Gene Therapy (2005) 12, 873–880

www.nature.com/gt

REVIEW

Intracellular trafficking of adeno-associated viral vectors

W Ding[1], L Zhang[1], Z Yan[1,3] and JF Engelhardt[1,2,3]
[1]Department of Anatomy and Cell Biology, University of Iowa School of Medicine, Iowa City, IA, USA; [2]Department of Internal Medicine, University of Iowa School of Medicine, Iowa City, IA, USA; and [3]Center for Gene Therapy, University of Iowa School of Medicine, Iowa City, IA, USA

Adeno-associated virus (AAV) has attracted considerable interest as a gene therapy vector over the past decade. In all, 85% of the current 2052 PubMed references on AAV (as of December 2004) have been published in the last 10 years. As researchers have moved forward with using this vector system for gene delivery, an increased appreciation for the complexities of AAV biology has emerged. The biology of recombinant AAV (rAAV) transduction has demonstrated considerable diversity in different cell types and target tissues. This review will summarize the current understanding of events that control rAAV transduction following receptor binding and leading to nuclear uptake. These stages are broadly classified as intracellular trafficking and have been found to be a major rate-limiting step in rAAV transduction for many cell types. Advances in understanding this area of rAAV biology will help to improve the efficacy of this vector system for the treatment of inherited and acquired diseases.
Gene Therapy (2005) **12,** 873–880. doi:10.1038/sj.gt.3302527; Published online 14 April 2005

Keywords: *adeno-associated virus; transduction; endosomal trafficking*

Introduction

Adeno-associated virus (AAV) is a small nonenveloped virus that belongs to the dependovirus genus of the parvovirus family.[1,2] AAVs share varying degrees of similarities to autonomous parvoviruses including Canine parvovirus (CPV), Feline parvovirus (FPV), and minute virus of mice.[3] As such, comparative biology of these related parvoviruses has been useful to the study of AAVs. AAV was initially discovered as a contaminant in adenovirus preparations.[4,5] Like other members of the parvovirus family, AAVs have a single-stranded DNA genome. The viral genome is approximately 4.7 kb in length, and is composed of two major open-reading frames encoding Rep (replication) and Cap (capsid) proteins. Flanking these two viral genes are the inverted terminal repeats (ITRs, 145 bp in length for AAV2) at either end of the viral genome.[6] Both positive and negative stands of DNA are packaged into AAV virions.[7]

One of the most unique components of the AAV genome is the self-complementary, CG-rich ITR. This stable T-shaped hairpin plays an important role in AAV genome biology and is the only necessary viral component in recombinant vector genomes. Included in the ITR are two motifs, a terminal resolution site and a Rep binding site, which play key roles in replication and encapsidation of the viral genome. Four Rep proteins (Rep 78, Rep 68, Rep 52, and Rep 48) are generated from the 5′ open-reading frame of wild-type AAV through the use of two different promoters and alternative splicing.[8] The 3′ open-reading frame of wild-type AAV generates three cap proteins (VP1, VP2, and VP3) through alternative mRNA splicing and alternative start codon usage.[9] The VP1, VP2, and VP3 proteins assemble at a ratio of approximately 1:1:10, respectively, to form a mature AAV particle approximately 26 nm in diameter.[10–12]

In the early 1980s, pioneering work on the successful cloning of AAV established the foundation of recombinant AAV vectors capable of expressing foreign genes in mammalian cells.[13,14] Currently, eight serotypes of AAV have been evaluated as recombinant vectors (AAV1–AAV8) and many more have been isolated from various species including non-human primates.[15,16] These AAV serotypes share a common genome structure, but have varying abilities to infect different cell types and tissue based on their capsid protein recognition by cell surface receptors. The repertoire of rAAV vectors has also been greatly expanded by the development of technologies to pseudo-package rAAV genomes,[17–20] package AAV genomes with two different ITR serotypes,[21] generate mosaic rAAV particles with more than one capsid serotype,[22–24] retarget AAV by generating rAAV capsid modification[25–29] and generate rAAV with chemically modified capsids.[30] These technologies have greatly expanded the ability to tailor rAAV for specific applications in gene therapy.[31]

Despite the great number of rAAV serotypes and rAAV variants available, several biologic barriers appear to limit the effectiveness of rAAVs for gene therapy. Understanding the fundamental basis of these biologic barriers have aided in establishing methods to improve the efficiency of rAAV-mediated gene delivery. As shown

Correspondence: Dr JF Engelhardt, Department of Anatomy and Cell Biology, University of Iowa School of Medicine, Room 1-111 Bowen Science Building, 51 Newton Road, Iowa City, IA 52242-1109, USA
Published online 14 April 2005

in Figure 1, these biologic barriers can be broadly divided into seven stages based on the rAAV infectious process. First, AAV must bind to the surface of the target cell. The most significant factor affecting viral binding includes the abundance of AAV receptors. It is this stage that can be most significantly influenced by the choice of rAAV serotype or type of capsid variant used to generate the recombinant virus. The second stage includes receptor-mediated endocytosis of the virus. This stage may be influenced by the abundance of AAV coreceptors on the surface of target cells and the activation of cellular pathways that trigger endocytosis. The third through fifth stages of rAAV infection involve rAAV movement to the nucleus. These stages are a major focus of this review and involve vesicular trafficking (stage 3), endosomal escape (stage 4), and nuclear transport (stage 5). As will be discussed, each of these stages appear to be interrelated and rate limiting for many AAV serotypes. The final postnuclear stages for rAAV transduction include viral uncoating (stage 6) and genome conversion (stage 7) of the single-stranded rAAV genome to double-stranded DNA intermediates (circular and linear) capable of expressing transgenes. Although these stages will not be reviewed here, it is noteworthy to mention that viral uncoating may be closely linked to processing of the virion through certain endosomal compartments.

