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Mechanisms of Endocytosis

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Key Words

caveolae, clathrin-mediated endocytosis, clathrin-independent endocytosis, dynamin, small G proteins

Abstract

Endocytic mechanisms control the lipid and protein composition of the plasma membrane, thereby regulating how cells interact with their environments. Here, we review what is known about mammalian endocytic mechanisms, with focus on the cellular proteins that control these events. We discuss the well-studied clathrin-mediated endocytic mechanisms and dissect endocytic pathways that proceed independently of clathrin. These clathrin-independent pathways include the CLIC/GEEC endocytic pathway, arf6-dependent endocytosis, flotillin-dependent endocytosis, macropinocytosis, circular doral ruffles, phagocytosis, and trans-endocytosis. We also critically review the role of caveolae and caveolin1 in endocytosis. We highlight the roles of lipids, membrane curvature-modulating proteins, small G proteins, actin, and dynamin in endocytic pathways. We discuss the functional relevance of distinct endocytic pathways and emphasize the importance of studying these pathways to understand human disease processes.

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INTRODUCTION

With the advent of electron microscopy (EM) came a general appreciation of the enormous complexity of cellular membrane anatomy. Membranes allow the compartmentalization of cellular chemistry, either by the specific accumulation of proteins on their surfaces (increasing the avidity of protein-protein interactions) or by creating diffusion barriers between their lumina and the cytoplasm. Changes in the distribution, protein and lipid composition, and luminal content of these membranes, through the highly dynamic fission and fusion reactions that occur from and between such compartments, are primary regulators of many, if not most, extranuclear cell biological processes. To determine how these changes are effected, a large membrane trafficking community has emerged and has contributed invaluably to the understanding of many fundamental aspects of cell biology and physiology, in addition to many disease processes. This review focuses on the molecular characterization of endocytic pathways and the interrelationships between these pathways.

The outer leaflet of the plasma membrane is the surface through which each cell communicates with its environment, and in order to appropriately respond to, or affect, its environment, its composition must be tightly regulated by the cell. Endocytosis describes the de novo production of internal membranes from the plasma membrane lipid bilayer. In so doing, plasma membrane lipids and integral proteins and extracellular fluid become fully internalized into the cell. It can be considered the morphological opposite of exocytosis, which describes the fusion of entirely internal membranes with the plasma membrane. In so doing, this process expels specific chemicals to the extracellular space and delivers lipids and proteins to the plasma membrane. It is the control (by cellular proteins) of endocytosis from, and exocytosis to, the plasma membrane that allows the interactions between the cell and its environment to be precisely regulated. For example, endocytosis of transmembrane receptors (which removes them from the surface where they are able to interact with extracellular cues) regulates the long-term sensitivity of cells to their specific ligands. However, endocytosis does not simply negatively regulate interactions with the external world. The processes, which

endocytosis plays a key role in regulating, continue to expand and include processes as seemingly disparate as mitosis, antigen presentation, and cell migration. Furthermore, it is becoming apparent that endocytosis plays key roles in the positive regulation of many intracellular signaling cascades (1). In addition, pathogens often exploit endocytic routes to mediate their internalization into cells (2). Although much is known about the cargoes for endocytic structures, the specific mechanisms by which these cargoes are recruited and internalized are less clear. There exists many routes of endocytic uptake into cells.

Much work has focused on clathrin-mediated endocytosis (CME). The mechanisms by which the proteins involved in this process recruit cargo into developing clathrin-coated pits (CCPs), and subsequently form clathrin-coated vesicles (CCVs), are becoming increasingly understood (3, 4). Many proteins that are involved in the formation of CME intermediates have been identified and characterized, and researchers in the field can therefore make use of specific markers along the pathway and specific molecular tools to interfere with the process in a defined manner. These tools include RNAi techniques, although it should be noted that clathrin and the adaptor AP2 are very stable, and multiple rounds of treatment are required to completely deplete cells of these proteins. Inadequate depletion, coupled with the fact that transferrin uptake (used widely to assess CME efficiency) is incredibly sensitive to perturbation of such proteins, has led to confusion in the endocytic community about the role of CME in certain processes, and therefore, caution is advised when interpreting data using these approaches. Even the most profound reductions in transferrin uptake are not directly indicative of a total loss of CME events. In our hands, a useful tool to inhibit CME is overexpression of the C terminus of AP180 (5). AP180 is a neuronal clathrin adaptor that links clathrin to PtdIns(4,5)P_2 in the membrane. The region being overexpressed is the clathrin-binding domain without the membrane anchor.

Alongside a frequent misconception that CME is the only major mechanism by which endocytosis occurs, for many years, the term receptor-mediated endocytosis (RME) has been used, often synonymously with CME. Owing to the clarity beginning to emerge from studies of endocytic events, we urge abandonment of the term RME because methods are readily available to easily dissect the method(s) of endocytosis used for the cargo of interest; a scheme for endocytic classification can be found in Reference 6 (see also **Figure 1** and **Table 1**). Although CME is certainly an extremely important endocytic mechanism, accounting for a large proportion of endocytic events, an ever expanding array of cargoes has been shown to undergo endocytosis in a clathrin-independent manner (7). EM techniques have suggested that much of the budding that occurs from the plasma membrane does not appear to require the formation of the clathrin coat, which is readily observable around CCPs and CCVs. Indeed, cells devoid of CME events are capable of endocytosis. Clathrin-independent endocytosis itself has been further dissected into seemingly distinct pathways, based on the reliance of these pathways on certain proteins and lipids, their differential drug sensitivities and their abilities to internalize particular cargoes (6, 8). Clathrin-dependent and -independent endocytosis are expected to be roughly equiprevalent modes of internalization in fibroblastic cells, and the relative proportions of each will presumably differ in other cell types owing to their adaptions for specific functions. Although we describe the molecular players of these pathways below (see also **Table 1**), it is important to ask: What are the functions of different pathways? And how are the different pathways adapted/specialized for function? These distinct pathways create endosomal compartments with distinct lumina and surfaces to allow the differential modulation of intracellular events, including the possibility of delivering cargoes to distinct intracellular destinations.

Here, we review what is known about endocytic pathways in mammalian cells. This review does not attempt to be exhaustive;

CME: clathrin-mediated endocytosis

CCP: clathrin-coated pit

CCV: clathrin-coated vesicle

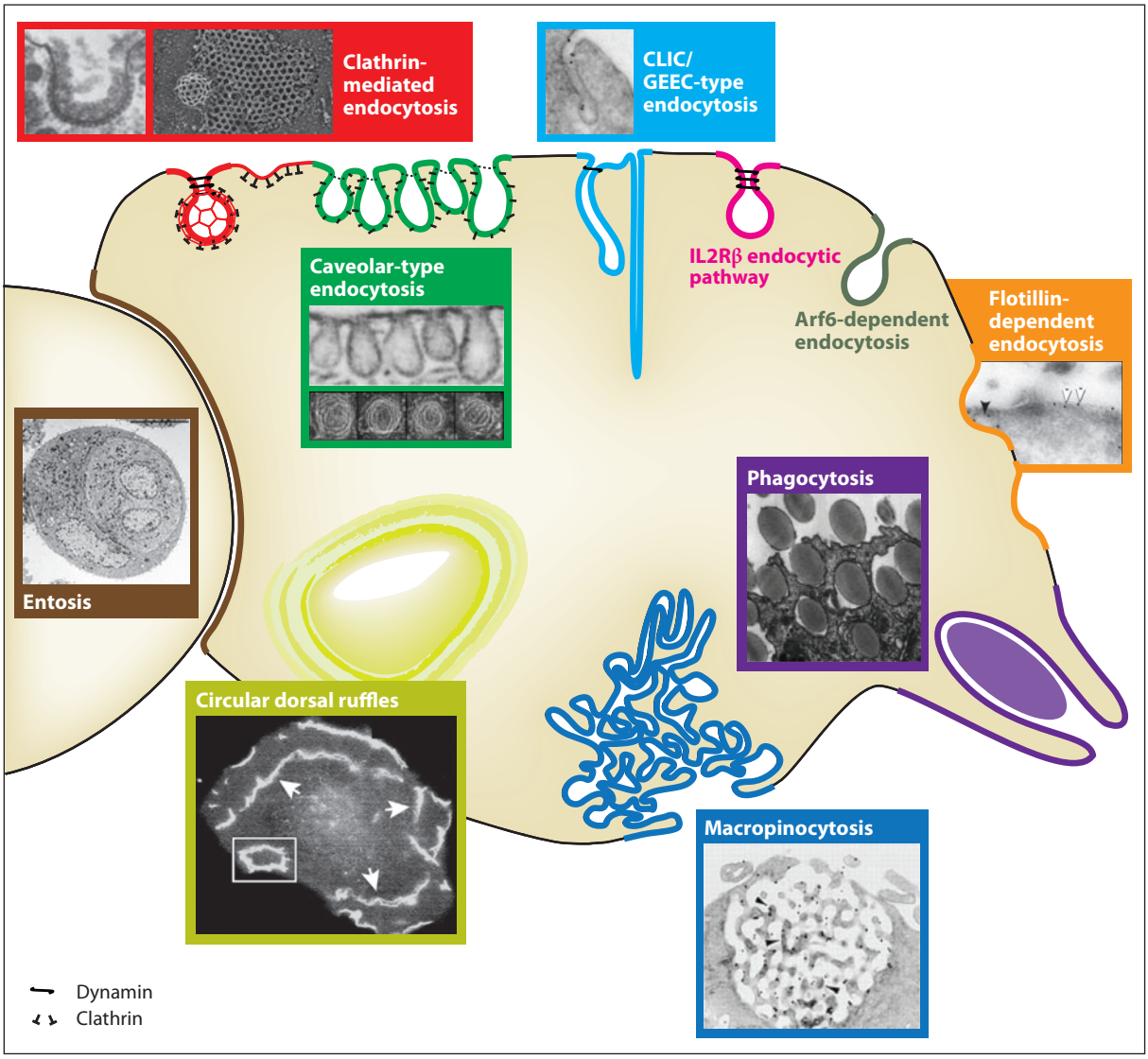


Figure 1

Putative endocytic portals. Transmission and scanning electron micrographs of structures known or thought to be involved in endocytic events. Note the morphological variability of these endocytic structures. Figures are reproduced or modified from the following sources: Clathrin-mediated endocytosis [provided by John Heuser (9)], caveolar-type endocytosis (11), CLIC/GEEC-type endocytosis (6, 7), the putative flotillin-associated endocytic structures (65), phagocytosis [provided by Robert North], macropinocytosis (104), circular dorsal ruffles (99), and entosis (120). The arrowheads in the flotillin-dependent endocytosis image point to immunoreactivity for green fluorescent protein (GFP) in cells expressing both flotillin1 and -2 GFP. Note that there is controversy surrounding the morphology of flotillin-positive regions of the plasma membrane and that the morphologies of the IL2Rβ pathway and arf6-dependent pathway are also unclear. The arrows in the circular dorsal ruffle image show ruffled structures that appear to evolve into circular structures such as that shown within the box. The arrowheads in macropinocytic picture indicate cytoskeletal elements.

