Adenovirus Endocytosis

Oliver Meier & Urs F. Greber[+)]

Zoologisches Institut der Universität Zürich, Winterthurerstrasse 190

CH-8057 Zürich, Switzerland

[+)] corresponding author:

Telephone: +41 1 635 4841

Fax: +41 1 635 6822

Email: ufgreber@zool.unizh.ch

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Abreviations:

AAV, adeno-associated virus; AP2, adaptor protein complex 2; Ad, Adenovirus; C3b, classical complement cascade product; CAR, Coxsackie virus B Adenovirus receptor; CED-1, cell death gene for scavenger receptor which serves the removal of apoptotic cells; CHO, chinese hamster ovary; CR, complement receptor; ECM, extracellular matrix; EEA1, early endosome antigen 1; EH, Eps15 homology; EGF, epidermal growth factor; EIPA, 5-(N-ethyl-N-isopropyl) amiloride; EM, electron microscopy; GAG, glycosaminoglycan; iC3b, alternative cleavage product of the complement cascade; IgG, Immunoglobulin G; Fcγ-R, immunoglobulin receptor; ITAM, immunoreceptor tyrosine-based activation motif; M-CSF, macrophage colony stimulating factor; MDCK, Madin-Darby canine kidney; PA, phosphatidic acid; Pb, penton base; PDGF, platelet derived growth factor; PH, pleckstrin homology; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; RTK, receptor tyrosine kinase; SH3, Src homology 3; src, non-receptor tyrosine kinase identified in sarcoma caused by Rous sarcoma virus; SYK, tyrosine kinase; UIM, ubiquitin-based intracellular network motif

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Summary

Pathogen entry into cells occurs by direct penetration of the plasma membrane, clathrin-mediated endocytosis, caveolar endocytosis, pinocytosis or macropinocytosis. For a particular agent, the infectious pathways are typically restricted, reflecting a tight relationship with the host. Here, we survey the uptake process of human Adenovirus (Ad) type 2 and 5 and integrate it into the cell biology of endocytosis. Ad2 and Ad5 naturally infect respiratory epithelial cells. They bind to a primary receptor, the Coxsackie virus B Ad receptor (CAR). The CAR-docked particles activate integrin coreceptors and this triggers a variety of cell responses, including endocytosis. Ad2/Ad5 endocytosis is clathrin-mediated and involves the large GTPase dynamin and the adaptor protein 2. A second endocytic process is induced simultaneously with viral uptake, macropinocytosis. Together, these pathways are associated with viral infection. Macropinocytosis requires integrins, F-actin, protein kinase C and small G-proteins of the Rho family but not dynamin. Macropinocytosis *per se* is not required for viral uptake into epithelial cells, but it appears to be a productive entry pathway of Ad artificially targeted to the high affinity Fcγ receptor CD64 of hematopoietic cells lacking CAR. In epithelial and hematopoietic cells, the macropinosomal contents are released to the cytosol. This requires viral signalling from the surface and coincides with particle escape from endosomes and infection. It emerges that incoming Ad2 and Ad5 distinctly modulate the endocytic trafficking and disrupt selective cellular compartments. These features can be exploited for effective artificial targeting of Ad vectors to cell types of interest.

Adenoviruses

Adenoviruses (Ads) are nonenveloped icosahedral DNA viruses of about 90 nm diameter. They produce progeny virions within the nucleus of infected cells and release the newly synthesized virions upon cell lysis [reviewed in 1]. The species C Ads, such as the human Ad2 and Ad5, are nononcogenic to humans and cause infections of the respiratory tracts. They are widely used as gene transfer vehicles or oncolytic agents in diseased somatic cells [2, 3]. The Ad particle is composed of an outer capsid and an inner DNA-associated core with a 36 kbp linear DNA, two terminal proteins and condensing proteins V and VII [4]. The chromosome also contains about ten copies of the cysteine protease p23 and is linked to the outer capsid by protein VI. The capsid consists mainly of hexon and is stabilized by proteins IIIa, VIII and IX. The vertices are made up of penton base (Pb) containing an exposed argine-glycine-aspartate (RGD) motive and the protruding fiber protein which attaches the particle to target cells [5].

Access to the plasma membrane

The plasma membrane of epithelial cells is protected by the extracellular matrix (ECM), a dense meshwork of proteins and sugars. The ECM has crucial functions in shaping the tissue architecture, controlling cell migration and surface receptors, and it regulates the activity of growth factors and hormones [6]. The ECM and the extracellular surface contain glycosaminoglycans (GAGs), i.e. highly sulfated, heterogeneous oligosaccharides, including heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid. GAGs can store chemokines and growth factors and also viral particles, such as herpesviruses [7], respiratory syncytial virus [8], human immunodeficiency virus [HIV, 9], adeno-associated virus [10] and foot-and-mouth disease virus [11]. Virus trapping in the ECM has been associated with a reduction [12] and in some cases with an enhancement of infection [13, 14]. The key to the plasma membrane is the viral docking to a selected cell surface receptor. This can trigger viral penetration of the plasma membrane or endocytic uptake, and thus not only overcomes the ECM but also provides opportunities to directly interfere with cell signalling [reviewed in 15].

Some Ad serotypes have been reported to bind to GAGs. Binding of the species C Ad2 and Ad5 to epithelial cells is partially inhibited by high concentrations of heparin [16]. The species D serotype Ad37 binds sialic acid linked to an oligosaccharide in an alpha 2-3 glycosidic bond [17, 18]. This residue is found on glycoproteins and glycolipids and could, in part, account for the ocular tropism of Ad37. Ad37 has recently been reported to also bind to a protein component of unknown identity [19]. Infections of polarized epithelial cells from the apical side with a variety of Ad serotypes of the species A, C, D, E and F appear to be inefficient [20]. These viruses are known to bind the Coxsackie virus B Adenovirus receptor CAR [21]. The same is true for amphotropic retroviruses [22], lentiviruses [23], adeno-associated virus (AAV) type 2 [24] and measles virus [MV, Edmonston vaccine strain, 25]. In the case of MV, the limiting factor does not seem to be the apically localized viral receptor CD46, but viral access to the plasma membrane through the glycocalyx or similar structures seems to be blocked [26, 27]. In contrast, AAV5 is reported to transduce airway cells from the apical side and it binds to alpha 2-3 linked sialic acid [24, 28]. Further experiments in HeLa cells suggested that AAV5 bound to apical microvilli and basolateral membranes with different kinetics and it was internalized from both membranes in clathrin-coated and non-coated vesicles [29]. Interestingly, a fraction of it trafficked to the Golgi complex, but the infectious pathway of AAV is presently unknown.

The Coxsackie virus B Adenovirus receptor (CAR)

Fibers of all Ad species except species B have been reported to bind the Coxsackie virus B Ad receptor [21], termed CAR [30-32]. Intriguingly, the enteric Ad41 of species F encodes a second fiber gene in the late region 5 which is shorter (20 nm) than its long fiber of 34 nm [33, 34]. While the long fiber binds to CAR, the receptor(s) of the short fiber, present in six trimeric copies per virion is unknown. It has been speculated that the short fibers may be responsible for the exceptionally high selectivity of Ad41 for the digestive tract [35, 36].