These multiple-step intracellular events can be regarded as barriers to AAV transduction. Increasing evidence has suggested that intracellular trafficking of rAAV may be a universal rate-limiting step in AAV transduction.[32–38] However, entry pathways of AAVs likely determine the paths by which these viruses move through the cell to the nucleus. As such, discussions of intracellular trafficking must be placed in the context of viral receptors and coreceptors that facilitate endocytosis and movement of AAVs through the endosomal compartment. This review will focus on the current understanding of rAAV transduction biology prior to uncoating of the virus in the nucleus.

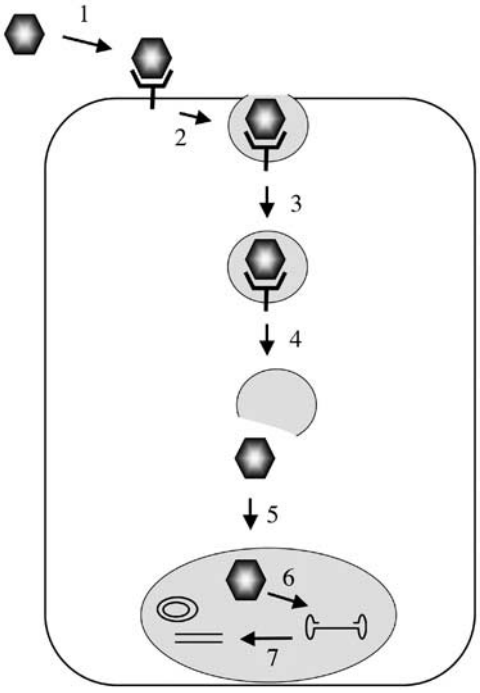

Figure 1 *Stages of rAAV transduction. Schematically shown are seven stages of rAAV transduction including: (1) viral binding to a receptor/coreceptor, (2) endocytosis of the virus, (3) intracellular trafficking of the virus through the endosomal compartment, (4) endosomal escape of the virus, (5) intracellular trafficking of the virus to the nucleus and nuclear import, (6) virion uncoating, and (7) viral genome conversion from a single-stranded to a double-stranded genome capable of expressing an encoded gene.*

AAV attachment receptors and coreceptors

As for most if not all viruses, infection with AAV is initiated by binding to cell surface receptors (Table 1). Heparan sulfate proteoglycan (HSPG) has been identified as one of the primary attachment receptors for AAV2.[39] To this end, soluble heparin can compete for binding of AAV2 to the surface of many cell types.[39,40] Mutagenesis and crystal structure studies have identified the amino acids in the capsid of AAV2 responsible for HSPG binding.[41,42] Heparin has also been reported to compete for AAV3 binding to cells, implying that AAV2 and AAV3 may share HSPG as an attachment receptor.[18,43] Despite the fact that HSPG appears to be a major AAV2 attachment receptor for many cell types, other model systems have demonstrated considerable uptake of AAV2 in the absence of surface HSPG expression.[32,44] Hence, attachment receptors for AAV2 other than HSPG may also exist, but have yet to be identified. In contrast to AAV2 and AAV3, sialic acid has been identified as a primary attachment receptor for AAV4 and AAV5. AAV5 primarily uses N-linked sialic acid for binding, whereas AAV4 preferentially uses O-linked sialic acid for attachment.[45,46] The attachment receptors for AAV1 and AAV6 have not yet been identified, but they do not appear to involve HSPG.[18,47,48] However, based on capsid homology between AAV1 and AAV6, it is likely they share the same attachment receptor for binding. AAV7 and AAV8 were rescued from non-human primate tissues and are the two newest AAV serotypes being evaluated as recombinant vectors.[49] Although the attachment receptors for these serotypes have yet to be identified, their distinct tissue tropisms suggest that they use different cell surface receptors than other AAV serotypes.

In addition to attachment receptors, AAVs also require coreceptors for efficient infection. For example, despite the fact that heparin can compete for rAAV2 and rAAV3 binding to cells, studies have demonstrated that rAAV3 can transduce hematopoietic cells not susceptible to transduction with rAAV2 vectors.[43] Such data suggest that alternative receptors (ie coreceptors) on the cell surface are required for infection with AAVs. Two coreceptors for AAV2 have thus far been identified. These include αVβ5 integrin[50] and fibroblast growth

Table 1 AAV attachment receptors and coreceptors

Serotype	*Attachment receptor*	*Coreceptor*
AAV1, 6, 7, 8	Unknown	Unknown
AAV2	HSPG[39]	α5βV integrin;[50] hFGFR1[53]
AAV3	HSPG[18,43]	Unknown
AAV4	O-linked sialic acid[45]	Unknown
AAV5	N-linked sialic acid[45,46]	PDGFR[54]

factor receptor type 1.[51] The level of αVβ5 integrin expression can significantly influence the efficiency of rAAV2 transduction in certain cell types; however, its functions as a coreceptor has been debated.[52,53] Nonetheless, inhibition of αVβ5 integrin with blocking antibodies can prevent internalization of bound rAAV2 on the surface of HeLa cells.[40] Similarly, platelet-derived growth factor receptor (PDGFR) has been considered the coreceptor for AAV5 and is also required for efficient infection with this serotype.[54] However, PDGFR itself is a sialo-glycoprotein containing both N- and O-linked oligosaccharide chains with sialic acid.[55,56] This suggests that PDGFR may be capable of acting alone as a receptor for AAV5.