Table 1 The molecular characteristics of endocytic events and the known morphological and molecular features of each of the endocytic pathways described in this review[a]

Endocytic mechanisms	Morphology	Implicated cargoes[b]	Small G-protein dependence	Dynamin implicated?	Other proteins implicated
Clathrin mediated	Vesicular	RTKs, GPCRs, transferrin receptor, anthrax toxin	Rab5, Arf6 implicated	Well established	Clathrin, AP2, epsin, SNX9, synaptojanin, actin amphiphysin, plus many others
Caveolae-/ caveolin1-dependent	Vesicular/ tubulovesicular	CTxB, SV40, GPI-linked proteins	Unclear (may regulate cdc42 activity)	Some evidence	Caveolins, PTRF, src, PKC, actin (many signaling proteins localize to these sites)
CLIC/GEEC	Tubular/ring like	Fluid phase markers, CTxB, GPI-linked proteins	Cdc42, Arf1	Not as yet	ARHGAP10, actin, GRAF1
IL2Rβ pathway	Vesicular?	IL2Rβ, FCεRI, Kir3.4, γc-cytokine receptor	RhoA, Rac1	Implicated	PAK1, PAK2
Arf6 dependent	Vesicular/tubular	MHC class I proteins, CD59, carboxypeptidaseE	Arf6	Not as yet	Unclear as yet
Flotillin dependent	Vesicular	CTxB, CD59, proteoglycans	Unclear	Implicated but unclear	Flotillin 1 and 2
Phagocytosis	Cargo shaped	Pathogens, apoptotic remnants	Arf6/cdc42/rac1/rhoA (depending on type)	Implicated	Actin, IQGAP1, amphiphysin1, Rho kinase, adhesion proteins
Macropinocytosis	Highly ruffled	Fluid phase markers, RTKs	Rac1	Not as yet (CtBP1/BARS implicated in scission)	Actin, PAK1, PI3K, Ras, Src, HDAC6
Circular dorsal ruffles	Highly ruffled	Fluid phase markers, RTKs	Unclear	Implicated	Cortactin, actin
Entosis	Cell shaped	Matrix-deligated cells	RhoA	Not as yet	Adherens junctions

[a]See the text for references.
[b]Abbreviations: CLIC, clathrin-independent carriers; GEEC, GPI-AP enriched early endosomal compartment; GPCRs, G protein--coupled receptors; GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex; RTK, receptor tyrosine kinase.

instead, we refer the reader to the ~64,000 papers identified by a PubMed search for endocytosis (as of January 2009)! Space limitations preclude a full discussion of all issues here, and we refer the reader to our **Supplementary Information** (follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**) where these discussions are continued, with a more extensive discussion of the importance to endocytosis of membrane lipids, membrane curvature-modulating proteins, cell adhesion, and cell migration. We begin with a discussion of endocytic membrane morphology before focusing on the molecular cell biology of distinct endocytic mechanisms. Much has been deciphered from studies following the uptake of bacterial toxins and pathogens, and these findings are also reviewed. We then discuss requirements for endocytic pathways and highlight the critical roles for lipids, membrane curvature-modulating proteins, small and large G

proteins, and the cytoskeleton in endocytic events. Finally, we discuss the function of endocytosis, the cell biological and physiological relevance of distinct endocytic pathways, and the relevance of endocytic mechanisms to the understanding and treatment of human disease. We must point out that great care should be taken when attempting to correlate observations that have been made in distinct cell types. Because distinct endocytic pathways will have their own functions, different types of specialized cells will therefore have varying proportions of plasma membrane turnover occurring by each of these mechanisms.

MORPHOLOGY OF PUTATIVE ENDOCYTIC PORTALS

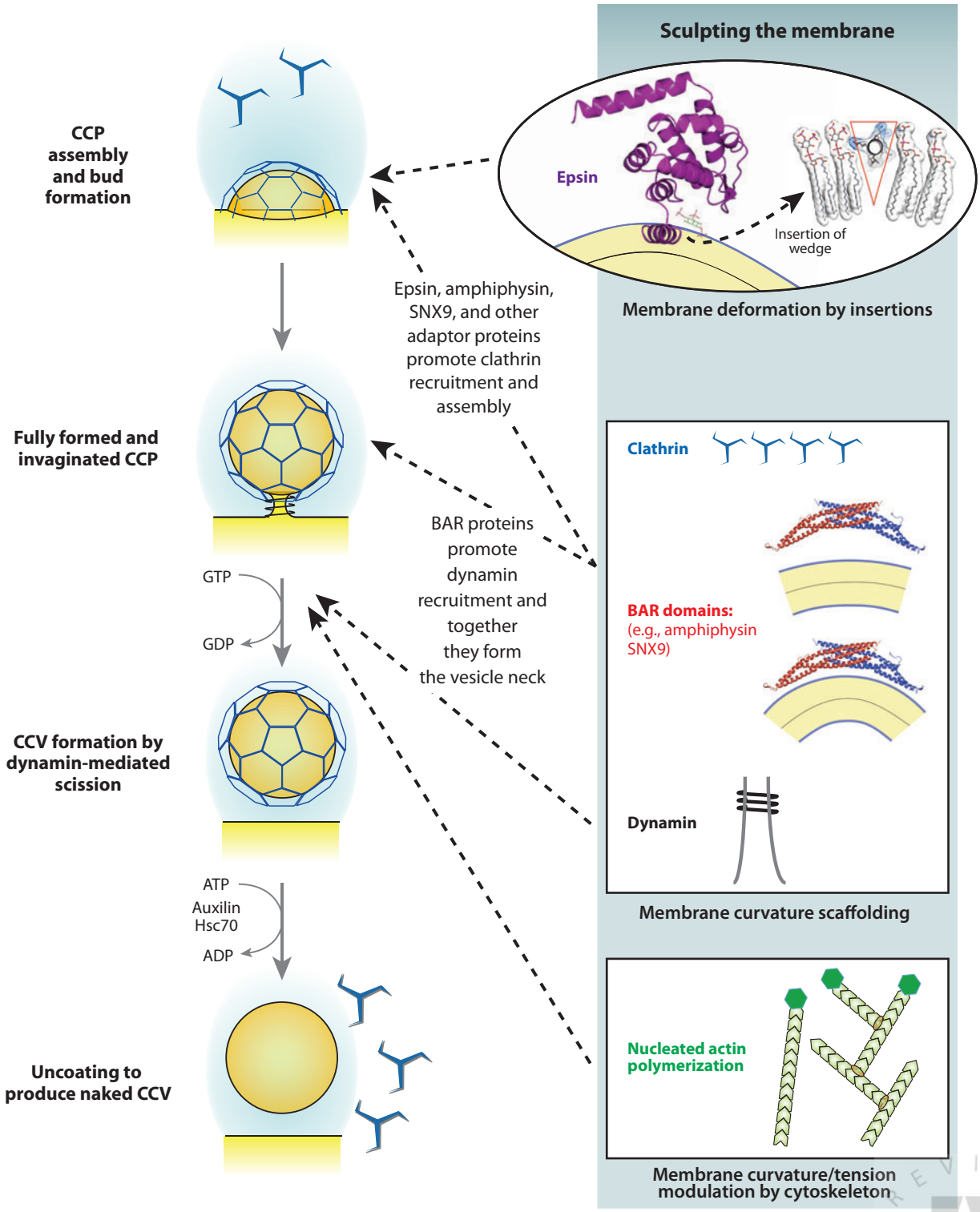
A budding structure from the plasma membrane is a necessary prerequisite for any endocytic pathway, and major known structures of this type are shown in **Figure 1**. Because clathrin coats are visible around spherical membrane buds and endocytosed vesicles by EM (**Figure 1**) (9), it is possible to distinguish clathrin-independent budding events by the absence of such a coat. CME proceeds through a series of well-defined morphological intermediates, whereby a CCP undergoes progressive invagination before scission from the plasma membrane to form a CCV (**Figure 2**) (4, 9). EM, however, requires fixed samples, so it is difficult to ascertain whether all invaginated CCPs, and other membrane invaginations observed by this technique, subsequently undergo scission from the plasma membrane. Only with the development of total internal reflection fluorescence microscopy (TIR-FM), which allows the visualization in real time of the behavior of fluorescent proteins (including fluorescently labeled clathrin) at, or very close to, the basal plasma membrane of cells, have some of these issues been addressed.

The most commonly reported nonclathrin-coated plasma membrane buds are known as caveolae (for "little caves") (**Figure 1**). These flask-shaped invaginations exist on the surface of many mammalian cell types, are around 60–80 nm in diameter, and can constitute approximately a third of the plasma membrane area of the cells of some tissues, being especially abundant in smooth muscle, type I pneumocytes, fibroblasts, adipocytes, and endothelial cells (10). Under certain conditions a filamentous, spike-like coat can be observed around caveolae (**Figure 1**) (11). Unsurprisingly perhaps given their morphology, these structures have been extensively implicated in endocytic events (10) and can be followed, using fluorescent caveolin1 expression, in real time. Multicaveolar assemblies, where many caveolae are connected together and to the plasma membrane in structures resembling bunches of grapes, can be commonly observed.

Internalization can also take place by macropinocytosis, which usually occurs from highly ruffled regions of the plasma membrane. Ruffled extensions of the plasma membranes form around a region of extracellular fluid, with apparent subsequent internalization of this complete region (**Figure 1**). The membrane may not always be as extensively ruffled as we depict. The other textbook method of internalization, which occurs in specialized cells, is phagocytosis, whereby opsonized and particulate material can be taken up into cells by the progressive formation of invaginations around the cargo destined to be internalized, with or without the growth of enveloping membrane extensions (**Figure 1**). These processes involve the uptake of larger membrane areas than CME and caveolar mechanisms,

Figure 2

Membrane curvature modulation during endocytosis. Schematic diagram (*left panels*) demonstrating the stages of, and some of the key protein players in, the budding and scission of clathrin-coated pits (CCPs). After budding to form a clathrin-coated vesicle (CCV), the clathrin basket is removed by uncoating proteins to form a naked vesicle. On the right are illustrations of different mechanisms of membrane curvature generation and stabilization.



allowing them to be followed by phase contrast microscopy.

It is becoming increasingly clear that endocytosis into tubular, in addition to vesicular, compartments is a common event in mammalian cells (8, 12), and views of cellular anatomy should be adjusted accordingly. Certain lipid compartments, and the distribution of their components, can be highly sensitive to fixation conditions (G.J. Doherty & R. Lundmark, unpublished results, and see References 13 and 14), and this may have hampered their discovery. The sections taken for EM visualization bias observations toward seemingly spherical/ovoid structures because tubular structures will usually appear thus in a cross section. Elegant EM studies have preserved and identified prevalent tubular endocytic intermediates responsible for the internalization of a variety of ligands (**Figure 1**) (8). As with macropinocytic structures, these structures appear to have no electron-dense coat when viewed by EM. These pathways can be followed in real time using fluorescently labeled cargo proteins.

Morphological analysis of other endocytic pathways has also revealed endocytosis associated with dorsal ruffles, putative flotillin-associated invaginations, and cell-in-cell invasion that happens after cell-matrix deligation known as entosis (**Figure 1**).

CLATHRIN-MEDIATED ENDOCYTOSIS

In CME, a wide variety of transmembrane receptors and their ligands are packaged with the use of cargo adaptors into CCVs (see **Figure 2**). In addition to those used in endocytosis, CCVs can also bud from various intracellular compartments with the use of different adaptor proteins. Thus, in the cell, there are various forms of CCVs. Even at the plasma membrane we view CCV production as a a collection of closely related endocytic pathways, linked by the use of the coat protein clathrin (4). For CCP and CCV formation from the plasma membrane, there is a wide range of possible cargo adaptors and accessory proteins that can be used, and the process is not hardwired, either in requiring the same complement of accessory proteins in every cell or in being limited to a small subset of membrane cargoes. The diversity of cargoes and the diversity of adaptor and accessory proteins used to implement vesicle formation reflect the pathways' adaptations to tools suited to the materials being packaged. In some cells, the cargo complement may be rather limited, whereas at synapses, at least 20 different cargoes of diverse functions are packaged in the same vesicle with high fidelity. The flexibility and fluidity in the system is inherent in the design, where there are overlapping and shared functions of various adaptors and accessory proteins. A likely corollary of this is that there will be a range of dependence of cargo internalization upon individual adaptors and accessory proteins (4). Although in some situations, the importance of AP2 adaptors in plasma membrane CCV formation may be fundamental; however, given the design, this dependence is unlikely to be universal. The design also allows us to consider some accessory adaptors and protein networks modulating actin polymerization as "add on" modules, where their involvement may help differentiate the closely related pathways.

Adaptor and accessory proteins coordinate clathrin nucleation at sites of the plasma membrane, which are destined to be internalized (15). This nucleation promotes the polymerization of clathrin into curved lattices, and this consequently stabilizes the deformation of the attached membrane. Clathrin polymerization (coupled with the action of other proteins) aids in the formation and constriction of the vesicle neck, helping to bring the membranes surrounding the neck into close apposition. The membrane scission protein dynamin is a large GTPase, which forms a helical polymer around this constricted neck and, upon GTP hydrolysis, mediates the fission of the vesicle from the plasma membrane (16), irreversibly releasing the CCV into the interior of the cell. The clathrin basket is subsequently released from the vesicle by auxilin and hsc70. This naked vesicle then undergoes further trafficking within the cell before appropriate delivery

of its cargo through fusion with a destination intracellular compartment.

