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Review

Signalling in viral entry

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Abstract. Viral infections are serious battles between pathogens and hosts. They can result in cell death, elimination of the virus or latent infection keeping both cells and pathogens alive. The outcome of an infection is often determined by cell signalling. Viruses deliver genomes and proteins with signalling potential into target cells and thereby alter the metabolism of the host. Virus interactions with cell surface receptors can elicit two types of signals, conformational changes of viral particles, and intracellular signals triggering specific cellular reactions. Responses by cells include stimulation of innate and adaptive immunity, growth, proliferation, survival and apoptosis. In addition, virus-activated cell signalling boosts viral entry and gene delivery, as recently shown for adenoviruses and adeno-associated viruses. This review illustrates that multiple activation of host cells during viral entry profoundly impacts the elaborate relationship between hosts and viral pathogens.

Key words. Entry; receptor; signal transduction; trafficking; virus infection.

Introduction

Viruses are tiny and are largely dependent on their hosts for survival. They propagate by replicating inside their host's cells. Viruses carry a minimal amount of information with them. A thin protein coat wraps around a small bag of genes. Unlike cellular genomes, which are protected and regulated by a variety of different polypeptides, viral genomes encode only a few coat and regulatory proteins [1]. Viruses use their proteins repetitively to build up regular geometrical 'capsids' [2], demonstrating that they have evolved effective use of their genetic and structural outfit [3].

Despite their apparent simplicity, viruses attract serious concern for the misery and illness they cause [4]. Most people regard viruses as uninvited and opportunistic guests which struggle with their host in an effort to replicate and spread their genetic material. Virally transmitted diseases range from the common cold to numerous forms of immunodeficiencies and cancer. Large-scale vaccination programmes have led to the prevention of some virus-caused diseases such as poliomyelitis, hepatitis B, virus-associated chronic liver disease and cancer of the liver [5]. On the other hand, the simplicity of viruses has made them useful instruments for the study of genetics, cell and structural biology, immunology and biochemistry [6]. Studies of viruses have also made major contributions to our understanding of principal cell functions, including the structure of DNA, genomic regulation, transcription, processing and transport of RNA, protein trafficking and evolution [3, 7]. Viral research has even contradicted a long-standing dogma of molecular biology through the discovery that RNA-containing retroviruses reverse the normal flow of genetic information by using an enzyme, reverse transcriptase, to transcribe single-stranded RNA into double-stranded DNA [8, 9]. In contrast to the standard view, viruses might even provide essential functions for the host. One hypothesis proposes that endogenous retroviruses (ERVs) of mammals locally suppress a cellular immune response

against the growing placental tissue, thus supporting acceptance of the allogeneic embryo in the mother [10]. A recent report goes further to suggest that a cellular gene captured by a human ERV serves an important function in the generation of trophoblast tissue by stimulating cell-cell fusion [11]. Another attractive speculation is that viruslike transposons are at the evolutionary origin of immunoglobulin heavy chain and the T-cell-receptor β-chain assembly in immature B and T cells, respectively [12].

But how does it happen that viruses utilize their targets so effectively, given that host benefits from viral infections seem to be scarce? The short answer to this question is that viruses are extremely well adapted to their hosts. A major part of this adaptation is that viruses manipulate host cell signalling. Biochemical and genomic analyses in the last decades have revealed that viruses harbor both genetic and nongenetic information with signalling potential [3, 13]. At least three principal mechanisms account for viral activation of cells (fig. 1). The delivery of signalling genes is probably the best-studied mechanism, whereas deposition of signalling proteins by incoming viral particles and activation of surface receptors are receiving increasing attention. This review aims to illustrate how viruses utilize signalling, with particular emphasis on how the process of cell entry by viruses can depend on signalling. A detailed review of viral structures and other aspects of viral life cycles is available elsewhere (for review, see e.g., [14–18]).

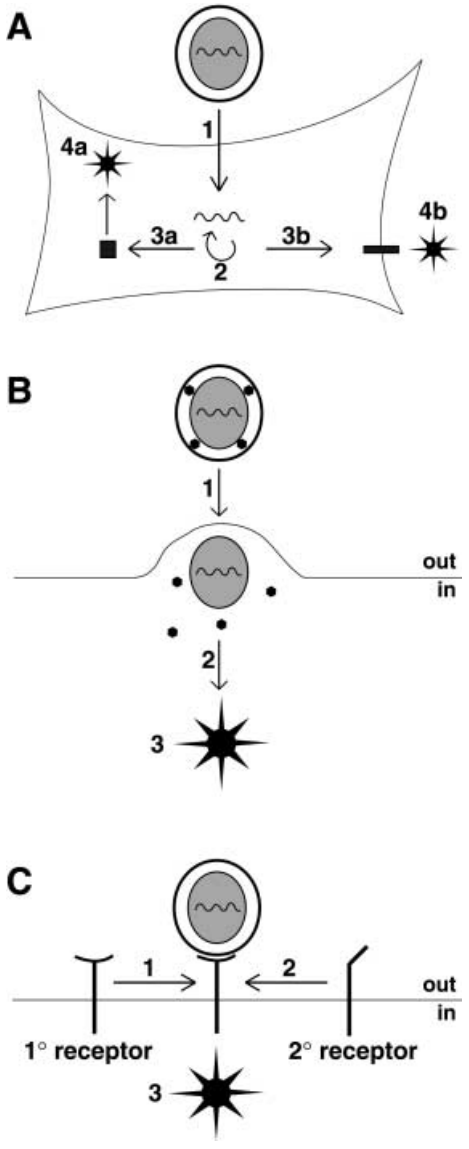

Figure 1. Three modes of viral activations of host cells. The most prevailing activation mode occurs by viral gene expressions (panel *A*). Incoming viruses deliver their genes to subcellular replication sites (steps 1 and 2) and from there drive the transcription of viral genes encoding intracellular (steps 3a and 4a) and extracellular signalling factors (steps 3b and 4b). Panel *B*: The second mode of signalling is a delivery of proteins or mRNAs by incoming viruses (step 1). These molecules can interfere with cell signalling (steps 2 and 3). Panel *C*: During entry, many incoming viruses recruit a primary receptor (step 1) and/or a secondary receptor with signalling functions (step 2) and thus lead to cell activation (step 3).

Delivery of signalling genes and proteins

The advent of high-resolution electron microscopy and reproducible cell-culture technology about 50 years ago enabled the tracking of intracellular viruses and viral genomes during infections. Sequencing of viral genomes and inventory of viral proteins have revealed that the genomes of certain retroviruses, for example, contain cellular genes of crucial signalling function that can lead to cell transformation and oncogenesis (fig. 1A). In fact, the first oncogene discovered was the cellular nonreceptor tyrosine kinase Src, pirated by the chicken Rous sarcoma virus [19]. Many other cell-derived oncogenes have since been identified in transforming retroviruses. The corresponding cell-owned protooncogenes are typically involved in mitogenic signalling and include peptide growth factors, growth factor receptors, protein and lipid kinases, G proteins and transcription factors (reviewed in [20–23]). Viral genomes also contain genes to counteract host defence mechanisms. They are necessary for viral survival in an infected organism, since acute infections are invariably countered by an innate immune response, mounting type I and type II interferons (IFNs), cytokines and increased levels of natural killer cell activity (reviewed in [24]). For example, the multifunctional cytokine tumor necrosis factor (TNF-α) is produced in activated monocytes and macrophages and activates caspases that lead to apoptosis of infected cells. Viral countermeasures include the downregulation of immune modulating proteins, inhibition of apoptosis, dysregulation of cytokine production and even destruc-

tion of immune cells (for reviews, see [25, 26]). Adenoviruses, for example, counteract the lytic action of TNF-α by a variety of proteins encoded in the E3 region [27]. Poxviruses produce a number of soluble cytokine receptors that act as decoy molecules to neutralize cytokines [28]. In addition, herpes- and poxviruses encode seven transmembrane G-protein-coupled receptors (GPCRs), presumably derived from the host chemokine system (for recent reviews, see [29, 30]). Although most of these receptors are still orphan receptors, the human herpesvirus 8 protein ORF74 seems to be positively modulated by angiogenic chemokines and is associated with cell transformation through phospholipase C and mitogen-activated protein kinase (MAPK) signalling pathways. It is likely that ORF74 has a major role in HHV-8-associated Kaposi's sarcomas. This example underscores the principle that viruses take control of cell proliferation by pirating and modulating cellular genes. These modulations involve cell-cycle control, intercellular signalling, subcellular trafficking of membranes and cell migration [31–34].

