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BREAK INS AND BREAK OUTS: Viral Interactions with the Cytoskeleton of Mammalian Cells

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■ **Abstract** The host cytoskeleton plays important roles in the entry, replication, and egress of viruses. An assortment of viruses hijack cellular motor proteins to move on microtubules toward the cell interior during the entry process; others reverse this transport during egress to move assembling virus particles toward the plasma membrane. Polymerization of actin filaments is sometimes used to propel viruses from cell to cell, while many viruses induce the destruction of select cytoskeletal filaments apparently to effect efficient egress. Indeed, the tactics used by any given virus to achieve its infectious life cycle are certain to involve multiple cytoskeletal interactions. Understanding these interactions, and their orchestration during viral infections, is providing unexpected insights into basic virology, viral pathogenesis, and the biology of the cytoskeleton.

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INTRODUCTION

In cells, the infectious cycle of all viruses can be divided into three essential steps: entry into a host cell, replication, and egress to ultimately infect another cell. This cycle is the crux of viral existence, and evolution has produced an amazing array of mechanisms to accomplish these steps in the face of significant host defenses. The limited coding capacity of viruses makes them dependent on cellular metabolic pathways to achieve each of these steps. Even the most complex viruses are dependent upon host ribosomes for protein expression. However, the dependence of viruses on host cell pathways is not limited to transcription, translation, and genome replication.

Many viruses interact with cytoskeletal elements, and these interactions are proving to be critical at almost every step along the infectious cycle. Viruses enter cells at the plasma membrane, depositing their genomes complexed with proteins in the cytoplasm. Virus replication often occurs in the nucleus or perinuclear region of the cytoplasm, usually some distance from the site of entry. Reaching these replication sites efficiently requires directed transport because diffusion through the cytoplasm, which has a high concentration of macromolecules often structured in large filamentous arrays, is not an effective mechanism for viral intracellular transport (Luby-Phelps 1994, Seksek et al. 1997). The complexity of the energy-transducing motor complexes needed to overcome diffusion barriers in the cytoplasm apparently precludes their encoding in viral genomes. Instead, viruses interface with and modulate host cytoskeletal transport machinery.

One common viral strategy uses microtubule-based transport to move virus components inside cells. Motion on microtubules is directional. Movement to the microtubule plus end requires a kinesin-family (KIF) motor, whereas movement to the minus end generally requires the dynein motor (and in all cases examined the dynactin complex as well). In most cell types, movement to the cell periphery (anterograde transport) requires plus-end directed motion, and movement into the cell interior (retrograde transport) requires minus-end directed motion. Exceptions include polarized epithelia, which have microtubules arrayed with their minus ends facing the apical membrane, and dendritic processes of neurons, in which microtubule orientation is mixed. Support for the assertion that viruses use microtubule transport machinery comes from several lines of experiments. First, microtubule destabilizing drugs, such as nocodazole or colchicine, interfere with the delivery of many unrelated viruses to the nucleus or perinuclear replication site. Second, introduction of dominant-negative proteins or overexpression of proteins that impair dynein motor function blocks delivery of virus to cellular locations. Third, in the case of neurotropic viruses where virus particles must travel from axon terminals to cell bodies that may be several feet away, rates of transport can be roughly estimated based on time of appearance of viral antigens in the cell bodies (Johnson 1982, Lycke et al. 1984). Viral transport in axons occurs by fast retrograde flow, a known microtubule-based mechanism (Hirokawa 1998). Finally, tracking of individual viral particles in living cells is providing further

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confirmation that an assortment of unrelated viruses is transported intracellularly along microtubules.

Interactions of viruses with the cellular transport machinery can be direct or indirect. For herpesviruses, the virus envelope (a lipid bilayer comprising the outer surface of some viruses) fuses with the axolemma. Therefore, the herpesvirus core (a complex of proteins surrounding the viral genome; commonly called the capsid, or collectively known as the nucleocapsid) is released into the cytosol and must engage motor complexes directly for retrograde transport. By contrast, poliovirus enters axons by endocytosis and is transported toward the neuronal cell body in an endocytic vesicle; the poliovirus particle does not directly engage the transport machinery (see below). Thus examination of neurotropic viruses has also revealed that viruses can be transported either as a proteinaceous complex or as a membrane-bound cargo along microtubules.

Viral interactions with the cytoskeleton are not done capriciously. The entering virus must engage and regulate endogenous microtubule motor complexes to reach the interior of the cell. Upon arriving at the cytoplasmic sites of viral replication, sometimes referred to as viral factories, other cytoskeleton interactions may occur as these sites are often enriched with intermediate filaments (Carvalho et al. 1988, Doedens et al. 1994, Garcia-Barreno et al. 1988, Murti et al. 1988, Nedellec et al. 1998). Components of the microtubule and actin cytoskeleton are essential cofactors for the replication and morphogenesis of some viruses (Burke et al. 2000, De & Banerjee 1999, Gupta et al. 1998, Hill & Summers 1990). Many viruses go so far as to induce the depolymerization of cytoskeletal elements to alter the diffusion properties of the cytoplasm or to compromise the integrity of the infected cell, facilitating viral egress. Others engage microtubule motor complexes and coerce their movement back to the cell periphery and sites of viral release. The virus can leave the cell to become a free infectious particle, or spread cell to cell without exposure to the extracellular environment. These final steps in the virus life cycle are sometimes achieved by inducing actin polymerization to propel viruses to adjacent cells.

The emerging theme is that all viruses must interact with the cytoskeleton, and probably do so at multiple stages throughout their infectious cycles. Consequently, the large number of virus families translates into a diverse repertoire of strategies and tactics used to exploit the cytoskeleton and produce more viruses. This review focuses on viral exploitation of the cytoskeleton to move within and between mammalian cells. While much has recently been discovered regarding the transport of viruses in plants, this work is beyond the scope of our discussion. We instead refer the reader to two recent reviews (Lazarowitz 1999, Lee & Lucas 2001). Furthermore, trafficking of viruses into the nucleus was recently reviewed and is not touched upon here (Cullen 2001, Whittaker et al. 2000). For interactions of *Autographa californica* M nucleopolyhedrovirus (AcMNPV) with the actin cytoskeleton of lepidopteran insects, we refer the reader to a review that discusses the topic (Cudmore et al. 1997). Lastly, several recent reviews are recommended for those wishing to gain additional insights into

intracellular viral transport (Ploubidou & Way 2001, Sodeik 2000, Stidwill & Greber 2000, Tomishima et al. 2001).