Dileucine- and tyrosine-based motifs on the cytoplasmic face of cargo molecules are both directly sensed by separate sites on the Adaptor Protein-2 (AP2) (previously Assembly Polypeptide-2) complex (3, 17), which links cargo to nucleating clathrin, such as is the case for the YTRF motif of the transferrin receptor (18), the internalization of which is a widely used specific marker for CME events. Different receptors can use many of the wide array of alternative adaptor and accessory proteins for the clustering and stimulation of their endocytosis (see below).

Theoretical considerations suggest that clathrin polymerization alone is insufficient for membrane curvature generation (19). Epsin family proteins, which can link cargo [for example, ubiquitinated cargo (20)] to clathrin, also bind directly to inositol lipids and can help drive membrane deformation in the assembling CCP through the insertion of a amphipathic helix (21). Where epsins are present, this will provide an initial membrane deformation in concert with clathrin polymerization to form a membrane bud (22). Other accessory proteins can also help deform the membrane (see below). Apart from epsins, G protein–coupled receptors (GPCRs) can be linked to clathrin similarly through the binding of β-arrestins, which interact with the phosphorylated receptors, PtdIns(4,5)P_2, clathrin, and AP2 (23, 24). Likewise, dab2 and ARH have been shown to function as cargo-specific adaptor proteins during low-density lipoprotein (LDL) receptor internalization (25), and numb appears to have a similar role during notch endocytosis (26). If multiple receptors are endocytosed by CME in the same cell, they may be sorted in endosomes. However, it is possible that the differential affinities of receptors (determined, for example, by phosphorylation or ubiquitination of the receptor) for specific adaptor proteins are responsible for their clustering into distinct CCPs/CCVs such that each vesicle can be formed in a kinetically distinct manner and then trafficked to its appropriate intracellular destination in a manner dependent on its cargo and their ligands.

Accessory proteins can participate in CME through aiding membrane deformation in the membrane bud (see below) by recruiting other participating proteins or by performing scaffolding/coordination functions within the endocytic process. N-BAR and BAR domain-containing proteins, such as SNX9 and amphiphysin, can generate and stabilize membrane curvature (and may thus aid in membrane deformation within the CCP), bind both clathrin and AP2, and recruit dynamin to the neck of the budding vesicle (27–31). Eps15 appears to act as a scaffolding protein and clusters AP2 appendages through a long, flexible C-terminal tail (15). Many more accessory proteins have been identified, including NECAP, intersectin, and stonin, as have several kinases that may play important roles during the endocytic process (15). On the basis of biochemical interrogations, our lab has proposed a model of how sequential interactions of increasing avidity of accessory proteins and clathrin for AP2 can drive CCP formation (15). Recent findings have shown that proteins originally shown to be important regulators of more diverse cellular phenomena, e.g., apoptosis (p53) (32), asymmetric cell division (numb) (26), or cell polarity (par proteins) (33), are also intimately involved in CME. Studies have also identified an increasingly large number of BAR superfamily domain-containing proteins involved in CME. These data suggest that the repertoire of proteins that are (or can become) involved in CME will be significantly larger than we currently appreciate, but whether such proteins are involved globally in CME, within particular subsets of CCPs, or even at all (because overexpressed proteins may localize artifactually to CCPs), remains to be definitively established.

It is becoming increasingly likely that distinct adaptor and accessory proteins control the internalization of distinct cargoes, that different subtypes of CCVs may subsequently be formed, and that different specialist proteins will be required to assemble each pit subtype. This may

seem like an obvious remark, given knowledge of the nature of the dynamic network of interactions that exists in this process, but conclusive proof that cargo-specific (or at least relatively cargo-specific) CCPs/CCVs exist is lacking (34), and clathrin-independent pathways do not appear to produce homogeneous endosomes (although these are not necessarily analogous structures). Because, in the main, morphologically indistinguishable structures appear to be responsible for the internalization of cargoes that enter via CME, and because single CCPs/CCVs have been shown (at least sometimes) to contain more than one type of cargo moiety, it is likely that each CCP/CCV contains a competitively produced population of cargoes and adaptors/accessory proteins. The exact composition of these CCPs/CCVs will depend on many factors, including (*a*) the concentration of each cargo type at the plasma membrane, (*b*) the specific activation state of each cargo, (*c*) the dependence on specific activation states for the recruitment of each cargo to CCPs, (*d*) the mobility of that cargo in the plasma membrane, (*e*) the overall affinity of adaptor/accessory protein binding to that cargo, (*f*) the concentration of adaptor/accessory proteins that can be used to recruit each cargo, and (*g*) the ability of that adaptor/cargo to interact with the clathrin polymerization machinery. This view of CME, however, poses a problem in cargo sorting for the cell. Whether sorting happens only in sorting endosomes or also in CCPs is unclear. The formation of cargo-specific CCVs, with cargo type-driven signals for further trafficking, would allow an additional layer of regulation. Cargo likely plays an important role in driving CCP/CCV formation because empty CCVs are unlikely to have useful functions. If cargo drives CCP formation, then it is also possible that each CCV will have a minimum number of molecules of each particular cargo. This would provide a quantum of cargo that may allow digital processing of cargo-associated information by the cell, especially if downstream consequences of endocytosis happen in a robust and stereotyped manner. It is important to address if cargo clustering drives CCP formation de novo or if clustered cargoes are recruited to sites where clathrin has already been partially assembled and completes this process of assembly.

The surface residence time of CCPs (and therefore membrane fission) is regulated by interaction with GPCR cargo (and this CCP formation is reversible) through interaction with the actin cytoskeleton (35). Furthermore, Transferrin Receptor Trafficking Protein (TTP) interacts with both dynamin and the transferrin receptor. The demonstration that, in the absence of transferrin receptor binding, TTP can negatively regulate the rate of GTP hydrolysis by dynamin provides a potential mechanism by which cargo can signal specifically and directly to the scission machinery (36). This might inform the scission machinery as to the state of cargo recruitment, and only when sufficient transferrin receptor has accumulated, will scission be permitted to proceed. This would automatically exclude the majority of other cargo proteins because cargoes will compete for similar binding partners and the avidity of these interactions will favor transferrin receptor incorporation once it has accumulated beyond a critical concentration. Space restrictions within the CCV will likely also select against hitchhiking by other cargoes. It remains possible that, just as many cargoes have specific adaptors, there might also exist cargo-specific interacting proteins that can interact with, and regulate, the scission machinery. Such an arrangement would certainly produce an elegant mechanism for the production of cargo-specific CCVs but remains unexplored. Another poorly understood issue is how CCP and CCV sizes are controlled. Furthermore, CCPs have been observed to form at the edges of flat clathrin sheets, suggesting that they may arise from the locally high concentration of coat proteins, or cargo in these regions, or perhaps even partially preassembled intermediates, but this remains uncertain.

The ligands internalized by CME are myriad, and it is likely that many more cargo-specific adaptor/accessory proteins will be discovered, each with core key features including

particular cargo affinities. Redundancy in the system allows for robustness; for example, close to 100% of receptor A may ordinarily be internalized through binding to a single type of adaptor protein, yet, in the absence of this adaptor, receptor A may use lower-affinity interactions to recruit the CME machinery. The view of CME as a dynamic network of protein-protein and protein-lipid interactions will help interpret overexpression and siRNA studies and provide novel insights into the regulation and spatiotemporal dynamics of this ubiquitous process (4).

CLATHRIN-INDEPENDENT ENDOCYTOSIS

Perturbation of CME affects the endocytosis of all the cargoes that have been mentioned in Table 1 row 1, at least in cell types in which this has been studied. However, the endocytosis of many other types of endogenous and exogenous cargoes is relatively unaffected by inhibition of this pathway. Furthermore, these types of cargo molecules are internalized, in the main, in a cholesterol-dependent manner, in contrast to CME cargo molecules. This strongly implies the existence of additional endocytic pathways that exist endogenously and that are independent of clathrin. However, it should be noted at this stage that using methods that inhibit CME as tools to discover new constitutive uptake pathways (that ordinarily exist and that are prevalent) is far from ideal because such treatment may functionally upregulate a pathway that is usually much more silent or even nonexistent. As well as the extent, the properties of this pathway may also change because interference with CME would have cell-wide consequences in terms of signaling levels, lipid and protein distributions, membrane tension, and stress responses. The discovery of novel pathways of endocytosis should therefore, where possible, rely on the use of unperturbed cells before using such techniques to probe their properties further.

Relatively few endogenous, noncargo proteins associated with clathrin-independent endocytic pathways have been discovered to date, and of those that have, little is known about how these might contribute to the mechanism of endocytosis. Perhaps this is owing to the absence of conserved core recruitment hubs for the concentration of cargoes, which would allow straightforward biochemical experiments to be performed. What is currently understood about these pathways has been derived mainly from study (in a variety of cell types) of the morphology of these endocytic structures by EM, from following the internalization of (often artificially clustered) endogenous markers or bacterial exotoxins predominantly by fluorescence microscopy, from determining the lipid requirements for these pathways, from examining their sensitivity to certain drugs, and from ascertaining their dependence on cellular proteins such as small G proteins, kinases, and dynamin. These pathways are discussed in the following sections.

CAVEOLIN1- AND FLOTILLIN-ASSOCIATED ENDOCYTIC MECHANISMS

There are three mammalian caveolin proteins. Although caveolin3 is muscle specific, caveolin1 and 2 are found widely in nonmuscle cells with only neurons and leukocytes apparently lacking caveolae. Caveolin1 is enriched in caveolae (11), with ~100–200 molecules per caveola (37), and cells that do not express this protein (and muscle cells not expressing caveolin3) are devoid of morphologically evident caveolae. Overexpression of caveolin1 in caveolae-deficient cells is sufficient to produce flask-shaped plasmalemmal invaginations morphologically indistinguishable from caveolae in normal cells (38). These data imply that caveolin1 is necessary (and perhaps sufficient) for caveolar biogenesis. Caveolin2 appears not to be necessary for caveolar formation but likely plays important roles in at least some such structures. Caveolin1 is capable of forming higher-order oligomers, is palmitoylated, and binds cholesterol and fatty acids, which stabilize oligomer formation (39, 40).

GSL: glycosphingolipid

CLIC: clathrin-independent carrier

GEEC: GPI-AP-enriched early endosomal compartment

This may be important both in ordering local lipids into invagination-competent compositions, as well as in the export of these lipids to the plasma membrane. Caveolin1 forms complexes composed of 14–16 monomers (41), and a model has been proposed by which caveolin1 could effect changes in membrane curvature (42) (see below). Caveolin1 binds the fatty acid tails of the glycosphingolipid (GSL) GM1 and can colocalize with GM1 and another "raft-associated" GSL, Gb3, in cellular membranes (43). However, GM1 and Gb3, and their ligands cholera toxin (CTx) and Shiga toxin (STx), respectively, are found on the extracellular leaflet of the plasma membrane, and caveolin1 is on the cytoplasmic side. Caveolin1 forms a hairpin structure that is embedded into the membrane, perhaps reaching through into the outer monolayer (and perhaps thereby contributing to the necessary cross talk across the bilayer). Both its N and C termini are exposed to the cytoplasm. It is thought that caveolins may contribute to the spike-like coat found on caveolae. In addition to this coat, high-resolution EM analysis of caveolar ultrastructure has revealed a ring-like density found circumferentially around the caveolar neck (44).

Caveolae (or rather precaveolae because they are not yet on the plasma membrane) appear to undergo formation in the Golgi complex, where they acquire their characteristic detergent insolubility and cholesterol association in concert with (at least partial) caveolin1 oligomerization (37). Cholesterol depletion flattens caveolae (11) and increases the mobility of caveolin1 in the plasma membrane. Despite these observations, the precise role of caveolin1, the specific lipid composition of caveolae, and the specific functional relevance of other caveolae-associated proteins remain to be firmly established.

