Insider information: what viruses tell us about endocytosis

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Viruses have long served as tools in molecular and cellular biology to study a variety of complex cellular processes. Currently, there is a revived interest in virus entry into animal cells because it is evident that incoming viruses make use of numerous endocytic pathways that are otherwise difficult to study. Besides the classical clathrin-mediated uptake route, viruses use caveolae-mediated endocytosis, lipid-raft-mediated endocytic pathways, and macropinocytosis. Some of these are subject to regulation, involve novel endocytic organelles, and some of them connect organelles that were previously not known to communicate by membrane traffic.

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Abbreviations
COPI coatomer complex I
GPI glycosylphosphatidylinositol
SV40 simian virus 40

Introduction

To multiply, viruses must deliver their genome and accessory proteins into host cells, and subsequently make use of the biosynthetic machinery of the host cell for replication. Because of their simplicity, they depend on assistance from the host organism in virtually all stages of the infection cycle. During millions of years of co-evolution with their hosts, they have acquired the relevant molecular 'passwords' and 'entrance tickets' to be able to exploit and control cellular functions. Therefore, by analysing virus–cell interactions, one can learn not only about the virus, but also about the cell.

In this review, we focus on what viruses are telling us about the different endocytic processes that they exploit to enter animal cells. The goal of a virus is to deliver its genome and accessory proteins into the cytosol or the nucleus, where the genome can be uncoated and replicated. While some viruses cross the plasma membrane directly, most depend on endocytosis. They deliver their genomes into the cytosol by penetration reactions that take place in endosomes or other intracellular organelles such as the *trans*-Golgi network (TGN) or endoplasmic reticulum (ER) [1–3]. As the current picture of endocytic trafficking is becoming increasingly complex, viruses are re-emerging as useful guides in the maze of pathways and organelles.

How viruses enter: the general picture

The journey of a virus particle from the cell surface to the cytosol and nucleus consists of a series of consecutive steps that move it closer to its site of replication [1,4–8]. At the same time, it receives cellular cues that drive it through a programme of disassembly and penetration. Many viruses are, moreover, capable of activating cellular signal transduction pathways after binding to the plasma membrane [9,10,11•]. This prepares the cell for the invasion, and, as discussed below, is in some cases essential for endocytosis of the virus.

One of the most crucial steps in the itinerary is the triggered penetration that allows passage of the viral genome, usually in the form of a capsid, through a cellular membrane into the cytosol. While enveloped viruses all penetrate by fusion of the viral membrane with the cellular membrane, non-enveloped viruses lyse the limiting membrane, or generate a pore through which the genome can enter the cytosol.

In Table 1, we have categorised animal virus families and individual viruses according to the current understanding of their entry mechanisms: what type of endocytosis they use; in which intracellular organelle the virus penetrates the membrane; and whether penetration is acid-activated or pH-independent. It should be noted that for many of the viruses included in the table, information about entry mechanisms is still incomplete and should therefore be viewed with some caution. The low pH in early and late endosomes is the most common cellular 'cue' that viruses use to enter into their penetration 'mode', and it is usually linked to clathrin-mediated endocytosis. However, as indicated in Table 1, there are numerous viruses that have acid-independent entry mechanisms. While some do not need endocytosis for entry, most of them are likely to use one or more of the various pathways of endocytosis now recognised in mammalian cells.

Different endocytosis pathways

There are several endocytic pathways a virus can choose (Figure 1); but to categorise them unambiguously is not so

Table 1

Means of cell entry for mammalian viruses.