SEEING VIRUSES ASSOCIATE WITH THE CYTOSKELETON

In general, most viruses are too small to be seen directly in living cells by light microscopy. The pox viruses are a remarkable exception: These 0.4-μm-long particles are just large enough to be resolved with transmitted light (for example see Cudmore et al. 1995). Historically, examination of viral/cytoskeletal interactions was based on electron microscopy studies. As early as the 1960s, studies of infected cells revealed associations of viruses with cytoskeletal filaments. These pioneering observations formed the foundation of much of what is known today and were extensively reviewed in 1982 (Luftig 1982). Since that time, the localization of viruses in relation to intracellular components has been further examined by immunofluorescence microscopy.

Although observation of fixed cells has contributed fundamental information on the infectious cycles of many viruses, new technological developments have recently made possible the tracking of individual viral particles in living cells. This was first achieved in a study examining adenovirus entry in A549 epithelial cells (Leopold et al. 1998). Because adenoviruses are not enveloped, infectious particles can be purified and chemically cross-linked to a fluorophore. Upon infection of cells, the fluorophore tag (Cy3 in this case) remains linked to the viral capsid. These tagged particles were imaged in cells by conventional epifluorescence microscopy using a back-illuminated CCD with frames captured at 4-s intervals. Using this method, individual particles (presumably representing individual adenovirus capsids) were tracked 10 min after infection (see below for further discussion of the biology).

An advance in the fluorophore-tagging method was recently accomplished using single-molecule imaging (Seisenberger et al. 2001). Detecting single fluorophores is complicated by several factors, including limited emissions, fluorophore decay, and the signal-to-noise ratio (the noise typically being autofluorescence, an issue particularly relevant to the imaging of living cells). These difficulties were recently overcome and adeno-associated viruses (AAV) bearing single molecules of Cy5 were imaged in living cells with 40-ms exposures. Previous studies had coupled hundreds of fluorophores to individual virus particles, possibly altering the infectious properties of the virus. Single-molecule imaging substantially reduces this concern. [Substantiating the concern of fluorophore interference requires further evaluation; previous studies of AAV using high-copy tagging methods did not track individual virus trajectories, preventing a comparison of transport dynamics with the single-molecule study (Bartlett et al. 2000, Sanlioglu et al. 2000).] Furthermore, by fitting the single-molecule fluorescence emission to a Gaussian function in two dimensions, the position of the fluorescent particle can be pinpointed to resolutions far greater than the spatial resolution of the microscope optics (40 nm

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in the case of the AAV study) (Seisenberger et al. 2001). This high-resolution positional information allows for detailed tracking of viral movement over short time periods, typically less than 10 s (limited by bleaching of the fluorophore).

Although fluorophore-tagged studies have been instrumental in observing the entry of some viruses into cells, the approach has some important limitations. Newly replicated virions inside a cell cannot be specifically labeled by chemical cross-linking, preventing studies of viral egress. Furthermore, enveloped viruses are not immediately amenable to this approach, as any fluorophores chemically cross-linked to their surfaces will be segregated from the unlabeled viral core upon membrane fusion. Single-molecule imaging of non-enveloped viruses during entry can also be complicated because some viral capsids, such as adenovirus, are dismantled on their way to the nucleus during entry (Greber et al. 1993). If the single fluorophore is cross-linked to a labile piece of the capsid, the observer may cease to track the viral capsid shortly after initial infection and instead follow a red herring.

Arguably, the greatest revolution in cell biology in recent years is the use of the green fluorescent protein (GFP) to image protein dynamics in living cells (Stearns 1995, van Roessel & Brand 2002). Although the ensemble behavior of many GFP-tagged viral proteins has been examined in both transfected and infected cells, the use of GFP to track individual viral particle transport in cells is gaining momentum. The key to success is not only the construction of a recombinant virus that expresses GFP fused to a viral structural protein; this fusion protein must also remain tightly associated with the viral components to be tracked, yet must not interfere with viral function. By using effective fusion(s) and proper imaging techniques, entry, uncoating, replication, assembly, and egress of single viruses all can theoretically be observed in living cells. Indeed, recombinant vaccinia, herpesviruses, and adenovirus expressing GFP fused to viral components associated with the viral capsid core, either directly or indirectly, have been tracked in cells (a report of HIV tracking is also expected; see below) (Bearer et al. 2000, Geada et al. 2001, Glotzer et al. 2001, Rietdorf et al. 2001, Smith et al. 2001, Ward & Moss 2001b).

One limitation of using GFP to label viruses is that multiple copies of the GFP fusion protein must be incorporated into a single viral particle. More GFP fusion proteins per particle results in greater fluorescence, which allows for shorter exposure times and greater temporal resolution. Of greater importance, higher-copy GFP incorporation also improves contrast in the presence of cellular autofluorescence, an issue for GFP studies more so than with the red fluorophores preferred in chemical cross-linking experiments. However, higher-copy GFP incorporation into virions could conceivably interfere with the very processes being studied. Thus we suggest that a standard requirement for GFP-labeling studies of viral movement should include evidence that recombinant fluorescent viruses behave like wild-type viruses in the process under study. Although this requirement is difficult to fulfill directly, demonstration that a GFP virus infects cells with wild-type efficiency, has the same particle-to-infectious-unit ratio, and has the same cell tropism or pathogenic profile is encouraging. Furthermore, as with chemical cross-linked fluorophores, if the GFP is not linked directly to the structure under investigation, such as to the genome in the viral core, evidence that the association

is not labile in the cell is essential. During the entry of some viruses, many viral proteins dissociate from the viral core. Similarly, during egress, a viral membrane protein fused to GFP could be imaged in a host transport vesicle or as part of a virion, and by time-lapse fluorescent microscopy it may not be immediately obvious which form is being tracked.

VIRAL ENTRY (BREAKING IN)

Viral entry has three general steps: binding the host cell, entry into the cytosol, and transport to the site of replication. A cell must express appropriate surface receptor(s) to be susceptible to infection. Identifying the host receptors that viruses use to enter cells is critical to understanding pathogenesis, as this information reveals tissue tropism as well as a target for intervention to help combat disease.

Receptor binding can trigger entry by at least two pathways. Some viral particles enter cells by endocytosis, and subsequent acidification of the endosome triggers release of the viral core into the cytosol. This entry pathway is used by some enveloped (i.e., influenza and rubella) and non-enveloped (i.e., adenovirus and AAV) viruses. The endocytosis of some viruses, such as adenovirus and some forms of vaccinia, is dependent upon actin polymerization (Li et al. 1998, Locker et al. 2000). Other enveloped viruses fuse with the plasma membrane of the host cell, directly releasing the viral core into the cytosol. Examples of the latter include herpesviruses and human immunodeficiency virus (HIV).

