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Review

Lipid Rafts and Caveolae as Portals for Endocytosis: New Insights and Common Mechanisms

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Clathrin-coated pits and caveolae are two of the most recognizable features of the plasma membrane of mammalian cells. While our understanding of the machinery regulating and driving clathrin-coated pit-mediated endocytosis has progressed dramatically, including the elucidation of the structure of individual components and partial *in vitro* reconstitution, the role of caveolae as alternative endocytic carriers still remains elusive 50 years after their discovery. However, recent work has started to provide new insights into endocytosis by caveolae and into apparently related pathways involving lipid raft domains. These pathways, distinguished by their exquisite sensitivity to cholesterol-sequestering agents, can involve caveolae but also exist in cells devoid of caveolins and caveolae. This review examines the current evidence for the involvement of rafts and caveolae in endocytosis and the molecular players involved in their regulation.

Key words: caveolae, **cholera toxin**, **endocytosis**, **GPI-anchored protein**, **raft**, **SV40**

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Caveolae and Caveolins

Caveolae were first described by early microscopists as smooth-surfaced flask-shaped pits of typically 55–65 nm diameter covering the surface of many mammalian cell types (1,2). Identification of a major protein constituent of caveolae, VIP-21 or caveolin-1 (3,4), provided the first molecular marker of caveolae and accelerated progress in the elucidation of caveolae formation and function. It was soon shown that cells, such as lymphocytes, that did not express caveolin-1 lacked morphological caveolae, and transient expression of the protein in these cells was sufficient to generate the characteristic structures identifiable by electron microscopy (5). Thus, caveolin-1 appears to have an integral role in the formation of caveolae. It is now known that caveolin-1 is expressed as alternatively spliced α and β isoforms (6), and that two other members of the family also exist; caveolin-2 (7) is coexpressed with and often associates with caveolin-1 in the same cell/tissue types (8), while expression of caveolin-3 (9) is found predominantly in skeletal and cardiac muscle. Caveolin-1 null mice lack caveolae in all cell types, except for differentiated striated muscle where caveolin-3 takes over the role (10–12). While caveolin-2 may facilitate caveolae formation in some cell types (13,14), caveolin-2 null mice have normal caveolae densities (15). Caveolins are integral membrane proteins consisting of a putative 33 amino acid central hydrophobic domain (which forms a hairpin loop in the lipid bilayer) flanked by cytoplasmically exposed N- and C-terminal domains (16–18). Caveolin-1 has been shown to bind cholesterol and fatty acids directly (19,20), is palmitoylated in its C-terminal domain (17), and forms high molecular weight oligomers (18). These properties are believed to be important in caveolae formation although the exact mechanisms involved remain unclear.

Unlike clathrin-coated pits, caveolae are abundant in some cell types (adipocytes, endothelia, muscle) but undetectable in others (lymphocytes, many neuronal cells). These observations, and the relatively mild phenotype of caveolin knockouts (21), raise the vital question of the role of the caveolae invagination in cellular function. How does a caveolin-1 null cell compensate for the lack of caveolae, which could comprise up to 35% of the plasma membrane surface, and why do only some cell types in an organism have caveolae? The highly conserved structure of caveolae in higher and lower eukaryotes suggests that the caveolar form plays some important role. One attractive possibility, suggested by their unique morphology, is that caveolae can bud off to form endocytic carriers. In this review we consider the evidence for caveolae involvement in endocytosis and draw comparisons with endocytosis mediated by related domains, lipid rafts. We will first briefly consider aspects of lipid rafts relevant to this field [for a more extensive review, see e.g. (22)].

Lipid Rafts

Over the last decade, evidence has accumulated for organization of the cell surface into lipid-based microdomains. Lipid rafts are envisaged as islands of highly ordered saturated lipids and cholesterol that are laterally mobile in the

plane of a more fluid disordered bilayer of largely unsaturated lipids (22–25). The straight acyl chains of the saturated polar lipids permit their close packing with cholesterol molecules to form liquid ordered microdomains (25). By comparison, the bent hydrocarbon chains of unsaturated phospholipids which make up the surrounding 'liquid-disordered' bilayer cannot pack so closely together. These properties contribute to different biochemical properties of raft and non-raft domains; they are relatively resistant to non-ionic detergents and are present in low-density fractions after density centrifugation. This can be used as a diagnostic tool for lipid raft association. As these properties depend on concentration of cholesterol within rafts, cholesterol-sequestering agents have been used to selectively disrupt rafts. This has been used to great effect to determine whether proteins are *bona fide* lipid raft components or merely copurified contaminants (26), as well as to determine the role of rafts in cellular processes (27). Raft residents include glycosylphosphatidylinositol (GPI)-anchored proteins, dually acylated proteins (e.g. the Src family tyrosine kinases and Gα subunits of the heterotrimeric G proteins) and cholesterol-associated and palmitoylated proteins (e.g. caveolins, Sonic Hedgehog) (22). Some molecules associate/dissociate from rafts in a regulated manner depending on their state of activation [e.g. H-Ras (28)].

The raft hypothesis provides a means to explain the spatial segregation of certain signaling pathways emanating from the plasma membrane. For example, signaling by H-ras, but not K-ras, to the same downstream effector is inhibited by agents which reduce surface cholesterol levels (29). However, visualization of native raft domains has been difficult and, despite the use of a range of techniques, a consensus on size and density of rafts is presently lacking (30–33). Surface labeling methods can also influence the segregation of markers into raft domains or caveolae, as outlined below. These difficulties have hindered progress in understanding the dynamics of lipid raft domains and, particularly relevant to this review, their involvement in endocytic events. However, recent studies have implicated lipid raft domains in specific clathrin-independent endocytic processes.

