Caveosomes and endocytosis of lipid rafts

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Summary

Endocytosis of various endogenous plasma membrane molecules, including signalling receptors, glycosphingolipids and glycosylphosphatidylinositol (GPI)-linked proteins, occurs in the absence of functional clathrin-coated pits. Most of these molecules are found in biochemically defined lipid rafts, which suggests that at least some clathrin-independent endocytosis may be raft specific or raft mediated. However, recent studies of the uptake of raft markers have revealed a diversity of internalization methods. Although lipid rafts may somehow be recognized by endocytic machinery, at this stage the data do not readily fit with the idea of a single raft-specific or raft-dependent endocytic pathway. Many studies report uptake of raft molecules by caveolar endocytosis (defined by sensitivity to cholesterol depletion and to overexpression of a specific mutant of dynamin 2). It is now apparent that this is a highly regulated process, and caveolin 1, one of the characteristic protein components of caveolae, might in fact act to slow or inhibit endocytosis. The molecular details of caveolar endocytosis have yet to be elucidated. Several sources indicate that clathrin-independent uptake to a distinct class of caveolin-1-containing endosome, termed the caveosome, allows different types of endocytic mechanisms to have different functional consequences for the cell. It is likely that there are mechanisms that allow recruitment and targeting of specific molecules to caveosomes.

Key words: Caveosome, Endocytosis, Lipid raft

Introduction

The term lipid rafts refers to putative membrane microdomains with a different composition to surrounding regions of the membrane. It is thought that lipid rafts are enriched in cholesterol, glycosphingolipids, sphingomyelin, phospholipids with long, unsaturated acyl chains, glycosylphosphatidylinositol (GPI)-linked proteins and at least some membrane-spanning proteins (Simons and van Meer, 1988; Simons and Ikonen, 1997; Brown, 1998; Simons and Toomre, 2000). A functional role for rafts has been invoked in a diverse array of cellular processes, the common theme being that rafts could provide sites of local enrichment of molecules that need to interact with each other or to be transported to the same place in the cell (Simons and Ikonen, 1997). The only widely applied assay for incorporation of molecules into raft domains is a biochemical one, revolving around resistance to extraction with non-ionic detergents such as Triton X-100 (Brown and London, 1998; Edidin, 2001; Heerklotz, 2002; Heerlotz et al., 2003). The nature and functional relevance of raft microdomains remains controversial, partly because of the obvious technical challenges of studying small, dynamic structures in the membranes of living cells. Several excellent reviews on lipid rafts that discuss in detail the limitations of biochemical approaches have recently been published (Anderson and Jacobson, 2002; Maxfield, 2002; Edidin, 2003). The terms DRM (detergent-resistant membrane) and DIG (detergent-insoluble glycolipid-rich membrane) are also used to describe biochemically defined lipid rafts. The term lipid raft is used in this article to denote those membrane components that are found in DRM preparations.

Endocytosis comprises multiple mechanisms that allow cells to internalize macromolecules and particles into transport vesicles derived from the plasma membrane (Conner and Schmid, 2003; Fig. 1). Together, these mechanisms have to control entry into the cell in a co-ordinated and specific manner, and they play a crucial role in many cellular processes. New data on endocytosis of raft markers not only provide insights into these mechanisms, but also constitute a good testing ground for the validity of competing models of lipid raft organization and function. The interplay between studies of specific endocytic pathways and more general consideration of the nature of lipid rafts and organization of the plasma membrane provides the basis of this Commentary. Recent data have also shed light on the role of rafts and caveolae in pathogen entry, and on post-endocytic trafficking of raft proteins and lipids. These topics are beyond the scope of this Commentary and are discussed elsewhere (Nichols et al., 2001; Puri et al., 2001; Lamaze et al., 2001; Nichols, 2001; Sabharanjak et al., 2002; Fivaz et al., 2002; Duncan and Abraham, 2002; Nabi and Le, 2003; Sharma et al., 2003; Chazal and Gerlier, 2003).