Regardless of which entry pathway is used, the viral core must travel to the site of viral replication, typically the nucleus or perinuclear region of the cytosol. In this regard, individual particles of several unrelated viruses have been imaged and tracked during initial infection of cultured cells.

Herpesviruses

Herpes simplex virus type 1 (HSV-1) capsids move from the cell surface to the nucleus using microtubule-based retrograde transport. The capsids colocalize with microtubules during entry into nonneuronal cells; overexpression of p50/dynamitin blocks viral transport to the nucleus in nonneuronal cells, and microtubule-disrupting drugs reduce transport (Kristensson et al. 1986, Sodeik 2000, Sodeik et al. 1997, Topp et al. 1994). Using GFP-fusion proteins, herpesvirus particles have been imaged during retrograde transport in neurons by time-lapse microscopy. By fusing GFP to a HSV-1 tegument protein, which is often associated with viral capsids during infection, HSV-1 particles were tracked in dissected giant axons from the squid, *Loligo pealei*, following detergent extraction of the viral envelopes and injection of the capsids into the axon (Bearer et al. 2000). In this model, GFP punctae were observed to move exclusively in the retrograde direction at an average rate of 2.2 μm/s. No transient pauses in motion or long-term losses of motility were observed, similar to motion of endogenous structures in squid axons (Allen et al. 1982, Brady et al. 1982).

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By fusing GFP directly to the capsid of pseudorabies virus (PRV; a neurotropic herpesvirus related to HSV-1), we observed viral transport from the axon terminal to the cell body in chick dorsal root ganglion neurons at rates averaging 1.3 μm/s (G.A. Smith & L.W. Enquist, unpublished observations). The transport is both saltatory and bi-directional, but with the vast majority of motion in the retrograde direction. The reason for the differences in transport of herpesviruses in squid axons and chick neurons is not clear, but may be accounted for by the different herpesviruses used, differences in the GFP fusion, or differences in the endogenous transport machinery in vertebrate and invertebrate neurons. However, the bi-directional transport of PRV in sensory neurons indicates that herpesvirus capsids associate with both a minus-end and plus-end directed motor during entry.

Adenoviruses

Individual adenovirus particles have been imaged in living cells by fluorophore tagging (Leopold et al. 1998, 2000; Suomalainen et al. 1999) and GFP fusion techniques (Glotzer et al. 2001). In the latter study, GFP fused to a DNA-binding protein was bound to newly replicating recombinant adenovirus genomes carrying multiple binding sites for the fusion protein during infection. The resulting mature virions carry up to 20 copies of GFP packaged with the viral genome inside the capsid. Consequently, there is little concern that the fluorophore inside the capsid could interfere with external viral protein interactions with host cell components during transport or that the fluorophore would be removed from the capsid during transport because of capsid disassembly. Using both methods, net retrograde transport to the nucleus was found to occur at rates 10–100-fold slower then herpesviruses' entry and transport. The slower rates result from a nearly equal contribution of fast anterograde and retrograde transport of individual capsids (Glotzer et al. 2001, Suomalainen et al. 1999). The relative contributions of dynein and the unidentified KIF are nearly equal, with short movements occurring at less than 1 μm/s in either direction; however, bursts of retrograde transport occur intermittently at peak speeds of 2–3 μm/s, resulting in net transport toward the nucleus (Suomalainen et al. 1999). Overexpression of p50/dynamitin to prevent dynein function in cells blocks the retrograde component of adenovirus movement, resulting in net anterograde viral transport. This result is particularly noteworthy because blocking dynein function interferes with both retrograde and anterograde transport of endogenous host vesicles (Valetti et al. 1999, Waterman-Storer et al. 1997), suggesting that adenovirus transport may be coordinated differently than trafficking of host cargoes inside cells. Therefore, like herpesviruses, adenoviruses associate with both minus-end and plus-end motors during entry, but the regulation of the motors by the two viruses appears to be distinct.

Adeno-Associated Viruses

Like adenovirus, AAV has been chemically tagged with fluorophores and examined in living cells (Bartlett et al. 2000, Sanlioglu et al. 2000). More recently,

individual AAV capsids tagged with a fluorophore in a single copy were tracked during entry (Seisenberger et al. 2001). Although only ~8% of cytoplasmic AAV moved directionally, this movement was sensitive to nocodazole, indicating the moving virus particles were transported by microtubule motors. Transport rates ranged from 1.8 to 3.7 μm/s. Unlike adenovirus and PRV, AAV transport was unidirectional, at least when in close proximity to the nucleus. Yet, transport to the nucleus following infection was slow, typically requiring 15 min to traverse the cytoplasm from the plasma membrane. Given that the displacement of the nucleus from the plasma membrane in the HeLa cells used in the study was likely never more than 10 μm, and viral entry into the cell was surprisingly quick (<0.1 s), active transport toward the nucleus must occur in infrequent bursts. This is consistent with the observation that the movement of the majority of capsids inside the cells was limited to diffusion. Based on diffusion constants, some of these capsids were determined to be in endosomes, whereas others had escaped the endosome and were exposed directly to the cytosol. Which of these forms moved along microtubules is not clear.

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Who's Next?

With the technology to track individual viruses becoming more accessible, examination of many additional viruses can be expected. In the case of HIV, Chicurel recently discussed unpublished work by T. Hope, who imaged viral particles with GFP fused to the Vpr protein during entry (Chicurel 2000). HIV was described as moving on microtubules immediately following infection. Surprisingly, the entry of vaccinia virus, which is arguably the most thoroughly studied virus in terms of egress, has yet to be examined at the single-particle level. Vaccinia does not replicate in the nucleus (unlike other DNA viruses, including herpesviruses, adenovirus, and AAV) but presumably undergoes retrograde transport during entry to reach the perinuclear region of the cytoplasm. This assertion is supported by nocodazole treatment and p50/dynamitin overexpression, both of which prevent perinuclear localization of virions near the microtuble organizer center (MTOC) 6 h post infection (Ploubidou et al. 2000). Although these results are suggestive of a role for microtubule-based retrograde transport during entry, examination of earlier time points, preferably by individual particle tracking, is required to address this possibility directly. Interestingly, nocodazole and p50/dynamitin overexpression also prevent perinuclear accumulation of African swine fever virus (ASFV), which is not related to vaccinia, but like vaccinia is a large DNA virus that replicates in the cytoplasm (Alonso et al. 2001). Although GFP-fusion studies of vaccinia have been successfully used to image viruses during egress, the viral proteins so far tagged with GFP are membrane proteins that may not remain associated with the viral core during the entry process (Geada et al. 2001; Hollinshead et al. 2001; Rietdorf et al. 2001; Ward & Moss 2001a,b). Studies of viral entry will likely require finding an appropriate core protein constituent to fuse to GFP.