Caveolae in Endocytosis

The endocytic function of caveolae still remains controversial half a century after their first description. If caveolae are an endocytic vehicle comparable to a clathrin-coated vesicle, then certain predictions can be made about caveolae endocytosis. Firstly, cargo molecules should be specifically concentrated in caveolae through specific sorting mechanisms (protein or lipid-based). Second, caveolae would be expected to be dynamic, with rapid replenishment of budded caveolae. Caveolin, as an integral membrane protein marker of caveolae, would be expected to cycle between the surface and internal compartments. Third, if caveolae are truly endocytic carriers analogous to clathrin-coated pits, then cargo molecules would not be efficiently internalized in cells lacking caveolae. In fact, very few studies have been able to show that any of these criteria are satisfied. Only a limited number of markers have been shown to be restricted to caveolae on the cell surface (as judged using non-perturbing methods, such as on-section post-embedding EM labeling). The non-caveolar pool of a putative caveolar marker could be rapidly internalized by clathrin-coated pits, making this a significant factor in studying caveolae-dependent pathways. The subcellular distribution of caveolin would also be expected to provide insights into the endocytic pathway. Caveolin-1 in most cultured cells is evident on the cell surface and within the Golgi complex (see Figure 1) with only partial colocalization with endosomal markers such as EEA1 (Figure 1A–C), a marker of the early sorting endosome (34). However, visualization of the intracellular itinerary of caveolin may be hampered by epitope masking in specific locations [e.g. (35)]. Caveolin tagged with fluorescent reporters appears to reveal more intracellular labeling but may not completely mimic the untagged protein (caveolin proteins are smaller than the GFP 'reporter'). The association of caveolin-1 with large structures which are apparently inside the cell but actually connected to the cell surface can also be misleading [see Figure 2 and (36)]. These considerations have made study of caveolae endocytosis difficult but, nevertheless, it is apparent that endocytosis via caveolae in most cells shows very different properties to that mediated by clathrin-coated pits.

Endothelial cells, in which up to 30% of the cell surface can be occupied by caveolae (1), provide the most compelling case for constitutive caveolae endocytosis. Early studies suggested a role for caveolae in transendothelial transport of fluid and solutes across the endothelial barrier (37), but failed to agree on the question of caveolae detachment (or budding) from the plasma membrane (38). Analysis of capillary endothelial cells by ultrastructural techniques revealed single caveolae or clusters of interconnected caveolae extending from either the lumenal plasma membrane (facing the capillary lumen) or the ablumenal surface (facing the interstitial space) almost to the opposite cell front (38). This prompted the proposition that interconnected caveolae may increase transendothelial permeability by forming channels from one side of the cell to the other (39). Subsequent work demonstrated the GTP-dependent budding of pre-formed caveolae from silica-coated endothelial plasma membranes in an *in vitro* assay (40) and identified the presence of molecules known to mediate budding, docking and fusion of transport vesicles in purified preparations of endothelial caveolae (40,41). The GTPase, dynamin, was also localized to the neck of the caveolae in endothelial cells by immunoEM (42) consistent with a role in regulating caveolae budding.

If endothelial cells rely on caveolae for transendothelial transport, is this process dependent on caveolae? The

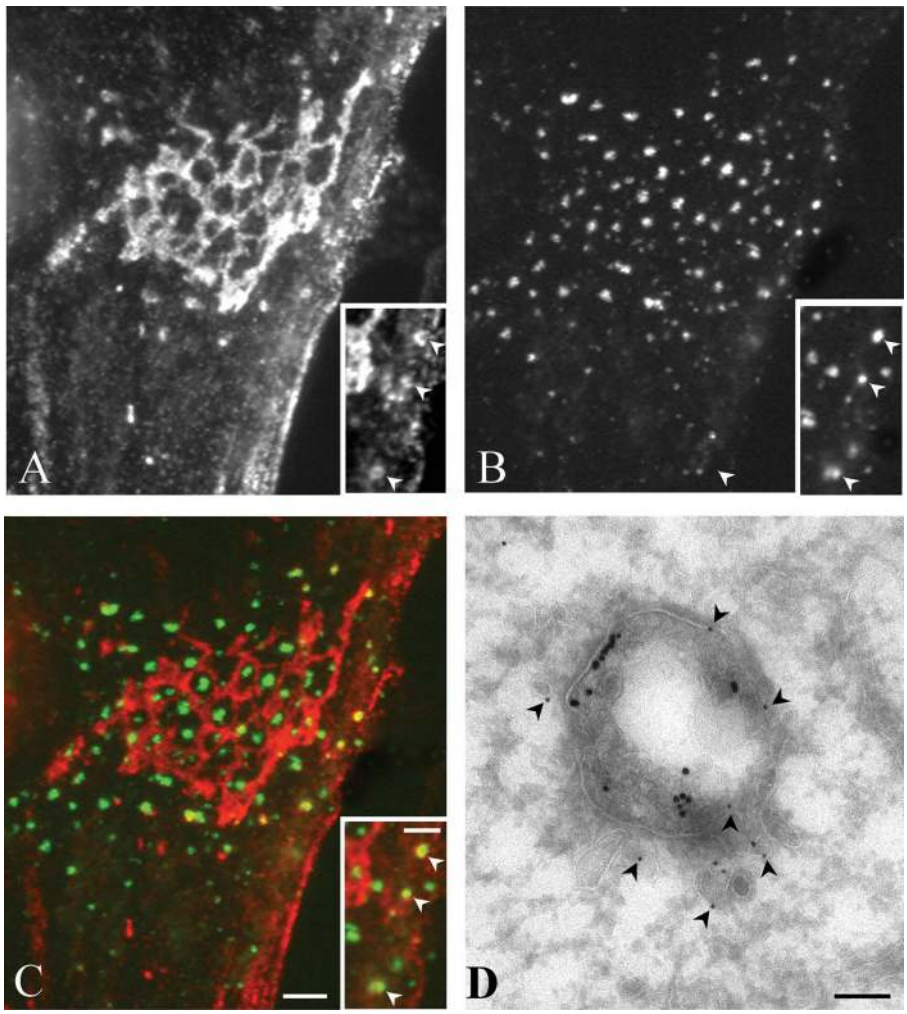

Figure 1: Caveolin-1 and endosomes in cultured human cells. Human primary skin fibroblasts labeled for endogenous caveolin-1 (A) and EEA1 (B, marker of the early sorting endosome) show labeling of caveolin-1 in the Golgi complex (reticular labeling) and at the cell surface whereas EEA1 is predominantly in distinct structures throughout the cytoplasm. However, a small number of puncta consistently label for both markers (merge panel C; arrowheads in insets which show higher magnification views from same cell). Panel D shows an A431 cell allowed to internalize 14 nm cholera toxin-gold for 10 min and then labeled for caveolin-1 (10 nm gold; arrowheads). The labeled structure has the classical morphology of the sorting endosome, and parallel labeling showed that these structures are positive for EEA1 [see (34)]. Bars A–C; 500 nm; inset 100 nm; D, 100 nm.

availability of caveolin-1 null mice lacking endothelial caveolae meant that this question could now be addressed experimentally. Caveolin-1 null mice showed no difference in albumin levels in cerebrospinal fluid, suggesting that transendothelial albumin transport was normal (10). However, other studies showed that endocytosis and transcytosis of albumin was significantly impaired in the endothelium of caveolin-1 null mice (11,43). BSA-gold perfused into the lungs of WT or knock-out (KO) mice was seen by transmission electron microscopy on both the capillary lumenal surface and ablumenal surface of endothelial cells in WT lungs but was excluded from the ablumenal surface of KO endothelial cells (43). Similarly, aortic ring segments from KO mice were unable to take up the labeled BSA at 37 °C, while competent uptake competed by unlabeled BSA was observed in WT aortic rings (43).