Virus family	Virus example	Site of entry/ penetration	References
Dependent on low pH for penetration			
Alpha	Semliki forest virus	CCP/early endosome	[20,58]
	Sindbis virus	CCP/early endosome	[59]
Flavi	Tick-borne encephalitis virus	Early endosome	[60]
Orthomyxo	Influenza A	CCP and non-CCP/late endosome	[61–63]
Rhabdo	Vesicular stomatitis virus	CCP/early endosome	[61,64]
Bunya	La Crosse virus	Endosome	[65]
	Hantaan virus	Endosome	[66]
Adeno	Adenovirus5	CCP/endosome	[67]
	Adenovirus2	CCP/endosome	[17••,68]
Filo	Ebolavirus	Caveolae/endosome	[69]
Irido	African swine fever virus	Endosome	[70]
Rubella	Rubella virus	Endosome	[71]
Parvo	Minute virus of mice	Endosome	[72]
Not dependent on low pH for penetration			
Paramyxo	Measles virus	Plasma membrane	[73]
Herpes	Herpes simplex virus I	Plasma membrane	[74]
Papova (some)	SV40	Caveolae/ER	[36••]
	Polyomavirus	Caveolae/?	[45]
Papilloma	Bovine papillomavirus	plasma membrane	[75]
Pox	Vaccinia virus	Plasma membrane/macropinosome?	[53]
Rota	Human rotavirus	Plasma membrane/lipid rafts?	[76]
Reovirus	Infectious subviral particles	Endosome	[77]
Hepatitis B	Duck hepatitis B virus	Early endosome	[78]
Retro (most)	HIV-1	Plasma membrane	[79]
Picorna (most)	Human rhinovirus 14	Dynamin-independent endocytosis/?	[80]
	Poliovirus	Dynamin-independent endocytosis/?	[6]
	Echovirus I	Caveolae/?	[43]
Corona (some)	Murine hepatitis virus 4	?/Endosomes	[81]

CCP, clathrin-coated pit.

straightforward. However, using dependence on clathrin, dynamin, caveolin-1 and lipid rafts as criteria, we come up with five types of relevant processes for which there is some evidence [12•,13–15]. Pathway A corresponds to the classical clathrin-coated pit pathway, and pathway B to the caveolar pathway (discussed in detail below). Pathway C represents a lipid-raft-mediated pathway present in cells that are devoid of caveolin, and possibly elsewhere, whereas pathways D and E represent still poorly characterised dynamin-independent pathways that are either lipid-raft-mediated or are independent of lipid rafts, respectively. The best-characterised pathway in one of these latter two categories is macropinocytosis, which is dynamin-independent [16,17••].

Although it is known that the clathrin-mediated pathway leads to early and late endosomes, lysosomes and the TGN, the downstream organelles in the other pathways are less well understood. It has been recognised recently that caveolar endocytosis links up with a new organelle, the caveosome, from which traffic can proceed to the ER. The other pathways listed might also transport to endosomes or caveosomes, but it is equally possible that they involve additional, still unrecognised endocytic organelles.

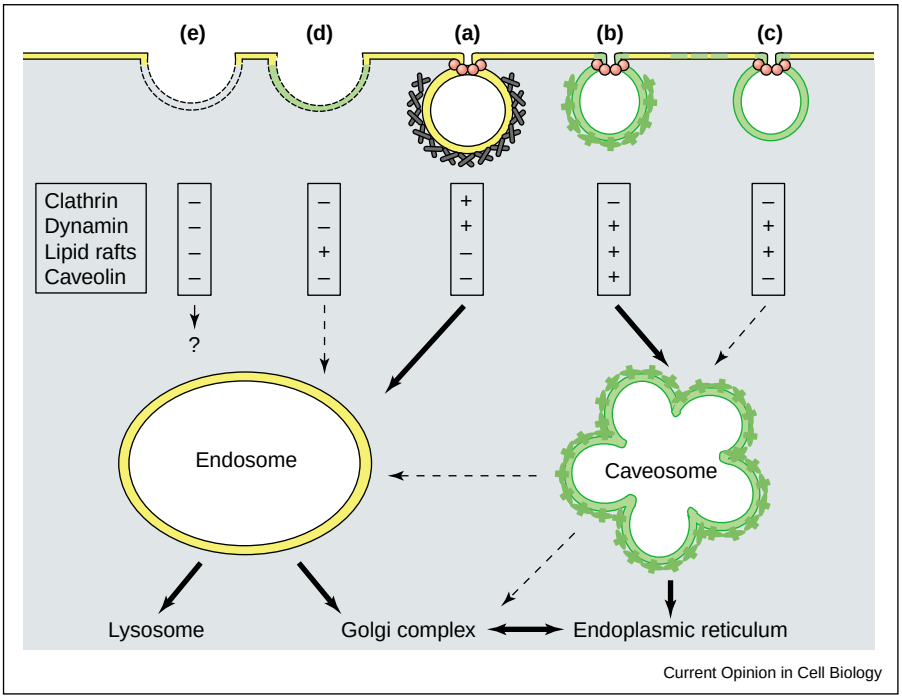
Why endocytosis?