The abundance of caveolae in many cell types is certainly consistent with a major role in trafficking, and certain markers have been rather convincingly shown to be capable of undergoing endocytosis into caveolin1-positive structures (which include large neutral pH intracellular structures, termed caveosomes, and smaller vesicles and tubules), such as SV40 virions, cholera toxin B subunit (CTxB), and glycosylphosphatidylinositol (GPI)-linked proteins (7, 10, 45). Such caveolin1-positive structures have been shown to have great variance in their life cycles and their abilities to fuse with each other and other organelles. However, a role for caveolae in the constitutive endocytosis of these markers is questioned by the artifactual clustering of glycolipid or protein receptors into caveolae that occurs when they are exposed to multivalent ligands, including antibodies and toxins. Moreover, although caveolar-type structures are certainly observed in regions of the cell distant from the obvious plasma membrane, it has been shown that many of these structures can still be connected directly to the plasma membrane and have thus not undergone scission from this site (46), making it difficult to assess the endocytic nature of caveolin1-positive membranes. Furthermore, Sandvig et al. (47) have convincingly argued that most caveolae appear surface connected when sections for EM are taken perpendicular to the plasma membrane, but not when sectioned otherwise. In addition, using ruthenium red treatment, they have also shown that large caveosomal/multicaveolar structures that appear distant from the plasma membrane are still surface connected (47). Although caveolae may or may not pinch off from the membrane, it still remains useful to discuss data derived from experiments examining caveolae because it is clear that the predominantly caveolae-associated protein caveolin1 is associated with endocytosis, and because experiments previously thought to be examining caveolar endocytosis may shed light on other endocytic pathways. As caveolin1-associated cargoes overlap with those of the clathrin-independent carrier (CLIC)/GPI-AP enriched early endosomal compartment (GEEC) pathway, perhaps manipulations previously thought to affect caveolar endocytosis are acting on this pathway. Consistent with this, a recent study has intriguingly found that a region of caveolin1 shows homology to a GDI sequence, and caveolin1 specifically binds GDP-bound cdc42 (48).

Caveolin1 depletion resulted in an increase in activated cdc42 levels at the plasma membrane. These intriguing data suggest that caveolin1 regulates the activity of cdc42, which is heavily implicated in CLIC/GEEC endocytosis. However, caveolin1 manipulations may also affect other pathways.

The internalization of very few markers has been shown to be caveolin1 dependent (7, 10), and perhaps, transcytosis across endothelial barriers, which appears to occur through caveolae, is currently the most convincing trafficking process associated with caveolae (49). Caveolin1-null mice are surprisingly normal macroscopically, despite being devoid of caveolae, but do have a reduced life span (50). They have defects in the regulation of vascular tone, which appears to be linked to endothelial cell hyperproliferation and microvascular hyperpermeability (51), which is eNOS dependent (52). They also have transcytotic defects and defects in lipid droplet formation and in liver regeneration after partial hepatectomy (10, 53).

Although overexpression of caveolin1 increases the number of caveolae, it can also profoundly inhibit the endocytosis of ordered lipid microdomains. Caveolin1 overexpression also inhibits the endocytosis of autocrine motility factor (AMF) to the ER and CTxB to the Golgi apparatus (54). Such a CTxB trafficking defect was also observed in caveolin1-null mouse embryonic fibroblasts (MEFs), which were also shown to bind the same levels of CTxB as wild-type controls (8). The internalization of both AMF and CTxB is sensitive to cholesterol depletion and tyrosine kinase inhibition. These two ligands do not colocalize intracellularly when cells are exposed to both, despite these accumulating together in caveolae on the surface (54). Furthermore, although caveolin1 actually facilitates dysferlin delivery to the plasma membrane (an opposing role to that in endocytosis), dysferlin enters cells by a clathrin-independent pathway more rapidly in caveolin1-null cells (55). These results, and others, suggest that either caveolae may be involved in anterograde trafficking, the protection of surface proteins from internalization, and/or competition with other pathways for particular essential lipids. Caveolae might induce the titration of endogenous binding partners or lipids necessary for endocytosis. Conversely, caveolin1 overexpression may reduce overall membrane fluidity and therefore even inhibit caveolin1-mediated internalization events. A very tight regulation of the levels of caveolin1 on the plasma membrane would consequently be required, and perhaps, this contributes to differences reported for caveolar dynamics using fluorescence approaches.

The presence of caveolin1 on apparently intracellular compartments that colocalize with recently internalized markers is consistent with the hypothesis that these represent caveolae that have been internalized whole. It also remains possible that caveolin1 may also mark (perhaps nonspecifically because there is an abundance of caveolin1 on the plasma membrane) other uptake pathways that are formed from noncaveolar regions of the plasma membrane. Consistent with this is the finding that caveolin1 can be found in the tubular CLIC/GEEC membranes but is unnecessary for this pathway to proceed (see below). We have also observed that under certain circumstances caveolin1 can be found in extensive tubular networks, and caveolin1 may prefer to locate to areas of such high membrane curvature (G.J. Doherty, unpublished observations).

Fluorescence recovery after photobleaching experiments have shown that caveolae can be very stable in unstimulated cells (56). However, using TIR-FM it has also been shown that many plasma membrane caveolin1-positive spots appear and disappear toward the cell interior (almost half were shown to be dynamic over 5 min) and seem to undergo kiss-and-run fusion and fission with the plasma membrane, although some were observed to travel longer distances intracellularly (37). Caveolin1 on intracellular vesicles retains its ability to provide stability to membrane microdomains, and this may influence subsequent sorting (57). Using EM techniques that can distinguish between surface-connected and nonsurface-connected

SL: sphingolipid

caveolar/caveosome-like structures, about 2% of the caveolar population (at least small-/single-caveolin1-positive vesicular structures) in MEFs were shown to have undergone apparent scission from the plasma membrane after warming for 1 min in the presence of CTxB (8). After a longer period of incubation, larger, tubulovesicular caveolin1-positive structures were shown to accumulate the toxin and appeared to be unconnected to the plasma membrane (8). This internalized population could be doubled in extent by treatment with the phosphatase inhibitor okadaic acid as well as by the addition of exogenous lactoside ceramide (LacCer). The mobility of caveolin1-positive structures is also increased by stimulation with SV40 virions, GSLs, or cholesterol (37, 58). Apparent internalized caveolae have been shown to be capable of fusing with both the classical early endosome as well as with caveosomal structures; both of these steps are rab5 dependent (57).

LacCer (at least in its BODIPY fluorescent form) traffics through caveolin1-positive compartments and subsequently reaches the Golgi apparatus (59). Total sphingolipid (SL) or specific GSL depletion results in the reduction of the amount of LacCer endocytosis, a reduction of caveolin1 levels at the plasma membrane, and accumulation of caveolin1 at the Golgi apparatus (60). C8-sphingomyelin and the ganglioside GM3 are each sufficient to restore caveolin1 export in SL-depleted cells.

Phosphorylation of caveolin1 appears to trigger the flattening and fusion of caveolae in cells expressing viral-sarcoma (v-src). Caveolin1 S80E (a phosphomimetic mutant) does not associate with detergent-resistant membrane fractions and is not found on the plasma membrane (61). The corresponding phosphomutant (S80A) has an affinity for cholesterol that is similar to that of wild-type protein and is found on caveolar membranes. Thus, dephosphorylation at this site, perhaps at the level of the ER/Golgi, might regulate the formation of caveolin1-dependent microdomains. Likewise, phosphorylation at the plasma membrane may then be responsible for their disassembly, which could be either a positive or negative regulator of endocytosis. A number of kinases have been shown to regulate caveolar/microdomain-dependent endocytosis, but how this might be temporally coordinated, and exactly which type of clathrin-independent endocytosis is affected in these assays, is unclear (37). There have also been a number of novel roles suggested for caveolae (see Reference 10 and below).

A large number of signaling proteins have been found associated with, and regulated by, caveolae, and these structures may act as signaling platforms (62). However, aside from the caveolins, little is known about proteins involved in caveolar genesis. Recently, PTRF (or cavin) was shown to associate with surface caveolae, to be necessary in both zebra fish and mammalian cells, and to be necessary for caveolar formation, with caveolin1 located instead on flat regions of the plasma membrane and undergoing quicker degradation (63), suggesting that caveolin1 is not sufficient for membrane deformation.

Flotillin proteins (also called reggie proteins) are not enriched in caveolae but are found oligomerized in distinct membrane microdomains (64). Their homology to caveolin1 suggests that they may play a role in the ordering of lipids in an analogous manner to caveolae (flotillins are also proposed to form a similar hairpin structure in the membrane and are palmitoylated). Indeed, it has recently been shown that flotillin1 and -2 are found in plasma membrane domains distinct from caveolae (65) and that flotillin1 is necessary for a portion of CTxB uptake (64). By TIR-FM, budding of flotillin1-positive structures into the cell was found to be rare in comparison with that of CCPs, similar to findings with caveolar-type endocytosis. Flotillin2 appears to undergo trafficking from the Golgi, and by TIR-FM, flotillin2-positive puncta were shown to undergo cycling to and from the plasma membrane in a manner similar to flotillin1-positive puncta (66). Flotillin2 cycling can be inhibited by cell-cell contact formation and stimulated by serum and epidermal growth factor (EGF), but was shown not to be associated

with epidermal growth factor receptor (EGFR), cellular prion-related protein (PrP_c), or GFP-GPI uptake. However, the GPI-linked protein CD59 is found in flotillin1+2-positive domains at 12°C and in internalized flotillin1-positive vesicles, and flotillin1 is necessary for CD59 uptake (65). Interestingly, flotillin1 is also necessary for the dynamin-dependent but clathrin- and caveolin1-independent uptake of cell surface proteoglycans, which it appears to deliver to late endosomes in flotillin1-positive compartments (67).

Although one study showed that co-overexpression of flotillin1 and -2 generated new plasma membrane microdomains in which they colocalize, another study showed that flotillin1 and -2 colocalize less impressively, perhaps reflecting different expression levels in these studies (65, 66). Flotillin1- and -2-positive domains contain about 95 molecules of each flotillin and have been suggested by correlative fluorescence and EM to be morphologically similar to caveolae (65). However, although expression of caveolin1/3, or even honeybee caveolin, results in the rescue of caveolae production in (caveolae-negative) caveolin1-null cells, co-overexpression of flotillin1 and -2 did not induce caveolae-like invaginations (68). Flotillin may mark a pathway separate from those discussed below, or may act upstream in domain organization for other clathrin- and caveolin1-independent pathways. Indeed, flotillin can be found concentrated in the membranes of early CLIC/GEEC intermediates (14).

CLATHRIN- AND CAVEOLAE/CAVEOLIN1-INDEPENDENT ENDOCYTOSIS

Endocytosis occurs in cells depleted of both CME events and caveolin1 in a manner dependent on cholesterol, implying distinct endocytic pathways that require specific lipid compositions. Such microdomain-dependent, but clathrin- and caveolin1-independent, endocytosis has been shown to internalize extracellular fluid, SV40 virions (69), CTxB, GM1, other SLs, GPI-linked proteins, as well as receptors for IL2, growth hormone, AMF, endothelin, and many other molecules (7). The dependence of these pathways on specific cell substituents, and their morphologies, is summarized in **Figure 1** and **Table 1**.

Proteins that attach to membranes by way of a glycosylphosphatidylinositol anchor (GPI-linked proteins) are enriched in detergent-resistant membrane fractions and are not normally concentrated into CCPs or caveolae. They undergo endocytosis via a cholesterol-dependent, clathrin-independent endocytic pathway (13). The internalization of these proteins is kinetically distinct from the uptake of transmembrane proteins and can bypass conventional rab5-positive endocytic compartments. One such pathway, which delivers cargoes to endosomes termed GPI-AP-enriched early endosomal compartments (GEECs), is seemingly regulated by the small G protein cdc42 and is not inhibited by the overexpression of dominant-negative dynamin proteins that inhibit CME (13, 70). Such an acidic, tubulovesicular compartment appears to account for a large proportion of internalized fluid phase markers (71). The recruitment of GPI-linked proteins into these endocytic structures is dependent on the GPI moiety, but it is currently unknown how this is regulated despite the active maintenance of nanoscale clusters of such proteins on the cellular surface that are dependent upon membrane cholesterol (58). A recent hypothesis incorporating the chirality and intrinsic tilt of molecules within such a cluster suggests that the membrane constituents alone are sufficient for membrane budding (72). The production of high membrane curvature in this way may allow cellular proteins that sense and generate membrane curvature, such as BAR domain-containing proteins, dynamin (although dynamin has not been found on GEEC endocytic intermediates), and their functional homologs, to induce further curvature generation to produce tubular/vesicular structures that are then delivered to GEECs, as well as to induce the ultimate scission of such invaginations from the plasma membrane.