CAR is an important receptor for Ad entry into a large variety of cultured cells, including respiratory endothelial and epithelial cells [20], although CAR mRNAs in human lung tissue are relatively low [37]. The mature human CAR is a type 1 transmembrane protein

of 346 amino acids. Based on sequence similarities, CAR has been placed into a conserved subfamilly of the Ig superfamily of proteins, together with the A33 protein of colon carcinoma cells and the CTX protein of *X. laevis* cortical thymocytes [reviewed in 37]. More recently, a new member of the Ig superfamily has been identified, termed BT-IgSF [brain- and testis-specific Ig superfamily, 38]. Its DNA sequence predicts it to be a type-1 transmembrane protein with extracellular V and C2-type Ig domains and significant homology to CAR and endothelial cell-selective adhesion molecule [ESAM, 39]. In humans, CAR is abundantly expressed in heart, pancreas, the central and peripherous nervous system, prostate, testis and also in lung, liver and intestine. Little or no CAR is found in B and T-cells, adult muscle and on many malignant cells. Noteably, the expression of CAR is developmentally regulated [40] and seems to be induced by mediators of inflammation [reviewed in 37]. There is also an intriguing inverse correlation of CAR expression and growth of prostate carcinoma cells [41]. The large cytoplasmic domain of CAR contains basolateral targeting information and might be involved in homotypic signalling, although the latter has not been directly demonstrated [42, 43]. The extracellular portion comprises 216 amino acids and is folded into a V-like immunoglobulin (Ig) fold comprising amino acid 1 to 120 and a C2-like fold of amino acids 124-210. The amino-terminal V-like Ig domain 1 (D1) functions as the Ad binding domain by binding the protruding fiber knob domain. This has been demonstrated upon expression cloning of the human and the mouse CAR in chinese hamster ovary (CHO) cells and NIH 3T3 mouse fibroblasts, respectively [30, 31]. X-ray cristallography studies subsequently showed that viral docking to D1 involves the same CAR interface as D1 dimerization [44-47]. This indicates that Ad may subverse the intrinsic property of CAR to oligomerize. Recently, it was shown that Ad2 fiber proteins that are known to be released from infected cells together with Ad particles interfere with CAR oligomerization in the infected respiratory epithelium [48]. It was suggested that fibers disrupt the adherens junctions of the epithelial barrier and promote viral release to the airway lumen, implying that CAR is also an exit receptor. Intriguingly, another study recently suggested that Ad2 Pb was exported from the infected cell prior to the release of Ad particles and, by doing so, Pb abduced the fiber protein to the surrounding cells of the monolayer [Fig. 1, and 49]. If this occurs in the infected respiratory epithelium, viral spreading is expected to be even more effective due to CAR blocking by free fiber-Pb complexes prior to the release of viral particles.

Adenovirus infection of the respiratory epithelium

But how do Ads infect the respiratory epithelium? This is a burning question for essentially every respiratory agent infecting the apical membrane of polarized epithelial cells. In polarized respiratory epithelial cells and in airway cultures, the Ad receptor CAR is localized in the tight junctions near the top of the cells and along the basolateral membranes [Fig. 1, and 42, 50]. The amount of tight junction association of CAR seems to depend on the particular cell type and the culture conditions and, accordingly, CAR has been found also in adherens junctions along the lateral sides of airway cells [48]. The absence of CAR in the apical membrane is thought to preclude Ad infection of polarized airway epithelia from the lumenal side. Interestingly, the expression of CAR mutants lacking the transmembrane or the cytoplasmic domains gave rise to both apically and basolaterally distributed CAR in polarized Madin-Darby canine kidney (MDCK) cells [42]. This constellation did not allow significant Ad5 transduction, unless the apical glycocalyx was experimentally removed. Another report, however, stated that in transfected airway cells apical CAR was sufficient for infection [51]. In any case, it is unlikely that incoming Ad binds to CAR in tight junctions of polarized cells. Rather, airway infections may start in specialized nonpolarized cells that express CAR on the lumenal membrane, or that lesions of the epithelium prior to infection expose basolateral membranes to allow viral docking to CAR or other receptors (Fig. 1). Such models would also be compatible with the basolateral localization of integrins, an Ad coreceptor involved in viral endocytosis. However, other types of infection cannot be excluded. One interesting concept of epithelial infection has recently been discussed again, transcytosis. *Streptococcus pneumoniae* are able to co-opt polymeric Ig receptors, which normally transport antibodies across mucosal epithelial cells as part of the host defense, and thus the bacteria gain entry into airway epithelial cells [52].

Endocytic uptake

Endocytosis serves to sample the extracellular environment and regulate the activity of cell surface receptors [for reviews, see e.g., 53, 54, 55]. Notably, all types of endocytosis can be abused by pathogens as gates into target cells [56]. Over the last decades, one particular type of receptor-mediated endocytosis was intensively studied, clathrin-

dependent endocytosis. It originates at specialized plasma membrane regions where the cytosolic adaptor protein complex 2 (AP2) binds the endocytic receptors and phosphoinositides and together with other cytosolic proteins mediates the formation of clathrin coats [57]. Coats function to deform the donor membrane to produce a vesicle, and they also assist in sorting of transmembrane receptors and their cargo. In addition to receptor-mediated nutrient uptake, clathrin-mediated endocytosis contributes to the initiation, propagation and downregulation of signalling [recently reviewed by 58, 59]. Less is known, however, about other receptor-mediated uptake processes, such as caveolar endocytosis and constitutive non-clathrin uptake [for recent reviews, see 60, 61]. Caveolae are flask-shaped invaginations that are formed and stabilized by the coat like protein caveolin-1. The recruitment of receptors is thought to occur by the cholesterol and sphingolipid-rich nature of caveolar membranes and this serves as a signalling platform at the cell surface. Similarily, clathrin and caveolin-independent uptake can be mediated by specialized cholesterol and sphingolipid domains, called lipid rafts.

In contrast, phagosomes and macropinosomes are designed to remove large particles, such as invading microorganisms. Phagocytosis often occurs upon activation of Fc-receptors [Fc-R, 62] and in extreme cases even appears to involve the recruitment of membranes from the endoplasmic reticulum to the plasma membrane [63]. Macropinocytosis is related to phagocytosis but is less specific, as first discovered by Lewis in 1931 [64]. It is a major endocytic pathway, found in epithelial cells, fibroblasts, neutrophils and macrophages [60, 65]. Typically, macropinocytosis is triggered by growth factor stimulation or downstream activated signalling molecules. It plays a key role in the entry of *Salmonella* and *Shigella* bacteria [66], and it is crucial for antigen presentation of dendritic cells and viral clearance [67-69]. Macropinosomes are dynamic structures formed by the closure of lamellipodia at ruffling membranes and can reach several µm in diameter. They assure the endocytic removal of large membrane domains, alter the adhesive and communicative properties of the cell and are involved in cell contraction and migration.

Clathrin-mediated endocytosis

Recent studies have revealed intricate details of the regulation underlying clathrin-dependent endocytosis. Initially, clathrin is recruited by the heterodimer Hip1/Hip1R which leads to an actin-dependent formation of flattened coated-pit zones at the plasma membrane [70]. Hip1 is termed according to its interaction with the Huntingtin protein and binds the adaptor protein AP2. AP2 is a heterotetramer comprising two large subunits, α and β2, and two smaller chains, μ2 and σ2 [57]. The μ2 subunit of AP2 recognizes a tyrosine (Y)-based signal (YxxØ) with a large hydrophobic residue (Ø) or a dileucine motif on cytosolic domains of plasma membrane receptors [71]. While the β2 subunit of AP2 binds the clathrin-triskelions, the α subunit is responsible for the interaction with a complex network of accessory proteins. These proteins interact with each other by various protein-protein recognition modules, including Eps15 homology (EH) domain that bind to NPF-motives on AP180/CALM, epsin and synaptojanin, and Src homology 3 (SH3) domains recognizing proline rich regions. The α-subunit of AP2 preferentially binds to membranes rich in phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) and $PI(3,4,5)P_3$. Oligomers of the transmembrane protein synaptotagmin are thought to cooperatively tighten AP2 membrane association [72]. In many cases, the coat contains the additional monomeric protein AP180/CALM that later in the process appears to define the size of the coated vesicle [73]. The membrane curvature of clathrin-coated pits is driven by epsin binding to $PI(4,5)P_2$ [74]. $PI(4,5)P_2$, in turn, is generated by PI(5)P-4-kinase and PI(4)P-5-kinase and stimulated by phosphatidic acid (PA). PA is produced by endophilin or generated by phospholipase D-mediated hydrolysis of phosphatidyl-choline. The enzymatic activity of PLD is enhanced by the small GTPase Arf6 and $PI(4,5)P_2$, thus generating a positive-feedback loop. This cycle can be inhibited by the phosphoinositide phosphatase synaptojanin and by amphiphysin [for a review, see 72]. $PI(4,5)P_2$ is also recognized by epsin amino-terminal homology domains, present in epsin, Ap180/CALM and Hip1R.

