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REVIEW ARTICLE

Dissecting virus entry via endocytosis

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Numerous virus families utilize endocytosis to infect host cells, mediating virus internalization as well as trafficking to the site of replication. Recent research has demonstrated that viruses employ the full endocytic capabilities of the cell. The endocytic pathways utilized include clathrin-mediated endocytosis, caveolae, macropinocytosis and novel non-clathrin, non-caveolae pathways. The tools to study endocytosis and, consequently, virus entry are becoming more effective and specific as the amount of information on endocytic component structure and function increases. The use of inhibitory drugs, although still quite common, often leads to non-specific disruptions in the cell. Molecular inhibitors in the form of dominant–negative proteins have surpassed the use of chemical inhibitors in terms of specificity to individual pathways. Dominant–negative molecules are derived from both structural proteins of endocytosis, such as dynamin and caveolin, and regulatory proteins, primarily small GTPases and kinases. This review focuses on the experimental approaches taken to examine virus entry and provides both classic examples and recent research on a variety of virus families.

Introduction

As obligate intracellular parasites, all viruses must have ways of entering target cells to initiate replication and infection. In animal cells, viruses can do this in two principal ways: by a direct mechanism at the cell surface (plasma membrane) or by following their internalization into cellular compartments (for example, endosomes). In the case of endocytic entry, internalization itself is generally not sufficient for productive infection, as incoming viruses are still part of the extracellular space while in endosomes. Therefore, endocytosed viruses must penetrate or fuse with the endosomal membrane to be released into the cytoplasm. In addition, the endocytic pathway is often used by viruses requiring a specific localization within the cell for a successful infection.

This review is intended as an update on the endocytic route of virus entry. It covers both the classical and the more recently described non-classical internalization and trafficking pathways utilized by animal viruses to gain entry into host cells. We use the term endocytosis to include not only internalization from the plasma membrane but also sorting and trafficking events within intracellular vesicles. We will focus on each of the endocytic pathways and examine the experimental approaches used – giving examples of the best-characterized viruses that utilize these routes for infection. For more detailed information on individual virus families, see previous reviews of virus entry by Marsh (1984), Marsh & Helenius (1989), Marsh *et al*. (1983) and Russell & Marsh (2001). For a review of the role of endocytosis in virus replication beyond virus entry see Marsh & Pelchen-Matthews (2000) and for information on post-endocytic events during virus entry, such as interactions with the cytoskeleton and nuclear import machinery and with signalling molecules, see Greber (2002), Sodeik (2000) and Whittaker *et al*. (2000). Several other excellent general articles on endocytosis have been published recently (Gruenberg, 2001; Mellman, 1996; Mukhergee *et al*., 1997) and in this review we will only include details on the various cellular pathways where it is pertinent to virus entry.

The majority of virus families utilize endocytosis as a means of entry into cells. This is not surprising, considering the many benefits that endocytosis offers. Many viruses have a low-pH-dependent conformational change that triggers fusion, penetration and/or uncoating and endocytosis is crucial to these viruses due to the acidification occurring within the endosomal pathway. It is also becoming appreciated that viruses without a strict low-pH step for entry also enter cells via endocytosis, as endosomes offer a convenient and often rapid transit system across the plasma membrane and through a crowded cytoplasm. For nuclear replicating viruses especially, the endosome can deliver its viral cargo to the vicinity of the nuclear pore, ready for translocation into the nucleoplasm (Whittaker & Helenius, 1998).