Different AAV serotypes preferentially transduce (ie express their encoded gene) different cell types. For example, comparative analysis of rAAV1, rAAV2, and rAAV5 transduction in the brain has demonstrated differences in cellular tropisms.[57] It is generally assumed that the abundance of known receptors and/or coreceptors correlates with tropism for certain AAV serotypes. However, this is not always the case. For example, in polarized human airway epithelia, rAAV2 transduction from the basolateral surface is 200-fold greater than following apical infection.[44] Although HSPG and αVβ5 integrin are absent on the apical surface and abundant on the basolateral surface, there is only a slight difference (three- to five-fold) in the extent of AAV2 endocytosis from apical and basolateral compartments.[32,44] This observation suggests that there are likely other AAV2 internalization pathways independent of known AAV2 receptors, and that the entry pathways for AAV2 may significantly influence the efficiency of intracellular processing of the virus.

Receptor-mediated endocytosis of AAV

Thus far, the endocytic processes that control AAV infection have only been studied for rAAV type 2 and type 5. HSPG-bound rAAV2 appears to enter cells through clathrin-coated pits in a dynamin-dependent process.[58,59] Although AAV5 utilizes a different primary attachment receptor (sialic acid), it also appears to enter the cell through clathrin-coated pits based on ultrastructure localization.[60] Studies on CPV and FPV also suggest that viral endocytosis is dynamin dependent and clathrin mediated.[61,62] Fluorescent-labeled AAV has been used intensively in pulse/chase experiments to study AAV trafficking. Single AAV particle real-time tracking experiments have shown that AAV uptake into cells occurs in a very rapid manner (within 100 ms).[63] However, studies in intact epithelia have suggested that endocytosis can be a rate-limited factor in rAAV2 infection, and that UV-irradiation can enhance endocytosis of virus.[44]

In a manner similar to ligand binding and activation of cellular receptors, AAV binding to cell surface receptors also triggers intracellular signaling pathways that stimulate receptor endocytosis. These processes have been most extensively studied for rAAV2. For example, rAAV2 binding to HSPG and αVβ5 integrin stimulates activation of Rac1, an intracellular small GTP-binding protein.[40] The activation of Rac1 appears to be necessary to initiate endocytosis of cell-surface-bound AAV2, since expression of the dominant-negative N17Rac1 mutant inhibits endocytosis of virus.[40] In this context, inhibition of either Rac1 or αVβ5 integrin prevents endocytosis of AAV2, implying a potential functional link between these two signaling pathways in AAV2 infection. The activation of Rac1 appears to be closely linked to subsequent activation of the phosphatidylinositol-3 kinase pathway that is required for movement of AAV particles to the nucleus along microtubules and/or microfilaments.[40]

Endosomal processing and trafficking of AAV

It has become increasingly recognized that following endocytosis, processing of rAAV virions through the endosomal compartment is intricately linked to transduction. There are several components of endosomal processing that likely determine the efficiency of transduction with a given serotype and receptor-entry pathway. First, processing of the capsid while within the endosomal compartment may be critical for activating 'competency' of viral particles for transduction. For example, CPV directly injected into the cytoplasm of cells fails to replicate.[64] We have observed similar findings with rAAV2; rAAV2eGFP virus injected into the cytoplasm of HeLa cells failed to express its encoded transgene (unpublished data). Interestingly, endosomal processing of rAAV2 also appears to be important for nuclear transport of the virus. When directly injected into the cytoplasm, Alexa568-labeled rAAV2 failed to accumulate in the nucleus as was seen following infection with rAAV2 (Figure 2). These findings suggest that endosomal processing of the AAV2 capsid may play an important role in priming the virus for nuclear transport

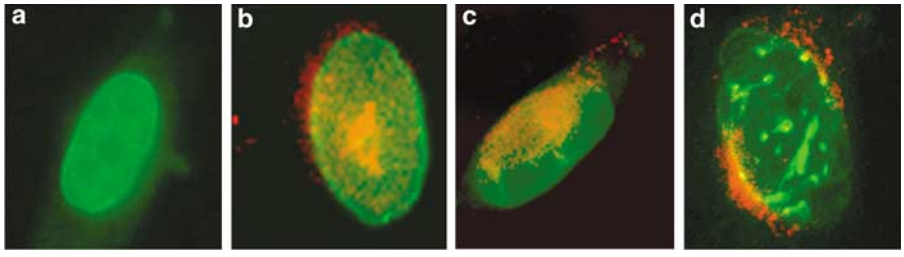

Figure 2 *Fluorescent detection of rAAV movement to the nucleus in HeLa cells. HeLa cells were transfected with an expression plasmid encoding a Lamin-B receptor-GFP fusion to mark the nucleus. At 48 h post-transduction, cells were either (a) mock infected (no virus), (b) infected with Alexa568-labeled AV2Luc, (c) injected with Alexa568-labeled AV2Luc into the nucleus, or (d) injected with Alexa568-labeled AV2Luc into the cytoplasm. Photomicrographs were taken at 24 h following viral infection or injection.*

and/or uncoating. Acidification inside the late endosomal and lysosomal compartments also appears to be another potential endosomal event critical for rAAV transduction. Several laboratories have demonstrated that when acidification of the endosomal compartment is blocked by bafilomycin A1, transduction with rAAV2 is significantly decreased.[33,38,65] However, a requirement for acidification within the endosomal compartment also demonstrates cell-type specificities.[38,65] Furthermore, acidification alone is not sufficient to promote transduction with CPV as demonstrated by microinjecting particles pretreated at pH 5.0.[64] Whether the AAV capsids are modified on the virion, while within the endosomal compartment, remains to be directly proven. However, evidence suggests that such modifications are likely part of AAV transduction biology.