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Are Microtubules Always Used During Entry?

It is much too early to defend the assertion that all viruses use microtubule-based, retrograde transport to reach their sites of replication after entry. The dependence on microtubules is not absolute for some viruses. In the presence of nocodazole, adenovirus no longer accumulates at the MTOC as it does normally prior to reaching the nucleus, but capsids still associate with the nuclear membrane and the viral genome is delivered into the nucleus (Glotzer et al. 2001). The same observation holds for AAV, herpesviruses, retroviruses, and ASFV, all of which can productively infect a cell in the presence of nocodazole or colchicine (Alonso et al. 2001, Saib et al. 1997, Sanlioglu et al. 2000, Sodeik et al. 1997). It is unclear whether these observations indicate that viruses can enter cells on apical surfaces where the plasma membrane is in close juxtaposition to the nucleus or that disruption of the microtubule cytoskeleton allows for increased cytoplasmic diffusion of viral capsids. The latter hypothesis is supported by the observation that secretory vesicles from the Golgi arrive at the plasma membrane in nocodazole-treated cells, although some targeting specificity is lost (Rogalski et al. 1984, Wacker et al. 1997). Additionally, overexpression of p50/dynamitin, unlike treatment with nocodazole, completely blocks infection with ASFV (Alonso et al. 2001).

The actin cytoskeleton must be considered an alternative transport pathway for entering viruses, and it appears to be used as such by an insect virus (Cudmore et al. 1997). However, although actin plays a role in the endocytosis of some viruses, as of now there is no direct evidence for a role of actin in transport of mammalian viruses during entry. The transport of viruses to the nucleus in the presence of nocodazole is probably not the result of actin-based transport because simultaneous treatment of cells with nocodazole and cytochalasin D does not block adenovirus movement (Glotzer et al. 2001). More work clearly remains to be done.

VIRAL EGRESS (BREAKING OUT)

Once a cell is productively infected, progeny virions must exit the cell to complete the infectious cycle. There are at least two recognized routes out of the infected cell: viral exocytosis and cell lysis. Virions bud or wrap into a lumenal/extracellular space in the exocytic route that ultimately results in either the release of a freely diffusing infectious particle into the extracellular space or direct transfer of virions to an adjacent uninfected cell. The phenomenon of cell-to-cell spread is poorly understood but is well documented for several viruses (Johnson & Huber 2002).

Cell lysis is the final component of the cytopathic effect (CPE) of many viruses and literally is the last gasp of the infected cell. Prior to lysis, CPEs often include gross changes in cell shape and architecture, which are concomitant with changes in the cytoskeleton. Disintegration of the cell's cytoskeletal and membrane integrity is to be contrasted with apoptosis, a process that also may occur in virus-infected cells. The concept that viruses lyse cells as a mechanism for egress originates from

infections of bacteria with lytic phage. In cell culture, mammalian viruses typically disrupt the host cell's integrity at the end of infection, resulting in release of virions into the culture medium. The significance of this process in animal infections is not clear. Although neurotropic herpesviruses cause significant CPE in cultured cells, cell lysis plays no role in the spread of virus from peripheral sites of inoculation to the brain. In fact, mutant herpesviruses unable to release infectious particles from cells nonetheless spread in the vertebrate nervous system identically to wild-type viruses by a direct cell-to-cell transmission pathway (Heffner et al. 1993, Mulder et al. 1996). Therefore, release of viruses by cell lysis is not required for the dissemination and pathology of at least some infections in animals. However, unlike enveloped viruses such as herpesvirus, non-enveloped viruses are generally regarded to have fewer options to exit a cell.

Because viral envelopes are acquired by traversing a host membrane during maturation, non-enveloped viruses are often assumed to achieve egress from cells exclusively by cell lysis. This model may have resulted from an overuse of Occam's razor, as observing non-lytic viral egress in cells exhibiting severe CPE may be difficult. In fact, some non-enveloped viruses release infectious particles from cells prior to lysis (Blank et al. 2000, Clayson et al. 1989, Jourdan et al. 1997, Tucker et al. 1993). In particular, hepatitis A virus is released from permissively infected polarized Caco-2 cells without any loss of cell viability or changes in transepithelial permeability (Blank et al. 2000). Furthermore, there is evidence that viruses do use novel pathways to move across membranes without becoming permanently enveloped. This is the case for rotaviruses, which bud through the endoplasmic reticulum (ER), gaining an envelope, but while in the ER, the envelope is subsequently removed by a mechanism not fully understood (Griffiths & Rottier 1992, Tian et al. 1996). Although rotavirus requires cell lysis to egress from the ER in nonpolarized cells, rotavirus particles are released from the apical surface of polarized Caco-2 cells by a non-lytic Golgi-independent pathway (Jourdan et al. 1997). As rotavirus replicates in the intestinal epithelium of animals, its infectious cycle may be adapted for polarized cells. These observations underscore the importance of studying the interaction of viruses with a cell type relevant to the pathogenesis of the virus, as dependence on cell lysis for egress in some cases may be a cell culture artifact. Whether viruses use CPE to increase diffusion in the cytoplasm and thereby reach a target membrane or rely exclusively on directed transport in natural target cells is of ongoing interest.

Leaving One at a Time: Virus Egress by Membrane Fusion

Viral envelopes are acquired by budding or wrapping into a host membrane; no viruses are known to encode their own membrane biosynthetic pathways, although there is a debate if vaccinia virus directs the formation of a lipid bilayer during an early stage of viral assembly (Sodeik & Krijnse-Locker 2002). Some viruses bud through internal membranes (i.e., herpesviruses, rotaviruses), whereas others bud directly out of a cell through the plasma membrane (i.e., HIV, influenza virus). Host actin is a known component of many released viruses that use a budding

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step during their egress, indicating that traversing the membrane may be an actin-dependent process (Damsky et al. 1977, Naito & Matsumoto 1978, Nermut et al. 1999, Ott et al. 1996, Ulloa et al. 1998, Wong & Chen 1998), possibly related to endogenous multivesicular body formation (Garrus et al. 2001). How viruses get to these membranes is only now being studied, and perhaps not too surprisingly, microtubule-based transport again is the emerging theme.