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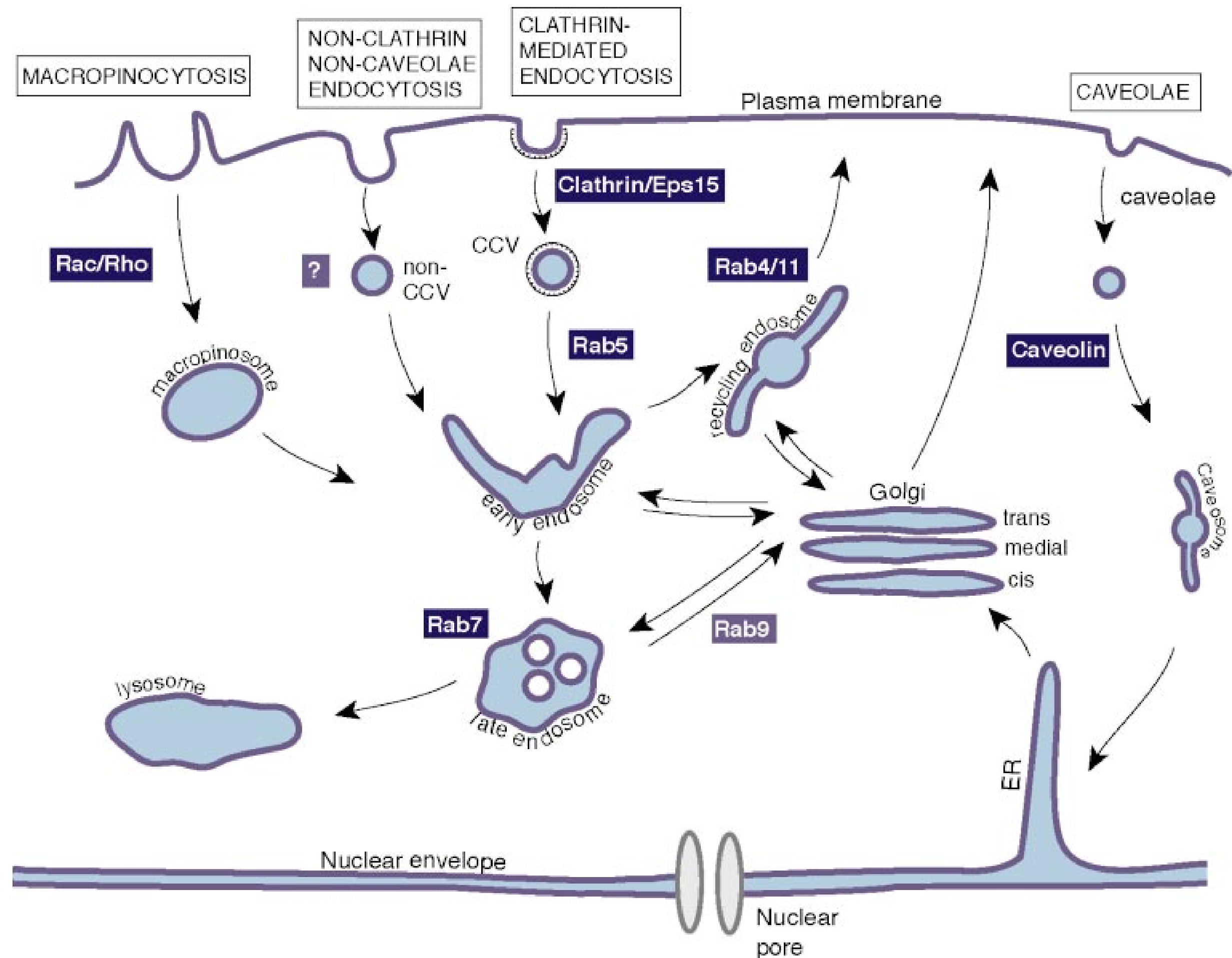

Fig. 1. Summary of the major routes of endocytosis used by viruses. The most established route of entry is via clathrin-coated pits, requiring Eps15 among other molecules. Clathrin-coated vesicles (CCVs) progress to early endosomes in a Rab5-dependent manner. Early endosomes can recycle back to the cell surface (controlled by Rab4 and/or Rab11) or progress to the increasingly acidic milieu of the late endosome/lysosome (controlled by Rab7). Other possible internalization pathways are non-coated pits (non-CCV), macropinocytosis (dependent on the Rac/Rho family of GTPases) or caveolae (dependent on caveolin). The Golgi apparatus is a central player in membrane traffic, affecting transport within both the secretory and the endocytic pathways.

The first molecule shown to be required for endocytosis was clathrin, which mediates the primary route of endocytic internalization into cells (reviewed by Brodsky *et al*., 2001). In response to an internalization signal (involving typically either a YXXΦ or di-leucine sequence in the cytoplasmic tail of a receptor), clathrin is assembled on the inside face of the plasma membrane to form a characteristic invagination or clathrin-coated pit. Clathrin also interacts with a number of other essential molecules, including Eps15, ampiphysin and the AP-2 adapter proteins, as well as the dynamin GTPase responsible for releasing the internalized vesicle from the plasma membrane (Marsh & McMahon, 1999). For some time now, other internalization pathways have been known to exist but these have remained elusive to molecular and biochemical analysis. Recent work, however, has begun to elucidate the molecular mechanism behind some of these pathways (reviewed by Nichols & Lippincott-Schwartz, 2001). Of the non-clathrin pathways, caveolae have featured prominently, in part because of their association with lipid rafts (detergent-resistant membrane domains) (reviewed by Harder & Simons, 1997). Other pathways include macropinocytosis and non-clathrin, non-caveolae-dependent endocytosis (Fig. 1).