Another interesting aspect of AAV infection is that proteasome inhibitors significantly augment transduction for at least two of the serotypes (AAV2 and AAV5).[32–35,66] The mechanism of enhanced transduction in the presence of proteasome inhibitors does not appear to be due to blocking AAV genome degradation.[32,66] Rather, inhibition of the proteasome enhances nuclear uptake of viruses in a number of cell systems.[32,35] These findings suggest that modulation of the ubiquitin–proteasome system might alter AAV intracellular processing at the level of endosomal trafficking, endosomal escape, nuclear transport, and/or uncoating of the virus. The enhanced transduction of rAAVs in the presence of proteasome inhibitors does not appear to be a direct effect of modulating second-strand genome synthesis, but rather a result of increasing the concentration of viral genomes in the nucleus.[34,35] Furthermore, there appears to be some level of cell-type specificity in the action of proteasome inhibitors since they do not enhance rAAV transduction in all cell types and tissues.[32,65] Although the mechanism of proteasome inhibitor action remains unclear, the finding that both rAAV2 and rAAV5 capsids are a substrate for ubiquitination *in vivo* and *in vitro*[32,66] suggests that the extent of capsid ubiquitination may be important in directing intracellular movement of the virion. Furthermore, *in vitro* ubiquitination assays have demonstrated that partially denatured purified AAV virions are preferred substrates for ubiquitination.[66] This result suggests that AAV processing inside the endosomes may be required for priming AAV capsids for ubiquitination once they enter the cytoplasm. Since increased capsid ubiquitination occurs when proteasome activity is inhibited, it is currently thought that ubiquitination may be a positive signal for productive transduction with rAAVs.[66] Similar studies with other parvoviruses also suggest that the ubiquitin–proteasome pathway plays an essential role in the life cycle of these viruses by facilitating capsid disassembly and/or nuclear transport.[67,68]

Previous reports have clearly demonstrated that intracellular trafficking to the nucleus for rAAV2 and CPV is a slow, rate-limiting process for certain cell types.[32,37,38,62] Conclusive determination of the endosomal trafficking pathways required for functional transduction with various AAV serotypes has been difficult. For example, cell-specific differences in the trafficking patterns have been observed for rAAV2, and even in the same cell type, differences in trafficking have been reported. In general, three endosomal trafficking

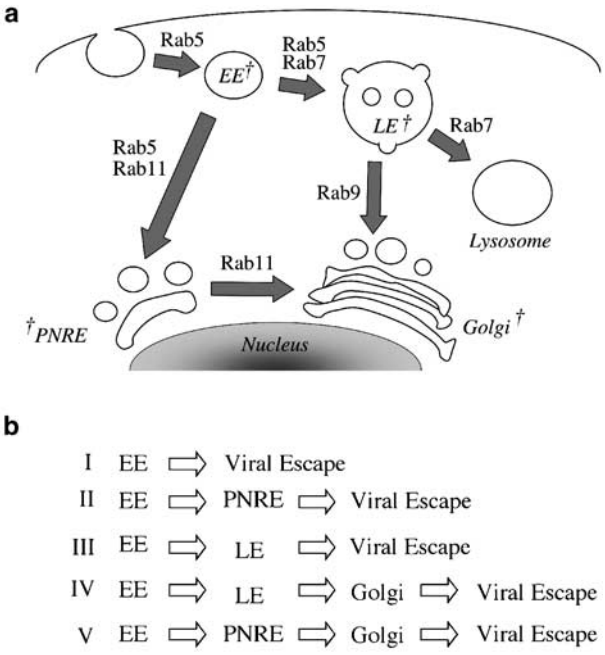

Figure 3 *Potential intracellular pathways for rAAV endosomal processing. (a) Schematically drawn intracellular pathways through which rAAVs may traffic. †marks potential sites of endosomal escape for rAAV2, AAV5, or CPV (a close relative of AAV), based on the present knowledge of where these viruses accumulate following infection.[33,36,38,58–60,62] Rab GTPases associated with the movement and location of various vesicular compartments are noted. (b) Five potential hypotheses for viral trafficking and endosomal escape are outlined that are consistent with the current knowledge of rAAV and CPV trafficking through cells. Potential sites of viral endosomal escape are inferred based on reports demonstrating viral accumulation in a given compartment, and/or through the inhibition of endosomal maturation. Compartments include the early endosome (EE), late endosome (LE), Golgi, and the perinuclear recycling endosome (PNRE).*

pathways have been described for AAVs and their CPV relative (Figure 3). As discussed above, all studied parvoviruses appear to enter into the early endosome through receptor-mediated endocytosis. This compartment is classified as containing the small GTPase Rab5. Rab proteins are a large family of small GTPases that regulate endosomal budding, sorting, movement and fusion within the cell.[69,70] As such, these proteins can act as 'zip codes' to facilitate endosome movement. Rab5 early endosomes can be routed to several destinations within the cell, of which the most relevant to AAVs are the late endosome (Rab7-positive compartment)[71] and the perinuclear recycling endosome (PNRE, Rab11-positive compartment).[72] The late endosomal pathway further branches off into two compartments: the lysosome or the trans-Golgi. Late endosomal movement to the lysosome is facilitated by Rab7,[71] while movement from the late endosome to the trans-Golgi is facilitated by Rab9.[73,74] Similarly, vesicular movement from the PNRE to the trans-Golgi can also occur through a Rab11-facilitated mechanism.[75] Although these described vesicular-trafficking pathways have been documented, it is important to recognize that the biology and boundaries of various Rab vesicular compartments within the cell are not black and white. The abundance, morphologic structure, and subcellular location for each of the vesicular compartments discussed can vary significantly

between different cell types. This influence of cell phenotype on the diversity of intracellular compartments almost certainly is reflected in the biologic complexity of AAV transduction.