There are several reasons why endocytic entry is advantageous for viruses: first, the particles are directed only into cells that have active membrane transport and not, for example, into erythrocytes, a dead end. Second, a particle can bind anywhere on the cell surface and rely on the endocytic processes to ferry it not only into the cell but also carry it past the cortical actin filaments and other cytoplasmic barriers to the perinuclear region [18]. In this way, a virus avoids having to diffuse through the cytoplasm by itself. Third, penetration from cytoplasmic organelles decreases the risk for immunodetection because no viral proteins remain exposed on the plasma membrane. Fourth, in the endocytic organelles, local cues such as low pH help the virus undergo its penetration programme. Not surprisingly, many protein toxins also make use of endocytic cell entry pathways for the same reasons [19].

Clathrin-mediated endocytosis

Semliki Forest virus was the first virus shown to use clathrin-mediated endocytosis for infectious entry [20], and its stepwise endocytic entry pathway is now well characterised (Figure 2). Many different viruses, both enveloped and non-enveloped, have been shown to use a clathrin mediated pathway with penetration in early or

Figure 1



Different endocytosis routes potentially used by viruses for infection. The spectrum of different endocytic pathways described in the literature depends on the cell type and the ligands followed, and it is not entirely clear how they relate to each other. In the classification above, we have chosen four criteria: the dependence on clathrin, caveolae, dynamin and lipid rafts. The best-characterised are **(a)** the clathrin-mediated pathway and **(b)** the caveolar pathway. The lipid-raft-mediated uptake pathways can be dynamin-dependent **(c)**, or dynamin-independent **(d)**. In addition, there are pathways that do not seem to depend on any of these factors **(e)**. The intracellular routing for each of the pathways is also shown, but for all except the clathrin-mediated pathway they are poorly defined (indicated by thin arrows).

late endosomes. Because it is the most common pathway for virus entry [1] (Table 1), viruses have often served as tools to study this classical pathway [21–24].

Caveolae-mediated endocytosis

Simian virus 40 (SV40), a simple non-enveloped DNA virus that replicates in the nucleus, was the first virus shown to enter via caveolae (Figure 2). Early electron microscopy studies showed the virus in narrow (50–70 nm), uncoated invaginations that gave rise to small tight-fitting, 'monopinocytotic' vesicles containing a single virus particle [25,26]. The invaginations were later shown to be caveolae [27,28]. (For recent reviews on caveolae, see [29–31].)

In contrast to clathrin-mediated endocytosis, the internalisation of caveolae is a triggered event [11•,32]. Triggering can occur by clustering of lipid raft components such as glycosylphosphatidylinositol (GPI)-anchored proteins and MHC class I molecules on the plasma membrane. The clusters are sequestered into caveolae and a signal transduction cascade is initiated [11•,28,33–35].