The folate receptor FRα, which likely increases local folate concentrations at the plasma membrane to allow its efficient uptake via folate transporters, is a GPI-linked protein and is only concentrated in caveolae after cross-linking (73). Binding of folate does not change the normal diffuse distribution of FRα at the plasma membrane, and further studies have ruled out a role for caveolae in FRα endocytosis (74, 75). KB cells endogenously express this receptor where it is found in noncoated membrane invaginations as well as in clathrin-coated structures (76). In CHO cells, the major endocytic pathway for stably transfected FRα delivers it to GEECs (13). The GEEC pathway is downstream of the highly prevalent CLICs that have been observed by EM to be predominantly tubular or ring like in morphology (8) and are discussed in detail below. Without ruling out the possibility that there are several distinct subtypes of this endocytic route, the pathway is referred to here as the CLIC/GEEC pathway. No cell physiological function has yet been reported for this prevalent endocytic route, despite the large amount of internal membranes that this pathway contributes. We have recently shown how the BAR domain-containing protein GRAF1 (a tumor suppressor in leukocytes) sculpts the ∼40-nm diameter tubular membranes of this prevelant endocytic route (14).

When the CLIC/GEEC pathway is disrupted, there appears to be compensatory endocytosis of GPI-linked proteins via CME (13), but whether this allows internalized proteins to be trafficked to their appropriate destinations is unclear. The intracellular destination for material endocytosed through the CLIC/GEEC pathway appears to differ between cell types, including lysosomal and pericentriolar recycling compartments (13, 77). If this reflects the presence of specific CLIC/GEEC subtypes or is due to other cell type-dependent factors is unknown.

Whether and how all GPI-linked proteins are endogenously regulated through endocytic mechanisms remains a mystery. The core structure of the GPI moiety is conserved among all GPI anchors, but the precise structures of their lipids and side chains are variable. These variabilities may modulate their abilities to become concentrated in, or escape from, specific types of membrane microdomains. If distinct types of microdomains are turned over by distinct endocytic mechanisms, then this would provide each subtype of GPI-linked protein with distinct turnover kinetics.

Both GPI-linked proteins and the IL2Rβ receptor are enriched in detergent-resistant membrane fractions (implying clustering into membrane microdomains), and cholesterol depletion abrogates the endocytosis of both types of protein (78). However, it has been demonstrated that a clathrin- and caveolin1-independent endocytic pathway, which is seemingly distinct from that of the CLIC/GEEC pathway, is responsible for the internalization of the IL2Rβ receptor after ligation and that this pathway is dependent upon the activity of the small G proteins rhoA and rac1, and the kinases PAK1 and PAK2 (79). At least some of the membranes internalized by this pathway communicate with clathrin-dependent endocytic compartments, and the pathway appears to be dynamin-dependent. A similar dynamin-dependent, clathrin-independent pathway appears to be responsible for the internalization of the γc cytokine receptor and the IgE receptor FcεRI (80, 81), but both the prevalence and the wider role of such a pathway outside of leukocytes are unclear.

It has been shown that the GPI-linked protein CD59 and major histocompatibility complex (MHC) class I proteins can be cointernalized into clathrin-independent, arf6-positive endosomes that appear to be distinct from CLIC/GEEC endocytic structures (14, 82). Arf6 is also heavily implicated in a recycling pathway to the plasma membrane (83). This arf6-associated endocytosis appears to be dynamin independent, and these endosomes can communicate with both transferrin-positive compartments and the arf6-dependent recycling pathway (82). This latter observation makes phenotypes downstream of arf6 activity modulation difficult to reliably interpret. The K^+ channel Kir3.4 also takes this

arf6-associated endocytic pathway, and a series of acidic motifs in these proteins enhance the association of the channel with members of the EFA6 family of arf6 GTP exchange factors (GEFs) (84). Whether these function analogously to sorting motifs in CME is unclear. Postendocytic clathrin-independent trafficking pathways are beginning to be resolved, and some degree of communication with the classical rab5-positive endosome is a feature of many, if not all, endocytic pathways (6). Although GPI-linked proteins have been shown to be internalized by the pathways described above, it has also been suggested that GPI-linked proteins are transported directly to the Golgi apparatus, and there is evidence for a constitutive clathrin-independent endocytic mechanism responsible for direct transport of luminal contents and GPI-linked proteins to this site (85, 86). The endocytic apparatus responsible for this is currently unclear, but may include CLIC/GEEC subtypes.

MECHANISMS OF INTERNALIZATION INVOLVING LARGER VOLUMES OF MEMBRANE

Although the mechanisms described in the preceding two sections can be described as pinocytic, macropinocytosis describes a form of larger-scale internalization that frequently involves protrusions from the plasma membrane that subsequently fuse with themselves (or back with the plasma membrane), resulting in the uptake of extracellular components trapped between these sites (**Figure 1**). This morphologically described process is both rac1- and actin-dependent, and many studies have linked it to the ability to form membrane ruffles. Although this is a well-known process, the molecules involved in the mechanism and regulation of macropinocytosis are elusive, as is any relationship between this process and clathrin-independent pinocytic processes. The kinase PAK1 has been heavily implicated in macropinocytosis and is necessary for, and sufficient to induce, the process (87). PAK1 binds rac1, which also activates it (88). Phosphatidylinositol-3-kinase (PI3K), ras, and src activities also promote macropinocytosis, presumably downstream of receptor ligation, and recent studies also implicate HDAC6 and its substrate hsp90 in this process (89–91), although how these contribute remains unclear.

Macropinocytosis is cholesterol dependent, and cholesterol is required for the recruitment of activated rac1 to these sites (92). Perhaps, because this process can be stimulated by the profound activation of receptors, it lies at one end of the same spectrum as other membrane microdomain-dependent endocytic pathways. Indeed, microdomain markers are enriched in membrane ruffles (93). The substantial local accumulation of specific lipids at heavily activated regions of the plasma membrane likely leads to dramatic intracellular changes at these sites (given the profound interplay that exists between plasma membrane organization and the cytoskeleton) and may account for the morphological features of this process.

It was shown as early as 1979 that receptor tyrosine kinases can be internalized by clathrin-independent mechanisms (94). The EGF and platelet-derived growth factor (PDGF) receptors have been shown to be present in caveolae, but overexpression of caveolin1 inhibits their activation, suggesting that residence in caveolae is inhibitory to their signaling (95–97), and perhaps this also inhibits their endocytosis. In the presence of high EGF concentrations and in concert with a population of receptors undergoing CME, there appears to be another endocytic mechanism that occurs in response to receptor ubiquitination. Upon treatment with EGF (or PDGF/hepatocyte growth factor (HGF) ruffled structures appear on the dorsal surface of receptive cells and appear to move or evolve along the dorsal surface (dorsal waves; cf. classical macropinocytosis, which occurs predominantly at cellular peripheries) (98, 99). The formation of such structures, known as circular dorsal ruffles (CDRs), appears to be cortactin and dynamin dependent. Several kinases and adhesion molecules are also found in these regions (100). Upon formation

of these structures by high EGF concentrations, EGFRs become predominantly concentrated in CDRs and appear to be rapidly internalized from these sites by tubular endocytic structures, which are independent of clathrin and caveolin1. Treatments that inhibit ruffle formation, however, have no effect on EGF-stimulated macropinocytosis, suggesting that ruffles themselves are not necessary for this process (101, 102). Rab5 (usually implicated in CME), and its effector rabankyrin5, have been shown to be involved in CDRs/macropinocytosis (103), but how these operate here is not known, and such observations may even reflect modulation of exocytosis to these sites. Treatment of PC12 cells and neurons with nerve growth factor induces the endocytosis of its receptor TrkA in a manner stimulated by, and dependent upon, the large GTPases Pincher (EHD4) and dynamin (104). This mode of internalization resembles that mediating EGF receptor uptake in macropinocytic ruffles. Although the functions of these endocytic modes are not established, these results intriguingly suggest that they may act to densensitize cells to large concentrations of growth factors through internalization of a large proportion of their specific receptors. This may allow processes such as gradient sensing to proceed.

In cell culture, CDRs can be differentiated spatially from the ventral location of similarly distorted plasma membrane regions associated with invadopodia and podosomes (105). Invading tumor cells extend long protrusions into the surrounding matrix, which results in the specific degradation of matrix at these sites through the specific enrichment of soluble and transmembrane matrix metalloproteinases. These structures, termed invadopodia, are capable of endocytosing matrix components, which are subsequently delivered to lysosomal compartments (105). The nature of these endocytic structures is unknown but they seem to contribute strongly to cell invasion. Invadopodia are actin-rich structures and possess many of the actin-regulating proteins present in podosomes as well as dynamin (106). The inhibition of dynamin function reduces local matrix degradation by invadopodia. Dynamin directly binds cortactin, which cross-links actin filaments, forming actin sheets (107).

Macrophages and osteoclasts endogenously produce podosomes (108), which are actin-rich and actin-dependent membrane extensions involved in the early steps of cell-matrix adhesion and possess focal adhesion components and the src tyrosine kinase (src family members are enriched in membrane microdomains) in addition to Arp2/3 and actin-regulating proteins that are absent from focal adhesions. They also contain dynamin and cortactin as well as the BAR domain-containing protein endophilin2 (109). Because all of these latter proteins have been implicated in endocytic events, and because these structures form tubular membrane structures, it is tempting to speculate that podosomes may, as well as likely playing an important role in cellular adhesion and migration, be sites of active endocytosis. However, there is currently no direct evidence to support this. The shared presence of dynamin, cortactin, and actin-regulating proteins suggests that CDRs, invadopodia, and podosomes may share some common underlying principles of function.

Professional phagocytic cells, such as macrophages, monocytes, and neutrophils, are dependent upon small G proteins for their clathrin-independent internalization of opsonized particles. Upon ligation of Fc receptors by the constant regions of antibodies, the phagocyte produces filopodial extensions around the particle in a cdc42-dependent manner with subsequent internalization being dependent upon rac1 (110–113). These proteins recruit N-WASP and the actin nucleating and polymerizing Arp2/3 complex to the phagocytic membranes, and actin polymerization is necessary for phagocytosis to occur (114). IQGAP1, and its interaction with the formin Dia1, is also required for phagocytosis (as well as cell migration) (115). By contrast, the internalization of particles opsonized with the complement effector fragment C3b occurs in response to the ligation of CR3 (a modified integrin, which undergoes internalization in a microdomain-dependent manner) in a rhoA- (and its effector

rho kinase-) dependent manner (116, 117). The phagocytosed particle is internalized into actin-lined membranes (116, 117). In this method of phagocytosis, membrane protrusions are not usually observed despite Arp2/3 recruitment (114). Amphiphysin1, usually associated with CME, is also required for phagocytosis in Sertoli cells (118). Vinculin and paxillin (markers of cell-matrix adhesion sites) are present at the regions where the particle is in contact with the plasma membrane (119). This link with adhesion proteins, coupled with the rhoA dependence of this process, suggests that the formation of local adhesive structures is instrumental in this mechanism of internalization, and phagocytosis may therefore bear mechanistic similarities to other internalization mechanisms described in this section.

Recently it was shown that upon detachment of cells from matrix, which usually triggers cell death, a still-living cell can be internalized whole into another in a rhoA- and actin-dependent manner before degradation by, or release from, the host cell (120). Imaging of the adherens junctions (which are necessary for this process) is reminiscent of the zippering mechanism of bacterial entry and CR3-associated phagocytosis, although more work needs to be done to determine if these have common links. More work also needs to be done to investigate the mechanisms of membrane rearrangements and fission in this system, as well those that occur during the rac1-dependent process of trans-endocytosis, where a small region of receptor-containing membrane is internalized by a neighboring cell. Clathrin, dab2, and dynamin have already been implicated in this latter process, at least in gap junction trans-endocytosis (121, 122).

