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Review

Secrets of caveolae- and lipid raft-mediated endocytosis revealed by mammalian viruses

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Abstract

In recent years, it has been unambiguously shown that caveolae and lipid rafts can internalize cargo upon stimulation by multivalent ligands, demonstrated by the infectious entry routes of certain non-enveloped viruses that bind integrins or glycosphingolipids. We currently understand little about the membrane trafficking principles of this endocytic route, but it is clear that we cannot use paradigms from classical membrane traffic. Recent evidence indicates that caveolae- and lipid raft-mediated endocytosis plays important roles in cell adhesion and anchorage-dependent cell growth, but the underlying mechanisms are not known. In this review, I will introduce new models based on current research that aims at identifying the core machinery, regulatory components and design principles of this endocytic route in order to understand its role in cellular physiology. Again, viruses are proving to be excellent tools to reach that goal.

Keywords: Caveolae; Caveosome; Simian Virus 40; Polyoma Virus; Echovirus 1; Endocytosis; Lipid raft; Integrin; Ganglioside; Membrane trafficking

1. Introduction

The plasma membrane is the cell's interface with the environment. It is therefore one of the most complex organelles of a mammalian cell with a high capacity to adapt to specific needs. It is the site at which most extracellular information is received and at which most intracellular information is sent out. To relay and process this information correctly, the plasma membrane is capable of compartmentalizing itself in various ways. A well-known mechanism for plasma membrane compartmentalization involves the internalization of parts of the plasma membrane, known as endocytosis [1], and their distribution to different sub-cellular locations. In fact, all organelles and membrane trafficking routes connected with the plasma membrane must together be seen as one complex system that allows the cell to transport and process material and information in order to respond to a given environment.

Mammalian viruses are superb navigators through this maze of membrane trafficking routes. Their dependence on reaching the intracellular host cell replication and translation machineries, and therefore to overcome the cell surface barriers, forced them to co-evolve with their hosts as they increased in complexity. As a result, viruses have adopted many different strategies to infect a host cell, including hijacking the different endocytic routes [2]. Up to the point of membrane penetration, most mammalian viruses do not use their own machineries (like enzymes incorporated in the envelope or capsid). They are thus completely dependent on cellular machineries and know how to activate the right pathways and mechanisms. By studying how a virus exploits a cell, we can thus learn much about the cell itself [3].

In this review, I will mainly focus on what mammalian viruses, particularly Simian Virus 40 (SV40), Polyoma Virus (Py) and Echovirus 1 (EV1), are teaching us about a poorly understood endocytic pathway, which involves the triggered internalization of caveolae and lipid rafts (see several reviews in this BBA issue for detailed descriptions of these structures). These specialized plasma membrane domains have been the subject of intense scientific scrutiny since the identification of caveolins and the development of biochemical techniques to enrich for them, and many ideas and models have been discussed [4–8]. It is now widely accepted that caveolae and lipid rafts have internalizing

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capacity activated by certain ligands, such as viruses [9]. Alternative views that regard caveolae as permanently static structures [7,10] have been extensively considered and rejected. I will not discuss other endocytic mechanisms that can internalize lipid raft components, such as clathrin-mediated endocytosis [11] and forms of (macro)pinocytosis [12], but I will discuss interconnections between endocytic pathways inside the cell. At the end, I will zoom out and discuss ways to dissect how endocytic pathways are coordinated and integrated into cellular physiology, also by use of viruses.

2. Defining endocytosis by caveolae and lipid rafts

The term 'lipid raft' [13] is a rather general definition for an assembly of specific lipids, usually glycosphingolipids and cholesterol, into a more ordered domain within the membrane bilayer (see reviews in this BBA issue for more details). This is proposed to occur independent of proteins, and relies on partitioning coefficients of particular lipid species. By no means does the term lipid raft itself specify a particular endocytic route. Multiple endocytic mechanisms can internalize lipid raft components or molecules that preferentially partition into rafts. Among these various mechanisms are, however, two particular types of endocytosis, which are highly dependent on large amounts of raft lipids. These have much in common, and are hijacked by the viruses mentioned before [14–16]. One is called caveolae-mediated endocytosis [9], and the other has no good name, but concerns a pathway that is regarded as not caveolae-mediated, since the initial plasma membrane invaginations involved do not contain Caveolin-1, -2 or -3 (Cav1, 2, 3). In this review, I will, however, use the term lipid raft-mediated endocytosis to particularly refer to the latter pathway. It can be argued (see below) that they in fact represent variations of a common endocytic route, and I will refer to this as caveolae/raft-mediated endocytosis [17]. The caveolar coat, of which Cav1 is just one component, has specific effects on the membranes of this pathway, but in the absence of Cav1 the route itself does not disappear [18]. Such a view is different from that on the role of classical coats in membrane traffic (Clathrin, COPI, COPII), and, as will be discussed, sorting by caveolar coats may indeed follow fundamentally different principles. Thus, the currently accepted idea that in the absence of Cav1 a complete organelle has been eliminated [19] is questionable, given that many of the morphological, biochemical and functional characteristics are still in place.