Other cell types such as adipocytes and fibroblasts also have abundant caveolae but a role for these structures in constitutive endocytosis is less clear. Early electron microscopic studies revealed gold-conjugated cholera toxin (CT) bound to its glycosphingolipid receptor, GM1, in uncoated invaginations of the PM (44,45), later identified as caveolae (46). After warming the cells, gold particles appeared in early endosomes, suggesting that the toxin is internalized by caveolae budding. Similar ultrastructural studies showed the GPI-anchored folate receptor in PM caveolae (27). The finding that GPI-anchored proteins are greatly enriched along with caveolin in detergent-resistant raft fractions from polarized MDCK cells (47) and the inhibition of folate uptake in cells depleted of cholesterol (48) appeared to strengthen the possibility of caveolae-mediated endocytosis. However, other electron microscopic studies revealed that GPI-anchored proteins such as the folate receptor were not restricted to caveolae but were present over the whole plasma membrane and were only partitioned into caveolae upon clustering by antibodies (49). These studies demonstrated the difficulties in analyzing the distribution of surface molecules by labeling with multivalent probes. In A431 cells, GPI-anchored alkaline phosphatase was only clustered into, and internalized by, caveolae after crosslinking (50). As in previous studies, CT-binding subunit (CTB)-coated gold particles were shown to be highly concentrated in caveolae and excluded from coated pits when bound to GM1 on the cell surface. In contrast, labeling for the GM1 ganglioside on sections

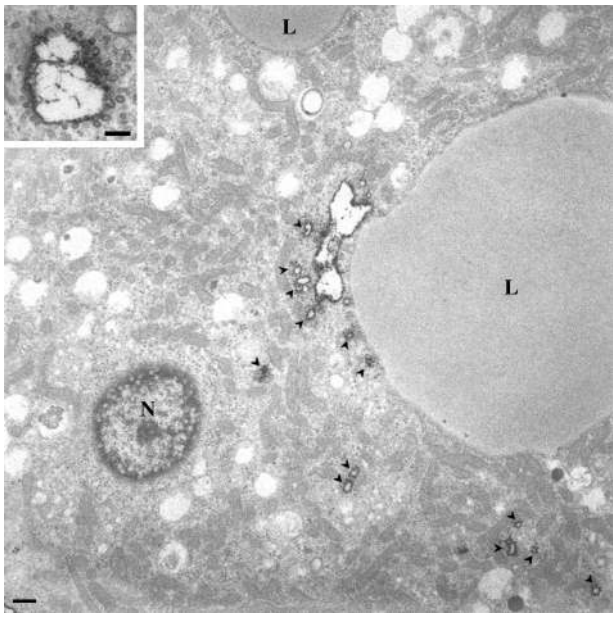

Figure 2: Surface-connected caveolae-covered domains in adipocytes. Low magnification image of a 3T3-L1 adipocyte which was surface-labeled at 4 °C with CTB-peroxidase. The labeled structures (termed 'caves', indicated by arrowheads) are surface-connected structures which have numerous caveolae, as seen in the inset (higher magnification view of a single cave), and some clathrin-coated pits [see (36)]. By light microscopy, the caves appear as intracellular caveolin-1 labeled ring-like structures. Note the proximity to lipid bodies (L). N, nucleus. Bars, main panel 500 nm; inset 200 nm.

after low-temperature embedding showed only a slight concentration of GM1 in caveolae as compared to coated pits. These studies suggest that crosslinking of GM1 not only causes concentration in caveolae but causes exclusion from coated pits. Recent studies using fluorescence resonance energy transfer (FRET) on living cells showed cholesterol-dependent clusters of GM1 (putative lipid rafts) labeled with multivalent CTB are also excluded from coated pits (51). Cholera toxin has been extensively studied as a caveolar/raft marker and analysis of the toxic entry pathway is providing new insights into a novel entry pathway.

Cholera Toxin Entry

Cholera toxin belongs to a family of structurally homologous hexameric AB_5 bacterial toxins which includes *Escherichia coli (E. coli)* heat-labile enterotoxin type I (LTI), Shiga toxin and Verotoxin (also called Shiga-like toxin) (52–54). These toxins consist of an enzymatic A (active) subunit anchored within the central pore of a pentamer of B (binding) subunits, each of which binds one or more molecules of the toxin receptor. The B subunits of LTI and CT both bind to the same glycolipid receptor, the GM1 ganglioside (55), while Shiga toxin and Verotoxin share a different glycolipid receptor, globotriaosyl ceramide (Gb3) (52,56). All four toxins must reach the endoplasmic reticulum (ER) from where their A subunits presumably translocate into the cytosol to mediate toxicity (57–59).

Early studies showing labeling of caveolae with CT-gold (44) have favored the frequent use of this toxin as a marker for caveolae. However, cells that do not express caveolin and have no morphological caveolae are also sensitive to CT internalization and toxicity (60). Thus caveolae are clearly not essential for cholera toxin entry. On the other hand, the raft-dependence of internalization of CT leading to toxicity has been clearly demonstrated (60,61). The latter study showed that CT bound to GM1 in polarized human intestinal epithelial cells was present in rafts, as determined biochemically, while the closely related *E. coli* heat-labile type II enterotoxin (LTIIb) which preferentially binds the ganglioside, GD1a, was found in detergent-soluble fractions (61). Although the enzymatic A subunits of both toxins are homologous, only the raft-associated toxin (i.e. CT) in these cells was able to elicit a cAMP-dependent Cl^--secretory response. Construction of a chimeric toxin composed of the enzymatic (A) subunit of CT and the B subunit of LTIIb demonstrated loss of the toxic response when CT-A was bound to GD1a. Reciprocally, a competent secretory response was initiated by the opposite chimera, composed of CT-B subunit and the enzymatic subunit of LTIIb, which was shown to be raft-associated (61). It is possible that CT localization in rafts at the PM causes activation of crucial signaling events leading to toxin endocytosis or it allows the toxin to follow a distinct post-endocytic trafficking pathway leading to toxicity.