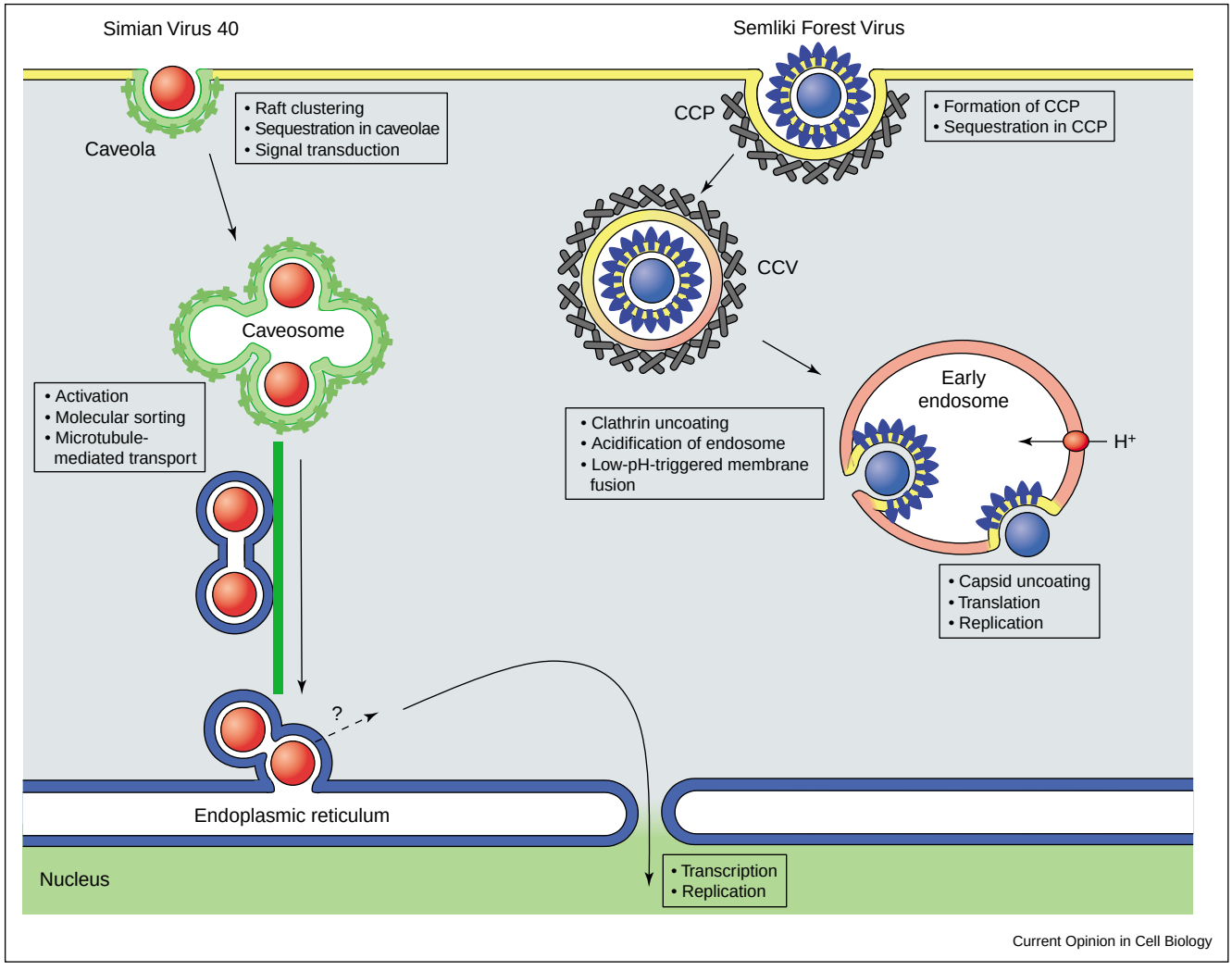
After binding to the cell surface via MHC class I molecules, SV40 moves laterally along the plasma membrane until trapped in caveolae [34,36••]. Furthermore, SV40 binding seems to recruit caveolin-1-containing vesicles to the membrane (L Pelkmans and A Helenius, unpublished data; Figure 3), indicating that in addition to the stable, stationary caveolae present on the cell surface at steady state, a second, more dynamic population is recruited upon ligand-induced triggering.