Like the SH3 domain of amphiphysin, Eps15 is an important diagnostic tool to discriminate clathrin-mediated endocytosis from other types of endocytosis. It was originally identified as epidermal growth factor (EGF) receptor pathway substrate clone 15 [75]. Benmerah and collegues showed that overexpression of an EH-domain deleted GFP-fusion protein inhibited transferrin uptake, whereas the deletion of the AP2 binding domain had no effect [76, 77]. Eps15 also plays a role in the recruitment of monoubiquitinated receptors [78].

Ubiquitin is a 76 amino acid peptide that becomes covalently attached to other proteins through an isopeptide bond between its C-terminus and a lysine residue of the target protein [reviewed by 79]. Monoubiquitination of receptors at the cytoplasmic tail serves as a signal for clathrin-mediated internalization [reviewed in 80]. In contrast, proteasomal degradation is mediated through polyubiquitination at the ubiquitin lysine residue 48 [for a review, see 81]. In the case of the alternative ubiquitination, a single ubiquitin-based intracellular network motif (UIM) is responsible for ubiquitin recognition and monoubiquitination. Two of these UIM's allow Eps15 to act as an adaptor between ubiquitinated membrane cargo and endocytic coats and their dual functionality leads to a amplification of this network in the endocytic system [78].

Another way to test if a ligand enters by endocytosis is to express dynamin mutants, such K44A-dynamin, defective of GTP loading and hydrolysis [82]. K44A-dynamin inhibits clathrin-mediated endocytosis, caveolar endocytosis and phagocytosis [reviewed by 83]. Dynamin is targeted to flattened clathrin lattices of the plasma membrane and is also found at the neck of emerging clathrin-coated vesicles by binding of its poly proline rich domain to the SH3 domain of amphiphysin and the pleckstrin homology (PH) domain recognizing $PI(4,5)P_2$ [84]. In the presence of GTP dynamins form oligomers and thus may act as a constrictase upon GDP-GTP exchange and GTP hydrolysis, thus leading to vesicle detachment. It was, however, also recognized that dynamin is a signal transducing molecule that activates downstream effectors rather than just being the constrictase. One of the dynamin effectors is endophilin which appears to exhibit a lysophosphatidic acid acyl transferase activity and may facilitate the formation of a strong membrane curvature [85]. Additional regulation is provided by the adaptor associated kinase 1 which phosphorylates the μ2 subunit of AP2 thus allowing initial concentration of receptors [86, 87]. Following dephosphorylation by an unknown phosphatase receptors are then rapidely internalized. Yet another regulatory mechanism can be activated by the cargo itself. Binding of EGF to the EGF receptor appears to lead to clathrin phosphorylation and facilitates EGF receptor uptake [88]. Furthermore, EGF activates the small GTPase Rab5, and provides a link between signalling and endocytic trafficking [89].

Clathrin-mediated endocytosis of Adenovirus

Initial electron microscopy (EM) studies of Ad entry had suggested that the virus penetrated the plasma membrane [90, 91]. Subsequent EM analyses detected incoming Ad2 and Ad5 in clathrin-coated pits and clathrin-coated vesicles [92-95]. It was also noted that endocytic uptake of Ad2 from noncoated membranes could occur at very high multiplicities of infection [94]. Several functional assays subsequently showed that the infectious entry pathway of Ad2 and Ad5 occurs either in clathrin-coated or non-coated membranes [96-99]. More recently, the use of dominant-negative expression constructs, including Eps15, clathrin fragments and K44A-dynamin underlined that the infectious entry pathway of Ad2 and Ad5 into epithelial cells is clathrin-mediated [100]. This and additional requirements of the infectious pathway in epithelial cells are depicted in Fig. 2. For additional information, the reader is referred to a recent review [15].

It had been noted earlier that viral endocytosis depends on the activation of α_v integrins [101], and several other integrins are also implicated in Ad endocytosis [for a review, see 15]. This suggests that Ad endocytosis is regulated. Most likely, Ad particles are endocytozed together with their integrin coreceptors, possibly by an NPXY motif present in the β_3 and β_5 subunits [102, 103]. NPXY motives have been implicated in the localization of certain receptors to coated pits [104]. That integrins bind to Ad particles has been directly shown by cryo-EM reconstructions and biochemical experiments using soluble $\alpha_v\beta_5$ integrin heterodimers attaching to the RGD domains of Ad2 and Ad12 pentons in a five-fold symmetrical manner [105]. The binding of RGD peptides to the extracellular domain of $\alpha_v\beta_3$ integrins leads to discrete conformational changes in the integrins [recently reviewed in 106]. This suggests that the incoming Ad particle activates integrins in a spatially controlled manner and triggers cell signalling. The internalization of Ad5 has been associated with an integrin-dependent activation of p85/p110 PI(3) kinase [PI3K, 107]. The products of p85/p110 activation, PI(3,4)P_2 and PI(3,4,5)P_3, have been proposed to act as second messengers for cell cycle progression and cytoskeletal changes underlying the cell plasma membrane and for regulating vesicular trafficking [108]. For instance, PI(3,4,5)P_3 can bind to and activate various isoforms of protein kinase C (PKC). Another target of PI(3,4)P_2 and PI(3,4,5)P_3 is the small GTP-binding protein Rab5, involved in coordinating viral endocytosis and homotypic vesicular fusion with early endosomes [109]. It was reported that the GTP-binding defective S34N-Rab5 mutant slightly reduced

Ad5 uptake and infection. Interestingly, members of the Rho family of small GTPase, Rac1 and Cdc42 acting downstream of PI3K induced actin polymerization and promoted Ad endocytosis [110], and viral endocytosis was recently found to be complemented by macropinocytosis [100].

Macropinocytosis

Macropinocytosis is the best studied type of receptor and coat-independent endocytosis, closely related to the Fc-R mediated phagocytosis [65, 111]. Depending on the trafficking of macropinosomes and the kind of receptor, macropinocytosis can be divided into a recycling type and a processive type (Fig. 3). Recycling macropinosomes are generated upon binding of a ligand such as EGF, platelet-derived growth factor or nerve growth factor to receptor tyrosine kinases and they can be induced by the activation of downstream activators, including GTP-Rac1 and GTP-Cdc42 [112-115]. In contrast, macrophage colony stimulating factor-triggered phagocytic cells generate processive macropinosomes that shrink in size and readily mix with late endosomes and lysosomes [116]. Processive macropinocytosis is mediated by a diverse group of receptors, including non-complement-receptor integrins, like $\alpha_v\beta_3/\beta_5$ and $\alpha_5\beta_1$, lectins such as mannose receptor, the lipopolysaccharide receptor CD14 and the *Caenorhabditis elegans* scavenger receptor CED-1, and it is involved in the uptake of particles, bacteria and apoptotic cells [62, 117]. Clearly, processive macropinocytosis has several features in common with recycling macropinocytosis, including F-actin-dependent surface ruffling, formation of large endocytic vesicles and a strong dependence on PKC. It is not sensitive to K44A-dynamin, as shown for macropinosomal uptake of *Chlamydia* bacteria [118]. PI3K is acting downstream of receptor tyrosine kinases and of tether/stimulating receptors, such as α_v integrins, and it is required for the completion of ruffles and the formation of macropinosomes [111, 119]. In addition, Rac1 and Cdc42 are needed for promoting actin polymerization.