After receptor-binding and internalization, CT-gold was seen by electron microscopy in an early endosomal compartment also positive for ligands internalized by clathrin-mediated endocytosis (44,45). This led to the proposition that the two endocytic pathways converge at the level of the early endosome. More recently, and consistent with those earlier findings, CT-gold-containing endosomes were shown to be EEA1-positive sorting endosomes [(34); also see Figure 1 for caveolin-1 and EEA1/CTB-gold distributions]. As CTB-gold is concentrated within caveolae to a greater extent than is GM1 (46), CTB-gold can be used as a marker of caveolae endocytosis, but it may not be representative of the native toxin, and certainly not of the receptor, GM1. In fact, consistent with the presence of GM1 in clathrin-coated pits, substantial evidence exists for clathrin-mediated internalization of the native toxin in addition to the non-clathrin raft-dependent pathway in a variety of cell types (60,62–64). However, unlike raft-dependent CT uptake (61), the productive toxicity of toxin molecules internalized by the clathrin route remains to be determined [see (65)].

After internalization, the toxin reaches the Golgi complex, from where it traffics to the ER by a coatomer (COP)

l-mediated retrograde pathway sensitive to brefeldin A and microinjection of antibodies to the coatomer component, β-COP (66,67). This transport step is facilitated by (although it is not dependent on) a KDEL retrieval motif in the A subunit of the toxin (57). Retrograde transport of the closely related LTI is also facilitated by an (R)DEL sequence (57); however, both Shiga toxin and Verotoxin use a KDEL-independent transport route (67,68) which is COPI-independent and Rab6-dependent (67). Once in the ER, redox-dependent unfolding of CT by protein disulfide isomerase (69) and reduction of the A subunit results in release of the active peptide CTA_1 which translocates into the cytosol via the sec61 protein translocation complex (70). Once in the cytosol, ADP-ribosylation of the α-subunit of heterotrimeric G proteins by the toxin results in persistent activation of adenylate cyclase and an elevation of cAMP in the cell (71). This pathway, from PM rafts to the ER and then cytosol, may be shared with other agents such as the virus, SV40.

Simian Virus 40

Simian virus 40 (SV40), first known as vacuolating virus (72), is a member of the papova (*pa*pilloma, *po*lyoma and *va*cuolating virus) family of DNA tumor viruses (73). The primary receptor of SV40 on the cell surface has been proposed to be the Major Histocompatibility Complex (MHC) class I molecule as specific antibodies to this protein inhibit virus adsorption to cell surfaces and subsequent internalization (74,75). After cell-surface binding, SV40 virions were observed by EM to be enclosed in close-fitting noncoated invaginations of the plasma membrane (76). These membranes were later shown to be caveolin-rich caveolae, proposed to form around the virus to mediate its internalization (75). At later times in the infection process the SV40 virions accumulate in a smooth-membraned subdomain of the ER [(76); see Figure 3]. The virus is then proposed to pass into the cytosol, by an unknown mechanism, before entering the nucleus via the nuclear pores (77).

The exact route taken by SV40 from cell-surface caveolae to the ER has until recently remained a mystery due to difficulties in identifying any other intermediates in the infection process. Real-time video microscopy observations have now provided exciting new insights into this process. SV40 was shown to be co-internalized with fluorescently tagged caveolin-1 (caveolin-1-GFP) into a nonclassical endosomal compartment. The 'caveosome', as it was termed, was shown to be negative for classical early endosomal markers such as EEA1 and transferrin and, moreover, was not acidic but of neutral pH. The caveosome was also clearly shown to be an internal compartment rather than a surface-connected domain of the plasma membrane by making use of the pH-sensitivity of fluorescein-labeled virus. This is an important consideration for

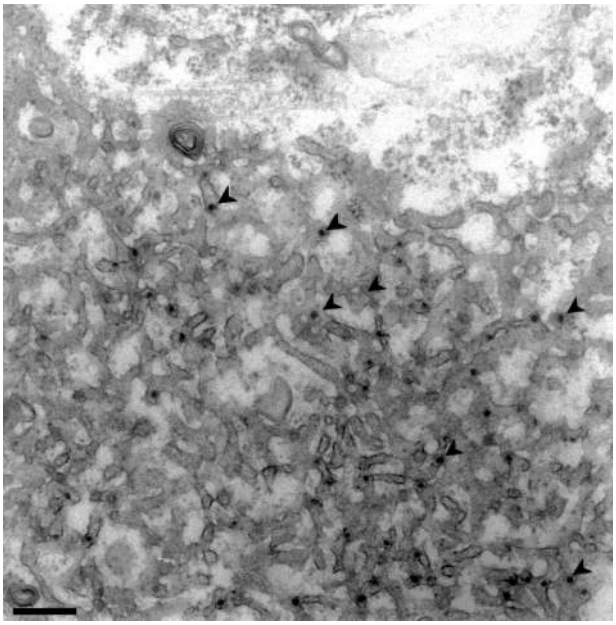

Figure 3: SV40 accumulates in domains of the RER. Image of the perinuclear area of a Vero cell incubated with SV40 for 24 h. Electron dense virions (e.g. indicated by arrowheads) accumulate in smooth-surfaced domains of the ER after transport from surface caveolae via caveosomes. From the ER, virions pass to the cytosol and then nucleus via as yet uncharacterized mechanisms. Bar, 500 nm.

other morphological studies of caveolae-associated endocytic pathways, as large caveolin-1-labeled structures connected to the cell surface are observed in many cultured cells [e.g. see Figure 2 and (36,46)]. Such structures, comprising a vacuolar domain studded with apparently fusing caveolae, can be assumed to be internal structures in the absence of surface markers. After delivery of SV40 and caveolin-1-GFP to the caveosome, the virus was sorted away from caveolin-1-GFP and was transported to the perinuclear region in a microtubule-dependent fashion (78). Dual immunolabeling for Golgi markers revealed no apparent colocalization with the virus-containing compartment. Instead, the virus was seen to colocalize with syntaxin 17, a marker for the smooth ER. This led the authors to propose a direct route for virus transport from the caveosome to the ER.