Clathrin-independent endocytosis

By far the best-characterized mechanism for internalizing both bulk membrane and specific proteins is by clathrin-coated pits, which are readily recognizable morphological entities now described in considerable molecular detail (Higgins and McMahon, 2002; Conner and Schmid, 2003) (Fig. 1). Indeed, until the mid-1990s, the extent to which mammalian cells internalize cell-surface components by alternative mechanisms to have different functional consequences for the cell. It is likely that there are mechanisms that allow recruitment and targeting of specific molecules to caveosomes.
Bück et al., 1993), eps15 (Benmerah et al., 1999), epsin (Chen et al., 1998) and AP180 (Ford et al., 2001), has allowed experiments that clearly demonstrate continued internalization of a range of molecules despite the absence of functioning clathrin-coated pits (Nichols and Lippincott-Schwartz, 2001). This has led to increasing acceptance that clathrin-independent endocytic mechanisms are functionally significant (Conner and Schmid, 2003). However, molecular details are still largely lacking. There are likely to be at least two types of clathrin-independent endocytosis that are separate from phagocytic engulfment of large particles: caveolar endocytosis, which is blocked by specific mutations in dynamin 2 (Nabi and Le, 2003), and macropinocytosis potentially associated with membrane ruffling, which is not blocked by specific mutations in dynamin 2 (Nabi and Le, 2003). The extent to which these different pathways require lipid rafts to operate, or are somehow selective for lipid rafts, is currently the subject of debate and active investigation.

A role for rafts?

Three main observations link clathrin-independent endocytosis with lipid rafts, but interpretation of each of them is by no means clear-cut (Lamaze et al., 2001; Nichols and Lippincott-Schwartz, 2001; Nabi and Le, 2003).

First, nearly all molecules that are known to be internalized independently of clathrin are found in biochemically defined lipid rafts, and archetypical substrates for uptake by clathrin-coated pits, such as the low-density lipoprotein (LDL) and transferrin receptors, are not (Nichols and Lippincott-Schwartz, 2001). Raft components might thus be taken up preferentially by clathrin-independent endocytosis, or might be excluded from clathrin-coated pits.

Second, one widely used criterion for a functional involvement of lipid rafts is pharmacological depletion of cholesterol from cell membranes. Such treatment reduces the propensity of proteins and lipids to accumulate in DRM fractions (Simons and Ikonen, 1997; Edidin, 2003), and cholesterol depletion blocks uptake of many of the molecules reported to be internalized independently of clathrin-coated pits (Lamaze et al., 2001; Nichols et al., 2001; Nichols and Lippincott-Schwartz, 2001; Purî et al., 2001; Sabharanjak et al., 2002; Di Guglielmo et al., 2003; Venkatesan et al., 2003). Interpretation of these data in terms of a functional role for lipid rafts is somewhat complicated by the facts that cholesterol depletion is likely to have more effects on cell physiology than specifically perturbing lipid rafts and, when carried out stringently enough, will also inhibit uptake by clathrin-coated pits (Rodal et al., 1999; Subtil et al., 1999). A sceptic might additionally argue that, even in those cases where a clear differential effect on clathrin-independent uptake has been observed, this does not directly demonstrate a requirement for clustering of the relevant molecules in the same microdomain. Nevertheless, the greater cholesterol sensitivity of clathrin-independent endocytosis is likely to reflect differences in sensitivity to biophysical properties of the plasma membrane and is consistent with a functional role for rafts.