Phagocytosis

In mammalian cells, the zippering phagocytosis is mechanistically similar to processive macropinocytosis (see Fig. 3). Both processes involve the sequential activation of

phospholipase Cγ and PKC, the activation of Ras and Src and the activation of PI3K to promote F-actin dynamics via Rac1 and Cdc42. Four steps can be distinguished during zippering phagocytosis [62, 120]. First, opsonized particles bind via the conserved Fc domains of Igs to the Fcγ-R, including the high affinity Fcγ-RI (CD64) and the low affinity Fcγ-RIIA (CD32) and IIIA (CD16). The interaction of Ig and the Fcγ-R triggers the phosphorylation of the ITAM (immunoreceptor tyrosine-based activation motif) which in turn provides docking sites for SYK, a tyrosine kinase, which activates PI3K. Interestingly, ligand induced monoubiquitination of the μ subunit of Fcγ-RI is thought to lead to clathrin-mediated endocytosis of soluble immune complexes, as shown for Fcγ-RIIA, whereas the nonubiquitinated Fcγ-RI appears to be involved in phagocytosis of opsonized particles [121]. The decision whether the Fcγ-RI receptor is internalized by the one or the other pathway may be taken according to a thresholding concentration of activated kinases, such as SYK, recuited towards the Fcγ-RI. Second, zippering drives the initial extension of membrane around the particle and this does not require actin polymerisation or signalling from the cytoplasmic domain of the receptors. Third, active signalling from the receptor leads to the recruitment of numerous cytoskeletal proteins, including the Arp2/3 complex, which nucleates actin filaments beneath the particle and thus pushes the plasma membrane further around the target in a process called pseudopod extension. Fourth, class I PI3K (p85α/p110β) activity leads to the generation of $PI(3,4,5)P_3$ and the closure of phagosomes. It is likely that $PI(3,4,5)P_3$ recruits proteins that control the actin cytoskeleton. Candidates are the PH-domain carrying Vav and ARNO, which bind $PI(3,4,5)P_3$ and act as GDP/GTP exchange factors for the small GTPases Rac1 and Arf6. By contrast, the class III PI3K (Vps34) product PI(3)P is required for phagosome maturation as it allows the recruitment of the Rab5 effector protein early endosome antigen 1 (EEA1) via its FYVE zinc finger [109].

One striking difference between macropinocytosis and zippering phagocytosis is the recruitment of dynamin to early phagosomes which requires PI3K and amphiphysin IIm [122]. Although dynamin is involved in the actin-based motility of macropinosomes [123], it does not participate in the initial internalization of macropinosomes [100, 118]. Phagocytosis differentiates also on the level of molecular scaffolds beneath the respective receptors which are responsible for actin remodelling. Integrins $\alpha_V\beta_5$ recruit the $p130^{cas}$-CrkII-Dock180 complex, which in turn triggers Rac1 activation and spacious phagocytosis of apoptotic cells in both professional and nonprofessional phagocytes [124]. Likewise,

the stimulation of Fcγ-RIIA leads to the recruitment of Crkl to the Cbl-Nck-Grb2 complex [125]. In this scaffold the downstream effector of Rac and Cdc42 GTPases, Pak1, binds to Nck and leads to actin remodelling in part through Grp2 mediated WASP/N-WASP interaction followed by Arp2/3 activation [120]. In contrast, the complement receptor (CR) 3 is coupled to the focal adhesion scaffold, including talin, α-actinin, vinculin and paxillin. Accordingly, CR-mediated phagocytosis is morphologically distinct from Fc-R mediated phagocytosis. Upon binding of iC3b-complement opsonized particles to CR3 ($\alpha_m\beta_2$ integrin) the actin cytoskeleton is reorganized into stress fibers as controlled by the Rho GTPase, and this triggers the sinking of the particle into the phagocyte. This type of endocytosis is exploited, e.g., by *Mycobacterium tuberculosis* for macrophage entry [62]. The signalling events involved in sinking phagocytosis seem to be different from triggering or zippering phagocytosis, as they include, e.g., inside out signalling of R-Ras on integrins via the small GTPase Rap1 [126]. Noteably, R-Ras has a high degree of sequence homology to Ras, yet it does not activate the ERK/MAP kinase pathway nor does it activate the JNK or p38/MAP kinase pathway.

Macropinocytosis in Adenovirus entry

Earlier studies had noted that endocytosed macromolecules were apparently released from pinocytic vesicles into the cytosol in the presence of Ad2 or Ad5 [127, 128]. It had been widely assumed that these macromolecules, including enzymes, dextrans and DNA, were codelivered with the incoming viral particles from receptosomes, i.e., endosomes that were bearing both the viral particle and the macromolecules [129-135]. Additional studies found that the delivery of the macromolecules was sensitive to lysosomotropic agents, such as chloroquin and ammonium chloride [136, 137]. Remarkably, the efficiency of fluid phase stimulation was high, i.e., in the range of 2 to 15 fold over the noninfected cells, depending on the cell type tested. This implied that Ad controlled an endocytic pathway. The nature of this pathway was recently shown to be macropinocytosis [Fig. 4, and 100]. Viral activation of macropinocytosis required signalling through α_v integrins, F-actin, the small Rho family GTPases and also PKC, and it was sensitive to amiloride, an inhibitor of the sodium / proton exchanger implicated in recycling and processive macropinocytosis [Fig. 3, and 138, 139]. In contrast to processive macropinocytosis, the Ad-induced macropinosomes were triggered to efficiently release their contents, i.e., more than 50% of

the cytoplasmic endosomes that were generated during the entry phase of Ad2 were destroyed [100]. These endosomes also included Ad-bearing endosomes, since Ad particles were released to the cytosol when macropinocytosis and pinosomal release occurred. Expression of the dominant-negative K44A dynamin further showed that the presence of Ad particles in macropinosomal membranes was not sufficient for rupturing the macropinosomes, but that discrete signals from the cell surface were involved in breaking the pinosomal membranes. These results imply that appropriate endocytic sorting of viral and cellular vesicles cooperates with cell signalling to mediate endosome rupture and viral escape.