ENDOCYTOSIS OF BACTERIAL TOXINS

Although many bacterial and plant toxins are capable of traversing the plasma [e.g., *Bordetella pertussis* adenylate cyclase (123)] or endosomal [e.g., *Clostridium botulinum* toxins (124)] membranes directly, others enter and traffic through cells without disrupting membranes and are thus useful probes for the dissection of endocytic routes. Anthrax toxin binds to transmembrane proteins TEM8 and CMG2 on the plasma membrane and is followed by cholesterol-dependent and clathrin-mediated internalization of the toxin (125–127). Toxins of the A-B_5 (such as CTx and STx, respectively) and A-B (such as the castor bean toxin ricin) types have been used to probe retrograde (plasma membrane to perinuclear compartment) trafficking pathways. The pentameric B subunits of A-B_5-type toxins have binding sites for specific glycolipids, which are resident on the cell surface. The glycolipid receptor for one such toxin, CTxB, is the ganglioside GM1 (128), which, as a GSL, has a high tendency to self-assemble into membrane microdomains, and the toxin may aid the partitioning of these lipids into such domains.

The transport of CTx is cholesterol dependent, and indeed, caveolin1- and clathrin-independent endocytosis has also been shown to be responsible for CTxB internalization. Some studies have shown that endogenous GM1 (the receptor for CTxB), and exogenously added CTxB, are found enriched in caveolae. However, CTxB can enter cells devoid of caveolae, and uptake of CTxB by caveolar-type mechanisms appears to be a minor mechanism for its internalization in certain cell types (7). In some cell types at least, CTxB can be internalized by CME (85). EM studies to determine the early carriers for CTxB have shown that (in conflict with other studies that have claimed that CTxB is a lipid microdomain/caveolar marker) the protein is only slightly enriched in these structures and is also found in CCPs, consistent with the demonstration that some CTxB enters via this pathway (8).

Analysis of the early clathrin-independent, CTxB-enriched, endocytic carriers in MEFs by EM demonstrated that these are tubular- and ring-shaped structures, belonging to the same pathway as GEECs (13). Consistent with this, GPI-linked proteins and fluid phase markers were also found in these structures (7, 8). These CLICs were shown to bud from the plasma

membrane and were the most prevalent early carriers of CTxB observed. These structures were also found to be cholesterol dependent. Some of these membranes contained caveolin1. However, this prevalent endocytic route is also found in caveolin1-null MEFs, and the morphology of structures involved in CTxB uptake and trafficking was found to be ultrastructurally identical in these cells, suggesting that the presence of caveolin1 on these compartments is not essential for their function. Overexpression of a dominant-negative eps15 protein in these cells, which blocked CME events, reduced the transport of CTxB to the Golgi apparatus by about 40% (8), consistent with the observation that CTxB can enter cells via CME (129). Methyl-β-cyclodextrin (MBCD) treatment, which had no effect on CME, reduced the Golgi delivery of CTxB in untransfected caveolin1-null cells by ~40% to 45%. Many studies of CTxB uptake by different endocytic pathways appear conflicting at first glance, but the heterogeneity of cell types used in these studies, with their intrinsic abilities to internalize by each type of pathway, may account for many of these differences. Furthermore, although combined inhibition of clathrin-, caveolin1-, and arf6-dependent endocytic events completely abolishes CTxB uptake, inhibition of any one of these pathways in isolation only slightly affects CTxB internalization, suggesting compensatory endocytosis through the others (130). Whether only a specific type of endocytosis is responsible for the appropriate subsequent trafficking of the toxin is unknown, and it appears that CTxB can take a direct route from the plasma membrane to the Golgi and that this can be unaffected by inhibition of its uptake through CME (85, 131, 132).

STx interacts with globotriaosylceramide (Gb3/CD77) through interactions with the trisaccharide domain of this GSL. The B_5 subunit of STx (STxB) can enter cells through both CME and clathrin-independent routes, undergoing retrograde transport in detergent-resistant membranes (133) and, from early endosomal structures, seems to traffic directly to the Golgi network. The binding of STxB to Gb3 results in cytoskeletal remodeling (134), and it has been suggested that STxB can promote its own (Syk-dependent) entry (135). The elicitation of cellular signaling downstream of simple binding would not be surprising. A single STxB protein could cluster up to 15 Gb3 molecules at a time (136), thereby presumably changing the face of the inner leaflet of the plasma membrane at this site. A variety of downstream events might ensue from the creation of such a domain. This may well be reflective of endogenous events, for example, at clustered sites of cellular adhesion or at sites where ligated receptors are recruited into membrane microdomains. A recent study has shown that STxB can be sufficient to induce its own internalization into model giant vesicles (137), but whether this can also happen in the absence of cellular proteins is unclear. This study showed that ATP is not necessary for the uptake of STxB in vivo (interpreted as being a similar process to internalization in their naked model membranes, although these have a specialized lipid mixture that is likely highly permissive for tubular invaginations, and perhaps similarly for scission, which was promoted in these assays by tension changes). However, cellular proteins can store a large amount of potential energy in their structures, and it would be surprising indeed if some of these were not required for internalization of the toxin.

The use of bacterial toxins, which bind to plasma membrane glycolipids, has certainly aided the study of clathrin-independent endocytic pathways. However, care must be taken when interpreting the pathways taken via these toxins as they may be sufficient to induce their own uptake via nonendogenous pathways. For example, ricin has two sites that bind to, and potentially cluster, β1–4-linked galactosides, which are displayed by a wide variety of cellular glycoproteins and glycolipids. This has, as yet, precluded the identification of any (if any exist) specific cellular glycoprotein/glycolipid receptors. Ricin, as well as entering via CME, was shown early to be internalized via a tubular clathrin- and dynamin-independent mechanism that appeared to converge with

clathrin-dependent endocytic markers soon after its internalization (138, 139), but it is unclear if this particular pathway is also used for endogenous cargoes.

ENDOCYTOSIS OF PATHOGENS

Although many pathogens can be taken up into immune cells by phagocytic processes, it is becoming clear that viruses and intracellular bacteria can hijack other endocytic pathways to gain access to different cells. Many viruses hijack the CME machinery in order to be internalized into host cells and, in the main, appear to induce their own CCPs rather than using preexisting ones. There appear to be at least eight pathways by which viruses can be internalized, but many of these may be manifestations of the same pathway, either being modified by the viruses themselves or dependent upon the specific cell type used (2). Markers used for these studies might also be nonexclusive for each putative pathway. It is clear however, that clathrin-independent mechanisms are important in the entry of many viruses. For example, although ~65% of influenza viruses enter through CME, the remainder enter via another clathrin- and caveolin1-independent route, which is involved in swift trafficking of the virus to perinuclear regions of the cell (140). Treatment of cells with eps15 dominant-negative overexpression (which profoundly abrogates uptake of transferrin but does not stop all CME), cholesterol sequestration with nystatin [although this was in contrast with another study in a different cell type (140)], or both simultaneously, had little effect on influenza infectivity (141). Epsin1 depletion did impede CME of the virus, which was still equally infective in depleted cells (142). These results suggest both that there is a cholesterol- and clathrin-independent viral uptake mechanism and that there is compensation for the loss of one uptake pathway by another [and this latter phenomenon can certainly occur in cells (143)]. Downstream redundancy in this system is certainly plausible because fusion of influenza virions with endosomal membranes to allow cytoplasmic release occurs similarly for both clathrin-dependent and -independent pathways (140). Influenza virons bind sialic acid residues on glycolipids/glycoproteins and appear to induce the formation of new CCPs at the site of binding (140). How the virions signal to induce this is unknown.

SV40 virions have regions on their capsid surfaces that bind to plasma membrane GSLs, including GM1 (the receptor for CTxB) to which it binds with high avidity in a similar manner to CTxB (144), enabling them to enter cells though clathrin-independent pathways. This may be due to the induction of lipid microdomain formation with the subsequent recruitment of clathrin-independent budding machinery. Incubation of receptive cells with the virus induced a doubling of the number of active caveolin1-positive structures at the cell surface, and these structures appeared to be capable of trafficking the virus into the cell (69). SV40 does not associate with CCPs. Using caveolin1-null cells, the virus appeared to undergo internalization through the progressive invagination of tightly associated clathrin-independent membranes (eventually ~60 nm in diameter) around the virus in a clathrin- and caveolae-independent, but cholesterol-dependent, manner. Uptake of virus was faster in caveolin1-negative cells than in caveolin1-positive cells and was not altered by the overexpression of dynamin2 K44A (in contrast to internalization in caveolin1-expressing cells) or dominant-negative/-positive arf6 (69). This uptake was sensitive to genistein, implying the necessity of cellular proteins dependent upon phosphorylation to allow functional internalization through this pathway. Internalized viruses continued to associate with detergent-resistant membranes, suggesting that they retained the plasma membrane lipids with which they originally associated. This tight binding may allow for the bypass of endogenous sorting mechanisms and perhaps also allows dissociation from coendocytosed proteins. Overexpression of caveolin1 into caveolin1-negative cells did not inhibit SV40 entry, and internalization could be seen to occur both in

caveolin1-positive as well as caveolin1-negative compartments (69).

The intrinsic curvature of viruses, and their abilities to act with membrane components directly and with high avidity, may immediately promote the production of membrane curvature at this site, with an invagination appearing at the site of contact. This may promote the accumulation of specific lipids, proteins that interact with these lipids, including signaling proteins (which may also be activated through receptor clustering), and membrane curvature-sensing molecules, all of which may synergize to stimulate a specific endocytic apparatus. Viruses are also capable of entering cells via a virus-induced, membrane bleb-associated, and PAK1-dependent macropinocytic pathway that is coupled with, and dependent upon, phosphatidylserine accumulation on the viral envelope, suggesting that this internalization may be similar to that involved in the uptake of apoptotic bodies (145).

Clathrin-independent endocytosis of lipid microdomains appears to bypass the canonical endosome-to-lysosome pathway, offering pathogens a potential route to avoid lysosomal enzymes. The first bacterium shown to invade cells using a lipid microdomain-based mechanism was *Escherichia coli*. The FimH adhesin on the tips of *E. coli* fimbriae is a mannose-binding lectin that binds the GPI-linked protein CD48 and allows the bacteria to be internalized with cholesterol-dependent lipid microdomains in macrophages and mast cells, thereby bypassing lysosomes (146). The entry of *Brucella abortus*, *Campylobacter jejuni*, and *Mycobacterium* spp. into cells has also been shown to be cholesterol dependent (147). Interestingly, clathrin accumulates at the sites where other bacteria, including *Listeria monocytogenes*, undergo internalization and is necessary for the entry of this bacterium into cells (148). This bacterium later traffics through cells by the induction of propulsive actin comets, allowing lysosomal bypass. The surface proteins of *L. monocytogenes*, InlA and InlB, bind to E-cadherin (triggering its phosphorylation and ubiquitination) and the HGF receptor Met, respectively, and, following their ubiquitination by Hakai and Cbl, the bacterium is internalized in a clathrin- and dynamin-dependent manner (148, 149). How this larger scale internalization corresponds sterically to what occurs during CME of nano-/pico-scale particles in noninfected cells may be difficult to imagine, but early studies on yolk protein uptake in mosquito oocytes showed larger CCVs than those routinely found in mammalian cells (9), and there may be poorly understood flexibility and robustness of the associated endocytic apparatus. It is also possible that these bacteria can induce nonendocytic clathrin lattices after binding to the plasma membrane; these have been observed endogenously and are of unknown function but may cluster proteins at particular sites. Indeed, it is unknown if clathrin plays any direct role in the membrane shape changes accompanying bacterial internalization or is simply permissive for entry by other means. Both clathrin and caveolin1 are recruited to E-cadherin-coated beads and are required for their internalization, as well as *L. monocytogenes* internalization (149), but whether and how these proteins communicate is uncertain. Clathrin even appears to be important in the formation of actin-rich pedestals by (noninvasive) enteropathogenic *E. coli* (150), which are induced by delivering (injecting) proteins into cells through type III secretion systems (151). The binding of such an apparatus to membranes (and thus delivery of effector proteins) is dependent on membrane cholesterol (152). Although some of the proteins delivered through this route can directly nucleate actin (153), others modulate the activity of rho family GTPases and thereby indirectly subvert the normal control of cellular actin dynamics (154, 155). Such proteins in *Salmonella typhimurium* [entry of which is cholesterol dependent (156)] include G protein–activating protein (GAP) and guanine nucleotide exchange factor (GEF) proteins that can be recruited to the plasma membrane by interactions with cdc42 (157, 158). Delivered proteins can result in the inhibition, or promotion, of bacterial uptake into cells (155, 159). Whether

such proteins promote bacterial internalization through nonendogenous pathways or modify endogenous endocytic routes is currently unclear.