Viruses not only pirate signalling genes but also borrow proteins (and lipids) and return them to the cell upon entry (fig. 1B). Although many cellular proteins can be found in highly purified enveloped viruses, only a few of them have been functionally characterized. One example is the cyclophilin A (CPA) captured by a retrovirus, the human immunodeficiency virus (HIV) [35]. CPA is a protein that helps maintain proper protein conformations by catalysing peptidyl-prolyl cis-trans isomerizations. It is required for infection by virtue of its binding to the viral Gag polyprotein [36]. Another example is MAPK, a component of the Ras signalling pathway. It is also captured by HIV and returned into the cell to potentiate cell growth and survival [37].

The importance of regulatory viral mechanisms designed to instantly interfere with host functions is underscored by the fact that herpesviruses deliver serveral hundred copies of viral transcription factors into a host cell (reviewed in [38]). Intriguingly, these viruses transfer yet another class of biomolecules into target cells. A recent study has shown that human cytomegalovirus (HCMV), a β-herpesvirus with an infectious DNA genome of about 200 different genes, also delivers at least four different viral RNA transcripts into the host cell [39]. One of these transcripts encodes a protein with a cleavable signal sequence targeting the secretory pathway. Although the precise functions of the viral messenger RNAs (mRNAs) remain to be determined, this strategy allows HCMV to express viral genes immediately after entry, in the absence of transcription. This significantly extends the viral potential to manipulate cell functions, independent of host control.

Receptors for viral attachment

Much work has been devoted to the identification of viral receptors. The receptor is of central importance for infection and typically serves to enrich virus particles on target cells. Viruses have selected their primary receptors on the basis of availability and accessibility, functional coupling to coreceptors and perhaps also downregulation to prevent superinfection and support viral release (reviewed in [40]). Members from one virus family can choose a large variety of receptors, as exemplified in a recent survey of retroviral receptors (reviewed in [41]). Broad availability is a prominent feature of large glycosamino-glycans, present in heparan sulphate and chondroitin sulphate proteoglycans. Glycosamino-glycans have been reported to serve as primary attachment sites for HSV-1 [42, 43], CMV [44], adeno-associated virus-2 (AAV-2) [45] and for the subgroup C adenoviruses Ad-2 and Ad-5 [46]. In many instances, the biological role of viral binding to glycosamino-glycans is still unclear, and it remains an open question whether heparan sulphate binding is a decisive property to determine tissue-specific viral replication. Interestingly, unmodified heparan sulphate proteoglycans are sufficient for HSV-1 binding but not for entry, which seems to require sulphation of specific glucosamine residues [47]. Along the same lines, sialic acid residues present on glycoproteins in an $\alpha 2-3$ glycosidic linkage appear to serve as primary receptors for the subgroup D adenoviruses Ad-8 and Ad-37 [48, 49] and also for AAV-5 [50], and it has been reported that Ad-37 binds a 50-kDa protein independent of sialic acid [51]. This suggests that more than the sugar moiety is required for surface binding. In any case, it is unknown at present whether viral binding to heparan sulphate proteoglycans or sugar moieties of other glycoproteins directly induces cell signalling.

Primary receptors can contain built-in signalling functions (fig. 1C). Examples include the acetylcholine receptor for rabies virus [52], the low-density lipoprotein receptor for minor group human rhinoviruses [53] and a tissue necrosis factor receptor-related protein for avian leukosis sarcoma viruses [54] and a recently identified junction-associated molecule (JAM) as a reovirus receptor [54a]. In many cases, the signals from activation of primary viral receptors are not sufficient to stimulate viral entry, as shown, for example, in case of the T-cell receptor CD4 of HIV (reviewed in [55, 56]). CD4 is a member of the immunoglobulin (Ig)-gene family of receptors that widely serve as primary viral receptors. Ig-family receptors also include the poliovirus receptor [57] and intercellular adhesion molecule 1 for major group rhinoviruses [58], a regulator of complement activation CD46 for the Edmonston strains and vaccine strains of measles virus (MV), and also human herpesvirus 6 infections [59]. Recently, a B and T cell receptor of the Edmonston strain and clinical stains of MV was identified,

the signalling lymphocyte activation molecule (SLAM) [60].

The coxsackie B virus adenovirus receptor (CAR) is yet another Ig-family member (see fig. 2, and [61, 62]). CAR is a broadly distributed type I membrane protein and serves as the major receptor for adenoviruses of subgroups A, C, D, E and F [63]. In highly polarized epithelial cells, the availability of CAR is restricted to the basolateral surface and tight junctions and thus limits access of subgroup C virus Ad-2 or Ad-5 to the basolateral membrane of normal bronchiolar epithelial cells [64]. Virus binding to CAR is very tight and most likely involves an avidity mechanism which trimerizes CAR [65]. The extracellular N-terminal Ig-like domain D1 of CAR is sufficient for binding the distal knob domain of the viral fiber protein, as indicated by functional analyses and X-ray diffraction studies [66-68]. The tight coupling of CAR to fiber has been exploited by generating fusion proteins of the extracellular domains D1 and D2 of CAR and various cellular ligands that bind surface receptors on particular cell types [69]. Using the Fc domain of a human IgG as a ligand, this strategy has recently allowed for highly efficient retargeting of adenovirus to human monocytic cells lacking CAR [70]. These results suggest that CAR is not required for adenovirus infections, provided that the virus is able to recruit cell functions that facilitate viral delivery. Similarly, neither the intracellular nor the transmembrane domains of CAR are needed for infection of nonpolarized cells with Ad-2 and Ad-5, as shown with glycosyl-phosphatidyl-inositol (GPI)-anchored, C-terminally truncated CAR [71]. Transgenic mice expressing truncated CAR in essentially all tissues allowed efficient infections of lymphoid cells that are normally resistant to Ad infections [72]. These results raise the possiblity that CAR is not directly involved in transducing signals for viral infection. It is possible that CAR cooperates with membrane-spanning proteins, such as integrin coreceptors [73], and thus generates signals boosting infection. Along these lines, the C-terminal cytosolic CAR domain harbors sorting signals for targeting to the basolateral membrane where integrin coreceptors are located [74]. Interestingly, apical expression of truncated GPI-linked CAR seems to support infection of human airway epithelial cells with apically applied adenovirus [75]. Since polarized airway epithelial cells are resistant to many viral and nonviral vectors added through the airways, it will be interesting to see whether truncated GPI-CAR is sufficient to achieve adenoviral gene delivery in vivo. Given the complexity of viral host interactions, it will, however, not be surprising, to find that surface receptors with signalling potential are needed for apical entry.