Third, caveolae, defined as morphological entities (small, uncoated invaginations in the plasma membrane), frequently contain the membrane protein caveolin 1 (Rotheberg et al., 1992; Harder and Simons, 1997), and the findings that caveolin 1 binds to cholesterol and is unusually resistant to detergent extraction (Sargiacomo et al., 1993; Murata et al., 1995) led to the suggestion that caveolae constitute a type of lipid raft (Harder and Simons, 1997). The discoveries that treatment with a phosphatase inhibitor (okadaic acid) apparently causes budding of caveolae and associated proteins into the cell (Parton et al., 1994; Thomsen et al., 2002) and that dynamin, a GTPase involved in budding of clathrin-coated pits, is, also found at the necks of caveolae (Oh et al., 1998; Henley et al., 1998) has generated much interest in the potential role of these structures as endocytic vesicles. Consequently, much research on cholesterol-sensitive, clathrin-independent endocytosis has focused on uptake in caveolae rather than other potential mechanisms (Fig. 1).

**Caveolar endocytosis**

Caveolae were identified morphologically a long time before the identification of caveolins as defining protein components of these structures (Stan, 2002). Expression of caveolin 1 is sufficient to generate caveolae in cells previously lacking such structures (Lipardi et al., 1998), and caveolin-null mice do not have obvious caveolae (Drab et al., 2001; Razani et al., 2001a). It has thus become commonplace to equate caveolar function with that of caveolins. This view has recently been challenged (Nabi and Le, 2003), and it is important to bear the distinction in mind. The appearance of caveolae as remarkably uniform invaginations with dynamin localized to their necks is easily reconciled with the idea that these structures frequently bud from the plasma membrane. However, although caveolar budding is widely thought to occur at a high rate in endothelial cells, the notion that this is generally the case was challenged by studies on the dynamics of green fluorescent protein (GFP)-tagged caveolin 1, which behaves in the same way as endogenous caveolin 1 (Pelkmans et al., 2001; Thomsen et al., 2002; Mundy et al., 2002). Van Deurs and colleagues observed that caveolin-1-GFP is largely immobile at the plasma membrane in standard tissue culture cells, and that exchange of caveolin-1-GFP between plasma membrane and

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**Fig. 1.** Different types of endocytosis. Four main types of endocytosis are shown (which might be an over-simplification). Caveolar endocytosis and clathrin-mediated endocytosis both require the GTPase dynamin 1, whereas other types of endocytosis do not. As implied by the figure, it is not clear how many different types of dynamin-independent pinocytic or macropinocytic mechanisms there are (discussed in the text).
intracellular pools is surprisingly slow compared with rates of clathrin-independent endocytosis (Thomsen et al., 2002). This then calls into question the extent to which caveolae constitutively bud from the plasma membrane in these cells. Moreover, caveolin 1 siRNA experiments suggest that caveolin 1 is not necessary for clathrin-independent endocytosis (Nichols, 2002), as does the fact that cholesterol-sensitive, clathrin-independent endocytosis occurs similarly in cell types that naturally lack both caveolin 1 and abundant caveolar structures (Orlandi and Fishman, 1998; Lamaze et al., 2001).

Two non-exclusive models go some way towards explaining these observations (Fig. 2). Experiments based largely on overexpression of caveolin 1 suggest that caveolin 1 negatively regulates caveolar budding. Uptake of gp60, a receptor for albumin localized to caveola in endothelial cells, is inhibited by overexpression of caveolin 1 (Minshall et al., 2000). Clathrin-independent uptake of cholera toxin B subunit (CTB), which binds to the sphingolipid GM1, is also decreased by such overexpression (Le and Nabi, 2003). Conversely, decreased expression of caveolin 1 in transformed NIH-3T3 cells correlates with increased clathrin-independent uptake of autocrine motility factor, and this effect is reversed when caveolin 1 expression is artificially increased (Le et al., 2002). These data led Nabi and Le to propose that morphologically defined caveolar invaginations in the plasma membrane that do not contain caveolin 1 exist as transient intermediates during budding into the cell, and that these intermediates are stabilized by caveolin 1, thereby slowing the rate of budding (Nabi and Le, 2003). This model is appealing because it provides a good explanation for the observation that cells lacking caveolin 1 lack abundant caveolae but maintain high rates of endocytosis of markers that, in caveolin-positive cells, are internalized by clathrin-independent, caveola-related mechanisms (Orlandi and Fishman, 1998; Lamaze et al., 2001; Nichols, 2002). A second way of explaining the data is to view caveolar endocytosis as a highly regulated process. There could, for example, be two functionally distinct populations of caveolin-positive caveolae, one population being largely static and the other actively budding from the plasma membrane. Little published data directly support this model, but live-cell microscopy does reveal the existence of a small pool of very active caveolin-containing vesicles (Mundy et al., 2002). It is worth reiterating that these models are in no way exclusive and emphasizing that both invoke currently uncharacterized machinery to generate caveolar invaginations, or to regulate budding of caveolin-containing caveolae (Fig. 2). Discovery of further proteins required for caveolar endocytosis is clearly required to facilitate further progress in this area.