3. Raft clustering and caveolae assembly

The first events in caveolae/raft-mediated endocytosis, and a topic of much current debate, are the clustering of lipid rafts and the formation and/or sequestration into caveolae. This is induced by virus particles after binding to the cell. A common property, and most likely a requirement, is that viruses hijacking caveolae/raft-mediated endocytosis are able to cluster components of lipid rafts or molecules with affinity for them, such as certain integrin combinations ($\alpha_2\beta_1$ in the case of EV1) [16] or glycosphingolipids (GM1 or GD1a in the case of SV40 or Py) [20,21]. That raft clustering is required for sequestration into caveolae is also indicated by several antibody- or protein A-gold-mediated cross-linking experiments of lipid raft-associated molecules [22–24]. Three different models can be proposed leading to the capture of raft-clustering virus particles in a membrane invagination.

In the simplest model (Fig. 1a), clustering of lipid rafts increases the local concentration of cholesterol, which can, at least in model membranes, lead to spontaneous curvature of the membrane [25]. It is, however, unclear how this, in cell membranes, would result in the specific inward curvature without the aid of additional factors. In the case of virus particles, the particle shape may aid this process, suggested by electron microscopy images that show a tight fit of the membrane around the particle [26]. Such 'custom-made invaginations' would form independently of cellular proteins, and may occur during the internalization of SV40 particles in cells not expressing Cav1, or even in parallel to virus particle sequestration into caveolae in cells expressing Cav1. However, this may be rather regarded as a favourable energetic state of the lipid bilayer, on which a variety of cellular proteins exert their modulating activities.

An extended, more developed version of the model involves the concomitant clustering of proteins associated with the cytosolic leaflet of lipid rafts (Fig. 1b). These could scaffold the membrane, and, by oligomerization, induce membrane curvature. Cav1 could have such a role [27,28]. It is an integral, non-spanning membrane protein (both N- and C-terminus are in the cytosol) [29], which is co-translationally inserted from the cytosol into the membrane of the endoplasmic reticulum (ER) after which it rapidly forms oligomers (perhaps 7 or 14 mers) and is transported to the cell surface [29]. It binds cholesterol in a 1:1 ratio [30] and can be cross-linked to exogenously added glycosphingolipid GM1 with a photo-reactive group at the end of one of its fatty acid tails [31]. This indicates that Cav1 directly interacts with certain gangliosides within the membrane, which may (GM1 is in the extra-cellular leaflet) contribute to the coupling and stabilization of the membrane leaflets. During virus-induced clustering of gangliosides (in the case of SV40 and Py), the Cav1 oligomers would concomitantly cluster on the cytosolic side, allowing them to form a larger polymer that forms the framework of a membrane scaffold. Cholesterol, which preferentially packs with gangliosides compared to unsaturated glycerophospholipids, and strongly binds to Cav1

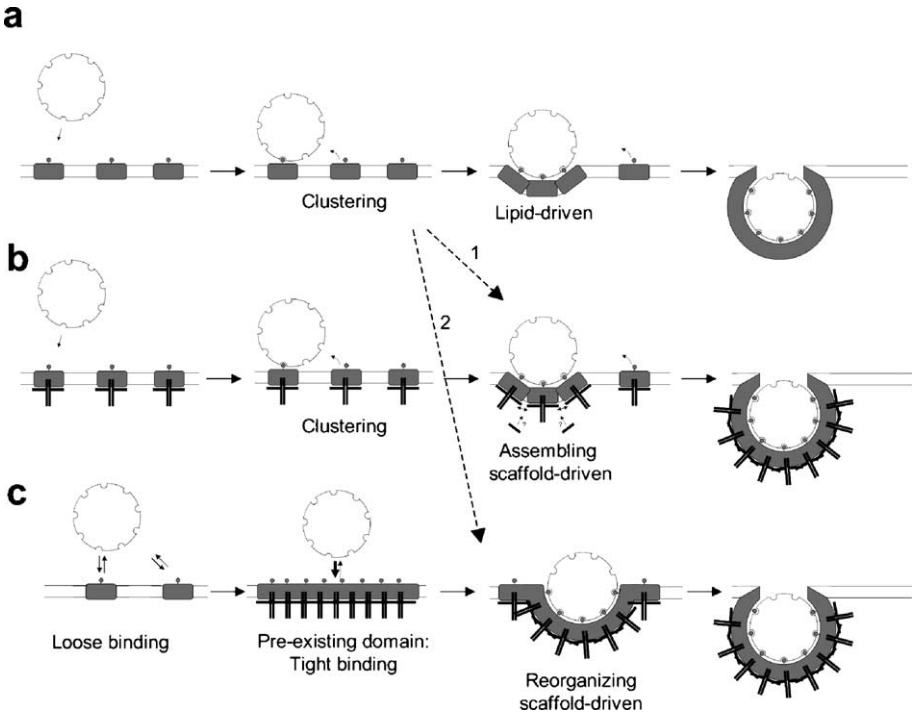

Fig. 1. Ways of virus particle sequestration into caveolae or invaginated lipid rafts. (a) The multivalent binding of virus particles to lipid raft components causes clustering of lipid rafts. This leads to the formation of a lipid domain with a tendency to bend. The binding of an increasing number of lipid raft molecules to the virus particle may contribute to the invagination process. (b) The virus-induced clustering of lipid rafts results in an increased concentration of membrane-integrated scaffolding molecules which then oligomerize on the cytosolic side. The geometrical structure of the scaffold may bend the membrane. The scaffold molecules could also be recruited after initial clustering (dashed arrow 1). (c) Virus particles bind to and dissociate from diluted lipid raft components. Upon encountering a pre-existing, scaffolded domain with a high concentration of binding sites the virus particles binds to many components simultaneously and becomes trapped in the domain. Alternatively, virus particles bound to clustered rafts have an increased affinity for a pre-existing scaffolded domain (dashed arrow). The entrapment starts the invagination process, which is allowed by the flexible scaffold. Virus particle binding may activate changes in the scaffold that promote a curved configuration.