Coreceptors

Viruses not only bind primary receptors, they also recruit and activate coreceptors at the cell surface, similar to bacterial pathogens and complex gamete interactions in metazoans. One of the first coreceptors has been found for Ad-2 and Ad-5, namely $\alpha_v\beta_5$ integrin (see Fig. 2, and

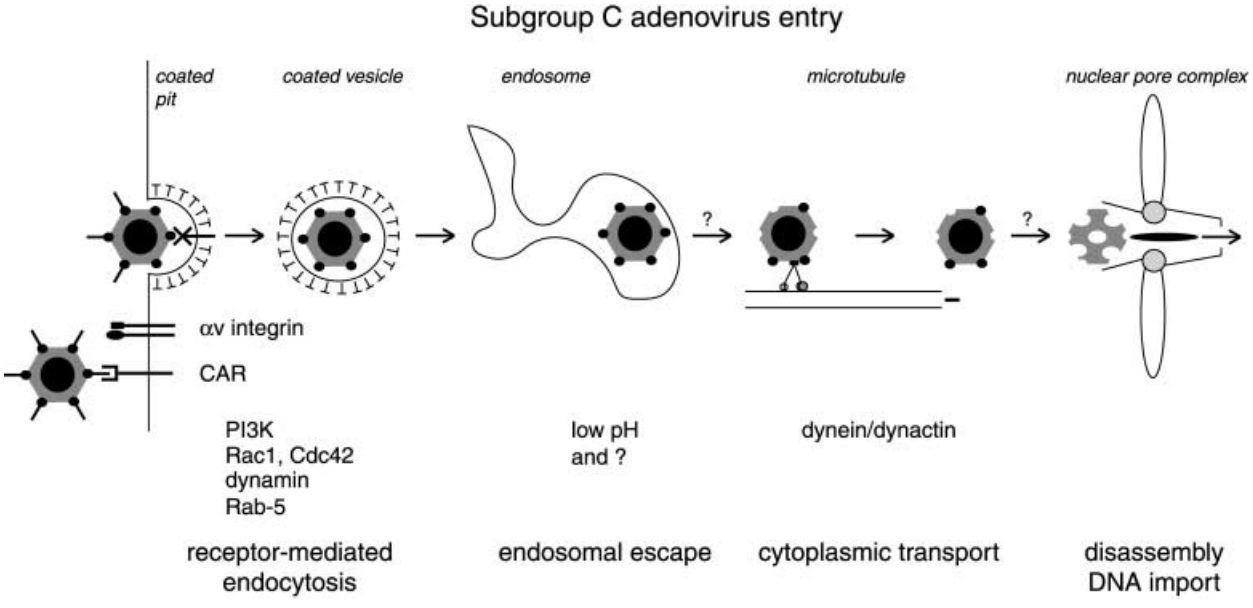

Figure 2. Generic view of subgroup C adenovirus entry. Ad-2 or Ad-5 bind to the coxsackievirus-adenovirus receptor (CAR) and the secondary receptor α_v integrin and enter cells by receptor-mediated endocytosis via coated pits and coated vesicles. Early entry steps require PI3K, Rac1 and Cdc42, and also dynamin. Virus then escapes from endosomes by an unknown mechanism, somehow assisted by low pH. Cytosolic virus particles are transported by the dynein/dynactin motor complex along microtubules in the minus-end direction towards the nucleus. Nuclear pore complex-docked virus particles are dismantled, and the DNA genome is released into the nucleus.

[73]). More recently, $\alpha_5\beta_1$ [76] and $\alpha_v\beta_1$ integrin coreceptors [77] were discovered. Keratinocytes and epithelial cells from β_5 integrin knockout mice were just as infected with Ad-5 as β_5-positive cells [78], suggesting that redundant integrin functions may coordinate viral entry. Coreceptors were also found for the lentivirus HIV-1 [79]; herpesviruses, including the α-herpesviruses HSV-1 and pseudorabies virus [80–82]; Epstein-Barr virus [83] and the retrovirus feline leukemia virus [84]. For AAV-2 two different coreceptors have been described, $\alpha_v\beta_5$ integrins [85] and basic fibroblast growth factor receptor 1 [86]. The involvement of integrin coreceptors in AAV-2 entry has recently been challenged [87].

Coreceptors typically bind particles with a lower affinity than the primary receptors and therefore act after viral binding to primary receptors. They are particularly important if the primary receptor is not coupled to virus uptake mechanisms or does not properly activate the target cell. In the case of HIV, the chemokine receptors CXCR4 (for T cell tropic viruses) and CCR5 (for macrophage tropic viruses) act in concert with primary receptors to mediate fusion of the viral and cellular membranes by activating the viral envelope protein (env) gp120/gp41 (reviewed in [56, 88]). The CXCR4 and CCR5 coreceptors are heptahelical transmembrane proteins. They are coupled to heterotrimeric G proteins and transduce a complex array of signals upon chemokine ligation (for review, see [89, 90]). Besides stimulating HIV entry and completion of early replication events, the CCR5 coreceptor appears to trigger intracellular-signalling events that boost both the recruitment of susceptible monocytes and macrophages to sites of viral replication [91, 92]. Although HIV infections stimulate the production of chemokines from a variety of cell types, chemokine signalling is not required for fusion of the viral membrane with the plasma membrane. Instead, chemokines can block contacts between viral glycoproteins and the coreceptor and have a role in coreceptor downregulation (reviewed in [93]). Exactly how coreceptor-induced signalling cooperates with signals from the primary receptor during HIV infections is a major challenge for future investigations.

Structural changes of viral particles upon entry

Among the first consequences of viral binding to surface receptors are changes in the structure of many virus particles. Capsid changes can be important for viral entry and are often followed by limited disassembly steps, necessary for complete dismantling of the particle and exposure of the genome at later stages [94]. Initial changes of viral structures occur upon binding of the primary or secondary receptors. In the case of nonenveloped viruses this can provoke conformational changes in the capsid or lead to loss of capsid components. Capsid destabilization at the cell surface is crucial for infections with picorna viruses, including polioviruses, rhinoviruses and echoviruses [95–98], reovirus [99] and also Ad-2 [100, 101]. Accordingly, small antiviral chemicals and peptides are being developed that bind to critical regions of viral capsids or, alternatively, to the viral receptor and thus prevent particle alterations and subsequent entry [102–105].

In the case of enveloped viruses, receptor binding can trigger a fusion reaction of the viral membrane with the plasma membrane. This has been shown for the avian sarcoma/leukosis virus (ASLV) [106, 107] primate retroviruses [108], lentiviruses including HIV (reviewed in [109]) and members of the herpesvirus family, such as HSV-1 (reviewed in [110, 111]). Other viruses, including influenza virus, vesicular stomatitis virus and Semliki forest virus, fuse their lipids with cellular endosomes upon exposure to low pH (for review, see [112]). This latter strategy enables viruses to take a ride within cellular vesicles and pass through the cortical actin barrier towards their replication site.

Cell activations during entry

Productive viral infections are typically associated with profound changes of the cellular transcription machinery often enhancing the expression levels of both cellular and viral genes (reviewed in [3]). In addition, levels of stress proteins are increased to facilitate replication and particle formation and also enhance the recognition of infected cells by the immune system [113–115]. One of the most immediate cell reactions to a viral infection is, however, the immune response.