Based on the above cell biology for vesicular trafficking, the literature currently supports five potential pathways for vesicular transport of AAV2, AAV5, and CPV (Figure 3a). Intracellular trafficking for other serotypes of AAV is yet to be reported. As mentioned earlier, CPV and AAV2 have both been demonstrated to be endocytosed through clathrin-dependent receptor endocytosis and processed through endosomal compartments in a manner similar to transferrin, but unlike fluid-phase markers such as dextran.[40,58,59,62] Transferrin trafficking has been extensively studied and shown to move through the early endosome to the PNRE.[76,77] The recycling of transferrin through the PNRE requires the coordinated interactions of several small GTPases (Rab5, Rab4, and Rab11) that direct the movement and fusion of early endosomes to the PNRE compartment.[77] The colocalization of CPV and AAV2 with transferrin has suggested the potential involvement of the PNRE in the processing of these viruses. Additionally, we have observed significant overlap with Cy3-labeled AAV2 with the Rab11 PNRE compartment in certain cell types (unpublished data). In contrast to these patterns of localization, other groups have used pharmacologic and biochemical subcellular fractionation approaches to suggest that rAAV2 may move through the late endosome to the lysosome pathway[33,38] or exit very early from the early endosome.[36] Lastly, it has also been suggested that both AAV2 and AAV5 traffic to the trans-Golgi network.[60,65] Although at face value it appears that results on AAV vesicular trafficking remain controversial, the results between laboratories are not entirely inconsistent. For example, movement to the Golgi compartment can be facilitated through either the Rab11 PNRE or the Rab7→Rab9 late endosomal pathway (Figure 3). Cell line-dependent differences in trafficking of AAV have also been reported,[65] and suggest that AAV can traverse several endosomal pathways. Furthermore, studies solely evaluating localization and bulk flow of AAV through the cell do not directly address what endosomal compartments are most important for functional processing of virions or the point of endosomal escape. Indeed, only a small fraction of input virions appear to lead to functional expression of a transgene. In future studies, more functional approaches aimed at modulating vesicular movement will be required to conclusively address the relevant pathways for AAV movement through the cell.

Endosomal escape and nuclear translocation of AAV

It is generally believed that AAV must exit from the endosome before it traffics to the nucleus. However, the point at which different serotypes enter into the cytoplasm has been difficult to address definitively. Studies demonstrating that bafilomycin A1 (an inhibitor of late endosomal acidification facilitated by the vacuolar H^+-ATPase) inhibits AAV transduction[33,38,59] suggest that AAV2 likely escapes from the late endosome prior to lysosomal maturation. The acid environment of the late endosome seems to be required for this process and for rAAV transduction. However, these findings could also be consistent with preventing late endosomal movement through the Rab9 compartment to the trans-Golgi network.

Interesting studies on endosomal escape for CPV have also discovered phospholipase A2 activity within the N-terminal domain of VP1.[78] This study suggests the intriguing hypothesis that acidification and phospholipase A2 activity of VP1 are required for CPV endosomal escape. Similar phospholipase activity has been seen in VP1 of AAV2 and also appears to be required for postendocytic processing of virus and efficient transduction.[79] Whether phospholipase A2 activity is a conserved feature of other AAV serotypes remains to be investigated. However, sequence analysis of the N-terminal region of VP1 between currently known AAV serotypes suggests that functional conservation of phospholipase A2 activity may indeed occur. Further research in this area may uncover unique mechanisms to enhance rAAV transduction by facilitating more efficient endosomal escape.

Once AAV has escaped from the endosome, it must traffic to the nucleus. Since disruption of both tubulin and actin filaments inhibits vesicular movement of AAV2,[40] dissecting the functional involvement of these cytoskeletal elements in the movement of cytoplasmic AAV has been difficult. However, real-time imaging of fluorescently labeled AAV has suggested that movement of free virus in the cytoplasm and nucleus may be facilitated by ATP-dependent molecular motors on tubular networks.[63] Although Brownian diffusion was also observed, perinuclear delivery of AAV occurred within seconds once anchored to the cytoskeleton.

It is generally believed that AAV particles are transported into the nucleus prior to uncoating, since fluorescent-labeled AAV virions are seen in the nucleus.[40,59,63] However, nuclear transport of AAV in most of the investigated cell lines appears to be a slow and inefficient process. In most studies, only a small portion of internalized AAV can be found in the nucleus at short time points postinfection. Several agents have been identified that promote nuclear accumulation of AAV and rAAV transduction. These include proteasome inhibitors,[32,33,35] hydroxyurea,[38] and adenovirus.[36] It is currently thought that these agents enhance endosomal and/or cytoplasmic processing of the virus rather than directly facilitating nuclear translocation.

Relatively little is known about the processes that control nuclear translocation of AAV across nuclear pores. Since the size of an AAV virion is comparable to the size of the nuclear pore, translocation has been proposed to be limited by diffusion. One study has evaluated nuclear translocation of rAAV2 in nuclei isolated from permissive and less-permissive cell types to determine whether nuclear translocation was a rate-limited step in transduction.[80] Findings from these studies suggested that AAV particles could efficiently enter the nucleus of both highly and poorly transducible cell types in a time- and temperature-dependent manner, suggesting that nuclear transport is not rate limiting for transduction. Interestingly, these studies suggested that AAV2 might enter the nucleus independent of classical nuclear receptor/nuclear pore complex entry pathways. Others have identified a nonconventional nuclear

localization signal in the capsid VP2 sequence.[81] This sequence is believed to be important for AAV viral packaging, but whether it plays a role in nuclear transport of intact AAV particles is unknown. It is also unclear if nuclear transport of AAV particles requires capsid modifications and/or association with transport receptors. In this regard, nucleolin (a protein that shuttles from the cytoplasm to the nucleus) has been shown to associate with AAV virions in the cytoplasm and nucleus.[82] Hence, nucleolin may act as a nuclear receptor for AAVs. Additionally, a correlation between increased ubiquitination of AAV capsid proteins in the presence of proteasome inhibitors and increased nuclear uptake suggests that the ubiquitination of AAV virions may facilitate nuclear transport of rAAV2 and rAAV5.[66] Other studies on minute virus of mice and CPV also support the functional involvement of proteasomes in nuclear transport.[68] However, these studies could find no direct evidence for capsid ubiquitination. Hence, although the ubiquitin–proteasome system appears to be linked to efficient cytoplasmic processing and nuclear uptake for several classes of parvoviruses, subtle differences in the mechanisms may exist.