A number of features of the pathway are worth noting. Firstly, as discussed below, endocytosis was a stimulated process triggered by the virus; in control cells caveolae were extremely static. This is consistent with detailed observations of caveolae using caveolin-1-GFP (79). Fluid phase markers could not be detected within the caveosome, consistent with caveolae transport to the compartment being a low-capacity selective pathway. Secondly, the study provided the first description of a novel organelle which may be of great importance in cellular function. It also raises a number of new questions. Can caveolae only

fuse with the caveosome or can they also fuse with the conventional sorting endosome (EEA1-positive), as suggested by studies with CT-gold? If so, what dictates traffic to the two compartments? A related question concerns the relationship between the caveosome and the conventional endocytic pathway. Caveolin-1 is detected in some elements of the sorting early endosome (Figure 1) as well as in recycling endosomes (80). Are there membrane-trafficking pathways between the caveosome and these 'conventional' endocytic compartments?

To address some of these issues, a recent study compared the SV40 trafficking pathway with the trafficking itinerary of CTB (81). CV1, COS7 and NRK cells expressing caveolin-1-GFP were allowed to bind and internalize fluorescently labeled CTB. The toxin was found to be internalized by either of two pathways; one involving clathrin-independent, filipin-sensitive entry to caveolin-1-GFP-positive endosomes [the 'caveosomes' of (78)], and the other route delivering the toxin to EEA1-positive early endosomes via clathrin-dependent uptake (63,81). Uptake of GPI-anchored proteins, GPI-GFP and endogenous CD59 [which had previously been shown to be co-internalized with CT by the clathrin-independent pathway (63)], also resulted in delivery to caveolin-1-positive structures but not to transferrin-positive endosomes in these cells (81). Since CT is internalized by both clathrin-dependent and -independent pathways, in order to selectively observe the clathrin-independent route taken by the toxin, cells were transfected with a dominant-negative truncation mutant of the clathrin adaptor protein, AP180, which specifically inhibits clathrin-mediated endocytosis. Transferrin uptake was efficiently blocked in these cells, as was CT uptake to EEA1-positive endosomes. The toxin accumulated almost exclusively in caveolin-1-positive endosomes and was still efficiently internalized to the Golgi (81). The two studies show significant differences. For example, the internalization of dextran to caveosomes in the latter studies suggests a constitutive fluid phase pathway to the caveosome.

A clathrin-independent, dynamin-mediated endocytic pathway similar to that taken by CT was also shown to mediate internalization of fluorescent analogues of glycosphingolipids, such as lactosylceramide and globoside (82). Endocytosis of these markers by human primary fibroblasts was found to be insensitive to inhibitors of the clathrin pathway (chlorpromazine and dominant negative eps15) but was greatly inhibited by cholesterol depletion (using nystatin), inhibition of tyrosine kinases (by genistein) and a dominant negative dynamin II mutant (K44A). An absence of colocalization of the glycosphingolipid markers with markers internalized by the clathrin pathway was also observed at early times, but these markers showed a striking colocalization with transfected caveolin constructs. Subsequently the markers were observed in transferrin-containing endosomes suggesting that caveolin-containing carriers, presumably budded caveolae, fuse with the conventional sorting endosome (83). This system, in which the lipid probes are internalized very efficiently via a clathrin-independent mechanism, should provide important new insights into this raft-dependent entry pathway.

Many similarities between the SV40 infectious pathway and the endocytic itinerary of CT leading to intoxication are apparent. Both markers are internalized within caveolae/rafts and are targeted to the ER. In addition, translocation of both the virus and the toxin out of the ER lumen into the cytosol appears to be necessary for productive infection and toxicity, respectively. In addition, both markers have now been shown to be internalized by a clathrin-independent pathway to the same caveosome compartment, at least in cells expressing caveolin-1-GFP constructs (81). These observations strongly suggest that SV40 infectious entry may occur by the same intracellular trafficking route as CT toxic entry. However, the SV40 entry pathway appears to differ from that of CT with respect to the involvement of the Golgi complex. Although CT is readily visualized within the Golgi complex by light or electron microscopy, SV40 virions are rarely observed in the Golgi complex. Recent studies (84,85) have found SV40 infection to be sensitive to the Golgi-disrupting drug, brefeldin A (BFA), as is the transport of CT from the PM to the ER. Other inhibitors of COPI-dependent retrograde transport previously shown to inhibit Golgi-to-ER delivery of CT (such as the GTP-restricted mutant of Arf1 (Arf1Q71 L) and microinjected antibodies to the coatomer subunit β-COP) also elicit a potent inhibition of productive infection by the virus (84). The inhibition, by these agents, of Arf1/COPI-dependent trafficking steps other than retrograde transport from the Golgi to ER cannot be excluded, however, and precise identification of the affected step in viral infection is now required. Interestingly, membrane-permeant TAT fusion proteins have recently been shown to follow a similar raft-dependent internalization step before BFA-sensitive intracellular trafficking (86). Like both CT and SV40, these agents also translocate to the cytosol, suggesting that this trafficking pathway may be important for the agent to reach the correct environment to allow translocation.

Bacterial Pathogens

Certain bacterial pathogens have also been shown to exploit caveolin-1-rich membranes for infectious entry into cells. FimH-positive strains of *E. coli* evade the immune system by infecting macrophages (87) and mast cells (88) through interaction of their cell-surface FimH molecules with the GPI-anchored protein, CD48, on the host cell membrane. This results in receptor clustering and enclosure of the bacterium in a tight-fitting caveolin-positive cell-surface-derived membrane compartment which displays all the characteristics of caveolar membranes, being enriched in GM1 and cholesterol (by immunoelectron

microscopy) and readily isolated in a detergent-resistant, low-density membrane fraction from infected cells (88). While the bacterium-containing membrane structure is clearly not a caveola according to the classical morphological definition of 'small, flask-shaped pits', the biochemical composition of both membrane compartments appears to be similar. The caveolin-rich bacterium-containing compartment is non-degradative, unlike the loose-fitting phagosomal compartment into which antibody-coated (opsonized) bacteria are internalized through interaction with Fc receptors on the immune cell PM (87,88). A similar pathway has now been described for non-degradative entry of another bacterial pathogen, *Chlamydia trachomatis*, into both epithelial cells and macrophages (89).