Caveolar budding might be regulated by reversible phosphorylation. Treatment with a phosphatase inhibitor (okadaic acid) causes massive mobilization of previously static caveolae, and a Src-family kinase inhibitor, genistein, blocks presumptively caveolar endocytosis (Parton et al., 1994; Puri et al., 2001; Thomsen et al., 2002; Sharma et al., 2003). How these effects are mediated is not clear. Caveolin 1 is phosphorylated on at least one tyrosine residue (Y14) in response to a variety of stimuli, including insulin, angiotensin II, osmotic shock and oxidative stress (Mastick et al., 1995; Aoki et al., 1999; Volonte et al., 2001), probably by non-receptor tyrosine kinases such as c-Fyn, c-Src and c-Abl (Sanguinetti and Mastick, 2003). However, caveolin 1 is thought to interact with several different signalling receptors (reviewed by Liu et al., 2002), and a direct functional role for caveolin 1 phosphorylation in endocytosis, rather than an interaction with signalling molecules (Cao et al., 2002), has yet to be demonstrated.

**Caveosomes**

Although the details of how caveolae bud from the plasma membrane need to be determined, it is now clear that uptake by such a clathrin-independent mechanism can lead to delivery to caveolin-1-containing endosomes, termed caveosomes (Fig. 3) (Pelkmans et al., 2001; Pelkmans and Helenius, 2002). Endocytosis to caveosomes is blocked by overexpression of a dynamin 2 K44A mutant and by cholesterol depletion, although neither of these treatments is likely to be highly specific (Pelkmans et al., 2001; Nichols, 2002). Caveosomes are separate from the early and recycling endosomes fed by uptake from clathrin-coated pits and were first characterized as intermediates in the trafficking of SV40 virus from the cell surface to the endoplasmic reticulum (ER) (Pelkmans et al., 2001). They are also likely to function constitutively in non-infected cells during transport of sphingolipids and GPI-linked proteins from the plasma membrane to the Golgi apparatus (Puri et al., 2001; Nichols, 2002). Several more recent papers have focused on the role of trafficking via caveolin-positive endosomes without using the term caveosome. For simplicity, I will refer to all caveolin-1-containing endosomes as caveosomes.

The emergence of caveosomes as a distinct organelle raises the question of the functional significance of uptake by a caveolar mechanism as opposed to clathrin-coated pits. The idea that different types of endocytosis have markedly different
functions is supported by work on the uptake of cholera toxin (Orlandi and Fishman, 1998; Nichols et al., 2001). For cholera toxin to be toxic it must reach the Golgi apparatus (Orlandi and Fishman, 1998). Several studies have now shown that cholera toxin is taken up into the cell by multiple mechanisms (Torgersen et al., 2001; Wolf et al., 2002). One key experiment, by Orlandi and Fishman, used pharmacological perturbation of uptake via clathrin-coated pits or caveolae to show that changes in total uptake of the toxin do not simply correlate with changes in its toxicity. Rather, a block in uptake via clathrin-coated pits has little effect on toxicity, whereas selective inhibition of caveolar (cholesterol-sensitive) endocytosis is sufficient to prevent toxicity (Orlandi and Fishman, 1998). These data argue against significant intracellular mixing of toxin taken up through caveolar endocytosis and clathrin-coated pits.