Endocytic compartments are pleiomorphic structures that fuse with one another to promote ligand trafficking. Subsequent to internalization, endosomes often undergo complex trafficking and sorting events (reviewed by Gruenberg, 2001; Mellman, 1996; Mukhergee *et al*., 1997). Two principal post-internalization endocytic trafficking routes exist in the cell, which can be termed recycling or lysosome-targeted (reviewed by Gruenberg, 2001). Regulation of sorting and trafficking is determined by inherent signals on the internalized receptor and by signalling events within the cell. One set of

crucial regulatory molecules are the Rab family of small GTPases and their accessory proteins (Collins & Brennwald, 2000; Somsel Rodman & Wandinger-Ness, 2000) and, in addition, the action of specific protein kinases is likely to be crucial for correct endocytic trafficking. The internalized vesicle acquires properties that are defined temporally and are thus termed 'early' and 'late' endosomes. The early endosome is an often pleiomorphic tubulo-vesicular structure (Tooze & Hollinshead, 1991) and is a major sorting station where internalized cargo can be delivered back to the plasma membrane (the recycling pathway) or can progress to the late endosome (Hopkins *et al*., 1990). Late endosomes, comparatively, have a mostly juxtanuclear distribution, are more spherical and contain internal vesicles – leading to the term multi-vesicular bodies (Piper & Luzio, 2001). They also differ from early endosomes in that they have a significantly lower pH (approximately pH 5·5 versus pH 6·2–6·5 in the early/recycling endosome). Late endosomes subsequently progress to lysosomes, which are characterized by the presence of degradative proteases and hydrolases, delivered by communication of endosomes with the *trans*-Golgi network (Kornfeld & Mellman, 1989).

Viruses not only depend on the machinery of the cell for internalization but also for trafficking within the cytoplasm and the ability to find the correct site for replication. For example, fusion of Semliki Forest virus (SFV) at the plasma membrane under extracellular low-pH conditions results in the internalization of the virus but does not necessarily result in a successful infection (Marsh & Bron, 1997). Such trafficking depends on specific post-internalization membrane sorting and, in some cases, direct interactions of virus particles with the cytoskeleton (Sodeik, 2000).

Internalization of viruses into endocytic compartments

Clathrin

As befits its major role in endocytosis, clathrin has been shown to play a major role in the internalization of many viruses. Traditionally, several viruses, including influenza virus, vesicular stomatitis virus and SFV were identified in clathrin-coated vesicles at early times of internalization (for example, 5 min) based on the presence of an electron-dense coat by transmission electron microscopy (Marsh & Helenius, 1980; Matlin *et al*., 1981, 1982). However, non-coated vesicles were also observed in these experiments, due either to the release of clathrin or to the presence of non-clathrin-coated pathways. Subsequently, a crucial role for clathrin in SFV internalization has been shown by microinjection of anti-clathrin antibodies (Doxsey *et al*., 1987).

Inhibition of clathrin function traditionally relied on three principle approaches – low-pH shock treatment, potassium depletion and treatment of cells with brefeldin A (BFA) (Brodsky *et al*., 2001) and more recently by the use of chlorpromazine (Wang *et al*., 1993). However, all of these treatments are non-specific; for example, BFA targets ADP-ribosylation factor (ARF) GTPase exchange factors in the secretory pathway and chlorpromazine targets many intracellular enzymes. As such, they have multiple effects on cell function and their use to inhibit virus infection should be treated with some caution. Chlorpromazine has been employed most extensively for studies of virus entry. It has been used to demonstrate the role of clathrin-mediated endocytosis in the uptake of influenza virus (Krizanová *et al*., 1982), the polyomavirus JC virus (Pho *et al*., 2000) and the picornavirus parechovirus type 1 (Joki-Korpela *et al*., 2001). Interestingly, a role for clathrin in the uptake of Epstein–Barr virus into B cells of the immune system, but not epithelial cells, has also been implicated through the inhibitory effect of chlorpromazine (Miller & Hutt-Fletcher, 1992). Similarly, human cytomegalovirus has been shown to enter epithelial cells by endocytosis but fuses at the plasma membrane of fibroblasts (Bodaghi *et al*., 1999), indicating that internalization pathways may be cell type-dependent. Chlorpromazine also blocks entry of the picornavirus hepatitis A virus (Bishop, 1998) but in this case its effects were ascribed to non-clathrin endocytosis. The alternative (and possibly more specific) technique of potassium depletion and hypotonic shock has been used to investigate the role of clathrin in human rhinovirus infection (Bayer *et al*., 2001; Madshus *et al*., 1987).

Dominant–negative mutant versions of cellular proteins provide a more specific way to analyse the function of defined pathways within the cell. When expressed at high levels, dominant–negative mutants act by overwhelming the wild-type protein and preventing its function. This type of approach has been used extensively in studying the function of GTPases within the cell (Feig, 1999), where a dominant–negative protein can lead to sequestration of effectors and regulatory molecules and effectively shut down the function of the endogenous wild-type protein. The only drawback is that inhibition at late time-points after initiation of the dominant–negative protein may be mediated by an indirect effect due to the inevitable network of interacting molecules.