Common Features of Caveolae and Raft Pathways; Stimulated Internalization of Caveolae

While it is still difficult to obtain a consensus view on caveolae internalization pathways, some common features of caveolae-mediated endocytosis are now emerging. Earlier studies in A431 cells found that antibody-clustered GPI-anchored proteins could be internalized by treatment with the general phosphatase inhibitor, okadaic acid, which was found to cause surface-connected caveolae to bud into the cell *en masse* (50). Staurosporine, a general kinase inhibitor, and (to a lesser extent) genistein, a tyrosine kinase inhibitor, blocked the okadaic acid-induced internalization of clustered alkaline phosphatase, as did the actin depolymerizing agent, cytochalasin D (50). Thus, the internalization of caveolae was proposed to be regulated by phosphorylation and to require the involvement of the actin cytoskeleton. These early observations are consistent with recent real-time studies of caveolin-1-GFP in control and okadaic acid-treated cells (79) and of SV40 entry (90). Exposure of cells to SV40 also leads to activation of a tyrosine kinase signaling pathway. This results in both recruitment of dynamin to virus binding sites on the PM, and cortical actin rearrangement and recruitment to these sites, to mediate budding of virus-containing caveolae into the cell. Thus dynamin may not be constitutively associated with the caveolae of non-endothelial cells, consistent with immunoEM observations in various cell types (R. Parton, unpublished observations). Treatment of cells with the tyrosine kinase inhibitor, genistein, abolished both the recruitment of dynamin and the rearrangement and recruitment of actin to virus binding sites on the membrane, resulting in a block in viral entry (90). Actin depolymerization (using Latrunculin A) and actin stabilization (using jasplakinolide) also inhibited virus entry. In contrast, an increase in binding and uptake of the virus was elicited by the tyrosine phosphatase inhibitor, vanadate, and by okadaic acid. Immunolabeling infected cells with a phosphotyrosine antibody confirmed the tyrosine phosphorylation of some component of virus-containing caveolae. Thus, binding of SV40 to MHC class I molecules in the membrane appeared to simulate the effects of crosslinking alkaline phosphatase, resulting in activation of a tyrosine kinase signaling pathway, which led to dynamin and actin recruitment and caveolae budding. Tyrosine kinase activity is now also implicated in CT internalization to the Golgi, since genistein inhibits this process (91). It remains to be determined whether the same mechanisms mediate FimH-dependent entry of *E. coli* within caveolin-1-positive membranes of macrophages and mast cells after cell-surface binding and clustering of the GPI-anchored FimH receptor, CD48 (87,88).

Recent years have seen the identification of other caveolae markers which follow an endocytic route closely resembling that of SV40 and CT. One such molecule is the autocrine motility factor receptor (AMF-R). By electron microscopy, this seven transmembrane domain receptor is localized to PM caveolae and membranes of the smooth ER at steady state (92). Endocytosis of AMF-R leading to ER delivery is sensitive to cyclodextrin treatment and the dynamin mutant, K44A, as well as inhibition of tyrosine kinases by genistein (93). However, unlike CT and SV40, AMF-R arrival to the ER is unaffected by both brefeldin A and nocodazole treatments (91), arguing against Golgi involvement in ER targeting of the receptor.

Tyrosine phosphorylation has also been implicated in the ligand-induced internalization of G protein-coupled receptors and receptor tyrosine kinases by dynamin-mediated endocytosis (94,95). While these events are clathrin-dependent, they display characteristics distinct from the constitutive clathrin-mediated uptake of nutrients such as transferrin. Agonist-stimulation of the EGF receptor (a receptor tyrosine kinase) and of β2-adrenergic receptor (a G-protein coupled receptor) results in activation of the nonreceptor tyrosine kinase, c-src, and phosphorylation of dynamin by this enzyme (94–96). Src-mediated tyrosine phosphorylation was shown to be critical for dynamin self-assembly (94) and resulting stimulated endocytosis of these agonist receptors (94,95). Interestingly, mutation of key tyrosine residues in dynamin, which are the phosphorylation targets of c-src, only inhibits agonist-induced endocytic events (like EGF receptor and β2 adrenergic receptor internalization) and appears not to perturb constitutive clathrin-dependent uptake of nutrient molecules like transferrin (97). The induced and constitutive pathways differ also in temperature sensitivity, since stimulated uptake of β2 adrenergic receptor was greatly inhibited at 16 °C, while transferrin receptor uptake was little affected (98). It is possible then that the same molecular mechanisms which regulate the agonist-induced clathrin-mediated internalization of hormones/growth factors are also in play for the regulation of caveolae-mediated endocytosis of SV40 and antibody-crosslinked GPI-anchored proteins.

The receptor-mediated uptake and transcytosis of albumin in endothelial cells has also been suggested to be

a stimulated process. Here also, a requirement for tyrosine phosphorylation mediated by Src kinase activation is suggested (99). Albumin uptake in endothelial cells depends on the albumin receptor, gp60, and antibodies to this receptor competitively inhibit specific binding of albumin to the endothelial cell surface (100). Ligand binding of gp60, which has been localized to caveolar membranes (40,101), results in physical association of the receptor with caveolin followed by activation of albumin internalization and transcytosis (99). Tyrosine phosphorylation of both caveolin-1 and gp60 was also found to be induced upon albumin binding of gp60 (102), and expression of dominant-negative Src kinase inhibited gp60-stimulated albumin uptake (99). These observations and the well-known enrichment of Src family kinases in rafts and caveolae make these enzymes likely candidates for the tyrosine phosphorylation events mediating SV40 uptake in non-endothelial cells.

Stimulated Internalization of Rafts

Are tyrosine phosphorylation and recruitment of dynamin and actin features only of caveolae-budding or can the same signaling pathways induce the budding of non-caveolar rafts? Antibody-induced clustering of the GPI-anchored protein, CD59, or of CT-bound GM1 molecules in raft domains of human lymphocytes (which neither express caveolin-1 nor display cell-surface caveolae) has also been shown to result in activation of the Src family kinases, Lck and Fyn, and in localized protein tyrosine phosphorylation (103). Again, accumulation of F-actin was observed in CD59 clusters and was inhibited by a specific inhibitor of Src kinases, PP2. By contrast, antibody crosslinking of a non-raft protein, the transferrin receptor, produced clusters which were not enriched in Src kinases or tyrosine phosphorylated proteins and did not accumulate F-actin. Ligand stimulation of the Interleukin-2 (IL2) receptor in lymphocytes also results in partitioning of this molecule into Triton X-100-insoluble lipid rafts, a phenomenon imitated by antibody crosslinking of the receptor at the cell surface (104). Subsequent internalization of the receptor occurred by a clathrin-independent pathway (unaffected by expression of a dominant-negative mutant of Eps15), which was, however, inhibited by the dynamin mutant, K44A (104). Internalization was also dependent on activation of the Rho family GTPases, Rac1 and Rho A, which may play a role in actin rearrangement and recruitment (105). Thus, it appears that the budding of non-caveolar rafts in caveolin-deficient cells occurs by very similar mechanisms to those described for caveolae-budding in caveolin expressors. What then is the role of caveolin itself in the endocytic process?