may facilitate and stabilize this event. Also integrins, which bind Cav1 via their cytosolic and perhaps transmembrane domain [32,33], could bring multiple Cav1 oligomers together when clustered by virus particles (in the case of EV1). The caveolar coat formed in this way could induce curvature in the membrane and a 'custom-made caveola' is formed. This model is most analogous to classical coats, since it would implicate that Cav1 oligomers function as subunits that are able to assemble (being clustered in the membrane) and disassemble (being released into the membrane) during the formation, transport and fusion of a caveolar vesicle from a donor to an acceptor compartment where the multivalent ligand is released. Some early electron microscopy images have suggested this mechanism for the internalization of SV40 particles [34], but there is currently no other experimental evidence that supports this model.

Increasing evidence suggests a third model. Photobleaching experiments [2,35], detailed analysis of caveolar vesicle fusion with the plasma membrane [36], and experiments monitoring the intermixing of two pools of Cav1 in a heterokaryon (A. Tagawa et al., submitted), indicate that the Cav1 polymer, which consists of 144±39 molecules (or 10±2 14 mers) of Cav1 [36], stays intact once formed at the Golgi complex. Caveolae apparently do not frequently form de novo on the cell surface, but result from the fusion of a previously assembled caveolar vesicle with the cell surface whereby its scaffold remains intact. Because virus particles initially bind anywhere on the surface [37], they must therefore become subsequently trapped in these pre-existing Cav1 domains. One possibility is that as virus particles bind an increasing number of sphingolipids and/or integrins, their affinity for caveolar domains increases and upon encountering one they become trapped. In this case, the caveolar scaffold must be able to exchange material to allow entry of and provide space for the cross-linked lipids and/or integrins. Alternatively, virus particles could transiently bind and release gangliosides (whose individual binding sites for gangliosides have only millimolar affinity) distributed on the surface and in this way move about the membrane. Only when the particle encounters a region where it is able to bind multiple gangliosides simultaneously, for instance upon encountering a caveolar domain, does it permanently bind and become trapped [37,38]. In both cases (Fig. 1c), the caveolar coat must be quite flexible to be able to sequester a spherical virus particle of 50 nm in diameter. The invagination must be able to change its shape, and perhaps even become flat. Electron microscopy images of caveolar coats do indeed suggest that they are flexible [39].

4. Activation of internalization

Regardless of which of the above-mentioned mechanisms is used, treating cells with genistein, a tyrosine kinase inhibitor, in all cases rapidly blocks the actual internalization of the invagination [38]. This implicates that, in vivo, the internalization is not completely lipid- and virus particle-driven, but involves proteins regulated by phosphorylation of tyrosines. Cav1 was originally discovered as a major substrate of a viral tyrosine kinase (v-Src) expressed by Rous Sarcoma Virus [28,40], and it was expected that the cellular homologue c-Src, is able to phosphorylate Cav1. Indeed, Src phosphorylates Cav1 on tyrosine 14 [41], and this has been indirectly linked to the endocytic activity of caveolae [42]. Loading the plasma membrane with exogenously added glycosphingolipids was shown to activate c-Src resulting in Cav1 phosphorylation and increased dynamics of caveolae [43]. This suggests that virus particles could stimulate c-Src at the site of glycosphingolipid cross-linking, by locally increasing the concentration of GM1. Consistently, c-Src is required for SV40 internalization and infection [36]. The dynamics of caveolae are, however, complex and multiple kinases and possibly other molecules play roles at different steps of the caveolar cycle.

5. The caveolar cycle and its regulation

Recent detailed total internal reflection microscopy and computational analysis of caveolar structures on the cell surface and the silencing phenotypes of kinases required for SV40 infection has revealed new concepts in caveolar trafficking, which are schematically depicted in Fig. 2. Caveolae are in equilibrium between individual and aggregated, multi-caveolar assembly states [36]. The former are dynamic, undergoing short-range cycles of fusion and internalization (kiss-and-run) just below the cell surface, while the latter are static and connected to the extra-cellular space. RNAi-mediated ablation of *SRC* and *MGC26597*, a putative phosphatidyl inositol 4-phosphate 5 kinase (not required for clathrin-mediated endocytosis), as well as the expression of a GTPase-deficient mutant of dynamin2 (dynamin2K44A), which also reduces SV40 internalization and infection [38], all result in the aberrant formation of large, immobile multi-caveolar assemblies on the cell surface [36]. This suggests that the equilibrium is regulated by local $PI(4,5)P_2$ synthesis which recruits dynamin2 to the membrane where it becomes phosphorylated/activated by c-Src [42]. Also, two serine/threonine kinases were identified (*KIAA0999* and *MAP3K2*) that specifically regulate the kiss-and-run dynamics. Their ablation led to accumulation of caveolae on the cell surface without affecting clustering. Intriguingly, these kinases are also required for SV40 infectious entry in cells not expressing Cav1 [44], indicating that, although Cav1 may itself be a target of phosphorylation by these kinases, their function to regulate endocytic activity does not solely act through Cav1.