In studies of virus entry, the most commonly applied dominant–negative approach has been to analyse the role of the dynamin, a GTPase required for the release of clathrin-coated pits (Hinshaw, 2000; Schmid *et al*., 1998). Dynamin exists in several forms in the cell, often with cell-type specificity. Dynamin-2 is expressed ubiquitously; however, the most-studied form of dynamin experimentally (dynamin-1) is normally found only in neurons. Although these proteins are similar, they do have distinct properties. Ideally, dominant–negative dynamin-2 should be used in most cell types, although, where examined, dominant–negative versions of dynamin-1 also appear show a strong phenotype in non-neuronal cells. The original expectation was that overexpression of a GTPase-deficient version of dynamin (for example, K44A) would result in a specific block of clathrin-mediated

endocytosis. However, as the study of dynamin has progressed, it is has become clear that it functions in many different membrane-traffic events. In addition to clathrin-mediated endocytosis, dynamin plays a role in formation of caveolae (Henley *et al*., 1998), budding of Golgi-derived vesicles (Jones *et al*., 1998; Nicoziani *et al*., 2000), phagocytosis (Gold *et al*., 1999) and non-clathrin endocytosis (Lamaze *et al*., 2001). The overexpression of dynamin K44A also results in an increase in endosomal pH (Huber *et al*., 2001), posing a possible complication when examining the internalization of pH-dependent viruses. The actions of mutant dynamin, therefore, need to be treated with some caution when interpreting effects on virus infection. A particularly illuminating study of the intracellular bacterium *Chlamydia* clearly showed an effect of dominant–negative dynamin on bacterial replication but without any effects on internalization (Boleti *et al*., 1999).

Nonetheless, the use of the dominant–negative dynamin K44A has proven valuable in our understanding of virus entry. The first report using this molecular inhibitor in studies of virus entry showed effects on SFV, Sindbis virus and rhinovirus but not poliovirus (DeTulleo & Kirchausen, 1998). Subsequently, an effect on rhinovirus internalization was confirmed (Bayer *et al*., 2001) but the effects of dynamin K44A were attributed to a change in endosomal pH (Huber *et al*., 2001). Other viruses where dynamin K44A is known to inhibit internalization include parvoviruses (Bartlett *et al*., 2000; Duan *et al*., 1999; Parker & Parrish, 2000), influenza virus (Roy *et al*., 2000) and adenovirus (Wang *et al*., 1998). Of particular note is the finding that the avian leukosis virus, a retrovirus, shows a 60–80% inhibition of infection in dynamin K44A-expressing cells (Mothes *et al*., 2000), although another retrovirus, Moloney murine leukaemia virus, showed no effects upon expression of dominant–negative dynamin (Lee *et al*., 1999). It is unclear if this apparently contradictory data for retroviruses represent differences in the individual viruses or cell types, under the assay conditions used, or if dynamin is required for other membrane-traffic events important for virus assembly.

Because of the pleiotropic effects of dynamin, investigators have searched for more specific molecular inhibitors of clathrin. One promising candidate is the Eps15 protein, which binds to the AP-2 adapter required for internalization through clathrin-coated pits (Benmerah *et al*., 1998). Deletion of the EH domain(s) of Eps15 produces a dominant–negative version of the protein that arrests clathrin-coated pit assembly (Benmerah *et al*., 1999) and prevents internalization of transferrin, a cellular marker of clathrin-mediated endocytosis. Other promising tools for clathrin inhibition are the overexpression of the clathrin hub domain, which prevents normal clathrin pit assembly (Brodsky *et al*., 2001), and expression of the μ2 subunit of AP-2 (Nesterov *et al*., 1999), which results in a very specific inhibition of AP-2-mediated endocytosis of receptors containing YXXΦ motifs, but not di-leucine-based signals. Of these clathrin/AP-2-specific approaches, Eps15 has received most attention as a way to block virus entry. Microinjection of purified Eps15 protein lacking the EH domains effectively blocked infection by Sindbis virus, even at the relatively high m.o.i. of 50 p.f.u. per cell (Carbone *et al*., 1997). In addition, overexpression of dominant–negative Eps15 has been shown to inhibit uptake of both SFV (unpublished results) and adenovirus (U. Greber, personal communication).