Herpesvirus Exocytosis

Herpesviruses assemble their capsids in the nucleus, but gain their mature envelope from the biosynthetic pathway downstream of the Golgi apparatus (Enquist et al. 1998, Granzow et al. 2001, Klupp et al. 2000, Skepper et al. 2001). In cultured neurons, one site for herpesvirus membrane acquisition is thought to be the axon terminal (Holland et al. 1999). The elongated nature of the axon has allowed for a detailed examination of HSV-1 egress prior to membrane acquisition. During egress, non-enveloped HSV-1 capsids are transported in axons in the anterograde direction to the axon terminal (Penfold et al. 1994). Transport is estimated to occur at rates consistent with microtubule-based fast axonal flow (2–3 mm/h) and is sensitive to nocodazole (Miranda-Saksena et al. 2000, Penfold et al. 1994). Thus like herpesvirus entry, egress is a microtubule-dependent process.

We have tracked individual herpesvirus capsids fused to GFP in cultured neurons from both chick and rat (Smith et al. 2001; G.A. Smith & L.W. Enquist, unpublished observations). Similar to viral entry, transport during egress is saltatory and bi-directional. Unlike viral entry, movement in both directions is processive (average anterograde velocity = 2.0 μm/s; average retrograde velocity = 1.3 μm/s), but net anterograde transport is highly favored because of the low frequency of capsids that move in the retrograde direction. Therefore, herpesvirus capsids have the remarkable ability to modulate microtubule-based transport to favor either retrograde movement or anterograde movement at different stages of their infectious cycle (see below).

Vaccinia Exocytosis

Current indications are that vaccinia virus uses microtubules at two distinct steps during assembly and egress. The maturation pathway of vaccinia is complex. Viruses begin their assembly in a perinuclear viral factory, where the viral genome is replicated. Intracellular mature virions (IMV) emerge from the factory. IMVs are infectious if released from cells by lysis, but can be wrapped by Golgi-derived membranes to egress by an exocytic mechanism. The wrapping results in the acquisition of two additional concentric membranes around the virion, resulting in a particle referred to as the intracellular enveloped virion (IEV) that can then exit the cell by fusion of the outermost membrane with the host cell plasma membrane. IMVs appear to transport from the viral factory to the Golgi-derived membranes along microtubules (Sanderson et al. 2000). A viral protein exposed on the surface of the IMV, A27L, is required for IMVs to leave the periphery of the viral factory

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and ultimately become further enveloped to form IEVs. Nocodazole treatment interferes with IMV migration from the viral factories to Golgi-derived membranes and subsequent IEV production. However, further confirmation of this process will require tracking of viral particles as they move from the factory to the Golgi-membrane envelopment sites, as A27L and microtubules could be necessary for a step in viral maturation independent of IMV migration.

IEVs must also overcome the cytoplasmic diffusion barrier to reach the plasma membrane. Such transport enables egress of the virus from the cell in a form that remains associated with the cell surface (cell-associated enveloped virus; CEV) or is released from the cell into the culture media (extracellular enveloped virus; EEV). Transport of IEVs to the plasma membrane was first proposed to occur by actin polymerization, similar to the movement of *Listeria* and *Shigella* bacterial pathogens (Cudmore et al. 1995). This mechanism is no longer in favor (Moss & Ward 2001), although actin polymerization does propel CEVs on the cell surface (Hollinshead et al. 2001, Rietdorf et al. 2001, Ward & Moss 2001a), similar to enteropathogenic *Escherichia coli* and so-called inductopodia (Forscher et al. 1992, Sanger et al. 1996).

Using GFP-tagged virions, Ward and colleagues first documented a role for microtubules in IEV transport to the plasma membrane (Ward & Moss 2001b). Subsequently, a number of research groups confirmed their findings and in most cases further demonstrated that IEV transport is exclusively microtubule-based, whereas actin-based motility is restricted to CEVs on the cell surface (Geada et al. 2001, Hollinshead et al. 2001, Rietdorf et al. 2001, Ward & Moss 2001a). These reports all use recombinant viruses expressing GFP fused to a protein that is a component of the IEV surface membrane: either the viral protein B5R or F13L. Both proteins are important in viral maturation, but the recombinant viruses have wild-type properties. Because IEV membrane proteins are found in Golgi-derived membranes prior to wrapping around IMVs, there is some concern that GFP punctae in these infections could represent vesicles carrying the fusion protein instead of the IEVs. This possibility was directly addressed by examining infected cells for colocalization of GFP punctae with DNA (Geada et al. 2001). The majority of GFP punctae were positive for DNA, which is consistent with the fluorescent signal originating from an IEV and not a vesicle. However, close examination of this data reveals GFP punctae that do lack DNA. Because individual punctae are sometimes seen moving to the plasma membrane where they suddenly elongate into filopodial extensions in bursts of actin polymerization, and because these filopodia are always associated with a single CEV particle at the tip, the GFP fusion protein clearly enables tracking of IEV egress in cells with the caveat that a minority of particles may be host vesicles carrying the GFP fusion protein (Ward & Moss 2001b).

Nocodazole treatment consistently inhibits IEV motion (Geada et al. 2001, Hollinshead et al. 2001, Rietdorf et al. 2001, Ward & Moss 2001a). Although this effect may be an indirect result of inhibition of IMV or IEV maturation (see above), recovery of individual particle movement upon nocodazole wash-out was also reported (Ward & Moss 2001b). IEV movement is saltatory and unidirectional. Rates

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of movement are consistent with microtubule motors, ranging from ~1 to 4 μm/s (Geada et al. 2001; Hollinshead et al. 2001; Rietdorf et al. 2001; Ward & Moss 2001a,b). Furthermore, IEVs colocalize with microtubules in fixed cells (Geada et al. 2001) and are observed to track along microtubules toward the periphery of living cells that are expressing GFP-tubulin (Rietdorf et al. 2001). Although IEVs are sometimes seen moving toward the nucleus, this motion likely comes from the microtubules that curve around in the cytoplasm because IEVs have not been reported to reverse direction (Geada et al. 2001). Kinesin colocalizes with IEVs, and overexpression of the TPR cargo-binding domain of the kinesin light chain 2 blocks IEV movement, implicating kinesin as the motor responsible for IEV transport (Rietdorf et al. 2001). While plus-end transport of IEVs along microtubules targets virions out of the cell, vaccinia interacts with the host cytoskeleton one additional way for a final push toward initiating another round of its infectious cycle.