Analogous to the cell surface, caveolar vesicles inside the cell are either part of larger, multi-caveolar assemblies, namely caveosomes (see below), or are dynamic, undergoing cycles of docking, fusion and release with intracellular organelles, like endosomes. It can be expected that these dynamics are also controlled by multiple kinases, perhaps in part by the same that control dynamics at the cell surface.

Interestingly, while in non-stimulated epithelial cells there is little exchange between these two pools of caveolar structures, this changes rapidly when cells are stimulated. Stimulation occurs by treatment with okadaic acid (a general phosphatase inhibitor) [35,45], or by binding of ligands to the cell surface [9]. It can also be mimicked by the ablation of the serine/threonine kinase *DYRK3* [36]. Upon binding of

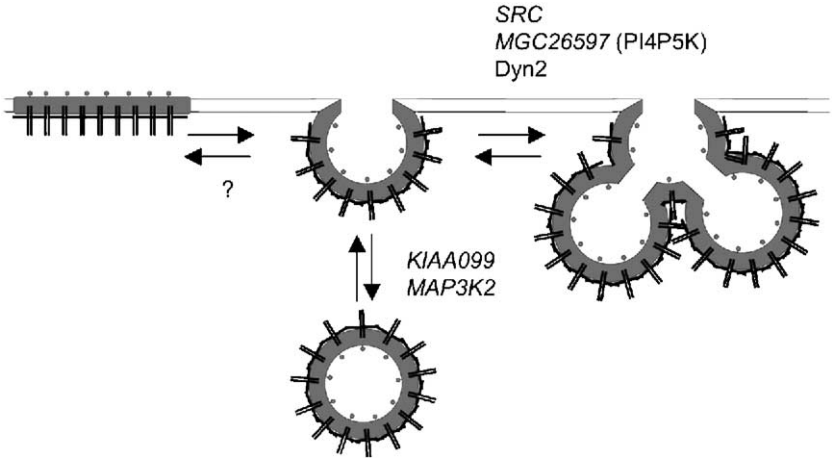

Fig. 2. The kinase-regulated caveolae cycle at the cell surface. Individual caveolae undergo continuous short-range cycles of internalization and fusion (kiss-and-run), or are stored in multi-caveolar assemblies that are static and connected to the surface. Formation and dissociation of multi-caveolar assemblies may be regulated by *SRC*, *MGC26597* (a PI4P5-K) and Dyn2, while kiss-and-run dynamics of individual caveolae are regulated by *KIAA0999* or *MAP3K2*. Perhaps, the caveolar coat is flexible and allows the membrane domain to become flat.

SV40 to the cell, a signalling cascade is activated that results in a transient loss of focal adhesions (at least the actin component of them) and actin stress fibers [38]. Concomitantly, the number of caveolae leaving the surface, but also the number of caveolae travelling to the surface is increased [36]. Those that internalize are now targeted to intracellular locations and those appearing at the surface come from inside the cell. As a result, there is net exchange between plasma membrane and intracellular Cav1 pools [35]. Activation does not involve stimulating the actual formation and fission of caveolar vesicles, but rather to change the mode of transport from short-range to long-range cycles. This switch is possibly established by a signalling cascade that eliminates the restrictions that the actin cytoskeleton imposes on caveolar dynamics at the cell surface and at the same time activates directional and long-range transport of the already locally dynamic caveolar vesicles. Given the involvement of microtubules in the latter event, components that coordinate actin turnover, the microtubule cytoskeleton, and transport along these filaments, are the most likely targets of this switch [46]. Notably, such components are concentrated and particularly active at focal adhesion sites. For EV1, the use of integrins as receptors fits perfectly in this model, and it is expected that EV1 activates the machinery involved in focal adhesion assembly and turnover. For SV40 and Py, the use of glycosphingolipids as receptors does not provide a simple explanation for the signalling cascades activated. However, given that integrin signalling is also required for SV40 infectious entry [44] (see below), a link between glycosphingolipid clustering and integrin signalling, possibly through c-Src, can be predicted.

There is a considerable lag time between binding of SV40 particles to the surface and the actual internalization of the viral particles [37]. This probably reflects the time needed to activate the above-mentioned events and is abolished by OA treatment [38]. Interestingly, untreated embryonic fibroblasts from a Cav1 null mouse do not show this delay in virus particle internalization [18]. Perhaps, the endocytic route is in a more active state when Cav1 is absent [17], comparable to its state in cells treated with OA, or these changes are not required in the absence of Cav1. This conclusion can however not be drawn from comparing two different cell lines, as it is likely that they differ in many aspects.