In caveolae, SV40 activates a signal that induces local tyrosine phosphorylation, depolymerisation of the cortical actin cytoskeleton, and local production of phosphatidylinositol 4,5-bisphosphate. Subsequently, both actin and dynamin 2 are recruited to the virus-loaded caveolae [11•] (L Pelkmans and A Helenius, unpublished data). Actin forms dynamic tail-like structures radiating from virus-loaded caveolae, whereas dynamin 2 transiently associates with the caveolae for about 8 s before dissociating again. These events lead to relatively slow but efficient invagination, internalisation and release of the caveolae as small, virus-containing vesicles into the cytosol.

Caveosomes

After internalisation, the caveolar vesicles deliver the viruses to membrane-bound cytoplasmic organelles, the caveosomes [36••]. These are pre-existing organelles with

Figure 2

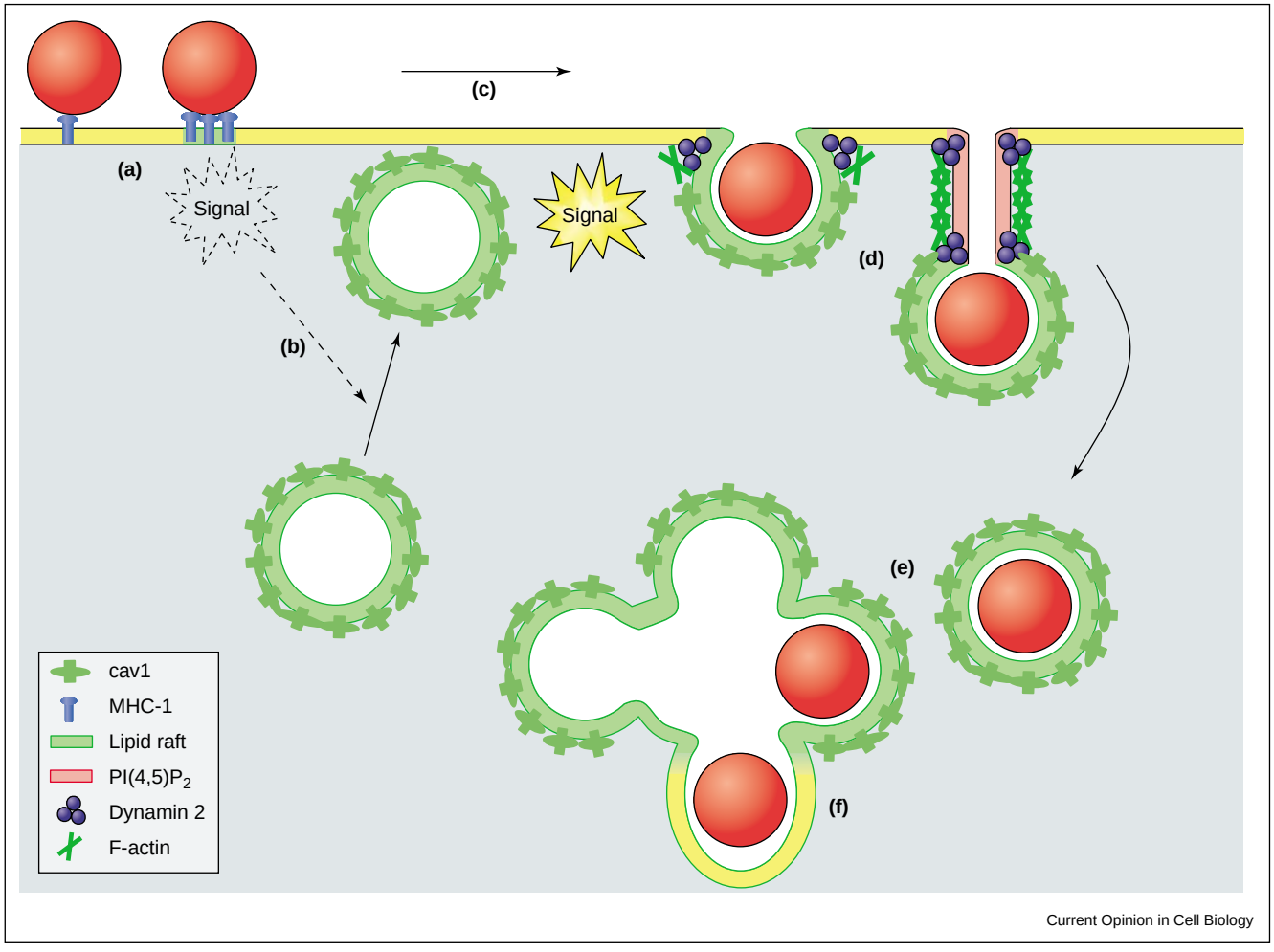


The entry pathways of Semliki forest virus and SV40. Entry of Semliki forest virus occurs in five steps. Multivalent binding to cell-surface molecules (i.e. virus receptors), which may involve consecutive or simultaneous interactions with several different receptor species. Lateral diffusion in the plane of the membrane and sequestration into clathrin-coated pits (CCP). Internalisation by receptor-mediated endocytosis in clathrin-coated vesicles (CCV) and delivery to early endosomes. Low pH triggers penetration through the endosomal membrane by viral spike glycoprotein-mediated membrane fusion, a process that serves to transfer the capsid to the cytosol and to remove the viral membrane. Uncoating of the viral plus-stranded RNA, translation, and replication in the cytosol. Entry of SV40 involves uptake via caveolae (see main text and Figure 3), traffic to caveosomes, sorting from caveosomes and transport to the smooth ER. In the ER, the virus penetrates the membrane in an unknown manner and travels through the cytosol to nuclear pores via which it enters the nucleus.