LIPID REQUIREMENTS FOR ENDOCYTOSIS

It has become apparent that the plasma membrane itself is not simply a passive component in endocytic processes. Indeed, the plasma membrane-enriched phosphoinositide PtdIns(4,5)P_2 is necessary for the invagination of CCPs, and many of the adaptor and accessory proteins involved in CME bind to this phospholipid. The regulation of and the functional roles of this phospholipid in CME are becoming increasingly understood and have been extensively reviewed elsewhere (160). An enzymatic translocation technique has recently shown how hyperacute plasma membrane PtdIns(4,5)P_2 depletion results in near complete AP2 depletion from the plasma membrane but had only minor effects on the assembly of clathrin, although this treatment blocked CME (161). This promising approach may allow further regulatory mechanisms of PtdIns(4,5)P_2 in CME and other pathways to be addressed.

In **Supplementary Information** (see also **Supplementary Figure 1**), we describe the role of other lipids in endocytosis, the nature of putative membrane microdomains/nanodomains, and how lipids may sense, or be involved in, the generation of membrane curvature.

Much work on the determination of lipid requirements for particular endocytic pathways has relied upon the use of agents that extract or sequester membrane cholesterol. Exogenously added MBCD results in the removal of cholesterol from the plasma membrane of cells and abrogates the isolation of ordinarily microdomain-associated proteins from detergent-resistant fractions (162). The hydrophobic core of this compound dissolves lipids by reducing the activation energy of their efflux from membranes. Agents that sequester cholesterol, such as filipin and nystatin, are also routinely used to functionally deplete cholesterol in the plasma membrane. Both extraction and sequestering agents have been used to disrupt endocytic events, but some findings are conflicting, and there remains some uncertainty about lipid requirements for endocytic pathways. For example, extraction of cholesterol with MBCD treatment has been shown to decrease the number of caveolae on the plasma membrane as well as having effects on CME, where treatment resulted in the flattening of CCPs together with a reduction in transferrin uptake (a specific marker for CME events). Such treatment had no effect on the endocytosis of the plant toxin ricin, and indeed, under certain conditions, cholesterol extraction can even increase ricin endocytosis (163). Cholesterol extraction produces a reduction in the amount of plasma membrane ruffling, coincident with reorganization of the actin cytoskeleton (92). In other studies, using concentrations of MBCD that did not have any effect on CME, clathrin-independent endocytic events were observed to be specifically disrupted (8). The use of nystatin/filipin has likewise been shown to specifically affect clathrin-independent endocytosis (69, 164, 165). Different findings with respect to the cholesterol dependence of endocytic pathways may simply reflect the type and concentrations of agents used, the cell types used, and the ligands used to assess clathrin-independent endocytic capabilities, and may also reflect the differential inhibition of truly unique clathrin-independent endocytic mechanisms. Nevertheless, the general consensus in the field remains that, although moderate depletion of plasma membrane cholesterol has little effect on CME, it profoundly affects the endocytosis of particular cellular proteins, as well as that of exogenous toxins and pathogens, and this distinction in lipid requirements has significantly contributed to the study of clathrin-independent endocytic pathways (7). However, as we have recently discussed, great care should be taken when using lipid-modifying agents because these can have knock-on effects on other lipids (166).

To determine the precise SL requirements for endocytosis of an array of markers, Cheng et al. (60) used a temperature-sensitive system whereby they could deplete total cellular SLs by incubation of mutant cells at a nonpermissive temperature, resulting in defective serine palmitoyltransferase activity. Such treatment resulted in a selective blockade of clathrin-independent endocytic events (as determined by LacCer, albumin, dextran, and IL2 receptor uptake assays) with no effect being observed on CME (as determined by transferrin and LDL uptake assays). Probing this further, by using cell-permeable inhibitors of sphingomyelin (SM) and GSL biosynthesis, they found that depletion of both SMs and GSLs inhibited the uptake of all of the studied markers of clathrin-independent endocytosis. Conversely, treatment with inhibitors that increase SM, but decrease GSL levels, specifically inhibited LacCer internalization, with the internalization of the other markers being relatively unaffected. Exogenous SLs were then added to SL-depleted cells to determine specific SL requirements for each cargo. Addition of GM3 or C8-LacCer was sufficient to restore the majority of LacCer endocytic potential, whereas C6-sphingomyelin had no effect on LacCer endocytosis but partially restored that of albumin, the IL2 receptor, and dextran. Although total SL depletion affects the endocytosis of these markers, this treatment increased the toxic effect of ricin through enhanced Golgi delivery in depleted cells (167). The total amount of ricin endocytosis in SL-depleted cells was shown to be normal. Incubation of cells with polyunsaturated fatty acids (which would be expected to interfere with lipid microdomain formation) dramatically reduces STx, but not CTx, uptake or ricin toxicity (168). Taken together, the data reviewed in this section highlight a key role for distinct lipids in the regulation of specific endocytic pathways, although for clathrin-independent endocytic pathways at least, this work remains in its infancy, and correlative uptake and ultrastructural studies after lipid modulation will help to address this in greater depth.

PROTEINS THAT DRIVE AND STABILIZE MEMBRANE CURVATURE

The common underlying feature of endocytic membranes, as opposed to other regions of the plasma membrane, is their necessary high curvature. Biophysical considerations tell us that membranes generally like to be rather flat. Indeed in recent years, a large variety of cellular proteins, including even proteins such as arf family small G proteins, were found to function at sites of specific membrane curvature, and many of these are also capable of, and necessary for, membrane curvature generation. We have already highlighted a role for epsin in CCP formation, where it inserts an amphipathic helix into the proximal lipid monolayer to induce membrane curvature that is concomitantly stabilized by clathrin. Such helices insert into the protein-proximal leaflet of the lipid bilayer, with the center of the helix at the glycerol backbone of the lipids. This results in the phospholipid moieties becoming splayed because the glycerol backbone is the most rigid point of the lipid (and acts as a fulcrum for lipid rotation). This splay, coupled with an increase in outer monolayer area relative to the inside, effectively induces membrane curvature generation.

The cytoskeleton is also likely to be important in membrane curvature regulation (166). In addition to pushing forces that might be contributed by actin polymerization (helping, e.g., to push neck membranes closer together), the cytoskeleton may also provide pulling forces to keep the neck under tension, which promotes dynamin's fission ability (169). Indeed, motor proteins such as myosin VI [which binds to the CME adaptor dab2 and PtdIns(4,5)P_2 (170)] and myosin 1E [which binds to dynamin (171)] have been implicated in CME.

The insertion of caveolin1 into the membrane likely plays a role in membrane curvature generation in caveolae, presumably through a poorly understood role for its membrane-inserted hairpin and oligomerization. However, flotillins, which are also proposed to have similar hairpins, do not appear to be sufficient to

induce membrane curvature in vivo (68). By replacing cholesterol with its precursor desmosterol, the number of caveolin1 molecules per caveola was decreased, and this resulted in a greater heterogeneity of caveolar sizes (172), consistent with caveolin1 being necessary for caveolar formation and its role in effecting membrane curvature changes (or stabilizing such changes) at these sites. Alternatively, caveolin1 may recruit other proteins responsible for the induction and stabilization of membrane curvature at these sites. Dynamin appears to play a role in caveolae membrane dynamics (see below), but given caveolar stability, these findings are at odds with the transient recruitment to, and function of, dynamin at other sites, and it is unclear how its scission activity would be inhibited at caveolae. Furthermore, although dynamin forms spiral oligomers, it appears that the densities at caveolar necks are more ring like (44), and it is unclear if dynamin contributes to these densities.

Other ways in which membranes can be deformed are schematized in **Figure 2**. In **Supplementary Information** (see also **Supplementary Figure 2**), we review the BAR superfamily of proteins, many of which are intimately linked with membrane trafficking events, including endocytic events, where they may help generate/stabilize the high membrane curvature of these sites and/or function in effector recruitment to sites of specific membrane curvature.

SMALL G PROTEINS AND CYTOSKELETAL REQUIREMENTS FOR ENDOCYTOSIS

Members of the arf, rab, and rho families of small G proteins appear to be very important in endocytic events. These proteins proceed through membrane-bound and cytosolic cycles in manners regulated by their intrinsic GDP/GTP-loading status and other cellular proteins. A typical life cycle for a rab family member is shown in **Figure 3*a***. Distinct endocytic pathways appear to require specific types of small G proteins, and the mechanisms by which these might promote endocytosis through effecting membrane curvature changes are shown in **Figure 3*b***. However, the observed small G protein dependence on the same underlying endocytic pathway may differ between cell types and may not be a very specific method (predominantly due to pleiotropic effects of interference with small G protein function) by which to initially categorize these pathways. However, in the absence of better markers and more mechanistic information as to how small G proteins contribute to endocytosis, such studies have proved very useful. The roles of some specific small G proteins known to be of relevance to endocytic pathways are now reviewed.

Of the six arf small GTPases, only arf6 is robustly found at the plasma membrane (173) and is enriched in membrane ruffles, where it is necessary for their rac1-dependent formation, perhaps by recruiting rac1 (174). Arf6 regulates at least one clathrin-independent endocytic pathway responsible for the internalization of cargoes, including CD59, MHC class I, IL2Rβ, mGluR7, carboxypeptidase E, and GPI-linked proteins (71, 82, 175). The turnover of GTP by arf6 also plays a key role in the cycling of synaptic vesicles (176). The arf6 effectors phospholipase D (which catalyzes the formation of phosphatidic acid) and phosphatidylinositol(4P) 5-kinase [which catalyzes PtdIns(4,5)P_2 formation] favor PtdIns(4,5)P_2 formation, which itself is an arf activator (177). Arf6 activation can thereby result in a rapid rise in PtdIns(4,5)P_2 levels at the plasma membrane. Arf6 also has a direct role in the regulation of the cytoskeleton, and local production of PtdIns(4,5)P_2 also regulates actin polymerization (for example, WASP and profilin are regulated by binding to this phospholipid) (178, 179). PtdIns(4,5)P_2 is enriched in the plasma membrane and is required for the invagination of CCPs (160). Overexpression of dominant-active arf6 produces ruffle formation and induces macropinocytosis (173). Many CME adaptor and accessory proteins bind to PtdIns(4,5)P_2 through PH, ANTH, or ENTH