6. Intracellular trafficking of caveolae and lipid rafts

The activation of the switch described above results in the internalization of small vesicles that carry little fluid phase and in virus-infected cells are fit tightly around the virus particles. Inside the cell, caveolar vesicles are continuously docking on and fusing with at least two endocytic compartments, caveosomes and early endosomes [2] (see Fig. 3). During transient interaction with endosomes, the caveolar vesicle maintains its identity. It docks on, fuses with and detaches from the endosomal membrane without disassembling the caveolar coat. This kiss-and-run type of interaction explains why there is only a limited amount of Cav1 on the endosomal membrane at a given time. The interaction can, however, be rendered more permanent by the expression of a dominant-active mutant of Rab5, the small GTPase which regulates membrane traffic towards the early endosome [2]. In analogy, expression of a dominant-active mutant of Arf1, the small GTPase that regulates membrane traffic to and from the transitional ER and *cis*-Golgi complex, results in entrapment of stable caveolar domains on an enlarged Golgi complex (unpublished results). In both cases, the normal infectious itinerary of SV40 is shifted to a non-infectious one [2,47], providing additional evidence that the infectious entry route bypasses the classical endocytic compartments and the Golgi complex.

Caveosomes are still poorly defined. They are assembled from multiple *intracellular* caveolar vesicles that cluster and partially fuse with each other [23,37]. Their grape-like appearance has often confused researchers to assume that these represent multi-caveolar assemblies on the plasma membrane. The lumen of caveosomes is, however, not accessible to small molecules (such as protons or membrane-impermeable reducing agents) from the outside [37,38] and electron microscopy images show no connection between caveosomes and the cell surface [23,26,45]. Caveosomes exist without the addition of ligands that stimulate the internalization of caveolae and the pH in the lumen of these organelles is close to neutral [37]. While caveosomes are certainly part of the interconnected system of endocytic organelles, these particular features, combined with the absence of markers for early, recycling and late endosomes, as well as ligands trafficking through these compartments, make them a new type of endocytic organelle. Importantly, they may accumulate some [2], but certainly not large amounts of fluid phase as previously suggested [48]. They are therefore not part of macropinocytosis-related endocytic routes that internalize certain lipid raft components [12].

Intriguingly, in cells devoid of Cav1, SV40 particles pass through an endocytic compartment with all the characteristic features of caveosomes [18]. It has a non-acidic luminal pH, does not contain markers of classical endocytic or macropinocytic pathways and is an intermediate station for SV40 particles on their way to the endoplasmic reticulum. Apparently, Cav1 is also not essential for the formation of downstream organelles of this particular endocytic route. Does this mean that the function of Cav1 is rather specific and restricted to the regulation of those molecules with which it directly interacts or does it, as membrane scaffold, play a role in membrane trafficking? The latter is suggested by observations that rapid, siRNA-mediated Cav1 silencing reduces the infectivity of SV40 by about 50% [2]. Similarly, Cav1 expression in cells with low basal levels of Cav1

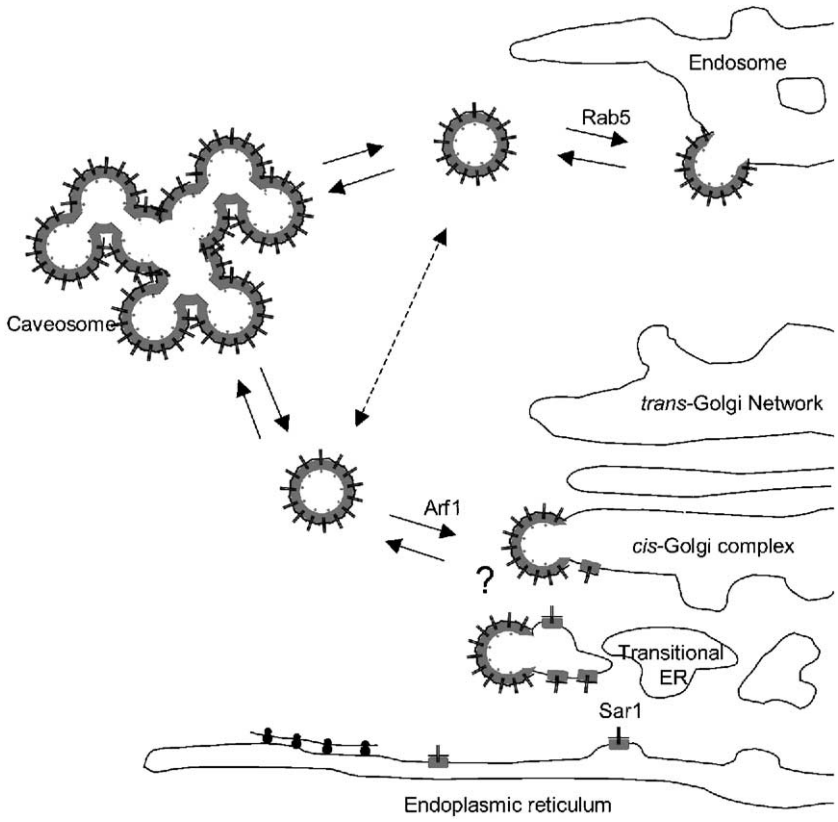

Fig. 3. Intracellular traffic to and from caveosomes. Caveosomes may be seen as intracellular distribution centres for individual caveolar vesicles. Caveolar vesicles transiently fuse with endosomes in a kiss-and-run manner under the control of Rab5, or with membranes of the early secretory pathway under the control of Arf1. The molecular details are not known, but it may well be that caveolar vesicles carry proteins that can interact either directly with activated Rab5 and Arf1 (i.e., effectors) or with molecules organized by these GTPases into domains on the target membranes. During these interactions the caveolar coat does however not dissociate. Caveolar coats are therefore not continuously and cyclically assembled after a round of transport, but only during the biosynthesis of a new caveolar vesicle, which likely occurs in the *cis*-Golgi complex. It involves the polymerization of several (10–20) caveolin-1 oligomers synthesized on the ER membrane.

increases the infectivity of Py [49]. In other words, Cav1 seems to enhance the efficiency of correct intracellular targeting of the viral particles.