Stimulation of the innate immune system

An initial antiviral response is mounted immediately after infection by setting off an innate immune response to protect cells and vital organs. Type I interferons (α- and β-IFN) are generated within hours upon infection by a large variety of viral agents. The type I INF response is crucial for restricting the exponential speed of viral spread, as indicated by type I INF receptor knockout mice, which exhibit a strongly increased sensitivity to viral infections [116]. The second branch of the immediate immune defence is the type II interferon (γ-IFN) system mounted upon activation of immune cells (reviewed in [117]). These activations can involve a large variety of cytokines and chemokines and many cell types including antigen-presenting cells, γ-IFN producing cells and natural killer cells (for recent reviews, see [118–120]).

The spectrum of induced cytokines and chemokines largely depends on the type of viral infection and the

types of recruited immune cells (reviewed in [121]). One important aspect of the innate immune system is activated by proinflammatory cytokines from macrophages and includes interleukin 1 (IL-1), IL-6 and TNF-α. IL-1 signals through the IL-1 receptor, the founding member of a diverse superfamily of receptors which share a cytosolic domain termed Toll/IL-1 receptor (TIR) domain. TIR couples to various adaptor proteins, small G proteins of the Rho family and receptor-associated kinases (IRAKs) and activates nuclear factor-kappa B (NFκB) and protein kinases, such as p38/MAPK. Interestingly, incoming adenoviral vectors elicit an early expression of a C–X–C chemokine (within 3 h) by activating NFκB in the absence of γ-IFN and TNF-α. They elicit a potent T-helper immune response consisting of CD8+-restricted cytotoxic T-lymphocytes that are directed against viral proteins [122]. Although signals upstream of NFκB are currently unknown, these results indicate that both immunogenic and signalling properties of the viral capsid have an early impact on the nature of host antiviral responses.

Triggering the p38/MAPK pathway

p38/MAPK is one of three major types of MAPKs in mammalian cells (for review, see [123]). It is triggered by diverse extracellular stimuli including UV-light, irradiation, heat shock, osmotic stress, proinflammatory cytokines and certain mitogens. These activate a cascade of upstream kinases and phosphatases and have a variety of physiological effects, including cytokine production, cytoskeletal reorganisation and apoptosis (for a review, see [124]). p38/MAPK activation has been documented for infections with rhinoviruses [125], herpesviruses [126–128], HIV [129], simian immunodeficiency virus (SIV) [130] and adenoviruses (see fig. 3, and [131]). Instillations of adenovirus into mouse respiratory tracts have shown, for example, that virus entered alveolar macrophages and within 30 min induced the transcription of TNF-α and IL-6 [132]. This suggested that the p38/MAPK pathway is part of an antiadenoviral defence system.

The p38/MAPK pathway is, however, also exploited by viral pathogens. For example, HIV utilizes p38/MAPK activations to control gene expression from its long terminal repeat [133]. Adenovirus usurps the p38/MAPK pathway to modulate the splice site selection on its immediate early E1A premessenger RNA, apparently by altering the nucleocytoplasmic distribution of cellular splicing facctors [134]. This was found in a reporter assay, where the induction of p38/MAPK by sorbitol gave rise to the long 12S and 13S E1A transcripts

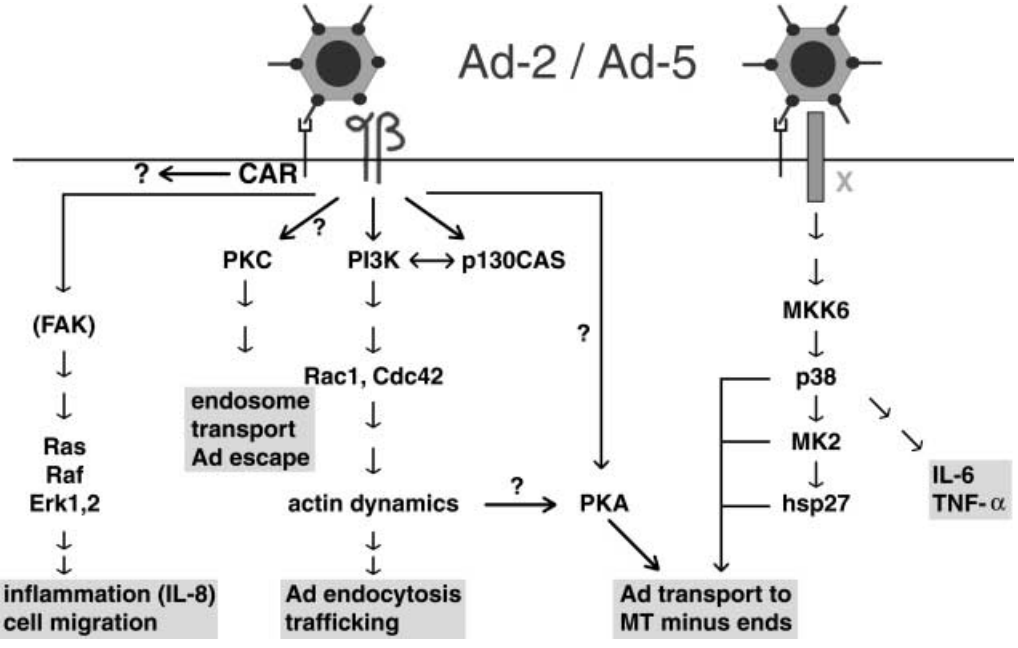

Figure 3. Cell signalling by incoming subgroup C adenoviruses. Ad-2 and Ad-5 activate α_v integrin heterodimerized to β_5. This stimulates PI3K, the adaptor protein p130/CAS, Rac1 and Cdc42, and promotes actin dynamics and endocytic trafficking of virus. In addition, incoming Ad-2 sheds its fiber proteins at the cell surface. This reaction depends on CAR and α_v integrins. Protein kinase C (PKC) is involved in endosome transport and viral escape. Whether the primary adenovirus receptor CAR alone has a signalling function is unknown. A second branch of integrin signalling occurs through Ras/Raf/ERK1,2, and leads to an inflammatory and migratory response. A further player downstream of integrins is protein kinase A (PKA). It is activated by incoming Ad-2 and stimulates viral transport along microtubules in the minus-end direction. An integrin-independent pathway signals through MKK6/p38/MK2/Hsp27 and stimulates viral transport to the nucleus. p38 activation by incoming virus also activates transcription of the cytokines IL-6 and TNF-α.

and reduced the levels of the short 9S transcript. Both 12S and 13S E1A transcripts are prevalent during productive adenoviral infections and induce early viral genes and also the 70-kDa heat-shock gene, albeit at different efficiencies [135]. Targeting the p38/MAPK pathway for antiviral therapies might thus be an interesting option.

Activation of ERKs

Besides triggering the innate immune system, many viruses activate extracellular signal regulated kinases (ERKs). ERKs belong to the MAPK family is activated by a large variety of growth factors binding to their cell surface receptors. The ERK pathway is downstream of Ras and regulates cell growth and differentiation (for a review, see [124]). The prototypic activation serves to regulate the activity of transcription factors including serum response factor, AP1 consisting of Fos and Jun subunits and cyclic AMP (cAMP)-responsive-element-binding protein (CREB) (for reviews, see [136, 137]). ERKs are also activated by many DNA tumor viruses, including HCMV [138], Kaposi sarcoma-associated herpesvirus (KSHV) [139], hepatitis B and C viruses [140, 141], papilloma virus [142], simian virus 40 (SV40) [143, 144] and adenoviruses [131, 145]. For example, HCMV stimulates sustained ERK activity, whereas KSHV expresses the highly polymorphic kaposin A type II transmembrane protein preferentially during the latent phase of B cell infections. Kaposin A is able to transform rat fibroblasts and directly interacts with cytohesin-1, a guanine nucleotide exchange factor of the small GTPase Arf-1. Activation of cytohesin then leads to ERK activation by a yet unknown mechanism. SV40 triggers ERK by virtue of the small tumor antigen which binds to and inhibits the protein phosphatase 2A, a deactivator of ERKs (reviewed in [143]). ERKs are also turned on in HIV infections by a complex series of reactions requiring the CD4 receptor, the tyrosine kinase Lck and the MEK kinase Raf-1, but in some cases also involve the coreceptors CXCR4 and CCR5 (for reviews, see [121, 129, 146]). Together with additional signals, these activations are thought to modulate the host immune response.