Adenovirus escape to the cytosol

Viral escape is one of the most crucial steps of entry but also one of the least understood ones. It is clear that the escape of Ad2 and Ad5 happens rapidely after endocytosis. It is also known that the delivery of the Ad particles into the cytosol is inhibited by lysosomotropic agents [95, 97] and that the Ad-mediated disruption of endosomes *in vitro* requires an acidic pH [140, 141], although a couple of studies reported rather weak effects of lysosomotropic amines on Ad infection [142, 143]. This implies then that low endosomal pH and the presence of Ad particles in endosomes are not sufficient for endosome disruption and additional factors must be involved. One of these factors has been identified as the $\alpha_V\beta_5$ integrin [144, 145]. The attachment of the Ad penton base protein to integrin $\alpha_V\beta_5$ at reduced extracellular pH promoted the permeabilization of the plasma membrane as measured by the release of small molecules into the extracellular medium. Another factor could be a spatially controlled activation of integrins, as suggested to be important for proper cell signalling through integrins [reviewd by 146]. While many signals emerge from the binding of wild type Ad2 or Ad5 to integrins [for a review, see 15], an Ad2 mutant called ts1 [for temperature sensitive 1, isolated by 147] elicits an incomplete spectrum of signals upon entry. Ts1 lacks the viral protease and contains a capsid which is not proteolytically processed and ts1 does not dissociate the fibers upon entry [reviewed in 148]. It binds to CAR, is endocytozed into the cells with indistinguishable efficiency and kinetics as the wild type virus, but it is unable to penetrate the endosomal membrane and ends up in late endosomes and lysosomes where it is degraded [149]. Interestingly, ts1 activates PI3K [data not shown in 107] but not the MAPK pathways ERK and p38 [150]

and also fails to trigger macropinocytosis [100]. These data support the notion that Ad precisely activates integrins preparing the host for its arrival. Precise activations seem to be also crucial for successfully retargeting of Ad particles to new receptors and cell types normally not affected by Ad. For example, Ad targeted to the transferrin receptor by an insertion into the HI loop of the fiber knob was able to transduce brain microcapillary cells to low levels but the virus was difficult to propagate and overall ineffective [151]. A more successful retargeting example is the delivery of Ad5 to the high affinity Fcγ-RI (CD64) of hematopoietic cells by virtue of a soluble adaptor consisting of the extracellular domains D1 and D2 of CAR and the constant region of a human Ig [152]. These hematopoietic cells are CAR-negative and express only very low levels of α_v integrins. Virus internalization and cytosolic delivery required a phagocytic mechanism and the transduction was effectively inhibited by anti-Fcγ-RI antibodies, particularly at low adaptor to particle ratios, i.e., when large virus aggregates had been formed. It remains to be tested if viral transduction via CD64 requires also a clathrin-dependent pathway or if it solely relies on Fcγ-RI mediated phagosome formation.

Conclusions

There are many ways to get into a cell, and pathogens essentially exploit every one of the entry pathways with astounding efficiency. However, under normal conditions, a particular pathogen usually takes only one defined entry pathway. This allows the exploitation of discrete signalling pathways at the cell surface and distinct intracellular sites. In the case of epithelial cells, it is clear that clathrin-mediated uptake of Ad2 or Ad5 together with signal stimulated macropinocytosis leads to infection. This does not preclude the uptake of viral particles by alternative routes, but these routes alone are considered to be noninfectious, unless the infectious route is used at the same time and the appropriate signalling switches are turned on. The lessons that pathogens teach us on their natural entry are crucial to further develop viral vectors into useful therapeutics.

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References

1. Shenk T. Adenoviridae. **In** *Fundamental Virology*. Knipe DM and Howley PM, editors. Lippincott-Raven, Philadelphia. 1053-1088. 2001.

2. Kochanek S, Schiedner G, and Volpers C. High-capacity 'gutless' adenoviral vectors. *Curr Opin Mol Ther* 2001; **3**:454-463.

3. Kirn D, Niculescu-Duvaz I, Hallden G*, et al.* The emerging fields of suicide gene therapy and virotherapy. *Trends Mol Med* 2002; **8**:S68-73.

4. Burnett RM. The structure of adenovirus. **In** *Structural biology of viruses*. Chiu W, Burnett RM, and Garcea RL, editors. Oxford Press, Oxford. 209-238. 1997.

5. Stewart PL, Chiu CY, Huang S*, et al.* Cryo-Em visualization of an exposed RGD epitope on adenovirus that escapes antibody neutralization. *EMBO J* 1997; **16**:1189-1198.

6. Bissell MJ, and Radisky D. Putting tumours in context. *Nat Rev Cancer* 2001; **1**:46-54.

7. Wudunn D, and Spear PG. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J.Virol.* 1989; **63**:52-58.

8. Krusat T, and Streckert HJ. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. *Arch Virol* 1997; **142**:1247-1254.

9. Mondor I, Ugolini S, and Sattentau QJ. Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. *J Virol* 1998; **72**:3623-3634.

10. Summerford C, and Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 1998; **72**:1438-1445.

11. Jackson T, Ellard FM, Ghazaleh RA*, et al.* Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. *J Virol* 1996; **70**:5282-5287.

12. Walker SJ, Pizzato M, Takeuchi Y*, et al.* Heparin binds to murine leukemia virus and inhibits Env-independent attachment and infection. *J Virol* 2002; **76**:6909-6918.

13. Spillmann D. Heparan sulfate: anchor for viral intruders? *Biochimie* 2001; **83**:811-817.

14. Liu J, and Thorp SC. Cell surface heparan sulfate and its roles in assisting viral infections. *Med Res Rev* 2002; **22**:1-25.

15. Greber UF. Signalling in viral entry. *Cell Mol Life Sci* 2002; **59**:608-626.

16. Dechecchi MC, Melotti P, Bonizzato A*, et al.* Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J Virol* 2001; **75**:8772-8780.

17. Arnberg N, Edlund K, Kidd AH*, et al.* Adenovirus type 37 uses sialic acid as a cellular receptor. *J Virol* 2000; **74**:42-48.

18. Arnberg N, Pring-Akerblom P, and Wadell G. Adenovirus type 37 uses sialic acid as a cellular receptor on Chang C cells. *J Virol* 2002; **76**:8834-8841.

19. Wu E, Fernandez J, Fleck SK*, et al.* A 50-kDa membrane protein mediates sialic acid-independent binding and infection of conjunctival cells by adenovirus type 37. *Virology* 2001; **279**:78-89.

20. Walters RW, Grunst T, Bergelson JM*, et al.* Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. *J Biol Chem* 1999; **274**:10219-10226.

21. Roelvink PW, Lizonova A, Lee JGM*, et al.* The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J Virol* 1998; **72**:7909-7915.

22. Halbert CL, Aitken ML, and Miller AD. Retroviral vectors efficiently transduce basal and secretory airway epithelial cells in vitro resulting in persistent gene expression in organotypic culture. *Hum Gene Ther* 1996; **7**:1871-1881.

23. Wang G, Williams G, Xia H*, et al.* Apical barriers to airway epithelial cell gene transfer with amphotropic retroviral vectors. *Gene Ther* 2002; **9**:922-931.

24. Zabner J, Seiler M, Walters R*, et al.* Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *J Virol* 2000; **74**:3852-3858.

25. Sinn PL, Williams G, Vongpunsawad S*, et al.* Measles virus preferentially transduces the basolateral surface of well-differentiated human airway epithelia. *J Virol* 2002; **76**:2403-2409.

26. Dorig RE, Marcil A, Chopra A*, et al.* The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell* 1993; **75**:295-305.

27. Blau DM, and Compans RW. Entry and release of measles virus are polarized in epithelial cells. *Virology* 1995; **210**:91-99.

28. Halbert CL, Allen JM, and Miller AD. Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *Journal of Virology* 2001; **75**:6615-6624.

29. Bantel-Schaal U, Hub B, and Kartenbeck J. Endocytosis of adeno-associated virus type 5 leads to accumulation of virus particles in the Golgi compartment. *Journal of Virology* 2002; **76**:2340-2349.

30. Bergelson JM, Cunningham JA, Droguett G*, et al.* Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**:1320-1323.

31. Tomko RP, Xu R, and Philipson L. HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl. Acad. Sci. USA* 1997; **94**:3352-3356.

32. Mayr GA, and Freimuth P. A single locus on human chromosome 21 directs the expression of a receptor for adenovirus type 2 in mouse A9 cells. *J Virol* 1997; **71**:412-418.

33. Pieniazek NJ, Slemenda SB, Pieniazek D*, et al.* Human enteric adenovirus type 41 (Tak) contains a second fiber protein gene. *Nucleic Acids Res* 1990; **18**:1901.

