revealed that CD46 acts as primary receptor for some species B AdVs such as HAdV-11, -16, -21, -35, and -50, but not for others such as HAdV-3, -7, and -14, which use CD46 as a low affinity, or secondary receptor [81,89,91,136].

A similar VOPBA approach was tried to identify HAdV-3 cell surface attachment factors and receptors without success [44]. This underlines the limitations of the VOPBA approach. First, certain attachment domains may be destroyed under the denaturing conditions of the SDS-PAGE. Second, interplay of several host proteins, that is, a receptor complex, may be required for virus attachment and such higher-order

protein complexes are again destroyed by gel separation. Third, affinities of VAPs, such as the fiber knobs for adenoviruses, to their cognate receptors can be too low for detection using VOPBA approaches. In fact, most viruses, non-enveloped and enveloped, profit from the avidity effect mediated by multiple VAPs per particle. Lastly, certain carbohydrate interactions are difficult to capture by VOPBA. While glycosylated receptor interactions can be captured by VOPBA as shown for the Lassa virus–dystroglycan interaction, HSPG interactions cannot as shown for HAdV-3, which strongly depends on HSPGs [44].

Alternatively, the receptor fishing probe used for the VOPBA approach may not be suitable. This was additionally the case for HAdV-3, where the fiber knob alone was insufficient to mimic binding to cellular proteinaceous receptors. The elusive receptor could be identified by using a different VAP probe, namely, recombinant HAdV-3 penton bases and fibers. These probes efficiently compete for whole HAdV-3 binding to susceptible cells and VOPBA could finally reveal the nature of the HAdV-3 receptor as DSG2 [93]. Binding assays including surface plasmon resonance and gain and loss of function assays with fully infectious HAdV-3 confirmed DSG2 as a HAdV-3 receptor. Interestingly, species B AdVs such as HAdV-7, -11, and -14 also use DSG2 as a cellular receptor. These results show that certain serotypes can use multiple receptors; for example, HAdV-3 can use CD46 and DSG2. Moreover, the study underlines the critical choice of VAP probe for receptor fishing expeditions.

An alternative to VOPBA is the immunoadhesin/co-immunoprecipitation technique. Here, the receptor binding domain of a VAP is fused to an antibody Fc domain. Such immunoadhesins can capture attachment factors and receptors at the cell surface or in cell lysates and purify them using a protein A/G affinity resin. Receptors for Nipah, Hendra, and arenaviruses were discovered using immunoadhesins [137–139]. Despite the fact that receptor capture can be performed in liquid state using physiological buffers, caveats such as inadequate choice of VAP probe and loss of avidity effects still apply to the immunoadhesin/co-immunoprecipitation approach.

The new shotgun proteomics developments may overcome some of the limitations of VOPBA and immunoadhesin approaches. A major advancement was achieved with the introduction of quantification methods in shotgun proteomics. Initially, these relied on isotope labeling of one or of two biological samples, resulting in light (corresponding to the natural isotope), heavy, and optionally medium mass-labeled proteins. The most common labeling method is stable isotope labeling of amino acids in cell culture (SILAC). Here, heavy isotope (C13 and N15) containing lysine and arginine is fed to cell cultures until nearly all proteins incorporated the isotope-labeled amino acids. This isotope labeling generates a characteristic mass offset

in the labeled proteins and peptides. Therefore, labeled and unlabeled samples could be processed and measured simultaneously and the relative abundance of labeled and unlabeled peptides quantitatively compared [140–143]. More recently, label-free quantification methods have been developed, which now allow for the quantitative analysis of primary cell material and tissues without chemical labeling. These rely either on quantification of spectral counts or on quantification of ion intensities [144,145]. In theory, these methods in combination with affinity enrichment hold the promise of purifying virus–receptor complexes from cell lysates after binding to intact cells. For a detailed description of affinity enrichment mass spectrometry (AE-MS) also referred to as immunoaffinity purification coupled to mass spectrometry, we refer the reader elsewhere [132,146]. In AE-MS, proteins of interest such as a VAP are purified together with their binding partners after binding to cell surfaces. Either antibodies raised against the endogenous VAP or epitope-tagged VAPs are used to co-purify attachment factors. In particular when using whole virus, this approach will prove valuable for interactions depending on avidity effects and on receptor clustering. Previously, AE-MS has successfully identified host factor interactions with viral non-structural proteins of virus families ranging from flaviviruses to herpesviruses (reviewed in Refs. [133,147–149]). The application to virus receptor discovery is now in reach (Fig. 3C).