7. Mechanisms of sorting by the caveolar coat

Caveosomes can be regarded as the intracellular distribution centres of caveolar vesicles that interact with target compartments in a kiss-and-run manner (Fig. 4). In this way, caveosomes connect with the plasma membrane, with early and perhaps also late endosomes, and with membranes of the early secretory pathway. Because the caveolar membrane scaffold stays intact during transient fusion with target compartments, there is little exchange of components. Therefore, no extensive compensatory mechanisms are required to maintain membrane homeostasis of the organelles involved, allowing the caveolar system to function independently of the major membrane trafficking pathways.

How then do caveolar vesicles transport and sort material? Classical membrane coats provide transport directionality by specifically assembling and sequestering ligands on donor membranes and dissociating once the vesicle is formed, allowing the membrane components of the vesicle to diffuse into the membrane of a target compartment after fusion [50]. As a result, ligands are specifically retrieved from the donor and transported to the target compartment. Sorting by caveolar coats is different, and two mechanisms may be proposed (Fig. 5).

Recent evidence suggests that ligand release from a caveolar domain is dictated by local cues, received at the target compartment. For instance, a large part of the B subunit of Cholera Toxin, which binds 5 GM1 molecules, is released from caveolar domains into the surrounding membrane in an acidic environment, while SV40 particles remain sequestered [2]. It is probably this large fraction of the internalized toxin that is subsequently transported to the Golgi complex and to lysosomes, but the small fraction escaping this release is eventually transported to the endoplasmic reticulum (ER). The itineraries of this toxin are extensively reviewed elsewhere (see this issue of BBA). Likely, it follows the same itinerary as SV40 and Py particles, but since the virus particles do not dissociate in endosomes, the majority reaches this destination. These observations indicate that certain ligands may use a local

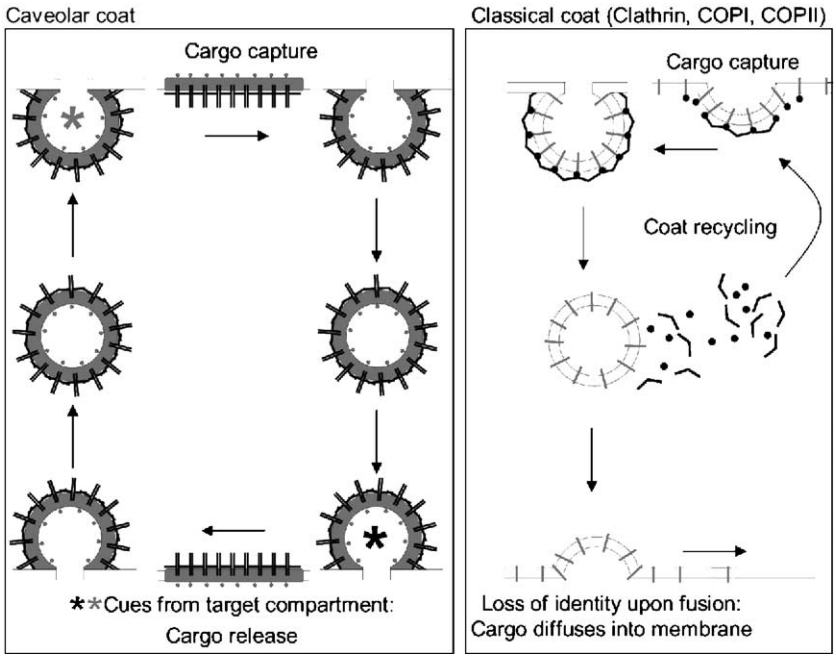

Fig. 4. Sorting by caveolar coats. (Left) A caveolar coat can capture cargo as outlined in Fig. 1, detach as caveolar vesicle, be transported to a target compartment where it fuses but keeps its identity. To release cargo, a reverse process as in Fig. 1 is proposed, triggered by the specific environment (such as low pH for the B subunit of Cholera Toxin) or the presence of specific factors in the target compartment: the 'local cue'. Such a mechanism of membrane transport prevents unwanted release of cross-linked non-membrane spanning molecules unless triggered. (Right) In the case of sorting by classical coats, adaptor molecules bind to the cytosolic domains of specific membrane-spanning cargo, and recruit coat subunits for the formation of an icosahedral cage (clathrin), which pulls the cargo into the pit and drives membrane invagination.

cue, such as low pH in endosomes, to become released from the permanently stable caveolar coat. In analogy, after ARF1-dependent transient fusion [47], ligands may be released from caveolar vesicles by the particular environment of the transitional ER, such as redox potential, high calcium or chaperone concentration. Alternatively, SV40 would trigger the disassembly of the caveolar coat via a mechanism discussed next.