34. Favier AL, Schoehn G, Jaquinod M*, et al.* Structural studies of human enteric adenovirus type 41. *Virology* 2002; **293**:75-85.

35. Allard A, and Wadell G. Physical organization of the enteric adenovirus type 41 early region 1A. *Virology* 1988; **164**:220-229.

36. Croyle MA, Stone M, Amidon GL*, et al.* In vitro and in vivo assessment of adenovirus 41 as a vector for gene delivery to the intestine. *Gene Ther* 1998; **5**:645-654.

37. Carson SD. Receptor for the group B coxsackieviruses and adenoviruses: CAR. *Rev Med Virol* 2001; **11**:219-226.

38. Suzu S, Hayashi Y, Harumi T*, et al.* Molecular cloning of a novel immunoglobulin superfamily gene preferentially expressed by brain and testis. *Biochem Biophys Res Commun* 2002; **296**:1215-1221.

39. Hirata K, Ishida T, Penta K*, et al.* Cloning of an immunoglobulin family adhesion molecule selectively expressed by endothelial cells. *J Biol Chem* 2001; **276**:16223-16231.

40. Tomko RP, Johansson CB, Totrov M*, et al.* Expression of the adenovirus receptor and its interaction with the fiber knob. *Exp Cell Res* 2000; **255**:47-55.

41. Okegawa T, Li Y, Pong RC*, et al.* The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. *Cancer Res* 2000; **60**:5031-5036.

42. Pickles RJ, Fahrner JA, Petrella JM*, et al.* Retargeting the coxsackievirus and adenovirus receptor to the apical surface of polarized epithelial cells reveals the glycocalyx as a barrier to adenovirus-mediated gene transfer. *J Virol* 2000; **74**:6050-6057.

43. Cohen CJ, Gaetz J, Ohman T*, et al.* Multiple Regions within the Coxsackievirus and Adenovirus Receptor Cytoplasmic Domain Are Required for Basolateral Sorting. *J Biol Chem* 2001; **276**:25392-25398.

44. Roelvink PW, Mi Lee G, Einfeld DA*, et al.* Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science* 1999; **286**:1568-1571.

45. Bewley MC, Springer K, Zhang YB*, et al.* Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. *Science* 1999; **286**:1579-1583.

46. Freimuth P, Springer K, Berard C*, et al.* Coxsackievirus and adenovirus receptor amino-terminal immunoglobulin V- related domain binds adenovirus type 2 and fiber knob from adenovirus type 12. *J Virol* 1999; **73**:1392-1398.

47. van Raaij MJ, Chouin E, van der Zandt H*, et al.* Dimeric structure of the coxsackievirus and adenovirus receptor D1 domain at 1.7 A resolution. *Structure Fold Des* 2000; **8**:1147-1155.

48. Walters RW, Freimuth P, Moninger TO*, et al.* Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell* 2002; **110**:789-799.

49. Trotman LC, Achermann DP, Keller S*, et al.* Cell exit of an adenovirus structural protein by a non-classical mechanism. *Traffic* 2003; **in press**.

50. Cohen CJ, Shieh JT, Pickles RJ*, et al.* The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci U S A* 2001; **98**:15191-15196.

51. Walters RW, van't Hof W, Yi SM*, et al.* Apical localization of the coxsackie-adenovirus receptor by glycosyl-phosphatidylinositol modification is sufficient for adenovirus-mediated gene transfer through the apical surface of human airway epithelia. *J Virol* 2001; **75**:7703-7711.

52. Kaetzel CS. Polymeric Ig receptor: defender of the fort or Trojan horse? *Curr Biol* 2001; **11**:R35-38.

53. Mellman I, and Warren G. The road taken: past and future foundations of membrane traffic. *Cell* 2000; **100**:99-112.

54. Gruenberg J. The endocytic pathway: a mosaic of domains. *Nat Rev Mol Cell Biol* 2001; **2**:721-730.

55. Qualmann B, and Kessels MM. Endocytosis and the cytoskeleton. *Int Rev Cytol* 2002; **220**:93-144.

56. Marsh M, and Pelchen-Matthews A. Endocytosis in viral replication. *Traffic* 2000; **1**:525-532.

57. Pearse BM, Smith CJ, and Owen DJ. Clathrin coat construction in endocytosis. *Curr Opin Struct Biol* 2000; **10**:220-228.

58. Sorkin A, and Von Zastrow M. Signal transduction and endocytosis: close encounters of many kinds. *Nat Rev Mol Cell Biol* 2002; **3**:600-614.

59. Entchev EV, and Gonzalez-Gaitan MA. Morphogen gradient formation and vesicular trafficking. *Traffic* 2002; **3**:98-109.

60. Nichols BJ, and Lippincott-Schwartz J. Endocytosis without clathrin coats. *Trends Cell Biol* 2001; **11**:406-412.

61. Pelkmans L, and Helenius A. Endocytosis via caveolae. *Traffic* 2002; **3**:311-320.

62. Aderem A, and Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 1999; **17**:593-623.

63. Gagnon E, Duclos S, Rondeau C*, et al.* Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 2002; **110**:119-131.

64. Lewis. Pinocytosis. *Johns Hopkins Hosp. Bull.* 1931; **49**:17-27.

65. Swanson JA, and Watts C. Macropinocytosis. *Trends Cell Biol.* 1995; **5**:424-428.

66. Nhieu GT, and Sansonetti PJ. Mechanism of Shigella entry into epithelial cells. *Curr Opin Microbiol* 1999; **2**:51-55.

67. Lanzavecchia A. Mechanisms of antigen uptake for presentation. *Curr Opin Immunol* 1996; **8**:348-354.

68. West MA, Prescott AR, Eskelinen EL*, et al.* Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. *Curr Biol* 2000; **10**:839-848.

69. Garrett WS, Chen LM, Kroschewski R*, et al.* Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* 2000; **102**:325-334.

70. McPherson PS. The endocytic machinery at an interface with the actin cytoskeleton: a dynamic, hip intersection. *Trends Cell Biol* 2002; **12**:312-315.

71. Kirchhausen T. Three ways to make a vesicle. *Nat Rev Mol Cell Biol* 2000; **1**:187-198.

72. Takei K, and Haucke V. Clathrin-mediated endocytosis: membrane factors pull the trigger. *Trends Cell Biol* 2001; **11**:385-391.

73. Zhang B, Koh YH, Beckstead RB*, et al.* Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron* 1998; **21**:1465-1475.

74. Ford MG, Mills IG, Peter BJ*, et al.* Curvature of clathrin-coated pits driven by epsin. *Nature* 2002; **419**:361-366.

75. Fazioli F, Minichiello L, Matoskova B*, et al.* eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Mol Cell Biol* 1993; **13**:5814-5828.

76. Benmerah A, Bayrou M, Cerf-Bensussan N*, et al.* Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J Cell Sci* 1999; **112**:1303-1311.

77. Benmerah A, Lamaze C, Begue B*, et al.* AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J Cell Biol* 1998; **140**:1055-1062.

78. Polo S, Sigismund S, Faretta M*, et al.* A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* 2002; **416**:451-455.

79. Hochstrasser M. Evolution and function of ubiquitin-like protein-conjugation systems. *Nat Cell Biol* 2000; **2**:E153-157.

80. Hicke L. Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol* 2001; **2**:195-201.

81. Pickart CM. Ubiquitin in chains. *Trends Biochem Sci* 2000; **25**:544-548.

82. Damke H, Baba T, Warnock DE*, et al.* Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *Journal of Cell Biology* 1994; **127**:915-934.