Future directions

With the increasing use of rAAV as a gene therapy vector,[31] it has become increasingly recognized that intracellular trafficking of this virus is often directly linked to the efficiency of transduction in many cell types and tissues. Furthermore, the intracellular trafficking of AAVs appears to be quite complex and influenced by cell phenotypes. Certainly, movement of AAV from the membrane to the nucleus is not the sole limiting factor that influences the efficiency of this vector system for gene delivery. Other aspects not directly discussed in this review, including genome conversion and stability, also play important roles in transduction, and in a given context can significantly limit the effectiveness of this vector. Furthermore, the process of virion uncoating in the nucleus may be equally important and indirectly affected by intracellular trafficking and processing of the capsid at certain intracellular locations such as the late endosome and/or proteasome. For example, a recent report has suggested that rAAV2 has a much slower rate of uncoating than rAAV6 and rAAV8 in hepatocytes.[83] Whether uncoating rates between these serotypes are a reflection of different routes of intracellular processing remains to be elucidated. A recent review has also suggested that the rate of intracellular processing and uncoating of viral vectors may critically affect host immunological response toward input capsids in the absence of new viral protein synthesis.[84] If this is true for AAVs, altering the rate of intracellular trafficking and uncoating of rAAVs using drugs or serotype modifications could directly influence the longevity of gene expression by reducing host immune responses that clear virus-infected cells.

Currently, our knowledge of AAV trafficking is based on a limited number of serotypes, AAV2 and AAV5. Relatively little is known about intracellular trafficking for any of the other AAV serotypes. With the increasing identification of new AAV serotype vectors, it is important to understand how intracellular processing for these different serotypes is similar and dissimilar in various cell targets. Current advances in technology have greatly increased our ability to dissect the complexities of AAV intracellular trafficking. With the latest advances in fluorescent microscopy, it has become possible to monitor the intracellular movement of single AAV virions in real time.[63] Studies focusing on AAV capsid structures have improved since the crystal structure of AAV2 was determined. Efforts to obtain capsid surface details for other AAV serotypes are accelerating[42,85] and have complemented genetic analyses of the AAV2 capsid that has revealed an enormous tolerance for large insertions such as GFP.[86] Such efforts to link sensitive fluorescent protein tags with AAV virions will enhance our capabilities to study AAV trafficking in real time. Another important area of study will be the development of *in vivo* imaging techniques for monitoring the process of AAV uncoating. A recent report adopting the use of tandem Lac repressor binding sites encoded in the AAV genomes to monitor AAV replication is another example of the trend toward real-time analysis of AAV biology[87] that may be readily applicable to the study of uncoating.

The success of the AAV field in developing numerous types of recombinant vector systems has greatly expanded the option for gene therapy of various organs. However, with this increased diversity come challenges and opportunities for dissecting AAV biology that are directly relevant to the efficacy of these vector systems. Few comparative studies on intracellular trafficking/processing characteristics between various AAV serotypes have made it difficult to dissect commonalities in transduction biology and differences that might be directly relevant to gene therapy. As discussed in this review, it is likely that many AAV serotypes share some common biology that influences intracellular trafficking and transduction. However, it is equally likely that comparative studies may uncover fundamental differences in trafficking biology related to differences in their receptor pathways for endocytosis. A better understanding of AAV intracellular trafficking for many serotypes will ultimately allow researchers to make informed choices about the most appropriate vector serotype and approach to treat disease in a given organ.

Acknowledgements

Research support in the area of this review has been derived from NIH RO1 HL58340 (JFE). We also thank Dr Greg Leno and Mariah Steele for editorial assistance with this paper.

References

1 Blacklow NR. Adeno-associated viruses of human. In: Pattison JR (ed). *Parvoviruses and Human Disease*. CRC Press: Boca Raton, FL, 1988, pp 165–174.
2 Berns KI, Giraud C. Biology of adeno-associated virus. *Curr Top Microbiol Immunol* 1996; **218**: 1–23.
3 Fisher RE, Mayor HD. The evolution of defective and autonomous parvoviruses. *J Theor Biol* 1991; **149**: 429–439.
4 Atchison RW, Casto BC, Hammon WM. Electron microscopy of adenovirus-associated virus (AAV) in cell cultures. *Virology* 1966; **29**: 353–357.