In the future we expect to see more studies of virus entry involving such molecular inhibitors. One note of caution concerning the clathrin-specific approaches outlined above is that, with time, cells expressing the dominant–negative proteins tend to compensate by upregulating non-clathrin-mediated pathways. Thus, experiments using these approaches need to be carefully executed and carried out under as short a time frame as possible. Another factor is that blockage of dynamin function can lead to an arrest of virus receptors at the cell surface and result in increased virus binding (Parker & Parrish, 2000).

Caveolae

Recently, caveolae have emerged as a route of entry for the polyomavirus, simian virus 40 (SV40). The uptake of SV40 is considered somewhat unusual, based on early electron micrographs showing accumulation of incoming virions in the smooth endoplasmic reticulum (ER) (Kartenbeck *et al*., 1989). This localization has been reinforced by the use of green fluorescent protein (GFP)-tagged caveolin-1 (a major component of caveolae) and video microscopy, which showed the delivery of SV40 from caveolae to the ER, bypassing the traditional endosome/lysosome system (Pelkmans *et al*., 2001). Compared to clathrin-mediated entry, caveolae perform internalization more slowly and the resulting vesicles do not become acidified. An additional difference between clathrin and caveolae is that internalization via caveolae is not a constitutive process (Thomsen *et al*., 2002) and only occurs upon cell stimulation. Caveolae are known to be major initiating centres for signalling within the cell (Ceresa & Schmid, 2000; Simons & Toomre, 2000). It is possible that partitioning of bound polyomaviruses into caveolae promotes their uptake and subsequent infection by activating downstream-signalling cascades (Chen & Norkin, 1999). Indeed, it has recently been shown that SV40-induced tyrosine kinase activity leads to recruitment of both actin and dynamin to virus-containing caveolae. This recruitment is necessary for formation of caveolae-derived endocytic vesicles and for SV40 infection (Pelkmans *et al*., 2002).

Colocalization studies with endogenous caveolin have also been used to implicate caveolae in the entry of SV40 (Norkin, 1999); however, conflicting reports for colocalization with murine polyomaviruses have been reported (Gilbert & Benjamin, 2000; Richterova *et al*., 2001). In these studies, murine polyomavirus was also shown to be independent of both clathrin and dynamin. Clearly, more studies are required to clarify the role of caveolae in the entry of the different members of the *Polyomavirinae*.

Several drugs have been used to inhibit endocytosis via caveolae selectively. The most effective way of disrupting caveolar function is with sterol-binding drugs that sequester cholesterol – a prominent component of lipid rafts involved in caveolae formation. Such drugs include nystatin, filipin and methyl-β-cyclodextrin (Neufeld *et al*., 1996; Orlandi & Fishman, 1998; Rothberg *et al*., 1992), none of which typically affect clathrin-mediated endocytosis. Along with colocalization studies, these drugs have been used in studies of both SV40 and murine polyomavirus (Anderson *et al*., 1996; Gilbert & Benjamin, 2000; Richterova *et al*., 2001). They have also been used to show the involvement of caveolae in the internalization of the picornavirus echovirus type 1 (Marjomaki *et al*., 2002) and the uptake of respiratory syncytial virus into dendritic cells (Werling *et al*., 1999).

The most characterized protein components of caveolae are the caveolins (Kurzchalia & Parton, 1999; Rothberg *et al*., 1992). For reasons that are still unclear, the caveolins require an intact N terminus for their function. Deletion of the N terminus of caveolin-3 results in a dominant–negative protein that inhibits both SV40 (Roy *et al*., 1999) and echovirus-1 (Marjomaki *et al*., 2002) when overexpressed. Similar findings were obtained with SV40 by tagging the N terminus of caveolin-1 with GFP, whereas fusion to the C terminus resulted in a wild-type protein (Pelkmans *et al*., 2001). It will be interesting to examine the effects of these dominant–negative caveolins on the uptake of other members of the *Polyomavirinae*.

Macropinocytosis

Macropinocytosis is considered generally to be a non-specific mechanism for internalization, in that it is not reliant on ligand binding to a specific receptor. Instead, formation of endocytic vesicles occurs as a response to cell stimulation, resulting in the closure of lamellipodia at the sites of membrane ruffling to form the large, irregular vesicles known as macropinosomes. Membrane ruffling is primarily actin-driven and macropinocytosis is, in terms of mechanics, similar to the process of phagocytosis that occurs in specialized immune system cells such as neutrophils and macrophages. The finding that macropinosomes have the ability to become acidified and can intersect with endocytic vesicles (Hewlett *et al*., 1994), makes them possible routes of entry for a wide variety of viruses.