83. Thompson HM, and McNiven MA. Dynamin: switch or pinchase? *Curr Biol* 2001; **11**:R850.

84. Simonsen A, and Stenmark H. PX domains: attracted by phosphoinositides. *Nat Cell Biol* 2001; **3**:E179-182.

85. Huttner WB, and Schmidt AA. Membrane curvature: a case of endofeelin' em leader. *Trends Cell Biol* 2002; **12**:155-158.

86. Ricotta D, Conner SD, Schmid SL*, et al.* Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals. *J Cell Biol* 2002; **156**:791-795.

87. Conner SD, and Schmid SL. Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. *J Cell Biol* 2002; **156**:921-929.

88. Wilde A, Beattie EC, Lem L*, et al.* EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. *Cell* 1999; **96**:677-687.

89. Barbieri MA, Roberts RL, Gumusboga A*, et al.* Epidermal growth factor and membrane trafficking. EGF receptor activation of endocytosis requires Rab5a. *J Cell Biol* 2000; **151**:539-550.

90. Morgan C, Rosenkranz HS, and Mednis B. Structure and development of viruses as observed in the electron microscope: X. Entry and uncoating of Adenovirus. *J. Virol.* 1969; **4**:777-796.

91. Brown DT, and Burlingham BT. Penetration of hst cell membranes by adenovirus 2. *J. Virol.* 1973; **12**:386-396.

92. Chardonnet Y, and Dales S. Early events in the interaction of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. *Virol.* 1970; **40**:462-477.

93. Patterson S, and Russell WC. Ultrastructural and immunofluorescence studies of early events in adenovirus-HeLa cell interactions. *J. Gen. Virol.* 1983; **64**:1091-1099.

94. Svensson U. Role of vesicles during Adenovirus 2 internalization into HeLa cells. *J. Virol.* 1985; **55**:442-449.

95. Pastan I, Seth P, FitzGerald D*, et al.* Adenovirus entry into cells: some new observations on an old problem. **In** *Concepts in viral pathogenesis II.* Notkins AL and Oldstone MBA, editors. Springer Verlag, New York. 141-146. 1986.

96. Varga MJ, Weibull C, and Everitt E. Infectious entry pathway of Adenovirus type 2. *J. Virol.* 1991; **65**:6061-6070.

97. Greber UF, Willetts M, Webster P*, et al.* Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* 1993; **75**:477-486.

98. Wang KN, Huang S, Kapoormunshi A*, et al.* Adenovirus internalization and infection require dynamin. *Journal of Virology* 1998; **72**:3455-3458.

99. Rauma T, Tuukkanen J, Bergelson JM*, et al.* Rab5 GTPase regulates adenovirus endocytosis. *J Virol* 1999; **73**:9664-9668.

100. Meier O, Boucke K, Vig S*, et al.* Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin mediated uptake. *J. Cell Biol.* 2002; **158**:1119-1131.

101. Wickham TJ, Mathias P, Cheresh DA*, et al.* Integrin alpha v beta 3 and integrin alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* 1993; **73**:309-319.

102. Suzuki S, Argraves WS, Pytela R*, et al.* cDNA and amino acid sequences of the cell adhesion protein receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion protein receptors. *Proc Natl Acad Sci U S A* 1986; **83**:8614-8618.

103. Ramaswamy H, and Hemler ME. Cloning, primary structure and properties of a novel human integrin beta subunit. *Embo J* 1990; **9**:1561-1568.

104. Mukherjee S, Ghosh RN, and Maxfield FR. Endocytosis. *Physiol Rev* 1997; **77**:759-803.

105. Chiu CY, Mathias P, Nemerow GR*, et al.* Structure of adenovirus complexed with its internalization receptor, alpha(v)beta 5 integrin. *J. Virol.* 1999; **73**:6759-6768.

106. Arnaout M, Goodman S, and Xiong J. Coming to grips with integrin binding to ligands. *Curr Opin Cell Biol* 2002; **14**:641.

107. Li E, Stupack D, Klemke R*, et al.* Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide-3-OH kinase. *J. Virol.* 1998; **72**:2055-2061.

108. Simonsen A, Wurmser AE, Emr SD*, et al.* The role of phosphoinositides in membrane transport. *Curr Opin Cell Biol* 2001; **13**:485-492.

109. Zerial M, and McBride H. Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2001; **2**:107-117.

110. Li E, Stupack D, Bokoch GM*, et al.* Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J. Virol.* 1998; **72**:8806-8812.

111. Amyere M, Payrastre B, Krause U*, et al.* Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of phosphoinositide 3-kinase and phospholipase C. *Mol Biol Cell* 2000; **11**:3453-3467.

112. Hall A. Ras-related GTPases and the cytoskeleton. *Mol Biol Cell* 1992; **3**:475-479.

113. Hewlett LJ, Prescott AR, and Watts C. The coated pit and macropinocytic pathways serve distinct endosome populations. *J Cell Biol* 1994; **124**:689-703.

114. Veithen A, Cupers P, Baudhuin P*, et al.* v-Src induces constitutive macropinocytosis in rat fibroblasts. *J Cell Sci* 1996; **109**:2005-2012.

115. Nobes C, and Marsh M. Dendritic cells: new roles for Cdc42 and Rac in antigen uptake? *Current Biology* 2000; **10**:R739-741.

116. Racoosin EL, and Swanson JA. Macropinosome maturation and fusion with tubular lysosomes in macrophages. *J Cell Biol* 1993; **121**:1011-1020.

117. May RC. Phagocytosis in C. elegans: CED-1 reveals its secrets. *Trends Cell Biol* 2001; **11**:150.

118. Boleti H, Benmerah A, Ojcius DM*, et al.* Chlamydia infection of epithelial cells expressing dynamin and Eps15 mutants: clathrin-independent entry into cells and dynamin-dependent productive growth. *J Cell Sci* 1999; **112**:1487-1496.

119. Araki N, Johnson MT, and Swanson JA. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* 1996; **135**:1249-1260.

120. May RC, and Machesky LM. Phagocytosis and the actin cytoskeleton. *J Cell Sci* 2001; **114**:1061-1077.

121. Booth JW, Kim MK, Jankowski A*, et al.* Contrasting requirements for ubiquitylation during Fc receptor-mediated endocytosis and phagocytosis. *Embo J* 2002; **21**:251-258.

122. Gold ES, Morrissette NS, Underhill DM*, et al.* Amphiphysin IIm, a novel amphiphysin II isoform, is required for macrophage phagocytosis. *Immunity* 2000; **12**:285-292.

123. Orth JD, Krueger EW, Cao H*, et al.* The large GTPase dynamin regulates actin comet formation and movement in living cells. *Proc Natl Acad Sci U S A* 2002; **99**:167-172.

124. Albert ML, Kim JI, and Birge RB. alphavbeta5 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. *Nat Cell Biol* 2000; **2**:899-905.

125. Izadi KD, Erdreich-Epstein A, Liu Y*, et al.* Characterization of Cbl-Nck and Nck-Pak1 interactions in myeloid FcgammaRII signaling. *Exp Cell Res* 1998; **245**:330-342.

126. Self AJ, Caron E, Paterson HF*, et al.* Analysis of R-Ras signalling pathways. *J Cell Sci* 2001; **114**:1357-1366.

127. Yoshimura A. Adenovirus-induced leakage of co-endocytosed macromolecules into the cytosol. *Cell Struct Funct* 1985; **10**:391-404.

128. Defer C, Belin M-T, Caillet-Boudin M-L*, et al.* Human adenovirus-host interactions: comparative study with members of subgroups B and C. *J. Virol.* 1990; **64**:3661-3673.

129. Willingham MC, and Pastan I. The receptosome: an intermediate organelle of receptor mediated endocytosis in cultured fibroblasts. *Cell* 1980; **21**:67-77.