A limitation of AE-IP in receptor discovery is the possibility that non-physiological interactions may occur after cell lysis, that is, after breakdown of the cellular compartmentalization. Therefore, stabilization of virus–receptor complexes before cell lysis and AE-MS analysis using specific crosslinkers is an attractive strategy. Crosslinking agents designed for ligand-based receptor capture include the compound TRICEPS, which comprises three functional groups and is specifically designed to capture glycosylated receptors for extracellular ligands including viruses [150,151]. The first functional group is an NHS ester for coupling of ligands, such as virus particles via primary amines. The second group is a protected aldehyde-reactive hydrazine for crosslinking of glycosylated receptors. The third functional group is a biotin for purification of captured receptors using streptavidin resins. A major advantage of this strategy is the covalent crosslinking of receptors and biotinylated ligands, which allows for digestions of proteins into tryptic peptides before purification and avoids losing interaction partners by a chosen method of cell lysis. TRICEPS has been shown to reliably identify receptors for insulin, transferrin, apelin, epidermal growth factor, the therapeutic antibody trastuzumab, and ErbB2 [151]. Importantly, attachment factors for vaccinia virus such as AXL, M6PR, DAG1, CSPG4, and CDH13 [151] as well as receptors for growth factors and the nerve binding peptide NP41 [152,153] were identified using this trifunctional crosslinker. The

discovery of AXL, which binds to phosphatidylserin in the viral envelope, highlights that crosslinking approaches have the potential not only to identify protein–protein interactions but also to proximal lipid–protein interactions. Similar compounds such as the aldehyde-reactive aminoxy group, sulfhydryl group, and biotin group containing compound (ASB) have recently been described and used to identify receptors for a peptide hormone and a monoclonal antibody [154]. The combination of such biocompatible ligand capture methods with shotgun proteomics is likely to allow fast and efficient identification of virus receptors in the future (Fig. 3D).

Apart from capturing primary interactions under physiological conditions, shotgun proteomics has the potential to map whole networks of proteins. Specifically, host protein interaction networks of virus receptors in the absence and presence of bound virus can be delineated. As receptor signaling and host protein–protein interactions are critical to guide productive virus uptake [155], unraveling higher-order receptor complexes will help understand virus host cell invasion. Upon virus binding to a receptor, intracellular protein networks may change and such alterations may trigger virus uptake. Clustering of virus receptors and recruitment of accessory factors is in this context a well-described phenomenon. Clearly, knowledge about receptor accessory proteins and about the changes in protein networks during virus entry can help better understand the molecular details of virus invasion.

An aspect of virus entry not yet addressed by shotgun proteomics is the usage of secondary intracellular receptors. In the past decade, it became evident that some viruses require not only cell surface receptors but also endosomal receptors. In particular Ebola virus and Lassa virus have been shown to undergo a receptor switch after endocytosis and this switch is critical to trigger membrane fusion [156,157]. Hence, proteomics approaches should in the future resolve the spatiotemporal interactions during virus entry. A solution to this problem may be the use of proximity labeling methods. Here proteins, which are expressed in distinct subcellular compartments, are fused to enzymes, which covalently link biotin or another probe to all proteins in close proximity (reviewed in Ref. [158]). This labeling then allows in a two-step purification protocol to enrich for organelle specific proteins and subsequently immunoprecipitate the bait such as the VAP. The feasibility of spatiotemporal resolution of protein networks was recently demonstrated for G-protein-coupled receptors [159]. Similar approaches will likely prove valuable for secondary virus entry receptor discovery and for the mapping of spatiotemporal changes in host cell protein networks induced during virus penetration.

Taken together, mass spectrometry-based proteomics has undergone massive technological advancements, which enable it in principle to discover virus–

protein interactions at the cell surface and in intracellular compartments. To date, these approaches have to be tailored to each virus. For HAdV-3 and -35, recombinant fiber knobs and penton bases and fibers proved suitable for receptor fishing. Future studies will reveal if these or novel probes can be used for other adenovirus types to discover proteinaceous cellular receptors.