Further illustrations of viral control over ERK activations have recently come from studies of RNA viruses, including the common cold-causing influenza virus [147], the neurotropic, noncytolytic Borna disease virus [148] and the paramyxovirus respiratory syncytial virus RSV [149]. Interestingly, the production of progeny influenza and Borna disease virions was substantially diminished in cells treated with inhibitors of the ERK-activating kinase MEK. Since cell integrity was apparently not affected by these inhibitors, the ERK pathway may be a potential target for antiviral therapies.

ERKs are also activated by incoming virus particles, as demonstrated with adenovirus. Activation of p42/44 ERKs depends on the viral coreceptor α_v integrin and eventually leads to the production of IL-8 [145]. It is expected that Ras and Raf are major contributors to early ERK activation by adenovirus, since incoming virus also activates protein kinase A [131], an antagonist of Ras-independent activations of Raf-1 [150]. In the case of adenovirus, ERK activation has also post-entry effects. It leads to phosphorylation of the E1A transactivator, which in turn activates transcription of the viral E4 genes [151]. Whether virally activated PKA has post-entry effects, for example activation of CREB, is not known, but it is known that PKA phosphorylates and activates p65/NFκB, which cooperates in Jurkat T cells with the transcriptional coactivator p300/CBP [152]. Interestingly, NFκB binds to the E3 region of the adenoviral genome in lymphoid cells and seems to enhance expression of E3 proteins which are required for immune evasion and viral persistance [153]. The action of NFκB is turned off by the early viral E1B 19-kDa protein, which ensures that the infected cell is not undergoing premature apoptosis [154].

Viral targeting to Ras/MAPK-activated cells

Besides driving the cell cycle and coordinating antiviral responses, an activated Ras/MAPK pathway can also have distinct proviral effects by rendering the target cell more vulnerable to lytic viral infections. Initial studies with NIH3T3 cells showed that reovirus, a double-stranded RNA virus, lytically replicated in cells with an activated Ras/MAPK pathway [155, 156]. In nonactivated cells reovirus replicated poorly, possibly due to interferon-induced protein kinase R (PKR). PKR phosphorylates the translation initiation factor eIF2α, which leads to a shutdown of host translation due to trapping of guanine nucleotide exchange factors. The activated Ras/MAPK pathway phosphorylates and inhibits PKR and thus permits translation of viral mRNAs. Interestingly, the unrelated HSV-1 takes advantage of the activated Ras/MAPK pathway in a way similar to reovirus and preferentially replicates in Ras-activated cells [157]. In addition to the cellular branch of PKR inactivation, HSV-1 takes advantage of a viral gene product, ICP34.5 to shut down PKR. ICP34.5 is thought to antagonize PKR by forming a complex with protein phosphatase 1 and triggering dephosphorylation of eIF2α [158]. Likewise, adenoviruses have developed further mechanisms including virus-associated RNAs (VA RNAs) to inactivate PKR (reviewed in [159]). It will be interesting to see whether oncolytic viruses in general rely on both a cellular and a viral branch to inactivate PKR and drive virus production.

Cell survival

While activation of cell growth and proliferation is crucial for many viral infections, it does not generally ensure that the infected cell survives long enough to give rise to sufficient amounts of progeny virus and sustain the infection. Cell survival pathways are conserved from *Drosophila* to humans, and phosphoinositide 3-kinases (PI3Ks) have a key role. They phosphorylate the 3′-OH position of the inositol ring of inositol phospholipids and give rise to three lipid products, PtdIns(3)P, PtdIns(3,4)P(2) and PtdIns(3,4,5)P(3). These lipids bind to the pleckstrin homology (PH) domains [160], FYVE-containing ring domains [161] and also PX domains of proteins (reviewed in [162]). Thus, they control the activity and subcellular localization of a diverse array of signal transduction molecules involved in cell survival and migration, vesicular trafficking and cytoskeletal organization. There are three classes of PI3Ks (for reviews, see [163, 164]). Class I PI3Ks are heterodimers of a catalytic (p110) and a regulatory subunit (p50–p85) binding via Src homology domain 2 (SH2) to phosphotyrosine residues of activated receptor tyrosine kinases. One of the PI3K isoforms does not bind phosphotyrosines but instead couples to β,γ subunits of heterotrimeric G proteins. All class I enzymes phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 in vitro with PtdIns(4,5)P_2 preference in vivo. The class II PI3Ks are distinguished by a PtdIns and PtdIns(4)P substrate preference and the presence of a carboxy-terminal C2 domain that binds calcium. The prototypic member of class III PI3Ks is the yeast Vps34p, which is mainly implicated in vesicular trafficking.

Adenovirus has been one of the first incoming viruses shown to activate PI3K (see Fig. 3, and [165]). Anti-phosphotyrosine immunoprecipitates from Ad-5-infected SW480 colon carcinoma cells contained enhanced activities of PI3K compared with noninfected cells. The assay measured the appearance of PtdIns(3,4,5)P(3) suggesting class I PI3K activation. Stimulation of PtdIns(3,4,5) P(3) formation was strongest during viral entry and was blocked by either wortmannin or overexpression of the SH2 domain of the regulatory protein p85. Since the purified capsid protein penton base alone enhanced PI3K activity, it is likely that penton base ligation of α_v integrins was sufficient for activation of PI3K. Whether virally activated integrins cross-talk with growth factor receptors or induce signalling on their own is not known. It is known, however, that the integrin-associated tyrosine kinase focal adhesion kinase (FAK) is not needed for adenovirus-triggered PI3K activation and entry [166]. The FAK-associated adaptor p130(CAS), however, binds p85 and supports receptor-mediated Ad-2 and Ad-5 entry. Molecular links between PI3K activation and endocytic uptake are emerging. Recent evidence shows that class II PI3Ks are activated upon binding to clathrin at the plasma membrane and in the trans-Golgi network [167]. This suggests that phosphoinositide lipids are generated at sites of clathrin coat formation, and this may coordinate the recruitment of the endocytic machinery. The broad spectrum of regulatory PI3K actions and the complexity of Ad entry, however, also suggest further roles of these enzymes in viral endocytosis, membrane trafficking and/or cytoplasmic transport.

A second virus particle shown to activate PI3K is AAV-2 (fig. 4 A, and [168]). AAVs are lipid-free single-stranded DNA parvoviruses (see below). Similar to Ad-5, AAV-2-mediated PI3K activation in HeLa cells was downstream of $\alpha_v\beta_5$ integrins, but surprisingly, and in contrast to Ad-5, Rac1 activation appeared to be upstream of PI3K. In both cases the actin cytoskeleton was required for viral uptake, but PI3K activation was not required for AAV-2-uptake. Instead, PI3K was involved in trafficking of AAV-2-containing endocytic vesicles towards the nucleus, consistent with the earlier notion that hVPS34 facilitated movement of Rab5-positive endosomes along microtubules [169].