130. FitzGerald DJP, Trowbridge IS, Pastan I*, et al.* Enhancement of toxicity of anti-transferrin receptor antibody-*Pseudomonas* exotoxin conjugates by adenovirus. *Proc. Natl. Acad. Sci. U.S.A.* 1983; **80**:4134-4138.

131. FitzGerald DJP, Padmanabhan R, Pastan I*, et al.* Adenovirus-induced release of epidermal growth factor and *Pseudomonas* toxin into the cytosol of KB cells during receptor mediated endocytosis. *Cell* 1983; **32**:607-617.

132. Cotten M, Wagner E, Zatloukal K*, et al.* High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proceedings of the National Academy of Sciences of the United States of America* 1992; **89**:6094-6098.

133. Cotten M, Wagner E, Zatloukal K*, et al.* Chicken adenovirus (CELO virus) particles augment receptor-mediated DNA delivery to mammalian cells and yield exceptional levels of stable transformants. *J. Virol.* 1993; **67**:3777-3785.

134. Yoshimura K, Rosenfeld MA, Seth P*, et al.* Adenovirus-mediated augmentation of cell transfection with unmodified plasmid vectors. *J. Biol. Chem.* 1993; **268**:2300-2303.

135. Carrasco L. Entry of animal viruses and macromolecules into cells. *FEBS Lett* 1994; **350**:151-154.

136. Seth P, Fitzgerald DJ, Willingham MC*, et al.* Role of a low-pH environment in adenovirus enhancement of the toxicity of a Pseudomonas exotoxin-epidermal growth factor conjugate. *J. Virol.* 1984; **51**:650-655.

137. Seth P, Rosenfeld M, Higginbotham J*, et al.* Mechanism of enhancement of DNA expression consequent to cointernalization of a replication-deficient adenovirus and unmodified plasmid DNA. *J. Virol.* 1994; **68**:933-940.

138. West MA, Bretscher MS, and Watts C. Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J Cell Biol* 1989; **109**:2731-2739.

139. Gekle M, Freudinger R, and Mildenberger S. Inhibition of Na+-H+ exchanger-3 interferes with apical receptor-mediated endocytosis via vesicle fusion. *J Physiol* 2001; **531**:619-629.

140. Blumenthal R, Seth P, Willingham MC*, et al.* pH-dependent lysis of liposomes by adenovirus. *Biochemistry* 1986; **25**:2231-2237.

141. Prchla E, Plank C, Wagner E*, et al.* Virus-mediated release of endosomal content in vitro: different behavior of adenovirus and rhinovirus serotype 2. *J. Cell Biol.* 1995; **131**:111-123.

142. Perez L, and Carrasco L. Involvement of the vacuolar H(+)-ATPase in animal virus entry. *J. Gen. Virol.* 1994; **75**:2595-2606.

143. Rodriguez E, and Everitt E. Adenovirus uncoating and nuclear establishment are not affected by weak base amines. *J. Virol.* 1996; **70**:3470-3477.

144. Wang K, Guan TL, Cheresh DA*, et al.* Regulation of adenovirus membrane penetration by the cytoplasmic tail of integrin beta 5. *J. Viol.* 2000; **74**:2731-2739.

145. Wickham TJ, Filardo EJ, Cheresh DA*, et al.* Integrin $\alpha v\beta 5$ selectively promotes adenovirus mediated cell membrane permeabilization. *J. Cell Biol.* 1994; **127**:257-264.

146. Liddington RC, and Ginsberg MH. Integrin activation takes shape. *J Cell Biol* 2002; **158**:833-839.

147. Weber J. Genetic analysis of adenovirus type 2. III. Temperature sensitivity of processing of viral proteins. *J. Virol.* 1976; **17**:462-471.

148. Greber UF. Virus assembly and disassembly: the adenovirus cysteine protease as a trigger factor. *Rev. Med. Virol.* 1998; **8**:213-222.

149. Greber UF, Webster P, Weber J*, et al.* The role of the adenovirus protease in virus entry into cells. *EMBO J.* 1996; **15**:1766-1777.

150. Suomalainen M, Nakano MY, Boucke K*, et al.* Adenovirus-activated PKA and p38/MAPK pathways boost microtubule-mediated nuclear targeting of virus. *Embo J* 2001; **20**:1310-1319.

151. Xia H, Anderson B, Mao Q*, et al.* Recombinant human adenovirus: targeting to the human transferrin receptor improves gene transfer to brain microcapillary endothelium. *J Virol* 2000; **74**:11359-11366.

152. Ebbinghaus C, Al-Jaibaji A, Operschall E*, et al.* Functional and selective targeting of adenovirus to high affinity Fcg receptor I positive cells using a bispecific hybrid adaptor. *J. Virol.* 2001; **75**:480-489.

153. Suomalainen M, Nakano MY, Boucke K*, et al.* Microtubule-dependent minus and plus end-directed motilities are competing processes for nuclear targeting of adenovirus. *J. Cell Biol.* 1999; **144**:657-672.

154. Leopold PL, Kreitzer G, Miyazawa N*, et al.* Dynein- and microtubule-mediated translocation of adenovirus serotype 5 occurs after endosomal lysis. *Hum Gene Ther* 2000; **11**:151-165.

155. Mabit H, Nakano MY, Prank U*, et al.* Intact microtubules support Adenovirus and Herpes simplex virus infections. *J. Virol.* 2002; **76**:9962-9971.

156. Trotman LC, Mosberger N, Fornerod M*, et al.* Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nature Cell Biology* 2001; **3**:1092-1100.

Figure Legends

Fig. 1: A hypothetical scheme of events leading to epithelial infection with Ad2 or Ad5.

Ad is thought to access the epithelial cells by virtue of cells displaying CAR receptors in the apical membrane. This first leads to a local infection and the spreading of the particles to basolateral membranes of epithelial cells. Viral spreading is aided by the release of fiber-penton base complexes which are thought to disrupt the tight junctions of epithelail cells prior to virus release by virtue of binding the CAR protein localized to cell-cell contacts. For further explanations, see main text.

Fig. 2: The infectious entry pathway of Ad2 and Ad5 into epithelial cells.

Ad fibers bind to CAR and locally activate α_V integrins which triggers clathrin-mediated viral endocytosis. This requires the large GTPase dynamin, PI3K, the small GTPases Rac1 and Cdc42 and also Rab5. Ad is then delivered to a slightly acidic intracellular compartment and escapes to the cytosol upon cell signalling. Additional signalling, including the activation of protein kinase A and the p38/MAPK cascade boost microtubule-dependent and dynein/dynactin-dependent viral transport towards the nucleus [150, 153-155]. Virus then docks to the nuclear pore complex receptor CAN/Nup214 and it disassembles by recruiting the nuclear histone H1 and the H1 import factors importin β and importin 7 [156].

Fig. 3: Mechanisms of macropinocytosis and phagocytosis.

While macropinocytosis is a spacious process, i.e., engulfing substantial amounts of extracellular fluid, phagocytosis tightly wraps plasma membrane regions around an extracellular particle, e.g., an opsonized bacterium. Recycling and processive macropinocytosis differ with respect to the trafficking of macropinosomes, while zippering and sinking phagocytosis differ with respect to the Rho GTPases involved. Common

signalling molecules to all four types of endocytosis include Ras, PI3K and PLCγ. Abbreviations see above.

Fig. 4: Ad2 or Ad5 triggered macropinocytosis.

Simultaneous with viral uptake by clathrin-coated pits and vesicles, the incoming Ad triggers macropinocytosis, independent of dynamin (Dyn), but depending on protein kinase C (PKC) and the sodium / proton exchange inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA). It appears that macropinosomes and virus-bearing endosomes simultaneously release their contents into the cytosol.