Alternative approaches for receptor discovery

Unquestionably, glycomics and proteomics are two of several technologies in the virologist's toolbox to discover host factors including virus receptors. Other powerful methods to be used in combination with glycomics and proteomics include RNA interference, CRISPR/Cas9 knockout, genome-wide haploid screens, cell-based arrays, drug-based approaches, and utilization of the microarray-characterized set of cells provided by US National Cancer Institute. We refer the reader elsewhere for further information on these methods [160–166]. A clear advantage of label-free glycomics and proteomics technologies is, however, the minimal system perturbation in particular when untagged endogenous bait glycans and proteins are used. In contrast, genetic and drug based approaches alter the biological system either by silencing/knocking out a gene of interest or by blocking its biological function with small molecules. For essential proteins, this may lead to cytotoxicity or to compensatory effects, that is, the upregulation of cellular pathways replacing the function of the knocked out or inhibited pathway. Moreover, many viruses are promiscuous and can use secondary entry pathways in the absence of the primary entry pathway. Notably, this phenomenon is independent of compensatory cellular mechanisms, questioning the suitability of single-molecule knockout/inhibition screens for such promiscuous viruses. Alternative entry pathway usage was clearly shown for IAV, which can use either clathrin-dependent or -independent uptake routes depending on the availability [167]. The notion of versatile virus entry strategies is further supported by the fact that IAV RNA interference screens revealed different host factor hits depending on the assay setup and target cell used [168]. Thus, glycomics and proteomics approaches seem particularly useful to analyze the physiological contribution of virus entry pathways in an unbiased manner. Another benefit of label-free glycomics and proteomics is the possibility to analyze primary cell and tissue material. In contrast, haploid screens can only be performed with specifically developed cell lines, which may not be productively infected by certain viruses. CRISPR/Cas9 knockout in turn is rarely amenable to primary cells, which often rapidly lose their differentiated state *ex vivo*.

Clearly, CRISPR/Cas9 and RNA interference are indispensable for follow-up analysis and validation of

glycomics and proteomics hits. Obviously, the mere interaction of a VAP with a host protein does not provide information on the function of the interacting factor in the entry process. Downstream analysis using one or several of the above mentioned genetic and pharmacological methods is critically required to distinguish between attachment factors, receptors and accessory molecules. Again, cytotoxicity and compensatory mechanisms after gene knockout are caveats for follow-up and RNA interference is therefore still an important alternative method, when carefully controlled.

Taken together, a smart combination of glycan- and protein-based screening technologies together with genetic and drug-based validation methods will draw a clear picture of virus invasion strategies and the underlying cell biological processes. Such systems biology view of virus entry will likely impact drug development and personalized medicine research [169].

Perspectives: Current challenges and future directions

Identification of the receptors is rarely simple or straightforward, and the outcome of such efforts is usually facilitated substantially by some pre-existing knowledge of the nature of the receptor(s), which can guide the strategy to be used. Despite the technological advances in glycomics and proteomics, a number of bottlenecks need to be overcome to allow usage of both technologies for virus entry factor discovery in a broad fashion.

Virus–cell interactions are seldom binary and a single step process. Many viruses, including AdVs, appear to use molecules as receptors, which are not or poorly expressed on the apical side of polarized/differentiated epithelial target cells and therefore not accessible initially. Specifically, molecules at cellular junctions and on basolateral sides of cells including JAM, nectins, CAR, ICAMs, and integrins are used as entry ports into cells. Examples are JAM-A usage by reoviruses [170], claudin-1 and occludin usage by hepatitis C virus [171,172], and CAR usage by Coxsackievirus and AdVs [61]. To overcome the problem of limited receptor accessibility, viruses developed strategies to disrupt tight junctions, use alternate receptors, transcytose epithelia via M cells, or induce a targeted transfer to receptors at tight junctions [173,174]. During AdV infection, certain adhesion molecules appear to re-localize to the apical surface in response to antiviral cytokines [175,176]. The re-localized adhesion molecules tether neutrophils that enhance initial infection of an intact epithelium [176]. Alternatively, certain splice variants of adhesion molecules are found on apical surfaces [177]. For Coxsackievirus B, the seeming conundrum of junctional molecule usage as entry receptor was solved by

seminal studies of Coyne and Bergelson. They demonstrated that Coxsackievirus B initially interacts with the GPI-anchored protein decay-accelerating factor at the apical surface of cells. This activates Abl kinase and triggers Rac-dependent actin rearrangements leading to virus translocation to the tight junction. At the junction, Coxsackievirus B can interact with CAR, which induces conformational changes in the viral capsid and cell entry [178]. Capturing such “late” interactions requires a synchronized infection and ideally the possibility to arrest entry at the site where the “late” entry factor is engaged. The same challenge exists for the characterization of interactions in endosomal compartments. While synchronizing apical binding can be achieved by cooling cells to 4 °C and thereby arresting trafficking, blocking entry at subsequent steps is not trivial and requires knowledge of the exact entry pathway and susceptibility to drugs, which may specifically block virus uptake. Arresting viruses in early endosomal compartments at 19 °C is one possibility.