Recent experiments following endocytosis and signalling from the transforming growth factor β (TGFβ) receptor also highlight a functional distinction between delivery to caveosomes and classical early endosomes (Di Guglielmo et al., 2003). Wrana and colleagues showed that the TGFβ receptor is constitutively endocytosed both by clathrin-coated pits into EEA1-positive early endosomes and by a clathrin-independent mechanism into caveosomes. Signal transduction by the TGFβ receptor is affected differently by blocks in these different modes of endocytosis: uptake by clathrin-coated pits promotes TGFβ signalling, whereas uptake into caveosomes leads to receptor turnover. Accordingly, accessory proteins involved in TGFβ receptor signalling, such as SARA and Smad2, are found in EEA1-positive endosomes, and proteins that target the receptor for degradation, such as Smad7 and Smurf2, are localized to caveosomes. TGFβ receptor can bind directly to caveolin 1, and this interaction could play a role in the targeting of TGFβ receptor to caveolin-1-positive organelles (Razani et al., 2001b). How this interaction might be regulated, and how a significant pool of TGFβ receptor remains free to enter clathrin-coated pits, is not fully understood. It seems unlikely that caveolin 1 is critical for TGFβ signalling per se, because TGFβ receptor presumably continues to function more or less normally in mice lacking caveolin 1, which are surprisingly normal developmentally (Drab et al., 2001; Razani et al., 2001a). In addition to TGFβ receptor, caveolin 1 is thought to interact with a diverse array of signalling receptors, including the insulin receptor (Mastick et al., 1995; Bickel, 2002). Endocytosis of many of these molecules into caveosomes has yet to be investigated, but the relatively subtle phenotypes of caveolin-1-null mice, in terms of, for example, insulin tolerance and fatty acid metabolism (Cohen et al., 2003), again suggest that the function of these various interactions may not be essential for signalling, but rather, as is apparently the case for TGFβ receptor, may be more to do with regulating receptor turnover. This suggestion is supported by the observation of significantly reduced levels of insulin receptor in caveolin-1-null mice. Moreover, this observation agrees well with the ideas that caveolin 1 negatively regulates endocytosis and that endocytosis to caveosomes is involved in targeting receptors for degradation (Nabi and Le, 2003; Di Guglielmo et al., 2003).

Studies suggesting that ligand-induced uptake of chemokine receptors CCR5 and CXCR4 occurs by different mechanisms provide an additional indication that uptake into caveosomes and by clathrin-coated pits have different consequences. CCR5 accumulates in caveosomes whereas CXCR4 uses clathrin-coated pits to enter the cell (Venkatesan et al., 2003). Venkatesan et al. suggest that this is important for modulating the signalling potential of the receptors. Uptake to caveosomes is a slow process relative to uptake by clathrin-coated pits, which is consistent with the study of TGFβ signalling described above, as well as previous work (Nichols et al., 2001; Nichols, 2002). Another set of experiments suggesting a specific function for uptake to caveosomes followed the uptake of the GPI-linked proteoglycan glypicin 1 (Cheng et al., 2002). Dissection of the endocytic cycle followed by glypicin 1 showed that delivery of glypicin 1 to caveosomes is specifically associated with exposure to heparanase activity and progressive S-nitrosylation of the protein core. This suggests that the appropriate enzymes are somehow recruited to caveosomes (Cheng et al., 2002).