Once an IEV reaches the periphery of the cell, its outermost envelope fuses with the plasma membrane. This is the final step in egress for the virus, which results in either a CEV or EEV particle. As mentioned above, CEVs are bound to the host cell surface and often extend outward on the tips of filopodia by actin-based motility. This process is required for wild-type virulence and likely allows for direct cell-to-cell transmission of infection (Rietdorf et al. 2001, Ward & Moss 2001a). Vaccinia filopodia extensions were first described by Stokes (1976). Further characterization confirmed the extensions were actin-based (Hiller et al. 1979), and time-lapse microscopy demonstrated that vaccinia associated with the tips of filopodia were actively undergoing actin-based motility to protrude away from the cell surface (Cudmore et al. 1995). Interestingly, similar viral extensions have been observed on the surfaces of cells infected with murine mammary tumor virus (MuMTV) and iridovirus (Damsky et al. 1977, Murti et al. 1985), and human respiratory syncytial virus (HRSV) has also been suggested to form surface extensions (Ulloa et al. 1998). It will be interesting to learn if these viruses also use actin-based motility to spread efficiently from cell to cell, and if microtubule-based motility also is used in these instances to first reach the plasma membrane.

Mass Exodus: Virus Egress by Cell Lysis

Compared with egress by exocytosis, much less is known regarding viral egress by compromising cellular integrity. Global changes in the cytoskeleton often accumulate as viral infections progress (for example see Belin & Boulanger 1987, Bowden et al. 1987, Carvalho et al. 1988, Chen et al. 1993, Dienes et al. 1987, Norregard Nielsen et al. 1987), but how these changes may lead to the rupturing of a cell is not clear. In cell culture, some viruses egress by exocytosis followed by cell lysis. In the case of vaccinia, virions released by exocytosis and lysis are distinct from one another, and these virions subsequently infect cells by different pathways (Locker et al. 2000). This finding adds credence to the idea that cell lysis is a functional form of egress in vivo. Thus cell lysis may provide a final escape route from the infected or compromised cell, but the ability to spread from cell to cell adds the advantage

of increasing the kinetics of egress and dissemination and possibly enables viruses to stay ahead of the immune response.

REGULATION OF DIRECTION

In general, viruses must interact with cytoskeletal transport machinery at multiple stages in their life cycles. In the simplest scenario, retrograde transport delivers virions to the site of replication during entry, and anterograde transport subsequently targets progeny virions to the appropriate host membrane for egress. This bi-directionality requires some form of regulation imparted by the virus. Because microtubules are emerging as the standard platform for intracellular virion transport, a more specific issue may be how viruses regulate plus-end (KIFs) and minus-end (dynein/dynactin) motor complexes at distinct stages of the infectious cycle. Such regulation is clearly relevant for herpesviruses, which are well documented to use microtubule transport for both entry and egress, and is likely the case for other viruses (Bearer et al. 2000, Lycke et al. 1984, Miranda-Saksena et al. 2000, Penfold et al. 1994, Smith et al. 2001, Sodeik et al. 1997, Topp et al. 1994).

Regulating direction of transport may be as simple as regulating binding of the virion to the necessary motor protein. However, the transport of adenoviruses during entry and herpesviruses during entry and egress is bi-directional, with individual virions often reversing direction instantaneously (or at least faster than the temporal resolutions of current recordings can resolve) (Smith et al. 2001, Suomalainen et al. 1999; G.A. Smith & L.W. Enquist, unpublished data). In these examples, plus-end and minus-end motors must be associated with individual virions simultaneously, which indicates that regulation of direction may occur by post-translational modification of the motor complexes. Although physical interaction between opposing cytoplasmic microtubule motors has not been demonstrated, there is compelling evidence for a bi-directional motor complex that is coordinately regulated (Bowman et al. 2000, Brady et al. 1990, Gross et al. 2002, Lippincott-Schwartz et al. 1995, Martin et al. 1999, Muresan et al. 1996, Reese & Haimo 2000, Valetti et al. 1999, Waterman-Storer et al. 1997).

For adenoviruses, the relative contributions of anterograde and retrograde movement on microtubules during entry are modulated by host signaling pathways triggered by the virus (Suomalainen et al. 2001). Integrins, such as $\alpha_v ß_5$, serve as receptors for adenovirus and upon binding trigger cAMP-dependent protein kinase (PKA) activity. PKA in turn enhances retrograde movement of capsids in the cytosol by an unknown mechanism. Similarly, p38/mitogen-activated protein kinase (MAPK) is activated during infection (independent of integrins and PKA), which also enhances retrograde movement. Inactivation of PKA with any of several inhibitors results in net capsid transport in the anterograde direction. Anterograde motion can also be made to predominate when p50/dynamitin is overexpressed in infected cells to prevent dynein function (Suomalainen et al. 1999). Similarly, AAV also triggers host signal pathways during entry that effect virion transport to the nucleus (Sanlioglu et al. 2000). Although adenovirus egress is generally thought

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to be dependent upon host cell lysis (Zhang & Schneider 1994), this conclusion is based on infections of nonpolarized cell lines. Similar to rotavirus (see above), adenovirus typically replicates in polarized epithelia in animal infections. A significant amount of time is seemingly wasted to get adenovirus to the nucleus during entry by competing plus-end and minus-end microtubule motors. This inefficiency may be explained if the virus also uses bi-directional microtubule transport to move to target membranes during egress, perhaps in polarized epithelia. Indeed, the system could be primed for differential targeting, as PKA and MAPK are specifically upregulated by initial infection, potentially allowing for movement in the reverse direction later in infection.

Herpesvirus transport in sensory axons is optimized for fast retrograde transport during entry (10–100 times faster than adenovirus), and slightly faster anterograde transport during egress (Bearer et al. 2000, Smith et al. 2001; G.A. Smith & L.W. Enquist, unpublished results). This effective transport regulation must differentiate between capsids during entry and egress, although the capsids being transported at these two stages are not known to be structurally different (Lycke et al. 1988, Penfold et al. 1994). Unlike adenovirus, herpesviruses encode protein kinases. HSV-1 expresses two serine/threonine protein kinases, encoded by the UL13 and US3 open reading frames. Both kinases are structural components of the herpes virion and are delivered to the cytosol along with the capsid upon infection (Coulter et al. 1993, Cunningham et al. 1992, Overton et al. 1992, Zhang et al. 1990). The UL13 kinase is known to be active when purified virions are permeabilized and reacted with ATP and Mg^{2+}, and phosphorylates viral structural proteins including itself (Morrison et al. 1998). Phosphorylation causes some viral proteins to dissociate from the capsid core; however, the phosphorylated isoform of the UL13 kinase remains attached to the capsid. The US3 kinase has not yet been examined in this regard. Because adenovirus can modulate microtubule movement to slightly favor net retrograde movement using host cell signaling pathways, it is conceivable that the more effective retrograde transport of herpesviruses is achieved by encoding a kinase(s) specifically adapted to interact with transport machinery and affixed to the surface of the capsid being transported. However, the role of the herpesvirus kinases in transport, if any, has yet to be examined.