Despite the enhanced throughput of proteomics and glycomics methods in virus entry research, we currently lack high-throughput techniques to address more than one question at a time, for example, the impact of plasma-membrane receptors, vesicle-based transport, soluble extracellular host factors, and of other microbes. Some of these bottlenecks may be overcome by the combination of APEX methods and labeling of subcellular compartments as described above. With regard to the impact of extracellular factors such as complement factors, soluble decoy receptors, and extracellular matrix molecules, recent efforts have demonstrated the usage of label-free MS for the identification and quantification of proteins in biological fluids including cellular secretions [179,180]. Meissner and colleagues [180] could detect thousands of secreted proteins with a concentration range spanning 4 orders of magnitude and discovered novel secretory proteins with yet unknown functions. It is expected that numerous biologically significant virus interacting secretory proteins and extracellular glycans shape virus infection. While interaction proteomics studies of viruses and secretory fluids are feasible, addressing the physiological function of secretory interactors will require more complex tissue culture models, for example, organoids or 3D cultures. In terms of throughput, proteomics analysis has become 96-well compatible [181]. Critically, establishment of a high-throughput compatible virus entry proteomics and glycomics pipeline would allow researchers to assess several cell types and virus serotypes at a time to draw a differential picture of AdV entry into host cells. Here the clear bottleneck is the establishment of 96-well compatible affinity enrichment methods.

A major challenge in the adenovirus field is the lack of small animal models in which adenoviruses from multiple species (not only species C AdVs) can

replicate to high titers, and that mimic the tropism and pathogenesis, for example, of EKC-causing AdVs. In the absence of such models, it is difficult to predict/select the main target cells in which AdVs replicate *in vivo*, and thereby, there is a risk that cells are used in attempts to identify novel receptors, which do not correspond to true target cells and thus do not express the correct receptor/co-receptor combinations.

In the absence of a human glycome “atlas,” a major limitation of most of the established glycan arrays is that they represent only a small fraction of the human glycome. Hitherto, most glycan libraries have been synthesized chemically or enzymatically or generated by purification from biological sources. However, recent progress in generation of glycan libraries from natural sources, using, that is, enzymes, hydrazinolysis, and/or oxidation-based approaches, open up possibilities to develop arrays with a more comprehensive coverage of the glycans in/on specific cells or tissues [182–184]. Here, a challenge will be to separate the large numbers of different glycans with similar/identical molecular weight from each other. Glycans with different molecular weight can, however, be purified and characterized by HPLC and matrix-assisted laser desorption/ionization-time of flight spectroscopy, respectively. Regardless of glycan library size, another limitation of glycan arrays is that they do not fully mimic the physiological environment with respect to membrane-mediated mobility and the local fluidic environment in the proximity of cells and tissues, and that current methods require labeling or antibody-based detection, as discussed above.

In addition to the challenges and bottlenecks described above, some recent discoveries provide novel insights, but also complexity, into the understanding of virus attachment to and entry into target cells. Among these discoveries is the ability of hepatitis A virus to be packaged in clusters within phosphatidylserine lipid-enriched vesicles and be released from cells in a non-lytic manner. Such enveloped forms of typically non-enveloped viruses display an increased infection efficiency compared with non-enveloped viral particles [185] and use alternative entry receptors. Another recent discovery challenging previous dogmas is that enteric viruses rely on other microbes for stability/survival and potentially also for attachment to and entry into target cells [186]. As described above, future research should also consider that even soluble host molecules (such as blood factors and lactoferrin) can affect attachment and entry mechanisms. Moreover, some viruses such as Ebola and Lassa virus require a “receptor switch” in intracellular compartments and thus use not only cell surface receptors but also secondary endosomal receptors [157,187]. Taken together, it is challenging to design and perform large-scale approaches that fully mimic the *in vivo*

situation of a given virus, with the aim to identify specific host molecules essential for virus attachment/entry. Researchers need to design and develop the experimental approach on a case by case basis and by considering the sum of prior knowledge on the structure, biochemistry, and tropism of a given virus.

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Abbreviations used:

HAdVs, human adenoviruses; LSFs, long-shafted fibers; SSFs, short-shafted fibers; SA, Sialic acid; IAVs, influenza A viruses; GAGs, glycosaminoglycans; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; HBGAs, histo blood group antigens; GSLs, glycosphingolipids; CAR, coxsackie and adenovirus receptor; VCAM-1, vascular cell adhesion molecule-1; MHC-I, major histocompatibility complex 1; CFG, Consortium for Functional Glycomics; SG, shotgun glycomics; ICL, Imperial College of London; VOPBAs, virus overlay protein binding assays; VAPs, virus attachment proteins; AE-MS, affinity enrichment mass spectrometry.

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