Another possibility, and perhaps more specific for transport to the ER under certain conditions, is that the caveolar coat itself disassembles, and is recycled by the secretory pathway as subunits to be re-assembled in a caveolar coat. Such a cycle has been, in essence at least, proposed previously [5,51]. Indeed, this may take place during the targeting of Cav1 from caveolae and caveosomes to lipid droplets during oleic acid and cholesterol feeding (S. Lelay and K. Simons, submitted). A recent study has shown that the antigenicity of Cav1 on lipid droplets is similar to that of newly synthesized Cav1, or of Cav1 on the surface after cholesterol depletion [52], which is known to disassemble the caveolar coat [28]. Furthermore, photobleaching experiments suggest that Cav1 on lipid droplets is monomeric (unpublished observations). Perhaps, internalizing caveolar vesicles, containing polymeric Cav1, carry cholesterol to lipid droplets, where the Cav1 scaffold disassembles to deposit cholesterol. Disassembled Cav1 is recycled via the early secretory pathway where it is reassembled into a coat. Such a system makes sense if polymeric Cav1 has higher affinity for cholesterol than monomeric Cav1, since that would allow vectorial transport of cholesterol. Finally, also in motile endothelial cells, it has been suggested that Cav1 exists in a polymeric state as part of a caveolar coat at the trailing edge, and in an oligomeric/monomeric state at the leading edge of the cell [53]. If these pools communicate with each other, also here a disassembly mechanism is required.

If and how polymer stability is regulated, and where this occurs in the cell, is not clear, but recent experiments provide the first hints. Detailed analysis of phenotypes from a screen for kinases functionally involved in SV40 infectious entry (see below), revealed that at least three kinases involved mitogenic signalling, *RAF1*, *ARAF1* and *VRK1*, are required for Cav1 polymer stability [36,44]. When silenced, Cav1 reached the cell surface, but predominantly in a monomeric/oligomeric state. SV40 infection was in two cases (*RAF1*, *VRK1*) enhanced, and in one case (*ARAF1*) reduced. Although it remains speculation what this means mechanistically, it suggests that the cell has the possibility to regulate caveolar coat stability, via mitogenic signalling cascades, either during assembly, or via active disassembly.

8. Zooming out: integrating caveolae- and lipid raft-mediated endocytosis in cellular physiology

Viruses are also great tools to obtain a comprehensive view on the functioning of caveolae/raft-mediated endocy-

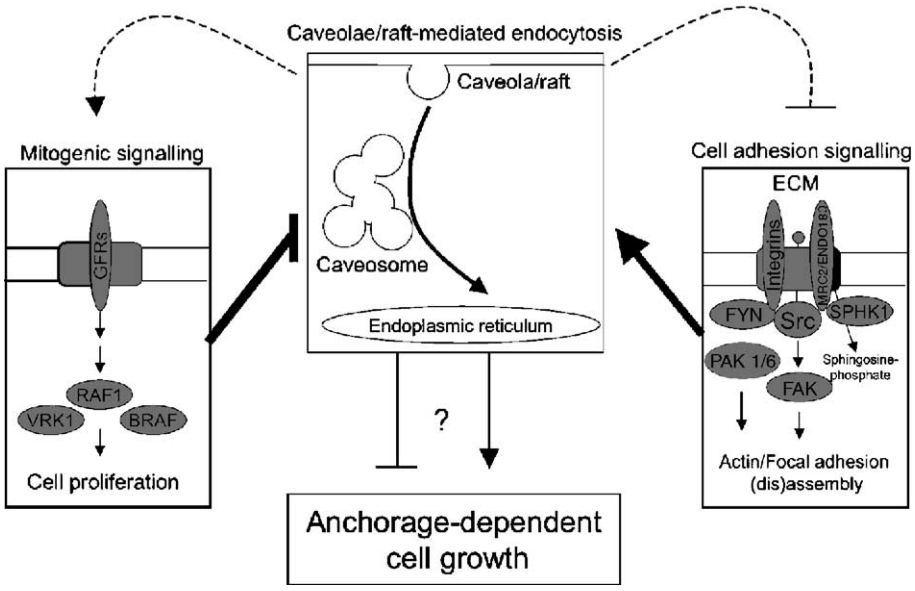

Fig. 5. Mitogenic and cell adhesion signalling controlling caveolae/raft-mediated endocytosis. RNAi-mediated depletion of depicted kinases involved in mitogenic signalling (left) stimulates caveolae/raft-mediated endocytosis, indicating that these kinases serve as suppressors of this endocytic route (depicted by suppressing arrow). RNAi-mediated depletion of depicted kinases involved in cell adhesion signalling (right) inhibits caveolae/raft-mediated endocytosis, indicating that they are required for or activate caveolae/raft-mediated endocytosis (depicted by stimulating arrow). In turn, activation of caveolae/raft-mediated endocytosis may be required to allow mitogenic signalling (depicted by dashed stimulating arrow) and to suppress integrin signalling (depicted by dashed suppressing arrow). This suggests a model whereby caveolae/raft-mediated endocytosis coordinates mitogenic and cell adhesion signalling, i.e., during anchorage-dependent cell growth.

tosis. Assays based on successful virus entry are easily automated and are robust. They are therefore well suited for high-throughput approaches to measure the functionality of the host machineries they hijack. This was recently done in a systematic analysis of the involvement of the human complement of protein, lipid and carbohydrate kinases (the kinome) in the infectious entry of SV40 and vesicular stomatitis virus (VSV), the latter hijacking clathrin-mediated endocytosis to late endosomes [44]. Combined with secondary assays analyzing the activity of caveolae/raft- and clathrin-mediated endocytosis, as well as the morphology and distribution of the organelles involved, several general conclusions about the regulatory principles and integration within cellular physiology could be drawn (Fig. 5).