The studies discussed above raise several intriguing questions about the properties of caveosomes. It seems likely that specific proteins can be recruited to caveosomes, but how this is achieved is not at all clear. Classical, transferrin-containing endosomes are characterized by the presence of phosphatidylinositol-3-phosphate (PtdIns3P) (Simonsen et al., 2001), but the fact that proteins containing a domain that specifically binds to this lipid, the FYVE domain (Misra et al., 2001; Di Guglielmo et al., 2003), are not recruited to caveosomes suggests a different mechanism. A related question is to what extent, and how, proteins and lipids are sorted during endocytosis to caveosomes.

**Rafts and sorting during clathrin-independent endocytosis**

Before trying to relate data on sorting into different endocytic pathways to potential roles for clustering in lipid rafts one should consider wider questions about the nature and function...
of lipid rafts. One principal requirement in this on-going debate is to establish a better understanding of how the membrane found in DRM fractions is organized in the intact plasma membranes of living cells. As already mentioned, there are various competing viewpoints. At one extreme, recent data, based mainly on nuclear magnetic resonance (NMR) and calorimetric measurements, suggest that addition of cold Triton X-100 is sufficient to segregate sphingomyelin and cholesterol from other lipids in previously well-mixed membrane preparations (Heerklotz, 2002; Heerklotz et al., 2003). The composition of DRM fractions might thus reflect properties of the detergent-lipid interaction, not pre-existing membrane domains (Edidin, 2001; Edidin, 2003). Separate experiments looking at the effects of different detergents on raft composition reveal that raft composition is highly sensitive to changes in detergent extraction procedure, which is certainly consistent with this view (Roper et al., 2000; Schuck et al., 2003). The other extreme view characterizes lipid rafts as stable microdomains ~50 nm in diameter, enriched in cholesterol, glycosphingolipids, sphingomyelin, GPI-linked proteins and at least some transmembrane proteins (Simons and Ikonen, 1997). In vivo evidence in support of this model comes from measurements of fluorescence resonance energy transfer (FRET) between GPI-linked proteins (Varma and Mayor, 1998) and between lipid-anchored proteins on the inner leaflet of the plasma membrane (Zacharias et al., 2002), and from measurements of local membrane viscosity of raft and non-raft probes (Pralle et al., 2000), as well as other sources (Simons and Ikonen, 1997; Maxfield, 2002). A third, intermediate model postulates that raft proteins are, in vivo, surrounded by a shell of glycosphingolipids or other raft lipids, and that this shell is sufficient to make the protein resistant to extraction from membranes with detergent. Thus, this ‘lipid shell’ model does not require raft proteins themselves to be functionally clustered although, given appropriate stimuli and crosslinking protein components, this could be the case (Anderson and Jacobson, 2002). The idea that membrane proteins interact with an annulus of surrounding lipid is an old one, and the properties of the protein membrane-spanning region, or indeed other regions of the protein, might well lead to preferential interaction with one type of lipid (Borbát et al., 2001).

One can of course imagine combinations of the above models, and there may be more than one type of lipid raft. A consideration of the way in which cells sort biochemically defined raft markers to different endocytic pathways is potentially useful in trying to begin to discriminate between the various possibilities. Some raft proteins and lipids – for example, TGFβ receptor and GM1, the sphingolipid to which CTB binds – enter cells by both clathrin-coated pits and uptake into caveosomes (Torgersen et al., 2001; Nichols, 2002; Di Guglielmo et al., 2003). However, others – for example exogenously added short-acyl chain fluorescent sphingolipid analogs (Sharma et al., 2003), chemokine receptor CCR5 (which is palmitoylated) (Venkatesan et al., 2003), and at least some GPI-linked proteins (Nichols, 2002) – are largely endocytosed into caveosomes. It has also been reported that GPI-linked proteins are taken up by a clathrin-independent mechanism into organelles devoid of caveolin 1 (termed GPI-enriched endosomal compartments, GEECs; Saharanjak et al. (Saharanjak et al., 2002); discussed further below]. The general picture that emerges is one in which molecules that can partition into biochemically defined rafts have a variety of different endocytic itineraries. Whether clathrin-independent endocytic mechanisms such as caveolar endocytosis are truly specific for raft proteins and lipids is not fully understood, because uptake of non-raft molecules has yet to be carefully studied in this respect. The best characterized is the transferrin receptor, which is largely excluded from caveosomes (Pelkmans et al., 2001). However, this may be because the receptor is largely concentrated within clathrin-coated pits, little free receptor being found in the rest of the plasma membrane. The issue of whether non-raft proteins are actively excluded during uptake to caveosomes thus remains to be addressed. Given the diversity of endocytic trafficking outlined above, raft molecules are unlikely to reside exclusively in the same stable microdomain. In addition, the finding that the same molecule can enter the cell by different mechanisms complicates the simple model where raft membrane is endocytosed to caveosomes and non-raft membrane enters by clathrin-coated pits. It is probably premature to postulate a single raft-specific or raft-mediated endocytic pathway, and better techniques to assay incorporation into rafts and to perturb potential raft functions are required.