Because of its strict requirement for actin, the most commonly used inhibitors of macropinocytosis are the cytochalasins, especially cytochalasin D (Maniak, 2001). Macropinocytosis is also highly dependent on the activity of phosphatidylinositol (PI) 3-kinase (PI3K) and the activity of Rho family small GTPases, which regulate actin rearrangements. Inhibitors of PI kinases, such as wortmannin and LY294002 (Araki *et al*., 1996; West *et al*., 2000), and Rho GTPases, such as toxin B (Just *et al*., 1995), along with amiloride, an inhibitor of Na^+/H^+ exchange (West *et al*., 1989), have all been employed to inhibit macropinocytosis in cells. As with the use of drugs to study other endocytic pathways, their specificity for macropinosomes may be limited. In particular, PI3K activity clearly affects early endosome dynamics (Corvera, 2001). It is also becoming increasingly apparent that actin is crucial for clathrin-dependent endocytosis, especially in polarized epithelial cells (Apodaca, 2001) – the *in vivo* target of many viruses.

Several promising molecular approaches in targeting macropinocytosis specifically have emerged recently, which target ARF- and Rho-family GTPases. Overexpression of ARF6 locked in its GTP-bound form (Nichols & Lippincott-Schwartz, 2001), dominant–negative forms of the Rho family GTPases (West *et al*., 2000) and the autoinhibitory domain of the Rac-dependent kinase PAK1 (Dharmawardhane *et al*., 2000), all result in an inhibition of macropinocytosis. However, none of these approaches have been used to study the role of macropinocytosis in virus entry and their usefulness in specifically targeting macropinocytosis remains to be established. At present, traditional approaches based on the visualization of virus particles at sites of membrane ruffles, visible by light or electron microscopy, or within large irregular vesicles may still be the best approach to demonstrate a role for macropinocytosis in virus entry. Such experiments have been used recently to demonstrate macropinocytic uptake of human immunodeficiency virus type 1 into macrophages (Marechal *et al*., 2001). Whereas most of the virions internalized in this way were degraded, some could in fact infect the cell.

Although macropinocytosis per se was not implicated, expression of dominant–negative Rho family members, as well as cytochalasin D, reduced significantly the entry of the intracellular mature virus (IMV) form of vaccinia virus (VV) (Locker *et al*., 2000). In these studies, the other infectious form of VV (the extracellular enveloped virus or EEV) was not affected. Other investigators, however, found that both EEV and IMV were sensitive to cytochalsin D, with differences occurring in the exposure of the two virus forms to a low-pH compartment (Vanderplasschen *et al*., 1998). The very large size and different morphological forms of VV (with possibly spatially restricted entry into conventional coated pits and vesicles), combined with the complex signalling network elicited upon infection, raises special challenges for a study of virus entry.

The fact that macropinocytosis appears to require cell stimulation raises issues of how a virus might induce signalling, and subsequently macropinocytosis, upon binding to the cell surface. Such virus-mediated signalling has recently been shown for adenovirus, where integrin-dependent virus binding activates macropinocytosis. However, in this case, macropinocytosis in not used for virus uptake but for escape from the endosome (U. Greber, personal communication). Another factor to bear in mind is the apparent lack of a specific receptor for macropinocytosis – a problem perhaps unimportant for

viruses without a specific receptor like the sialic acid-binding influenza virus.

Finally, macropinocytosis is known to be a major route of entry into antigen-presenting cells like dendritic cells (DCs) and in this case it is constitutive in that it does not require cell stimulation (Garrett *et al*., 2000; West *et al*., 2000). As DCs are one of the first immune system cells to encounter an invading virus, they have special importance for virus pathogenesis. The virus could productively infect the DC or could internalize the virus and then 'regurgitate' it to infect other cells at a distant site. Such regurgitation could occur via a process involving the release of so-called exosomes, derived from specialized multilamellar endocytic vesicles present in DCs (Denzer *et al*., 2000; Kleijmeer *et al*., 2001). The ultimate importance of macropinocytosis in virus infection may therefore turn out to be in the presentation of the invading virus to the immune system and in the spread of virus through the host, rather than in its primary infection of epithelial surfaces.

Non-clathrin, non-caveolae-dependent endocytosis

Beyond the established roles of clathrin and caveolae, and of macropinocytosis in stimulated cells, there exists an ill-defined route of non-clathrin, non-caveolae-dependent endocytosis in resting cells (Bishop, 1997; Nichols & Lippincott-Schwartz, 2001). Although the entry of many viruses has been implicated to occur through these pathways, based mainly on the presence of viruses in non-coated vesicles by electron microscopy (for example, influenza and Sendai viruses) (Marsh & Helenius, 1989), the paucity of specific information or cellular markers for these pathways has not allowed unequivocal conclusions to be drawn.