a neutral pH and multiple flask-shaped caveolar domains enriched in caveolin-1. They are devoid of markers of the classical endocytic and biosynthetic organelles, including the known endocytic Rab GTPases 4, 5, 7, 9 and 11. They are stable, stationary, cholesterol-enriched structures that, under normal conditions, exchange caveolin-1 with the plasma membrane only slowly [37].

Fluorescence recovery after photobleaching (FRAP) experiments have confirmed that caveolin-1 is remarkably static in caveosomes. When the caveolin-1–GFP (green fluorescent protein) signal in one-half of a caveosome is bleached, the caveolin-1–GFP in the unbleached half fails to diffuse into the bleached area (L Pelkmans, A Mezzacasa and A Helenius, unpublished data). Together with other results, these observations have led us to speculate that caveolae and caveolar vesicles might travel between compartments as stable entities and do not exchange caveolins with each other — unlike clathrin-coated vesicles that are assembled and disassembled for each use. The characteristic protein scaffold in caveolae is thought to consist of fibres of caveolin-1 heptamers [38], which most likely defines the size of the static membrane units.

Figure 3



Early events in caveolae-mediated endocytosis of SV40 (red). **(a)** After multivalent binding to receptors, including MHC I, SV40 partitions into lipid rafts, where it might induce a signal. **(b)** This putative signal recruits new caveolin-1-positive vesicles to the membrane, into which SV40 is sequestered. **(c)** From caveolae, a tyrosine kinase signal is activated that leads to cortical actin depolymerisation and the recruitment of an actin patch. **(d)** Local production of phosphatidylinositol 4,5-bisphosphate (PI[4,5]P_2), the formation of a small actin tail and the recruitment of dynamin 2 lead to internalisation of the caveola. **(e)** Inside the cytosol, caveolar vesicles fuse with pre-existing caveosomes. **(f)** By an unknown process, SV40 is sorted into tubules devoid of caveolin-1, which travel to the smooth ER.