More recently, incoming HCMV was found to activate PI3K in quiescent fibroblasts [170]. As in Ad-5 and AAV-2 infected cells, PI3K activation was transient, peaking at 15–30 min post-infection. Interestingly, the cellular kinases Akt and p70/S6K and the transcription factor NFkB were activated in a PI3K-dependent manner, suggesting that CMV induces cell survival pathways during entry. In the replicative phase, there was a second burst of PI3K activity which was maintained throughout the infection. PI3K inhibitors had no effect on viral uptake but inhibited replication. This situation is reminiscent of platelet-derived growth factor (PDGF)-activated HepG2 cells, which exhibited an initial peak of PI3K activity for early stimulations and a later PI3K peak for S-phase induction [171].

Signals boosting viral entry

Activation of the Ras/MAPK pathway serves multiple viral and cellular objectives, but there is little evidence that it affects viral entry. This raises the question whether uptake of viral particles is regulated or rather occurs constitutively, under stealth conditions. An example of a silent strategy is provided by one form of vaccinia virus, the extracellular enveloped virus (EEV), but entry of the intracellular mature virus (IMV) activates a signalling cascade involving Rac and protein kinase C [172]. The viral benefits of signalling-independent entry are unknown. On the other hand, emerging evidence demonstrates that cell signalling during entry facilitates viral uptake and appropriate intracellular targeting.

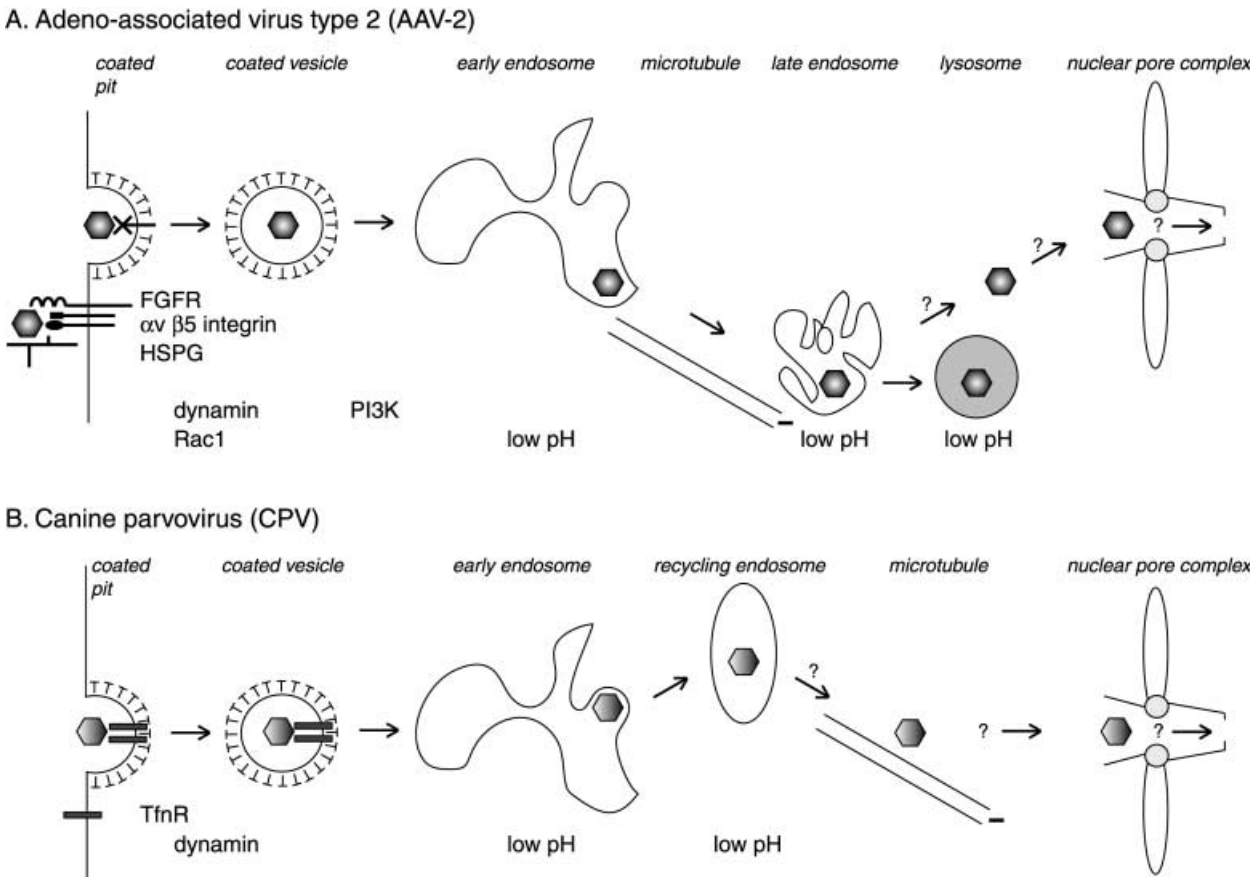

Figure 4. Trafficking of incoming parvoviruses. Panel *A*: The dependovirus AAV-2 binds to cell surface heparan sulphate proteoglycans (HSPG), $\alpha_v\beta_5$ integrins and fibroblast growth factor receptor (FGFR) 1 and is internalized by receptor-mediated endocytosis in a dynamin- and Rac1-dependent manner. PI3K appears to regulate endosome trafficking. AAV-2 is transported from early endosomes to late endosomes and lysosomes depending on microtubules. Virus escapes and releases its single-stranded DNA genome into the nucleus. Panel *B*: The helper-independent canine parvovirus (CPV) binds to the transferrin receptor (TfnR) and is internalized presumably by clathrin-dependent endocytosis. In early endosomes, it is sorted to recycling endosomes from where it may be released into the cytosol and reach the nucleus by microtubule-dependent trafficking. As in the case of AAV-2, it is unknown how its single-stranded DNA genome is imported into the nucleus.

Regulated endocytic uptake

Many enveloped and nonenveloped viruses utilize an endocytic shuttle mechanism for entry into host cells (reviewed in [173]). This ensures viral delivery to metabolically active cells and helps to overcome a major cellular barrier, the cortical actin cytoskeleton [174]. However, actin does not play an obligatory role in receptor-mediated endocytosis, but has one or several important accessory roles [175, 176]. Enveloped RNA viruses, such as the alpha viruses Sindbis and Semliki Forest virus and the orthomyxovirus influenza virus enter independently of actin, presumably by constitutive endocytosis, that is through receptors that are internalized from clathrin-coated pits in the absence of ligand [177]. In contrast, most signalling receptors undergo ligand-induced endocytosis, as originally discovered with the epidermal growth factor (EGF) receptor [178]. Emerging evidence, however, suggests that constitutive clathrin-independent endocytosis is effectively used by certain receptors, such as the IL-2 receptor [179]. Endocytosis assures that activated receptors are removed from the surface, and enables the cell to discriminate between old and new signals. In addition, endocytozed signalling receptors appear to have the ability to emit signals that are distinct from those generated at the cell surface (for reviews, see [180, 181]). It remains to be shown, for example, if and how these signals are connected to the large GTPase dynamin, which is implicated in multiple endocytic and intracellular trafficking events. Nonetheless, the endocytic pathway emerges as an important target for viral manipulation during entry.

Adenoviruses

Human adenoviruses are small nonenveloped viruses with a capsid diameter of about 90 nm and are causative agents of widely different diseases (reviewed in [182]).