5 Hoggan MD, Blacklow NR, Rowe WP. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proc Natl Acad Sci USA* 1966; **55**: 1467–1474.
6 Lusby E, Fife KH, Berns KI. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *J Virol* 1980; **34**: 402–409.
7 Rose JA, Berns KI, Hoggan MD, Koczot FJ. Evidence for a single-stranded adenovirus-associated virus genome: formation of a DNA density hybrid on release of viral DNA. *Proc Natl Acad Sci USA* 1969; **64**: 863–869.
8 Mendelson E, Trempe JP, Carter BJ. Identification of the trans-acting Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. *J Virol* 1986; **60**: 823–832.
9 Trempe JP, Carter BJ. Alternate mRNA splicing is required for synthesis of adeno-associated virus VP1 capsid protein. *J Virol* 1988; **62**: 3356–3363.
10 Buller RM, Rose JA. Characterization of adenovirus-associated virus-induced polypeptides in KB cells. *J Virol* 1978; **25**: 331–338.
11 Muzyczka N. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr Top Microbiol Immunol* 1992; **158**: 97–129.
12 Linden RM, Berns KI. Molecular biology of adeno-associated viruses. *Contrib Microbiol* 2000; **4**: 68–84.
13 Samulski RJ, Berns KI, Tan M, Muzyczka N. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci USA* 1982; **79**: 2077–2081.
14 Laughlin CA, Tratschin JD, Coon H, Carter BJ. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. *Gene* 1983; **23**: 65–73.
15 Grimm D, Kay MA. From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. *Curr Gene Ther* 2003; **3**: 281–304.
16 Mori S, Wang L, Takeuchi T, Kanda T. Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. *Virology* 2004; **330**: 375–383.
17 Duan D *et al*. Enhancement of muscle gene delivery with pseudotyped adeno-associated virus type 5 correlates with myoblast differentiation. *J Virol* 2001; **75**: 7662–7671.
18 Rabinowitz JE *et al*. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* 2002; **76**: 791–801.
19 Auricchio A *et al*. Noninvasive gene transfer to the lung for systemic delivery of therapeutic proteins. *J Clin Invest* 2002; **110**: 499–504.
20 Grimm D *et al*. Preclinical *in vivo* evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Blood* 2003; **102**: 2412–2419.
21 Yan Z, Zak R, Zhang Y, Engelhardt JF. Inverted terminal repeat sequences are important for intermolecular recombination and circularization of adeno-associated virus genomes. *J Virol* 2005; **79**: 364–379.
22 Bowles DE, Rabinowitz JE, Samulski RJ. Marker rescue of adeno-associated virus (AAV) capsid mutants: a novel approach for chimeric AAV production. *J Virol* 2003; **77**: 423–432.
23 Hauck B, Chen L, Xiao W. Generation and characterization of chimeric recombinant AAV vectors. *Mol Ther* 2003; **7**: 419–425.
24 Rabinowitz JE *et al*. Cross-dressing the virion: the transcapsidation of adeno-associated virus serotypes functionally defines subgroups. *J Virol* 2004; **78**: 4421–4432.
25 Girod A *et al*. Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med* 1999; **5**: 1052–1056.
26 Buning H *et al*. Receptor targeting of adeno-associated virus vectors. *Gene Therapy* 2003; **10**: 1142–1151.
27 Grifman M *et al*. Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. *Mol Ther* 2001; **3**: 964–975.
28 Wu P *et al*. Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol* 2000; **74**: 8635–8647.
29 Bartlett JS, Kleinschmidt J, Boucher RC, Samulski RJ. Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab′gamma)2 antibody. *Nat Biotechnol* 1999; **17**: 181–186.
30 Ponnazhagan S *et al*. Conjugate-based targeting of recombinant adeno-associated virus type 2 vectors by using avidin-linked ligands. *J Virol* 2002; **76**: 12900–12907.
31 Carter B, Burstein H, Peluso RW. *Adeno-Associated Virus and AAV Vectors for Gene Delivery (in Series: Gene and Cell Therapy : Therapeutic Mechanisms and Strategies)*. Marcel Dekker: New York, 2004, pp 71–101.
32 Duan D *et al*. Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. *J Clin Invest* 2000; **105**: 1573–1587.
33 Douar AM, Poulard K, Stockholm D, Danos O. Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol* 2001; **75**: 1824–1833.
34 Ding W *et al*. Second-strand genome conversion of adeno-associated virus type 2 (AAV-2) and AAV-5 is not rate limiting following apical infection of polarized human airway epithelia. *J Virol* 2003; **77**: 7361–7366.
35 Yan Z *et al*. Distinct classes of proteasome-modulating agents cooperatively augment recombinant adeno-associated virus type 2 and type 5-mediated transduction from the apical surfaces of human airway epithelia. *J Virol* 2004; **78**: 2863–2874.
36 Xiao W *et al*. Adenovirus-facilitated nuclear translocation of adeno-associated virus type 2. *J Virol* 2002; **76**: 11505–11517.
37 Hansen J *et al*. Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts. *J Virol* 2000; **74**: 992–996.
38 Hansen J, Qing K, Srivastava A. Adeno-associated virus type 2-mediated gene transfer: altered endocytic processing enhances transduction efficiency in murine fibroblasts. *J Virol* 2001; **75**: 4080–4090.
39 Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 1998; **72**: 1438–1445.
40 Sanlioglu S *et al*. Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. *J Virol* 2000; **74**: 9184–9196.
41 Opie SR *et al*. Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding. *J Virol* 2003; **77**: 6995–7006.
42 Xie Q *et al*. The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc Natl Acad Sci USA* 2002; **99**: 10405–10410.
43 Handa A *et al*. Adeno-associated virus (AAV)-3-based vectors transduce haematopoietic cells not susceptible to transduction with AAV-2-based vectors. *J Gen Virol* 2000; **81**: 2077–2084.
44 Duan D *et al*. Polarity influences the efficiency of recombinant adeno-associated virus infection in differentiated airway epithelia. *Hum Gene Ther* 1998; **9**: 2761–2776.
45 Kaludov N *et al*. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J Virol* 2001; **75**: 6884–6893.
46 Walters RW *et al*. Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. *J Biol Chem* 2001; **21**: 21.