Incorporation of kinases into virions is not limited to large DNA viruses. HIV packages host ERK2/MAPK, and upon infection, this kinase effectively targets the viral core (referred to as the pre-integration complex) to the nucleus (Bukrinskaya et al. 1996, Cartier et al. 1997, Jacque et al. 1998).

Additional forms of transport regulation are exemplified by the use of kinesin and actin polymerization during successive steps of vaccinia egress (Geada et al. 2001; Hollinshead et al. 2001; Rietdorf et al. 2001; Ward & Moss 2001a,b). The transition from microtubule transport to actin-based motility is mediated by phosphorylation of the A36R viral protein by Src kinase at the plasma membrane (Frischknecht et al. 1999a,b; Ward & Moss 2001a). Although how phosphorylation of A36R halts microtubule transport is not yet clear, the induction of actin polymerization is achieved by recruiting host factors to the phosphorylated tyrosine

residues, which act as SH2 binding sites for Nck, which in turn recruits N-WASP (Frischknecht et al. 1999b). N-WASP recruits the Arp2/3 complex to nucleate actin polymerization (Moreau et al. 2000).

FINDING THE EFFECTORS

The imaging of viral entry and egress is now yielding exciting insights into transport dynamics and regulation. Coupled with drug and inhibitor studies, this body of work is defining the cellular mechanisms used by viruses to infect cells. However, understanding the viral/host interactions at the molecular level requires detailed biochemical analysis.

The search to identify viral proteins that interact with the cytoskeleton was ongoing prior to the first imaging of individual adenoviruses in living cells. Although a number of viral/host protein interactions have been described, proof of the role and requirement in transport has been difficult to obtain. Imaging virus movement in live cells is a powerful technology, but the challenge is to define the interactions responsible for the newly described transport phenotypes revealed by imaging single virus particles.

The best-described viral factor that acts as an effector for transport is the A36R protein of vaccinia. A36R is required for actin polymerization and for the extension of vaccinia CEVs on the tips of membrane projections (see above for a description of A36R function) (Rietdorf et al. 2001, Rottger et al. 1999, Ward & Moss 2001a, Wolffe et al. 1998). Furthermore, expression of A36R alone in cells results in filopodia formations at the plasma membrane with A36R at the tips (Lorenzo et al. 2000). A36R is also postulated to be the effector of vaccinia IEV kinesin-directed movement to the plasma membrane, although a direct interaction of kinesin and A36R has not been demonstrated (Rietdorf et al. 2001). In fact, although a number of viral interactions with microtubule motor proteins have been observed biochemically, no viral protein has been demonstrated to effect viral capsid transport by microtubule motors.

For those viruses that have been tracked in living cells, no viral proteins of adenovirus or AAV are as of yet implicated in capsid transport. However, the UL34 protein of HSV-1 does bind a dynein intermediate chain (IC-1a) (Ye et al. 2000). Whether UL34, which plays an important role in herpesvirus transport from the nucleus to the cytoplasm during egress, is involved in capsid cytoplasmic transport remains to be addressed directly (Klupp et al. 2001, Reynolds et al. 2001, Roller et al. 2000). UL34, a type-II tail-anchored membrane protein, is present in membranes of infected cells (Purves et al. 1992, Shiba et al. 2000). This is not expected for a protein involved in capsid transport during entry because the herpesvirus envelope is not transported with the capsid. Furthermore, UL34 is not a structural component of extracellular PRV virions (Klupp et al. 2000). Although HSV-1 and PRV have morphologically identical infectious cycles (Granzow et al. 2001), distinct mechanisms for association with dynein between the two remains a possibility. Nevertheless, UL34 is not a conserved structural component of

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herpesviruses particles, whereas retrograde transport during entry is a conserved event of the infectious cycle. One clue to the identity of the capsid dynein-binding protein comes from immunogold studies that show dynein associated with HSV-1 capsid vertices (the 12 tips of the icosahedral capsid) (Sodeik et al. 1997). The capsid vertices are composed of the VP5 protein, which was also found to bind dynein IC-1a, although this interaction was suggested to be indirect (Ye et al. 2000). In addition to VP5, a subclass of viral structural proteins called the tegument is associated with the capsid vertices. The tegument protein immediately bound to VP5, and therefore a good candidate for a dynein-binding protein, is postulated to be VP1/2 (also referred to as VP1-3) (Zhou et al. 1999). Further examination of UL34, VP1/2, and VP5 will be necessary to better understand the interactions of capsids with dynein, as well as KIFs.

Additional viral/motor interactions have been found by yeast two-hybrid screens. The murine leukemia virus (MuLV) Gag protein binds KIF4 (Kim et al. 1998). This interaction may be important for targeting progeny virions to the plasma membrane, the site of retrovirus budding, but its significance has not yet been directly addressed. Similarly, the hepatitis B virus core protein was found to bind the actin-binding protein, ABP-276/278 (Huang et al. 2000).

Yeast two-hybrid assays have revealed interactions between the LC8 light chain of dynein with protein components of rabies virus (and the related Mokola virus) and ASFV (Alonso et al. 2001, Jacob et al. 2000, Raux et al. 2000). The LC8-binding site in the rabies virus P phosphoprotein was mapped to a consensus sequence (DxxQ) that is present in two host proteins, nNOS and Bim, that bind LC8 (Poisson et al. 2001). Although there is good evidence that ASFV uses dynein for retrograde transport during entry, the DxxQ motif is not present in the ASFV p54 protein that binds LC8 (Alonso et al. 2001). However, the motif is present in proteins of two other viruses: the VP4 protein of rotavirus and the poliovirus VP2 protein (Poisson et al. 2001). While the significance of this observation remains to be determined, the rotavirus VP4 protein does, in fact, co-localize with microtubules late in infection (Nejmeddine et al. 2000). However, the significance of the poliovirus VP2 DxxQ motif in particle transport is questionable. Although poliovirus is sometimes neurotropic and spreads from axon terminals to neuronal cell bodies by fast retrograde transport, movement occurs while the particle is in an endosome. Viral components, including VP2, cannot be in direct contact with cytosolic proteins such as dynein. An alternative idea is that the cytosolic tail of the host receptor for poliovirus, CD155, may direct axonal transport of poliovirus endosomes via an interaction with the dynein light chain, Tctex-1 (Mueller et al. 2001).