The arrival of SV40 particles from the surface induces a change in the behaviour of caveosomes. Live recordings show that after a few hours, caveosomes become more dynamic and begin to release virus-filled membrane tubules devoid of caveolin-1. These carriers travel along microtubules to the smooth ER [36••]. The separation of viruses from caveolin-1 in caveosomes, and the formation of transport vesicles is microtubule-dependent, and induced by the incoming virus particles [36••]. This second transport step, from caveosomes to the ER, can be inhibited by nocodazole, brefeldin A, and dominant-negative mutants of Arf1 and Sar1 (small GTPases involved in retrograde and anterograde membrane transport in the early secretory pathway, respectively) [36••,39•]. Together with the reported presence of coatomer complex I (COPI) on caveosomes [3], this suggests that transport from caveosomes to the smooth ER is COPI-mediated.

Arrival in the smooth ER is a prerequisite for SV40 infection [36••]. Partial disassembly of the particle occurs in this compartment [3], and it is likely that membrane penetration into the cytosol occurs from the ER [40]. SV40 belongs to those viruses that are not activated by low pH (Table 1). What triggers penetration is not yet known.

Traffic between caveosomes and endosomes

That SV40 bypasses the classical endocytic organelles does not mean that caveosomes do not communicate with

endosomes. In fact, endosomes contain some caveolin-1 [41,42], and our video recordings show that caveolin-1-containing vesicles can associate with endosomes in a kiss-and-run fashion. Overexpression of dominant-active mutants of Rab5 leads to entrapment of these vesicles, and the caveosome pool is consumed into enlarged endosomes (L Pelkmans, T Bürli and A Helenius, unpublished data). Under these conditions, SV40 is diverted from its normal itinerary: it gets trapped in endosomes, and fails to infect the cell.

The membrane traffic connection between caveosomes and endosomes demonstrated by these observations may play a role in the recycling of caveolae to the plasma membrane or in the sorting and trafficking of lipid-raft components to and from endosomes. A connection between caveolae and endosomes might be important for viruses that are internalised through caveolae but that need a low-pH environment for membrane penetration (see below).

Other viruses entering via caveolae

Echovirus 1 has also been reported to enter via caveolae (Table 1) [43]. It binds to certain integrins known for their ability to activate a variety of downstream signals [44]. Mouse polyomavirus, closely related to SV40, was originally shown to enter via similar uncoated plasma membrane invaginations and 'monopinocytotic' vesicles, like SV40 [26]. More recent literature indicates that the virus might use two distinct entry pathways: a caveolar pathway similar to SV40 and a lipid-raft- and dynamin-independent uptake mechanism. First, polyomavirus-like particles were seen in caveolin-1-decorated invaginations and intracellular vesicles, and the virus-induced changes in the actin cytoskeleton seemed similar to those in early stages of SV40 infection [45]. Interestingly, brefeldin A, an inhibitor of Arf1 guanine nucleotide exchange factor and thus COPI-mediated traffic, did not block infection, as is the case for SV40 [46]. This work indicates that the virus enters via caveolae but departs from the SV40 pathway at later stages. Indeed, it seems to travel to recycling endosomes, perhaps using the link between caveosomes and early endosomes [46]. The second pathway reported for polyomavirus deviates even more from the SV40 pathway. According to observations by Gilbert *et al.* [47,48], the virus utilises a clathrin-, caveolae-, lipid raft-, and dynamin-independent uptake mechanism (pathway E in Figure 1). The mechanism is also not related to macropinocytosis because it does not depend on a functional actin cytoskeleton. It will be interesting to find out if this virus is indeed capable of using such diverse entry mechanisms.