What Is the Role of Caveolin in Endocytosis?

The availability of cells lacking caveolin-1 and caveolae provides an excellent opportunity to dissect the role of caveolin, and the caveolar invagination, in specific endocytic events (21). If the caveolar invagination is required for internalization, then endocytosis should depend on caveolin-1, as shown in endothelial cells for albumin transendothelial transport (43). However, in only a limited number of cases has this been demonstrated.

SV40 currently provides one of the best markers for endocytosis by caveolae. Caveolin mutants inhibit SV40 infection (29), but this alone does not prove a dependence on caveolae *per se* [e.g. the caveolin truncation mutant, cavDGV, generally perturbs lipid raft domains (29)]. In fact, recent studies suggest that SV40 infection proceeds efficiently even in the complete absence of caveolae although, unlike internalization in caveolae-containing cells, in caveolin-null cells the entry process is dynamin-independent (Damm, E, Pelkmans, L. and Helenius, A., manuscript in preparation). Our recent studies also show that CT internalization, via a clathrin-independent pathway, occurs normally in caveolin-1 null embryonic fibroblasts which completely lack caveolae (Kirkham, M. and Parton, R.G., unpublished results). Although these findings contradict a recent report (106), they are consistent with the reported sensitivity of other caveolae-deficient cells to cholera toxin (60) and with studies employing more acute means of down-regulating caveolin-1 expression (81). In the latter study, the internalization of CT into SV40-containing caveosomes occurred in cells cotransfected with an AP180 truncation mutant (which abolished clathrin-mediated uptake) and caveolin-1 specific duplex RNA oligonucleotides which reduced caveolin-1 expression to background levels (81). Thus, in this system, uptake of CT was supported equally well by a non-caveolar, clathrin-independent pathway as by caveolin-1-positive membranes.

Reduction of caveolin-1 levels has proven either to have no effect on, or to accelerate, raft-mediated uptake of other markers of caveolae such as the AMF-R. The function of caveolin in endocytic uptake of AMF-R was examined in ras- and abl-transformed NIH3T3 cells which, as a result of transformation, expressed reduced levels of caveolin-1 and fewer caveolae (93). Surprisingly, instead of an inhibition of cyclodextrin-sensitive AMF-R internalization to the ER, an increase was observed in the transformed cells. Furthermore, overexpression of caveolin-1 in these cells decreased the rate of internalization to that in untransformed cells (93). These findings point to a role for caveolin-1, not as part of the essential machinery mediating raft/caveolae-dependent uptake but rather as a negative regulator of raft-mediated uptake. The presence of caveolin in endocytic raft domains may stabilize these structures at the PM and reduce their budding frequency, in keeping with the findings of Thomsen et al. that cell-surface caveolae are largely static (79). A similar observation was reported in endothelial cells where overexpression of caveolin-1 inhibited receptor-mediated uptake of albumin (99). In this case, sequestration of Gαi by overexpressed caveolin-1

and the resulting impedance of signal activation was proposed to be the mechanism of inhibition (99). Taken together, these studies support a model of raft-mediated endocytosis that is not dependent on the presence of caveolin-1 in raft microdomains, but instead may be negatively regulated by this cholesterol-binding integral membrane protein.

Different Types of Rafts Mediate Distinct Endocytic Events

The examination of many raft-dependent pathways in different cell types (summarized in Table 1) has uncovered a common theme of Src kinase activation and tyrosine phosphorylation, leading to actin rearrangement and recruitment and dynamin recruitment and activity. However, not all raft-dependent pathways conform to this description. A novel raft-dependent internalization pathway has recently been described which is clearly distinct from the pathways discussed above. This is a micropinocytic pathway specific for unclustered GPI-anchored proteins, which is not dependent on ligand binding and is also independent of both clathrin and dynamin function (107). GPI-anchored GFP (GFP-GPI) and native GPI-anchored proteins like the folate receptor and decay accelerating factor were all found to be in a compartment distinct from transferrin-containing classical sorting endosomes after 2 min at 37 °C. This compartment was acidic and did not label for endogenous caveolin-1 or for transiently transfected caveolin-1-GFP, unlike the 'caveosome' compartment to which both SV40 and CT are internalized [see (78,81)]. However, the fluid-phase marker, dextran, was internalized to the 'GPI-anchored protein-enriched early endosomal compartments' (GEECs) and together with the folate receptor was found to colocalize with transferrin in recycling endosomes after 20 min. Clathrin- and dynamin-independence of internalization to GEECs was demonstrated using dominant-negative mutants of Eps15 and dynamin 2, respectively, and RhoA and Rac1 dominant-negative mutants [which inhibit raft-dependent IL2 receptor uptake (104)] were also shown to have no effect on internalization to GEECs. On the other hand, internalization to this compartment was inhibited by a dominant-negative mutant of another Rho family GTPase, cdc42, which caused the redirection of GPI-anchored proteins into sorting endosomes. The lack of colocalization of fluorescent dextran and the folate receptor with GFP-rab5, GFP-rab4 or endogenous rab7, and the failure of rab5 dominant-negative or dominant active mutants to perturb GEECs, further confirms that this compartment is not a classical endosomal compartment. Neither is this a non-specific pathway for molecules excluded from the clathrin internalization pathway, since simply removing clathrin-targeting sequences from the transferrin receptor was not sufficient to direct this molecule into GEECs. On the other hand, the presence of a GPI anchor was found to be essential for targeting to GEECs and substitution of a transmembrane domain for this lipid anchor resulted in exclusion from this compartment.