First of all, the pathways are highly specific, with the majority of kinases regulating one pathway only. Second, of those regulating both pathways, the majority has opposite effects, i.e., they suppress one pathway while being required for the other. This indicates that the cell possesses a machinery of coordinating the endocytic pathway activities. Third, patterns that emerged from the identified kinases by computational analysis revealed that specific signalling pathways are integrated with specific endocytic pathways. For instance, a significant kinome-wide negative correlation was found between SV40 infectious entry and cell proliferation. This indicates that mitogenic signalling and caveolae/raft-mediated endocytosis are coupled events. It is known that signalling receptors in caveolae and lipid rafts, both at the surface and after endocytosis, have different downstream cascades or are completely shut-off [6,54], but it was not known that mitogenic signalling in turn regulates caveolar coat stability and endocytic activity. This suggests feedback mechanisms that can now be tested (Fig. 5). Furthermore, kinases downstream of integrin- and cell adhesion-dependent signalling were found to be specifically required for caveolae/raft-mediated endocytosis and SV40 infectious entry. It is known that Cav1 binds integrins and is required for downstream integrin signalling [33,55] and it has recently been shown that loss of integrin-mediated adhesion results in the internalization of rafts and caveolae, which reduces Rac1 targeting to the plasma membrane [56]. Thus, caveolae/raft-mediated endocytosis and integrin signalling/anchorage-dependent cell growth are also integrated events which feedback on each other (Fig. 5).

9. Outlook

Caveolae/raft-mediated endocytosis is rapidly being implicated in many different physiological roles. To avoid the proverbial ‘not being able to see the forest through the trees’, future work should aim at answering the many remaining fundamental questions about the nature of this endocytic pathway. Much work is required before comprehensive models can be formulated, and these are required for sensible predictions on the role of caveolae/raft-mediated endocytosis in cellular physiology.

One major task will be the detailed characterization of the caveolar coat, its assembly, its constituents, its mechanisms of ligand sequestration and release. Quantitative

analysis of coat constituents will be crucial, as well as the design of assays that allow coat assembly in vitro. For instance, knowing that only 144±39 molecules of Cav1 are incorporated in a caveolar coat [36] may suggest possible coat designs, but also indicates that a substantial number of other proteins are involved. Ultra-structural analysis of the coat will be essential, with an atomic model as the ultimate aim, in analogy to the clathrin coat. It is expected that this requires new methods in protein–lipid crystallization and NMR analysis of such complexes, since many of the important interactions take place within the lipid bilayer. Only such information will be able to end speculation about ligand sequestration and release.

Another major task concerns the comprehensive analysis of physical parameters (velocity, sizes, numbers of molecules, directionality, association/dissociation rates) of this endocytic route and its machinery under different circumstances, and to understand the regulatory principles controlling these parameters. High-resolution time-lapse imaging of the dynamics of multiple pathway components combined with computational image analysis will be required. Where in the cell is the caveolar coat assembled, how and where can it be disassembled and under which circumstances? Which molecules are sequestered by caveolar coats, which molecules are in equilibrium between caveolar and non-caveolar lipid rafts? This analysis must be carried out in several cell types, including epithelial, endothelial and cancer cells. A very important question will be to characterize in detail the effects of Cav1 polymers on the dynamics and itineraries of internalization of lipid rafts [17] and to come up with a model that combines both aspects (namely internalization of Cav1-scaffolded and Cav1-devoid membrane domains). Perhaps, cells have developed the caveolar scaffold to counteract the spontaneous internalization of clustered lipid rafts, allowing more control over localization of clustered raft domains.

As discussed, it is becoming clear from different sides that integrin signalling and caveolae/raft-mediated endocytosis are extensively integrated. Glycosphingolipid clustering appears to be functionally linked to the regulation of cell adhesion molecules and vice versa, and the viruses discussed all likely hijack this link. It will be important to elucidate how this is established. It may involve proteins with multivalent lectin domains that are part of cell adhesion complexes. In general, the role of membranes in focal adhesion assembly and turnover is still unknown, as are the effects of cell adhesion on membrane traffic. It can be expected that studies addressing these fundamental aspects allow us to place the role of caveolae/raft-mediated endocytosis in cellular physiology. Especially, the kinome-wide negative correlation between this endocytic activity and cell proliferation is an important piece of information. Feedback loops established by the effects of cell adhesion and mitogenic signalling on caveolae/raft-mediated endocytosis and the effects of the latter on integrins and signalling receptors must be analyzed and integrated into models for anchorage-dependent cell growth. I expect that viruses will continue to help us to reach these ambitious goals.

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