47 Negishi A *et al*. Analysis of the interaction between adeno-associated virus and heparan sulfate using atomic force microscopy. *Glycobiology* 2004; **14**: 969–977.
48 Halbert CL, Allen JM, 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. *J Virol* 2001; **75**: 6615–6624.
49 Gao GP *et al*. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* 2002; **99**: 11854–11859.
50 Summerford C, Bartlett JS, Samulski RJ. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med* 1999; **5**: 78–82.
51 Qing K *et al*. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat Med* 1999; **5**: 71–77.
52 Qiu J, Mizukami H, Brown KE. Adeno-associated virus 2 co-receptors? [letter]. *Nat Med* 1999; **5**: 467–468.
53 Qiu J, Brown KE. Integrin alphaVbeta5 is not involved in adeno-associated virus type 2 (AAV2) infection. *Virology* 1999; **264**: 436–440.
54 Di Pasquale G *et al*. Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med* 2003; **9**: 1306–1312.
55 Hosang M. Characterization of a platelet-derived growth factor receptor on Swiss 3T3 cells by affinity crosslinking. *J Recept Res* 1988; **8**: 455–466.
56 Daniel TO, Milfay DF, Escobedo J, Williams LT. Biosynthetic and glycosylation studies of cell surface platelet-derived growth factor receptors. *J Biol Chem* 1987; **262**: 9778–9784.
57 Burger C *et al*. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol Ther* 2004; **10**: 302–317.
58 Duan D *et al*. Dynamin is required for recombinant adeno-associated virus type 2 infection. *J Virol* 1999; **73**: 10371–10376.
59 Bartlett JS, Wilcher R, Samulski RJ. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* 2000; **74**: 2777–2785.
60 Bantel-Schaal U, Hub B, Kartenbeck J. Endocytosis of adeno-associated virus type 5 leads to accumulation of virus particles in the Golgi compartment. *J Virol* 2002; **76**: 2340–2349.
61 Parker JS *et al*. Canine and feline parvoviruses can use human or feline transferrin receptors to bind, enter, and infect cells. *J Virol* 2001; **75**: 3896–3902.
62 Parker JS, Parrish CR. Cellular uptake and infection by canine parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking. *J Virol* 2000; **74**: 1919–1930.
63 Seisenberger G *et al*. Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science* 2001; **294**: 1929–1932.
64 Vihinen-Ranta M *et al*. Intracellular route of canine parvovirus entry. *J Virol* 1998; **72**: 802–806.
65 Pajusola K *et al*. Cell-type-specific characteristics modulate the transduction efficiency of adeno-associated virus type 2 and restrain infection of endothelial cells. *J Virol* 2002; **76**: 11530–11540.
66 Yan Z *et al*. Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. *J Virol* 2002; **76**: 2043–2053.
67 Ros C, Burckhardt CJ, Kempf C. Cytoplasmic trafficking of minute virus of mice: low-pH requirement, routing to late endosomes, and proteasome interaction. *J Virol* 2002; **76**: 12634–12645.
68 Ros C, Kempf C. The ubiquitin–proteasome machinery is essential for nuclear translocation of incoming minute virus of mice. *Virology* 2004; **324**: 350–360.
69 Zerial M, McBride H. Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2001; **2**: 107–117.
70 Pfeffer SR. Rab GTPases: specifying and deciphering organelle identity and function. *Trends Cell Biol* 2001; **11**: 487–491.
71 Bucci C *et al*. Rab7: a key to lysosome biogenesis. *Mol Cell Biol* 2000; **11**: 467–480.
72 Trischler M, Stoorvogel W, Ullrich O. Biochemical analysis of distinct Rab5- and Rab11-positive endosomes along the transferrin pathway. *J Cell Sci* 1999; **112**: 4773–4783.
73 Diaz E, Schimmoller F, Pfeffer SR. A novel Rab9 effector required for endosome-to-TGN transport. *J Cell Biol* 1997; **138**: 283–290.
74 Barbero P, Bittova L, Pfeffer SR. Visualization of Rab9-mediated vesicle transport from endosomes to the trans-Golgi in living cells. *J Cell Biol* 2002; **156**: 511–518.
75 Wilcke M *et al*. Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-golgi network. *J Cell Biol* 2000; **151**: 1207–1220.
76 Ren M *et al*. Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. *Proc Natl Acad Sci USA* 1998; **95**: 6187–6192.
77 Sonnichsen B *et al*. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J Cell Biol* 2000; **149**: 901–913.
78 Suikkanen S *et al*. Release of canine parvovirus from endocytic vesicles. *Virology* 2003; **316**: 267–280.
79 Girod A *et al*. The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *J Gen Virol* 2002; **83**: 973–978.
80 Hansen J, Qing K, Srivastava A. Infection of purified nuclei by adeno-associated virus 2. *Mol Ther* 2001; **4**: 289–296.
81 Hoque M *et al*. Nuclear transport of the major capsid protein is essential for adeno-associated virus capsid formation. *J Virol* 1999; **73**: 7912–7915.
82 Qiu J, Brown KE. A 110-kDa nuclear shuttle protein, nucleolin, specifically binds to adeno-associated virus type 2 (AAV-2) capsid. *Virology* 1999; **257**: 373–382.
83 Thomas CE, Storm TA, Huang Z, Kay MA. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. *J Virol* 2004; **78**: 3110–3122.
84 Lowenstein PR. Input virion proteins: cryptic targets of antivector immune responses in preimmunized subjects. *Mol Ther* 2004; **9**: 771–774.
85 Walters RW *et al*. Structure of adeno-associated virus serotype 5. *J Virol* 2004; **78**: 3361–3371.
86 Warrington Jr KH *et al*. Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. *J Virol* 2004; **78**: 6595–6609.
87 Fraefel C *et al*. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. *J Virol* 2004; **78**: 389–398.