FUTURE DIRECTIONS

Although microtubule motors are emerging as common transporters for viruses to reach their sites of replication during entry and sites of budding during egress, this hypothesis will undoubtedly be further scrutinized by studies of additional viruses in cells (Table 1). Fluorescently tagged viruses that track viral movements in cells,

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TABLE 1 Directional movement of viruses during entry and egress

	ENTRY (retrograde transport toward nucleus)				EGRESS (anterograde transport toward cell periphery)			
Virus	**Motion[a]**	**Avg. minus-end velocities[b]**	**p50/dynamitin[c]**	**Nocodazole[c]**	**Motion[a]**	**Avg. plus-end velocities[b]**	**TPR KLC2[c]**	**Nocodazole[c]**
AAV	Uni-directional	2.8 μm/s	Sensitive	Sensitive	?	?	?	?
Adenovirus	Bi-directional	0.3–0.5 μm/s	Sensitive	Mixed results	?	?	?	?
Herpesvirus	Bi-directional	1.3–2.2 μm/s	Sensitive	Sensitive	Bi-directional	2.0 μm/s	?	Sensitive
Vaccinia	?	?	Sensitive	Sensitive	Uni-directional	0.3–1.0 μm/s	Sensitive	Sensitive

[a]Bi-directional motion is defined by individual particles being able to reverse direction during observation. Because reversals can be short lived, as is the case for PRV during entry, slow frame rates may result in motion erroneously being classified as uni-directional.

[b]Average velocities are listed for the dominant direction of motion only. Ranges are given for viruses that have been examined by more than one laboratory.

[c]Virus motion is considered sensitive to p50/dynamitin overexpression, kinesin light chain 2TPR cargo-binding domain overexpression, or nocodazole treatment if directed motion of individual particles is inhibited or if accumulation of virions near the nucleus (entry) or periphery (egress) is inhibited.

in combination with drug and inhibitor studies, are revealing aspects of cellular pathogenesis that previously could only be modeled from images of fixed cells.

Characterizing the mechanisms viruses use to move in cells will undoubtedly shed light on the workings and regulation of cellular transport machinery, as well as provide novel targets for treatment of disease. Viral models of intracellular transport have the potential to become powerful tools to study these cell biological processes. Many viruses are easily manipulated, and viral proteins are made in high quantity, simplifying their isolation and characterization. Furthermore, viral infections are the ultimate pulse-chase experiment to examine transport of newly made proteins in cells.

Many of the outstanding questions in viral transport relate to current questions in cell biology.

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HOW DO VIRUSES BIND MOTOR COMPLEXES? The identification of endogenous motor-docking proteins used to transport cellular cargoes is providing new insights into intracellular trafficking (Klopfenstein et al. 2000). Yeast two-hybrid screens and pull-down experiments have identified viral proteins that bind motor components, and examination of viruses with mutations in these proteins by individual particle tracking is beginning to reveal the biological significance of these interactions. Because viruses interact with the cytoskeleton in many ways, a diversity of interactions is expected to be found.

HOW DO VIRUSES REGULATE MOTOR COMPLEXES? From the few observations currently available, it is already clear that viruses regulate microtubule motors in a variety of ways. Kinases, either triggered by viral infection or encoded by viruses, are likely to be common effectors of viral transport regulation. However, the mode of regulation can result in uni-directional transport of some viruses (i.e., vaccinia and AAV), bi-directional motion with inefficient net displacement (i.e., adenovirus), or bi-directional motion translating into efficient transport in opposing directions at various times in the infectious cycle (i.e., herpesviruses). Currently, little is known about how opposing motors bound to the same cargo coordinate their activities to target distant cellular structures. Relating viral transport phenotypes with viral/motor interactions will be revealing of both viral pathogenesis and endogenous motor function.

HOW DO VIRUSES TARGET DIFFERENT PARTS OF THE CELL? Virus movement is not limited to transport to the nucleus and plasma membrane. Different viruses use different membranes as budding sites; for example, rotavirus buds into the ER, coronavirus into the intermediate compartment, vaccinia into Golgi, herpes into the *trans*-Golgi network, and influenza into the plasma membrane (reviewed in Griffiths & Rottier 1992). Therefore, in addition to hijacking motor proteins, viruses must use components of cellular targeting pathways to thread their way through the cytoplasm.

Intracellular viral trafficking has significant implications in disease outcome. Viruses typically come into contact with polarized epithelia upon infection of an

animal. Following replication, egress from the epithelial cells is an important determinant of pathogenicity. Virion release from apical or lateral surfaces confines the infection to the exposed tissues, whereas basal release often results in dissemination to deeper tissues and systemic spread. Similarly, neurotropic viruses can exit from peripheral or central branches of neuritic processes with markedly different outcomes for the host. Transport and release of viruses from peripheral axons is a way out of the nervous system and into surface-exposed tissues that ultimately allow for release of the virus into the host population. Conversely, egress from central nervous system processes can result in uncontrolled spread of virus in the brain.

Additional Issues

The methods used to track individual virions in the cytoplasm can also be applied to viral dynamics in the nucleus. Viruses replicate in specific regions of the nucleus, but how viral genomes become localized to particular domains is unknown. Furthermore, egress of these viruses from the nucleus is particularly complex. In the case of non-enveloped viruses that replicate in the nucleus (e.g., adenovirus, AAV, SV40, papillomavirus), progeny virions must breach at least three membranes for egress out of the cell, but little is known about the mechanisms involved.

Addressing many of these issues will require new variations on the current methods being used to image individual viruses in cells. For instance, spectrally shifted variants of GFP may allow for simultaneous tracking of distinct viral structures, or of viral and cellular structures, during infection. The DsRed protein from Discosoma coral may prove to be useful in multi-spectral applications and for single-molecule imaging. However, long maturation times of the protein and multimer formation are issues to be addressed for most applications (Baird et al. 2000, Gross et al. 2000). Whatever the methods used, the relationships between the different forms of viral transport and the various interactions with motor complexes will be key to understanding the pathogenesis of most, if not all, mammalian viruses.

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