Recent reports, however, have begun to clarify some of these non-clathrin, non-caveolae pathways. As with caveolae, it is likely that such pathways are intimately linked to the presence of detergent-resistant microdomains on the plasma membrane. The interleukin-2 (IL-2) receptor is perhaps the best-characterized marker for these pathways. Based on dominant–negative approaches in caveolin-negative T cells, IL-2 receptor uptake and delivery to late endosomes and lysosomes is clearly independent of clathrin, yet, like caveolae, requires dynamin and specialized membrane domains (Lamaze *et al*., 2001). Another pathway has been studied using GFP-tagged glycosyl PI-anchored proteins. This cholesterol-sensitive pathway delivers material directly to the Golgi, bypassing both clathrin and early endosome markers (Nichols *et al*., 2001).

Save for a report on picornaviruses that utilized physiological means of inhibiting clathrin (Madshus *et al*., 1987), little specific information is available on non-clathrin routes of virus entry. Potentially complicating factors include the likelihood that non-clathrin-dependent uptake of a particular ligand may well be occurring simultaneously with clathrin-mediated endocytosis and that multiple non-clathrin pathways exist (Dautry-Varsat, 2001). Overall, non-clathrin routes of entry may only be able to be examined once clathrin is inhibited specifically – a finding observed with uptake of certain lipid analogues (Puri *et al*., 2001) and should be carefully examined alongside known cellular markers. The availability of such markers, along with specific molecular targets for clathrin and caveolae (see above) now makes an examination of non-clathrin-dependent virus entry more meaningful.

Trafficking and sorting of endocytic compartments

Rab proteins and endocytosis

A major class of molecule that regulates membrane-traffic events is the Rab family of small GTPases (Collins & Brennwald, 2000). For endocytosis, four principal Rab proteins are involved – Rab5 (needed for formation of early endosomes), Rab7 (required for early to late endosome/lysosome traffic) and Rab4 and Rab11 (involved in the recycling pathway) (Somsel Rodman & Wandinger-Ness, 2000). Rab9 GTPase is also part of the endocytic network as it controls traffic between the endosome and the *trans*-Golgi network. For all of these Rabs, well-characterized point mutations exist which can be overexpressed as dominant–negative proteins to block a specific point in the endocytic pathway.

Such dominant–negative Rabs are becoming utilized in studies of virus entry. As with the use of many other molecular tools, the adenovirus system has led the field in this regard. Adenovirus uptake was increased by overexpression of wild-type Rab5 and decreased by dominant–negative Rab5 (Rauma *et al*., 1999). Rab proteins are also convenient markers for colocalization studies, as they are organelle-specific in their localization. For example, Miyazawa *et al*. (2001) used Rab7 to show the localization of adenovirus subgroup B (Ad7) to late endosomes. In contrast, these authors showed that the related (and more commonly studied) adenovirus subgroup C does not enter the late endosome but seems to penetrate from the early endosome. Thus, very similar viruses may show selective endocytic traffic, which could have profound implications for their pathogenesis. One unusual feature of Ad7 is that endosomal trafficking is very slow (Miyazawa *et al*., 2001). Other non-enveloped viruses, such as canine parvovirus, also show much delayed trafficking through endosomes (Parker & Parrish, 2000). Whether or not these viruses enter any kind of recycling compartment before finally penetrating into the cytoplasm remains to be seen.

Rho proteins and endocytosis

In addition to the established roles of the Rho family members, Rac, RhoA and Cdc42, in macropinocytosis, the Rho B and D proteins are important players in clathrin-mediated endocytosis (Ellis & Mellor, 2000). In contrast to the Rab proteins, which control membrane fusion and remodelling

Table 1. Toolbox of reagents targeting the endocytic pathway

Pathway	Inhibitor	Specificity	Reference
Clathrin	Anti-clathrin antibodies	+++	Doxsey *et al.* (1987)
	Low pH shock	+	Brodsky *et al.* (2001)
	Potassium depletion	++	Brodsky *et al.* (2001)
	Brefeldin A	+	Brodsky *et al.* (2001)
	Chlorpromazine	+	Wang *et al.* (1993)
	Dominant–negative dynamin mutant	+	DeTulleo & Kirchausen (1998)
	Dominant–negative Eps15 mutant	+++	Benmerah *et al.* (1999)
	Clathrin hub domain	+++	Brodsky *et al.* (2001)
	AP-2 μ2 subunit	+++	Nesterov *et al.* (1999)
Caveolae	Sterol-binding drugs	++	Neufield *et al.* (1996); Orlandi & Fishman (1998); Rothberg *et al.* (1992)
	Dominant–negative caveolin mutant	+++	Pelkmans *et al.* (2001); Roy *et al.* (1999)
Macropinocytosis	Cytochalasin D	++	Maniak (2001)
	PI3K inhibitors	+	Araki *et al.* (1996); West *et al.* (2000)
	Toxin B	++	Just *et al.* (1995)
	Amiloride	+	West *et al.* (1989)
	ARF6 GTPase mutant	?	Nichols & Lippincott-Schwartz (2001)
	Rho family GTPase mutants	?	West *et al.* (2000)
	PAK1 autoinhibitory domain	?	Dharmawardhane *et al.* (2000)
Endosomes	Rab GTPase mutants	+++	Somsel Rodman & Wandinger-Ness (2000)
	Rho family GTPase mutants	?	Ellis & Mellor (2000)
	PI3K inhibitors	?	Li *et al.* (1998b)
	PKC inhibitors	?	Nakano *et al.* (2000)