Lipid-raft-mediated endocytosis

Lipid-raft-dependent (pathways C and D in Figure 1) but caveolae-independent internalisation pathways have been mainly studied in cells that do not express caveolin-1 and thus do not display caveolae on their surface [49••]. It is becoming increasingly apparent that these pathways, too, can support entry of some viruses. First, the entry pathway used by SV40 into cells devoid of caveolin-1 has characteristics similar to those in cells that express caveolin-1, and thus similar to pathway B (E Damm, L Pelkmans, T Kurzchalia and A Helenius, unpublished data). Second, it is likely that some picornaviruses, papillomaviruses, filoviruses and retroviruses use such mechanisms for infectious entry (Table 1). Echovirus 11, for instance, clusters the GPI-anchored protein decay accelerating factor (DAF), leading to lipid-raft-dependent internalisation [50].

Lipid-raft-mediated endocytosis appears to be dependent on raft clustering. That such clustering can indeed dictate the mode of internalisation has recently been directly studied for the retrovirus avian sarcoma and leukosis virus (ASLV) [51•]: Binding to a transmembrane form of the receptor results in rapid internalisation via clathrin-coated pits, but binding to a GPI-anchored form of the receptor leads to slow, lipid-raft-mediated internalisation. Both routes, however, direct the virus to endosomes, where membrane penetration occurs. Whether the slower lipid-raft-mediated route first passes through caveosomes is not known.

Macropinocytosis

Macropinocytosis is a triggered process used by cells to internalise large amounts of fluid and membrane [52]. Large vacuoles are formed by closure of plasma membrane ruffles. The process is dependent on actin polymerisation but does not need dynamin. Because it is a rather non-specific process, not many viruses are known to use macropinocytosis for infectious entry. It seems, however, to be used by vaccinia virus, which is too large to enter clathrin-coated pits [53]. HIV1, which normally enters cells by direct fusion with the plasma membrane, can also fuse with the membrane of macropinosomes in macrophages, leading to infection [54]. This is not a very efficient pathway, however, since most HIV1 particles internalised by macropinocytosis end up being degraded.

Adenovirus 2 stimulates macropinocytosis not for uptake but to enhance its acid-activated penetration from endosomes [17••]. Activation of macropinocytosis occurs when the virus binds to a co-receptor, α_V integrin, that transduces a signal to the cell via phosphatidylinositol 3-kinase, protein kinase C and the Rho GTPase Rac1. The result is increased actin polymerisation and formation of ruffles and macropinosomes. How macropinocytosis contributes to the lytic penetration of the virus particles internalised by clathrin-coated vesicles is not clear, but perhaps macropinosomes and endosomes form joint vacuoles that are more easily lysed.

Conclusions and perspectives

It is now clear that besides the classical clathrin-mediated pathway, viruses can utilise caveolae- and lipid-raft-mediated endocytosis pathways, macropinocytosis, and

presumably other endocytic processes for infectious entry and post-entry events. Uptake is often activated by signals generated when the incoming viruses bind to cell-surface receptors. In some cases, further signalling is induced from intracellular compartments. Since the endocytic pathways are complex and tightly regulated, following the fate of viruses and analysing how they manipulate membrane traffic during entry provides an attractive experimental approach. Studying early virus–cell interactions will provide insights not only into the infectious cycle and thus into potential antiviral strategies, but also into unsolved secrets of the mammalian cell.

In addition to the extensive 'insider information' that viruses can reveal to us about cells, they have other advantages as a research tool. Viruses are easily manipulated genetically and biochemically. They are easily recognised by electron microscopy without labelling, and when coupled with fluorescent probes, they can be individually tracked as single particles using video microscopy in live cells [36••,55,56]. Non-hazardous virus-like particles that faithfully mimic the entry of infectious viruses can often be produced recombinantly [57]. Moreover, infection is an easily measured function that can be exploited in a screen for high-throughput functional genomics, a new challenge for cell biologists in the post-genomic era.

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