They deliver a linear double-stranded DNA genome of about 36 kb into the nucleus of target cells of a large range of tissues (see fig. 2). Human adenoviruses are classified into six distinct subgroups, A–F, with at least 51 serotypes. For example, serotypes Ad-2 and Ad-5 (subgroup C) are associated with upper airway infections, as is serotype Ad-3 (subgroup B). Other serotypes (e.g. subgroup D) are linked to epidemic keratoconjunctivitis, pneumonia (subgroup E), enteric infections (subgroup A) or gastroenteritis (subgroup F). The subgroup C adenoviruses are internalized by receptor-mediated endocytosis [183], requiring Rab-5 [184] (fig. 2). Rab-5 is a regulator of clathrin-coated vesicle formation, homotypic early endosome fusion and early endosome trafficking on microtubules (reviewed in [185]). Interestingly, the guanine nucleotide dissociation inhibitor (GDI) of Rab-5, which enhances the extraction of the inactive Rab-5-GDP from membranes and boosts endocytosis of fluid phase markers, has been reported to be activated by p38/MAPK [186]. Activated p38/MAPK is not, however, required for adenovirus endocytosis per se or endosomal escape, as indicated by quantitative electron microscopy and cell surface trypsinization assays [131]. This suggests that Rab-5 activation is not a limiting factor for adenovirus endocytosis. Viral endocytosis is, however, coordinated by α_v integrins [73], requires the large GTPase dynamin [187] and depends on actin-modulating G-proteins [101, 188]. The precise role of actin in viral endocytosis is yet unknown. Actin could be involved in membrane invagination, coated pit formation and sequestration, detachment of the newly formed vesicle or vesicular motility. Given that incoming adenovirus activates PI3K and stimulates membrane trafficking, it is likely that adenovirus endocytosis is ligand induced and tightly controlled. Endocytic control mechanisms might be involved in releasing viral particles from endosomes since protein kinase C inhibitors block viral escape and restrict incoming viruses to peripheral endosomes [101].

Less is known about the endocytic uptake of other adenovirus serotypes than subgroup C. The subgroup B viruses Ad-3 and Ad-7 utilize a different receptor than CAR for entry and are eventually targeted to late endosomes and lysosomes, from where they appear to deliver their genome to the nucleus [189, 190]. The lysosomal localization of subgroup B viruses is reminiscent of an Ad-2 mutant, the temperature-sensitive mutant 1 (ts1) [191]. At the nonpermissive temperature, ts1 particles lack a functional protease and contain nonprocessed precursor polypeptides [192]. Ts1 particles are 100–1000 times less infectious than wild-type Ad-2, but nonetheless enter cultured cells on a CAR and α_v integrin-dependent manner [193] and also trigger PI3K [165]. However, they seem to enter cells without detectable activation of MAPK pathways [131] and end up in late endosomes and lysosomes, where they are degraded [193, 194]. These data suggest that endocytic sorting of viral vesicles is regulated by cell signalling, and may hold key to determining the early course of infections.

Parvoviruses

Parvoviruses are very small animal DNA viruses with diameters of 18–26 nm. Members of the parvovirinae subfamily infect vertebrates and replicate in the nucleus of proliferating cells, either autonomously or nonautonomously (reviewed in [195]). The latter require coinfection with an unrelated virus (e.g. adenovirus or herpesvirus) and are called dependoviruses or AAVs. Although several clinical disorders are linked to parvovirus infections, for example, infections with the human blood progenitor cell-tropic B19 isolate, little is known about the entry mechanism of human parvoviruses.

More entry information is available, however, about autonomous animal parvoviruses. For example, canine parvovirus (CPV) and feline panleukopenia virus (FPV) are pathogens of dogs and cats. They are more than 99% identical in DNA sequence, but differ in host range due to variations of surface-exposed capsid loops [196]. An important determinant of CPV and FPV entry appears to be the transferrin receptor (see fig. 4B, and [197]), a constitutively internalized receptor without particular signalling properties. Transferrin uptake occurs via clathrin-coated vesicles, and incoming viruses colocalize with internalized transferrin [198]. Accordingly, CPV uptake was inhibited by dominant-negative dynamin K44A. Lysosomotropic agents inhibited DNA amplification and viral protein expression, suggesting that at least one low-pH compartment is required for infection [199]. Neutralizing cytoplasmic anti-capsid antibodies inhibited infection as late as 6 h post inoculation, suggesting that escape of CPV from endosomes is slow [200]. The escape mechanism and further downstream events, including nuclear import, are unknown, but it has been suggested that transport of cytoplasmically injected CPV requires intact microtubules. Less is known about other animal parvoviruses. Electron microscopy suggested that mouse minute virus (MMV) enters cells via coated pits [201]. By virtue of its capsid proteins VP1 and VP2, it somehow reaches the nuclear membrane and releases its genome into the nucleoplasm [202].

The dependo-virus AAV is a single-stranded DNA-containing human parvovirus with low pathogenicity. It has the potential to infect a large number of cell types and is currently tested in clinical gene delivery trials. Like Ad-2 and Ad-5, AAV-2 seems to utilize α_v integrins to coordinate endocytic uptake (see fig. 4A, and [85]), in a dynamin-dependent manner [203, 204]. AAV-2 activates PI3K and Rac1. Like ts1 Ad-2, AAV-2 slowly escapes from the endocytic compartment [168]. Inhibition of PI3K with wortmannin or blocking Rac1 with dominant-negative N17Rac1 inhibited AAV-2-mediated transgene expres-

sion. It is clear that activations of PI3K and Rac1 are not sufficient to specify the subcellular fate of endocytozed viral particles, since fluorescent AAV-2 did not colocalize with fluorescent Ad-5 [204]. AAV-2 remained in the endocytic pathway considerably longer than Ad-2. Percoll density centrifugations of cell lysates showed, for example, that incoming AAV-2 preferentially cofractionated with a lysosomal marker in permissive human 293 cells, but cofractionated with transferrin when internalized into the nonpermissive mouse NIH-3T3 cells [205]. Furthermore, the proton pump inhibitor bafilomycin A1 strongly delayed AAV-2 infection in human cells, suggesting the involvement of an acidic compartment [204–206]. In addition, the depletion of microtubules with nocodazole also inhibited AAV-2 transgene expression [168], consistent with the requirement of microtubules for endosomal trafficking. Interestingly, incoming AAV-2 is ubiquitinated and degraded by the proteasome [206, 207]. Potentially, this pathway might serve as an uncoating or an antiviral defence mechanism. How AAV-2 is sorted to late endosomes and lysosomes and how it reaches the cytosol and imports its DNA genome remain outstanding questions.

Other viruses

Papovaviruses are small icosahedral DNA viruses (50–60-nm capsid diameter), grouped into the genera papillomaviruses and polyomaviruses. Simian virus 40 (SV40) is their best characterized member in terms of entry and signalling. Unlike adenoviruses and parvoviruses, SV40 enters by caveolar endocytosis, as indicated by morphological analyses and expressions of dominant-negative caveolin [208–211]. Caveolin is a major component of the detergent-insoluble, cholesterol-rich caveolar membranes [212]. Binding of SV40 to target cells occurs via major histocompatibility class I molecule. SV40 entry into growth-arrested cells is associated with an immediate transcriptional upregulation of c-myc and c-jun, independent of protein synthesis and viral transcription [144]. Interestingly, these activations did not appear to affect Raf and ERKs but were sensitive to inhibitors of tyrosine phosphorylation and protein kinase C. Blocking tyrosine phosphorylation inhibited SV40 entry but apparently had no effect on viral partioning into detergent-insoluble membrane fractions, suggesting that tyrosine phosphorylation may originate in caveolae [213]. It will be important to sort out how these observations relate to the emerging notion that caveolin-containing microdomains are vital organizers of cell signalling (for a review, see [214]). Further along the entry pathway, SV40 particles are shuffled to perinuclear smooth endoplasmic reticulum (sER) [215], and somehow deliver their DNA genome into the nucleus (reviewed in [15, 216]). It can be expected that these sorting events are coordinated by cell signalling, although the reasons for SV40 targeting to the sER are currently not understood. It is worth noting that a virus unrelated to SV40, the human parechovirus (HPEV) of the picornavirus family of RNA viruses replicating in the cytoplasm has been found in the ER as well [217]. HPEV is thought to enter cells by clathrin-mediated endocytosis, presumably in an α_v integrin-dependent manner, passes through EEA-1-positive early endosomes and is routed to late endosomes and lysosomes. This illustrates once again that viruses subtly regulate subcellular trafficking.