events, the endosomal Rho proteins apparently regulate the rate of vesicle traffic along cytoskeletal tracks (Murphy *et al.*, 1996). Rho D localizes to early/recycling endosomes (Murphy *et al.*, 1996), while Rho B and its associated kinase PRK-1 are likely to control entry into multivesicular bodies (Gampel *et al.*, 1999). As an example of the convergence of endocytic pathway regulatory molecules, the effects of dominant–negative Rac and Cdc42, as well as toxin B (which targets Rho GTPases) and cytochalasin D, have all been shown to inhibit adenovirus endocytosis (Li *et al.*, 1998a). On the one hand, this set of experimental conditions could suggest macropinocytosis as a route of entry for the virus, yet the effects of these reagents are attributed to PI3K-related effects on clathrin-mediated endocytosis (see below).

Kinase-mediated regulation of endocytosis

As befits a role as a major signalling event in the cell, virus endocytic trafficking can be regulated by phosphorylation. Attention has focussed on two kinase families, PI3K and protein kinase C (PKC). For adenovirus, the initial interaction of the virus with cell surface integrins activates PI3K, which in turn modulates virus endocytosis. Addition of the PI3K inhibitors wortmannin or LY294002 reduced adenovirus internalization to approximately 30% of control samples (Li *et al.*, 1998b). It has not been shown at which point in internalization adenovirus internalization is arrested by PI3K inhibition but the kinases are believed to act upstream of small GTPases in the signalling cascade (Kotani *et al.*, 1995). Similarly, endocytic trafficking of adeno-associated virus type 2 is dependent on integrin/Rac-dependent PI3K activation (Sanlioglu *et al.*, 2000).

For many years, PKC has also been implicated in the processes of virus entry. The entry of several enveloped viruses, including rhabdoviruses, alphaviruses, poxviruses and herpesviruses, have been proposed to require PKC based on the action of protein kinase inhibitors, such as H7 and staurosporine (Constantinescu *et al.*, 1991). More recently, it has been shown that the successful entry of adenovirus type 2 requires PKC. In the presence of calphostin C, an inhibitor of the classical and novel PKC isoforms, adenovirus is prevented from escaping endosomes and accumulates in cytoplasmic vesicles near the cell periphery (Nakano *et al.*, 2000). Influenza virus has the ability to activate PKC upon binding to host cell-

surface receptors (Kunzelmann *et al.*, 2000) and we have described previously that bisindolymaleimide I, a specific PKC inhibitor, prevented influenza virus entry and subsequent infection (Root *et al.*, 2000). A role for PKC in virus entry is reinforced by recent data from our laboratory using cells overexpressing a phosphorylation-deficient form of the PKC βII isotype. In these cells, influenza virus entry was arrested at the level of the late endosome (S. B. Sieczkarski, H. A. Brown and G. R. Whittaker, unpublished data), without any apparent defect in endosome acidification. It remains to be seen which isoforms of PKC are involved in the entry of different viruses and at which point in endocytic trafficking they act.

Perspectives

As research into the mechanisms of endocytosis progresses, the tools to study these processes are becoming increasingly precise. The development of molecular inhibitors in the form of dominant–negative molecules has surpassed the use of chemical inhibitors in terms of decreasing non-specific effects (Table 1). The use of molecular tools with such precise specificity to individual cellular functions allows the defined examination of endocytic pathways. Such techniques are invaluable in our study of virus infections. However, even with these advances, ambiguity and a reason for caution still exist. The plasticity of many of cellular pathways means that virus entry may be impacted by an indirect mechanism rather than by direct inhibition. Additionally, it is possible that viruses make use of multiple internalization pathways. The next advancements in the study of virus entry are the developments in real-time single molecule imaging of virus infections, examples of which may be seen in Seisenberger *et al*. (2001). Such experiments allow an extra level of sophistication; for instance, it is now possible to view, in real-time, the entry and subsequent trafficking of viruses into live cells expressing dominant–negative molecules and to monitor each component by double-label microscopy. Such techniques enable virus endocytosis and subcellular trafficking to be examined with exquisite clarity.

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