Experiments carried out in our laboratory have used measurements of FRET as a tool to follow organization of GPI-linked proteins and CTB relative to clathrin-coated pits and caveolin in the plasma membrane of live cells (Nichols, 2003). A significant fraction of total membrane CTB is likely to be present in cholesterol-sensitive clusters, and these clusters are quite efficiently excluded from clathrin-coated pits. However, some CTB is clearly internalized by clathrin-coated pits, which leads to the hypothesis that CTB is found in two pools within the plasma membrane – clustered and unclustered – and that only the unclustered CTB can enter clathrin-coated pits. Surprisingly, no change in the distribution of CTB appears to be associated with regions of the plasma membrane containing a high concentration of caveolae (see Parton, 1994). These data are then consistent with a model where exclusion from clathrin-coated pits is an important factor in the sorting of raft markers – indeed, exclusion from clathrin-coated pits might be sufficient to generate apparently selective uptake by other mechanisms. At least some GPI-linked proteins are also likely to be excluded from clathrin-coated pits (Nichols et al., 2001), but the extent to which GPI-linked proteins are themselves clustered in the plasma membrane is controversial and requires further investigation (Kenworthy and Edidin, 1998; Varma and Mayor, 1998; Kenworthy et al., 2000; Dietrich et al., 2002). Thus, although one can conclude that clusters of lipid are excluded from clathrin-coated pits, the question of whether these clusters contain more than one protein molecule remains to be addressed, and all of the current data are as consistent with the lipid shell model outlined above as with the more typical view of 50 nm rafts.