Regulation of cytoplasmic motility

Initially, video-enhanced microscopy and digital image processing were used to observe enveloped viruses budding from the cell surface in living cells [218]. The first fluorescent viruses were the nonenveloped viruses adenovirus and reovirus, which allowed the visualization of overall viral entry features, such as capping of particles at the cell surface [189, 219, 220], and cytosolic virus motility by time-lapse fluorescence microscopy [221]. Major progress in fluorescence-imaging technologies during the last decade has allowed more accurate and less-invasive procedures [222–224], and analyses of numerous viruses either labelled with small fluorescent molecules or with green fluorescent protein (GFP) are under way in many different laboratories [e.g. 132, 168, 204, 211, 225–231].

From measurements of the fluorescence recovery after photobleaching of microinjected dextrans and ficoll beads, it can be expected that the diffusional properties of viruses are considerably smaller in the cytoplasm than in aqueous media [232, 233]. If these particles do not bind to slowly diffusing cytoplasmic structures, this means that cytoplasmic crowding with large obstacles, such as filaments and organelles, might restrict passive diffusion of cytosolic virus particles. Organelle motion is often dependent on molecular motors that convert the energy of ATP into mechanical work and move along cytoplasmic filaments. This is reflected by a large body of live cell experimentation in continuous mode, indicating that organelles are moving in a stop-and-go fashion with periods of motility and inactivity (for a review, see [234]). Since active motor complexes seem to be relatively rare and may be activated or recruited to a large variety of cargo by positional information, it is expected that movement of viruses is tightly controlled. Indeed, intracellular motility of virus particles turns out to be an important determinant of infections (for recent reviews, see [235–237]).

Adenovirus

Ad-2 and Ad-5 escape from the endosomal pathway long before virus-bearing endosomes have arrived in a perinuclear location (see fig. 2). Texas-Red labelled viruses and quantitative microscopy in both static and time-lapse modes combined with electron microscopy revealed that cytosolic Ad-2 uses bidirectional microtubule-dependent

cytoplasmic transport to reach the nuclear membrane [227, 238, 239]. The population speed of virus targeting to the nucleus was in the order of μm min^{-1}, but peak speeds reached 2.6 μm s^{-1}. Virus transport to the minus ends of microtubules near the nucleus is mediated by the cytoplasmic dynein/dynactin motor complex, which operates at velocities of μm s^{-1}. A first hint on possible regulatory mechanisms of viral motility came from the observation that microinjected native Ad-2 was essentially immotile but could be stimulated to move when the cell was challenged by an authentic Ad-2 infection [131]. Further experiments revealed that incoming Ad-2 induced two discrete signalling pathways from the cell surface that modulate viral motility (see fig. 3). The first pathway activated protein kinase A (PKA) and required upstream α_v integrins. The second pathway was independent of integrins and activated the p38/MAPK cascade, a central component of innate immunity and host cell defence. Knocking out the p38-activating MAPK kinase MKK6 by overexpression of dominant-negative MKK6 strongly inhibited nuclear targeting of Ad-2 and resulted in viral accumulation in the periphery. p38 stimulation by Ad-2 appeared to be required to suppress plus end-directed transport towards the periphery, and the p38 target MAPKAP kinase 2 (MK-2) to boost the frequencies of minus end-directed transport steps. These results demonstrate that incoming Ad-2 not only modulates the transcriptional environment of an infected nucleus but also tips the activities of cytoplasmic transport machineries to favor the infection. In analogy with lipid droplet movements in *Drosophila* embryos [240, 241], one might speculate that Ad-2 utilizes bidirectional microtubule-mediated transport to stay on track and to possibly overcome a limiting processivity of motor proteins. On the other hand, plus end-directed transport along microtubules might be important for the virus to reach particular subcellular locations, such as the plasma membrane during viral egress. A switching mechanism of transport could be directly or indirectly regulated by PKA, p38 and MK-2, and perhaps additional factors such as the viral structure. That minus end targeting is regulated by α_v integrin-dependent and independent pathways correlates with the notion that Ad-2 uses multiple secondary receptors for entry [78]. It will be interesting to see how other viruses that encode their own kinases manipulate the cellular signal transduction machinery during entry.

Other viruses

Vaccinia virus of the poxvirus family replicates its DNA genome in the cytoplasm, and takes advantage of cytoplasmic trafficking to facilitate spread and cell exit. Microtubules and the dynein/dynactin motor complex have a role in the formation of intracellular mature virus (IMV) and also intracellular enveloped virus (IEV) [242]. Recent studies show that intracellular vaccinia virus utilizes microtubule-dependent motility to reach to the cell surface [243–245], and actin comet tail formation was observed on newly synthesized vaccinia virus [246]. Although actin tail formation is not necessary for cytoplasmic motility, it clearly occurs when extracellular virus attaches to the plasma membrane. This depends on two critical tyrosine residues (112 and 132) of the viral protein A36R [247, 248]. Although the microtubule-dependent motors and the viral binding partners driving virus to the periphery are currently unknown, these results show that vaccinia virus alters the function of microtubules and utilizes actin polymerization to support spreading from cell to cell. Further examples of viral particles utilizing microtubules for trafficking include the enveloped DNA virus african swine fever virus [249], HSV-1 [250], the retrovirus foamy virus [251] and bovine papilloma virus [252]. In addition, a number of viruses encode proteins that either stabilize [253] or reorganize microtubules [254, 255], or bind to microtubule-associated proteins [256–259], actin [260] or intermediate filaments [261] and alter transport functions [262, 263]. Clearly, viruses exploit a wealth of opportunities to interfere with cell trafficking and architecture. We are just beginning to understand the underlying mechanisms.

Perspectives

Viruses are versatile, ubiquitous carriers of genetic material. Their mission is to replicate and produce progeny. Viral entry is a most critical process, setting off infection. It is highly regulated and often coordinated by signalling of cellular and viral factors. Prevailing evidence indicates that signals generated by virus-receptor interactions at the cell surface affect both viral and cellular gene expression. Signals from incoming virus particles bear on the efficiency of early entry steps, including virus uptake, escape to the cytosol, cytoplasmic transport and nuclear import. A major challenge will be to integrate cell signalling into transport and anchoring functions of the cytoskeleton and determine how cargo trafficking feeds back into amplification or extinction of signals. Viruses may well be a role model in such investigations. Further understanding of virus-cell signalling will broaden our knowledge of virally caused disease and eventually lead to new antiviral agents. A more detailed understanding of entry will enhance the development of retargeted viral vectors for selective therapeutic applications. Viruses with a flexible lipid envelope or with a modular lipid-free structure are initially preferred targets, but recent developments of soluble linker proteins may also allow efficient retargeting of rigid viral capsids. In any case, retargeted viruses may ideally activate cell signalling at the time of vector binding to the target cell, similar to native infections. This will fine-

tune both viral entry and transcriptional control of the transgene to yield optimal and sustained gene expression.

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