The existence of two distinct raft-mediated internalization pathways which are differentially regulated and result in delivery to distinct subcellular compartments prompts questions as to what factors distinguish these pathways and the raft domains involved. Could it be that rafts of differing protein (and lipid?) composition selectively mediate internalization of different sets of cargo to preferred subcellular destinations? Alternatively, the cargo itself may dictate the regulation of a raft-mediated endocytic event (e.g. through interaction of sequences in raft-associated receptors with specific regulatory molecules) to determine subcellular targeting. Certainly, different species of lipid raft domains, distinguished by their associated proteins, are known to be present in cellular membranes (108).

Conclusions

It is now clear that a number of clathrin-independent endocytic pathways exist in mammalian cells, but the relationships between the different pathways are still unclear. A recent review proposes that internalization via caveolae or internalization via lipid rafts are fundamentally similar processes (109). In this model caveolin/caveolae would not be required for endocytosis but would stabilize a potentially endocytic raft domain and decrease endocytosis. However, caveolae and caveolin can clearly be internalized in response to specific stimuli, as shown with SV40 (78) or by treatment with okadaic acid (79). To combine these observations, it might be envisaged that caveolin-1 normally acts as a negative regulatory mechanism preventing internalization via raft domains. However, to allow caveolae budding in response to a specific stimulus (e.g. SV40 binding), the inhibitory effect of caveolin would have to be overcome, presumably through phosphorylation of caveolin and/or associated proteins and the recruitment of other proteins such as dynamin. In caveolin-1 null cells these constraints would be absent. It is certainly evident that in most systems the caveolar invagination is not required for endocytosis, raising the question of what other mechanisms are involved in generating the endocytic carrier. Is it a property of the lipid raft domain itself and, if so, how is the process regulated in the absence of caveolin?

Further work should elucidate the properties of the caveosome, its role in normal cellular function, and how this compartment is linked to the better-characterized endocytic compartments in the clathrin pathway which are involved in sorting, recycling, and degradation of internalized components. Recent studies have suggested that the caveosome might also play a role in receptor degradation. Internalization of transforming growth factor (TGF)β receptors was shown to occur by two distinct endocytic

Table 1: Summary of known lipid raft/caveolae-dependent endocytic pathways

Endocytic marker/pathway	Caveolae/raft-dependence determined by:	Dynamin dependence	Src kinase/ tyrosine kinase-dependence	Actin dependence	Delivery to:
Cholera toxin	Filipin-sensitivity of internalization (60)	Yes – dynamin I (K44A) (42) – micro injection of anti-dynamin (111)	Yes Endocytosis to the Golgi inhibited by genistein (91)	–	Classical early endosomes and caveosomes (44,45,81). Eventually to Golgi and (by COPI-dependent retrograde transport) to ER (66,67)
Ab-clustered GPI-anchored protein (alkaline phosphatase)	EM localization in caveolae (50)	–	Yes – endocytosis sensitive to genistein, stimulated by okadaic acid (50)	Yes – inhibited by cytochalasin D (50)	Classical endosomes (50)
Simian Virus 40 (SV40)	Nystatin-sensitivity (112) or Nystatin/progesterone sensitivity (90) of internalization EM localization in caveolae (75). Infection is unaffected by dominant negative eps15 (90)	Yes – dynamin II (K44A) (90)	Yes – genistein or staurosporin inhibit infection (90)	Yes – latrunculin A or jasplakinolide inhibit infection (90)	Caveolin-1-GFP positive caveosome (78). Eventually to ER (76,78)
Albumin	Presence of gp60 receptor in endothelial budded caveolae fraction (40). Filipin sensitivity of internalization (99). Co-immunoprecipitation of gp60 with caveolin-1 in albumin-stimulated cells (99)	–	Yes gp60-activated uptake and transcytosis sensitive to herbimycin A and genistein (102)	–	–

AMF-R	Localization in caveolae by EM (92). Internalization to the ER is sensitive to methyl-β-cyclodextrin (92)	Yes – dynamin (K44A) inhibits internalization to the ER (93)	Yes Genistein inhibits internalization to the ER (91)	–	ER (91-93)
IL2-R	TritonX-100-insolubility of IL2-R (104). Absence from clathrin-coated profiles at PM. Internalization insensitive to inhibitory eps15 mutants (104)	Yes – dynamin I (K44A) inhibits internalization (104)	–	Possibly – dominant negative mutants of RhoA and Rac1 inhibit internalization (104)	–
Unclustered GPI-anchored proteins (Folate-R, CD59, DAF, GFP-GPI)	TritonX-100-insolubility (113). Internalization unaffected by K^+-depletion or dominant negative eps15 (107)	No – dynamin II (K44A) had no effect on internalization to GEECs (107)	–	Possibly – internalization is inhibited by dominant negative mutant of cdc42 but not dominant negative mutants of Rac1 and RhoA (107)	GPI-anchored protein enriched early endosomal compartment (GEEC) but not caveolin-1-GFP positive caveosomes (107)
Glycosphingo-lipids (Lactosylceramide and globoside)	Nystatin-sensitivity of internalization (82). Internalization not inhibited by chlorpromazine, K^+-depletion or inhibitory mutants of eps15 (82)	Yes – internalization inhibited by dynamin II (K44A) (82)	Yes – internalization inhibited by genistein (82)	–	Caveolin-1-GFP-positive structures (82) and then to transferrin positive endosomes (83) before Rab7-, Rab9- and PI3Kinase-dependent transport to Golgi complex (114)

Abbreviations: Ab, antibody; DAF, decay accelerating factor.

pathways, one mediated by clathrin-coated pits and leading to productive signaling in EEA1-positive endosomes, and a second involving caveolin-1-positive vesicles and leading to Smad7–Smurf2-dependent receptor degradation (110). The data suggest that internalization via the clathrin-coated pit pathway sequesters receptors to the signaling pathway, so avoiding degradation in the lipid raft-dependent, caveolin-stimulated pathway. Precise characterization of the compartments and mechanisms involved in TGFβ receptor degradation will be of considerable interest.

The complexity of lipid raft-dependent pathways appears to be increasing as new studies with specific markers reveal pathways with differing properties. However, common themes are emerging, and new studies should be able to establish whether the underlying mechanisms involved in apparently different pathways are actually the same. It is clear that the molecular mechanisms involved will be quite distinct from those characterized so thoroughly for clathrin-coated pit-mediated internalization, and understanding the properties of specific lipids and lipid microdomains will be of great importance. Dissection of the molecular basis for these novel endocytic mechanisms now presents an exciting challenge for the cell biologist.

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