It may be that crosslinking of membrane components by other factors is sufficient to generate raft-like clusters. Two separate papers show that specific, protein-mediated, crosslinking of membrane proteins [anthrax toxin receptor (Abrami et al., 2003) and the B cell receptor (Stoddart et al., 2002)] can cause incorporation into biochemically defined rafts, and is associated with uptake by clathrin-coated pits. However, it is hard to be confident that acquisition of resistance
Further clathrin-independent endocytosis has focused on caveolae and caveolin-containing endosomes. It is unlikely that this is the whole story. Several studies on caveolar endocytosis agree that this mechanism is sensitive to overexpression of the K44A mutant of the GTPase dynamin 2 (Pelkmans and Helenius, 2002; Nabi and Le, 2003), but there are several reports of endocytosis that is independent of both such overexpression and of clathrin-coated pits. The small GTPase ARF6 regulates recycling of membrane between the plasma membrane and endosomal compartments during macropinocytosis, which may be an important mode of endocytosis in resting as well as in activated or ruffling cells (Radhakrishna and Donaldson, 1997; Radhakrishna et al., 1999). Overexpression of either constitutively active ARF6, or the EFA6 guanine nucleotide exchange factor for ARF6, results in both ruffling and the accumulation of macropinosomes (Brown et al., 2001). In unstimulated cells, ARF6 is found at least in part on endosomes distinct from those labelled by transferrin, and is likely to regulate traffic from these ARF6-bearing endosomes to the classical endosomal pathway (Naslavsky et al., 2003). How membrane is constitutively delivered to ARF6-bearing endosomes is not clear, but overexpression of the dynamin 2 K44A does not block this process (Naslavsky et al., 2003), and it may be that machinery similar to that which generates macropinosomes or indeed phagosomes in activated cells is used (Niedergang et al., 2003). Proteins used as markers for trafficking to ARF6-bearing endosomes are generally not found in rafts. However, endocytosis of GPI-linked proteins independently of dynamin has been reported, and GPI-linked proteins appear to accumulate in separate endosomal structures, as well as in caveosomes (Sabharanjak et al., 2002). The requirement for Cdc42 in this process, as well as the fact that such GPI-enriched endosomes (GEECs) readily accumulate fluid phase, hints at a relationship to ruffle-associated macropinosomes, but the role of ARF6 in uptake to such GPI-positive endosomes has not been investigated in detail. In any event, there must be at least one constitutive endocytic pathway that, unlike caveolar/caveosomal endocytosis, is not blocked by overexpression of the dynamin 2 K44A mutant. Whether recruitment to lipid rafts is required for this process is unknown; if it turns out that ARF6-bearing endosomes and GEECs are related or equivalent, then this is unlikely, but the situation might well be more complex. In any event, at this stage, one can conclude that there are probably at least two clathrin-independent endocytic pathways operating constitutively in many cell types, and that the use of overexpressed dynamin 2 K44A offers a way of discriminating between these pathways (Fig. 1).

Overexpression of dominant-negative mutants of the small GTPases RhoA, Rac and Cdc42 has also been used as a tool to distinguish different endocytic pathways. As mentioned above, dominant-negative Cdc42 blocks uptake of GPI-linked proteins to GEECs (Sabharanjak et al., 2002). Internalization of interleukin 2 (IL-2) receptors, which like GPI-linked proteins are found in biochemically defined rafts, is specifically blocked by the equivalent mutant of RhoA, which indicates another potential endocytic mechanism (Lamaze et al., 2001). IL-2 receptors are internalized normally in cells lacking caveolin 1, but the suggestion that caveolar endocytosis does not necessarily require caveolin 1 protein (Nichols, 2002; Nabi and Le, 2003) means that IL-2 receptors might still use this pathway and accumulate in caveosomes; this question remains to be answered. The complexities in the use of Rho GTPases as specific reagents to block endocytosis (Qualmann and Mellor, 2003) are highlighted by the findings that constitutively activated RhoA and Rac can downregulate clathrin-mediated uptake (Lamaze et al., 1996) and that dominant-negative Rac (but not Cdc42 or RhoA) can block clathrin-independent uptake of the ATPase ATP7A (Cobbold et al., 2003). The identification of effectors, exchange factors or activators for these GTPases specific to a particular endocytic pathway would be a significant step forward.

Conclusions
The emergence of caveosomes as functionally significant organelles in membrane signalling and trafficking processes highlights the importance of clathrin-independent endocytosis, and raises the as-yet-unanswered question of whether endocytosis to these organelles is in any way specific for, or dependent on, raft microdomains. The finding that clathrin-independent endocytic pathways are apparently more sensitive to depletion of cholesterol from cell membranes than is uptake by clathrin-coated pits suggests that clathrin-independent endocytic pathways have extra requirements in terms of biophysical properties of the membrane itself. This observation is compatible with the notion of raft-specific or raft-mediated endocytosis, but is open to other interpretations that do not invoke lipid rafts. Data from a variety of laboratories imply that lipid raft markers are taken up by multiple pathways, not just to caveosomes, and it is not really possible at this stage to label one particular endocytic pathway as raft specific or raft mediated. There is a clear requirement for better definition of molecular mechanisms for clathrin-independent endocytosis and for alternatives to the biochemical definition of lipid rafts.

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