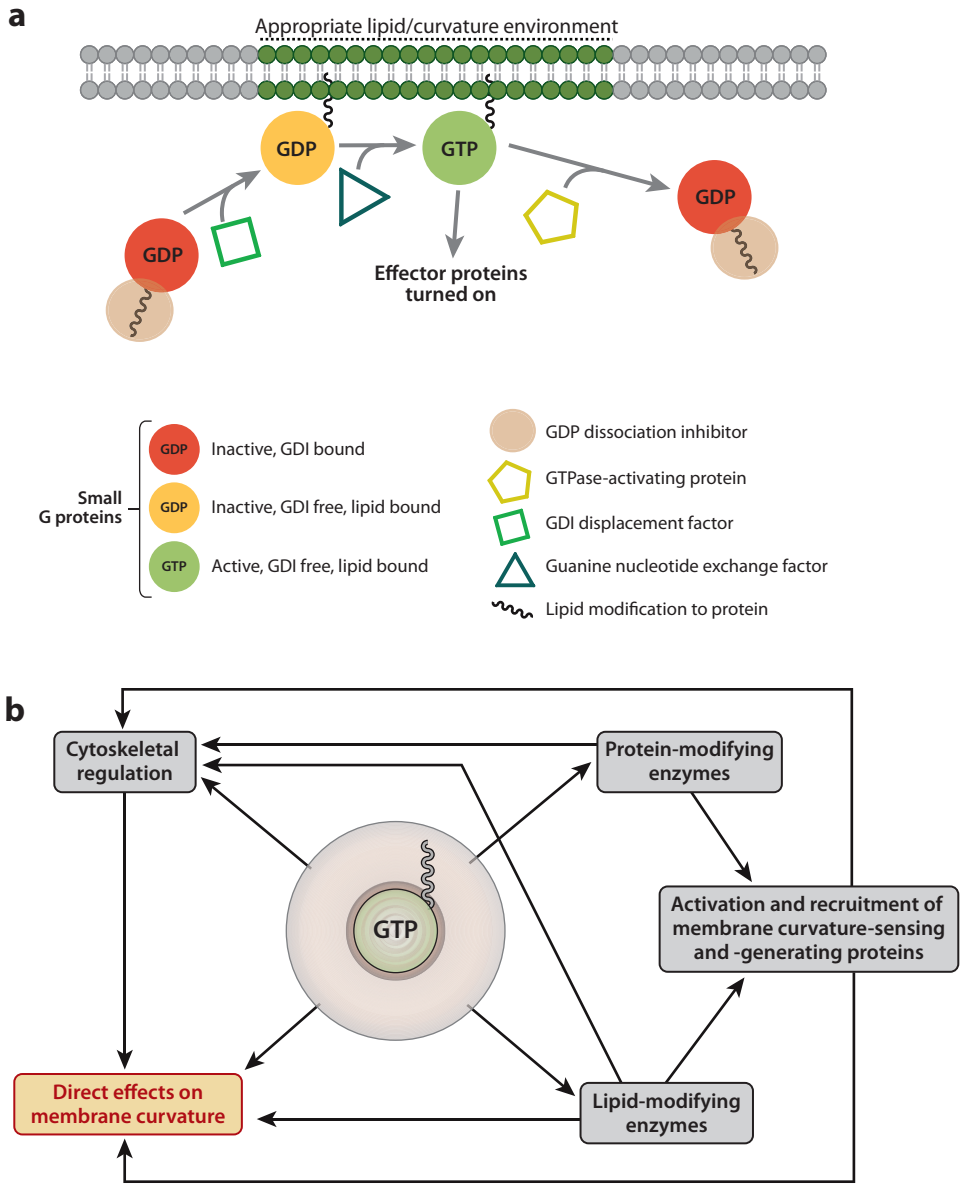


Figure 3

The small G protein life cycle and ways in which these proteins can modulate endocytic events. (*a*) Schematic diagram showing a typical life cycle for a small G protein. In the case of arf family G proteins, activation will take place preferentially on a highly curved membrane. (*b*) Effects of small protein activation, with a focus on how these proteins can have effects on membrane curvature.

domains and positively charged patches (in the case of AP2) (160). A direct role for arf6 in CME is elusive, as there seems to be little biochemical evidence to support a link specifically to this process, although activated arf6 can recruit AP2 to membranes in vitro (180). However, in some cell types at least, arf6 regulates CME of receptors, such as angiotensin type I, and

vasopressin type 2 receptors (181), and the arf6 GAP, SMAP, which interacts with clathrin, appears to have effects specifically on CME (182).

Recently, we have shown how arf and arf-like proteins may contribute to endocytic regulation in a novel manner (183). GTP-loaded arf1 and arf6 (their active forms) are capable of generating positive membrane curvature through the insertion of an N-terminal amphipathic helix into the proximal lipid monolayer (183). The hydrophobic surface of this helix is unavailable for membrane binding in GDP-bound arf but becomes released upon GTP loading (184). Furthermore, these proteins load GTP in a curvature-sensitive manner (loading is stimulated by high positive curvature). These characteristics are positively reinforcing: Exchange of GTP results in helix insertion, which, in addition to decreasing the off rates of these proteins from the membrane, results in greater membrane curvature and more GTP loading. Active arfs thus become clustered on membrane buds where they activate their effectors. It is unclear how this process is spatiotemporally regulated by arf GTPase-activating protein (arfGAPs) and arf guanine nucleotide exchange factors (arfGEFs), but several of these proteins are curvature sensitive (through BAR domains). It may be therefore that curvature is the primary regulator of the GDP-GTP cycle of arf proteins.

Arf1, which is usually resident at the Golgi, and the PDZ domain-containing rhoGAP ARHGAP10 that it recruits have been shown to be necessary for the CLIC/GEEC endocytic pathway (185). ARHGAP10 depletion also reduces α-catenin recruitment to adherens junctions and *L. monocytogenes* invasion (186). Given that arf6-associated clathrin-independent endocytic membranes share many properties with CLIC/GEEC membranes, it is possible that arf1 and arf6 differentially regulate CLIC/GEEC subtypes, or such membranes in different cell types. How arf1 contributes to CLIC/GEEC endocytosis is uncertain, but its activity is necessary for the regulation of the plasma membrane residence time of cdc42 [perhaps through ARHGAP10, which has GAP activity for cdc42 (186)], which is known to be important for CLIC/GEEC function. Furthermore, dominant-active arf6 suppresses lipopolysaccharide-induced macropinocytosis, and arf6 hyperactivity or inactivity both cause dramatic inhibition of podosome formation in, and migration of, primary dendritic cells (187), perhaps through lipid or cytoskeletal effects, but is unclear precisely how this is mediated.

Each rab GTPase (including specific isoforms) appears to highlight and regulate a distinct intracellular trafficking compartment. Although rab5 plays important roles in the pathway of CME, rab8 associates with macropinosomes and the seemingly endocytic, arf6-associated, tubular structures that emerge from these (188). Rab8 is also necessary for CTxB transport to the Golgi, and β1 integrins have been found in rab8-positive tubules. This is consistent both with observations that β1 integrin trafficking is arf6 dependent and that rab8 overexpression/depletion has effects on cellular morphology (188, 189). Niemann-Pick C (NPC) cells have abnormal storage of intracellular cholesterol and exhibit defects in CTxB uptake and trafficking to the Golgi apparatus. Remarkably the abilities of these cells to traffic CTxB and remove accumulated cholesterol are restored by rab8 overexpression, despite the fact that this is not the genetic deficit in these cells (190). Rab8 depletion also results in intracellular cholesterol storage. These results might imply that a membrane trafficking pathway regulated by rab8 allows bidirectional transport (either directly or through appropriate lipid homeostasis). We have recently shown that rab8 also localizes to CLIC/GEEC endocytic membranes (14).

Members of the rho family of small G proteins—rhoA, cdc42, and rac1—have been extensively implicated in endocytic regulation as well as in controlling cytoskeletal changes and signaling events within the cell (191). The pathway responsible for IL2Rβ endocytosis is dependent on rhoA and rac1 (78, 79), whereas rac1 is heavily implicated in macropinocytosis (102). Heterologously expressed nicotinic acetylcholine receptor (AChR) is internalized

by tubular intermediates in a Rac- and actin-dependent endocytic manner (192). Although overexpression of a constitutively GDP-bound mutant of rhoA specifically inhibits albumin and IL2R endocytosis, inactive cdc42 specifically inhibits fluid phase internalization (60). The CLIC/GEEC pathway requires cdc42, and perhaps its functions there include the promotion of actin polymerization, which appears to be required for endocytosis through this pathway (70, 71). RhoA and cdc42 bind to lipids and preferentially to SM- and cholesterol-enriched membranes. Cells depleted of SLs have less rhoA and cdc42 present at the plasma membrane, and this effect is also observed upon SM depletion (which may account for some of the observed lipid dependence of endocytic pathways described above). The localization of dominant-active rhoA/cdc42 to the plasma membrane can be restored in these cells by C6-SM, which also partially restores the endocytosis of albumin, dextran, and the IL2R. Therefore, membrane recruitment of small G proteins to specific membrane regions from which endocytosis appears to occur seems to be important for their functions in endocytic regulation. Cholesterol depletion, however, stabilizes cdc42 at the plasma membrane and reduces its activation (70), perhaps accounting for some of the effects of this treatment on clathrin-independent endocytic events. How small G protein–regulatory events in endocytosis are spatiotemporally coordinated, and the roles and nature of their important effectors in each endocytic process, remains incompletely characterized. The schematic in **Figure 3*b*** may provide a framework for the study of the roles of these proteins in endocytic regulation.

Rho family small G proteins are extensively implicated in cytoskeletal regulation. We have previously discussed the role of both small G proteins and lipids in modulating cytoskeletal polymerization and the important way in which membrane-cytoskeleton interactions are produced and controlled (166). These issues are not discussed here. It should, however, be noted here that, although endocytic pathways may be differentially modulated by agents that disrupt the actin cytoskeleton, whether such requirements for actin are due to the need to coordinate its polymerization at the site of an internalizing membrane is not understood because these agents have profound effects on other parameters, e.g., plasma membrane tension, which would have knock-on effects on endocytosis. Constitutively active rhoA can even inhibit CME, although it is not likely that rhoA plays a direct mechanistic role in this pathway (78). Such requirements may be dependent on cell type; as well as on the substrate on which the cells are grown; and whether one is imaging/recording endocytosis at the coverslip face of the cell, the dorsal surface, or more globally (166). Although most studies on macropinocytosis have found it to be actin dependent, the NKG2A inhibitory receptor was found to be internalized by a rac1-dependent, actin-independent macropinocytic process (193), suggesting that there may be several variants of this type of process. We and others have discussed the various roles actin may play at the site of endocytosis, and we refer the reader to these reviews (166, 194, 195).

Despite the importance of small G proteins and actin, focused study of these molecules may ultimately not allow the definitive dissection of endocytic pathways. The identification of specific markers that surround, and that are necessary for the formation of, the newly forming endosomal membranes will allow such questions to be addressed more directly. The identification of GRAF1 as a protein directly involved in, and necessary for CLIC/GEEC endocytosis goes someway toward this goal (14).

Because researchers are predominantly dependent on cultured cells for the study of endocytic pathways, as well as the caveats already discussed, the route to senescence that these cells usually undergo through repeated passage may likewise determine the relative proportion of endocytic events at any given passage number. Studies must therefore depend on the isolation and characterization of distinct pathways and their specific endogenous markers in

homogeneous cells before the true relevance, overlap, and function of these pathways in vivo can be addressed by tools such as mutant small G proteins.

DYNAMIN AND FISSION OF ENDOCYTIC MEMBRANES

Dynamin is a large molecular weight GTPase, which can bind and tubulate liposomes, and, upon GTP hydrolysis, is capable of stretching these tubulated liposomes through changes in the conformation of its spiral oligomer (196). Dynamin1 preferentially binds PtdIns(4,5)P_2, which promotes dynamin oligomerization, a process regulated by other curvature-sensing proteins, such as the BAR domain-containing proteins SNX9 and amphiphysin1 (197). Dynamin1 is found around the neck connecting invaginated CCPs and the rest of the plasma membrane and is necessary for CME (198, 199). It has also been proposed that, as well as this pulling force which brings the membranes of the neck into close apposition, a twisting force exerted by dynamin1 on tubular membranes is responsible for their vesiculation/fission (169) and that this may contribute to overcoming the high activation barrier presented to membrane fission (**Supplementary Figure 3**). It might also provide a reason why actin is involved in endocytic events—here it may provide tension to the neck of the vesicle. Membrane fission must occur during the last stage of any endocytic event. Fission could occur from the "ripping" and subsequent resealing of membranes. This is unlikely because not only does ripping membranes require a great deal of energy, it would also have to occur circumferentially around the membrane at once. Furthermore, it allows transient electrochemical communication between the extracellular and intracellular fluids. The most likely mechanism by which membrane fission occurs is via the transient fusion of membranes, with proximal monolayer mixing and point-wise formation of a hemifusion intermediate, and subsequent expansion of this point to form a hemifusion diaphragm before full fission. It is extremely unlikely that this will occur spontaneously (within normal thermal fluctuations) without the input of energy in one form or another from cellular proteins. The published dynamin dependencies of endocytic pathways are summarized in **Table 1**. Specific findings that have led to some of these conclusions are now reviewed.

At first glance the CLIC/GEEC pathway appears to be dynamin independent because dominant-negative dynamin still allows apparent internalization and because dynamin does not localize to these early endocytic structures by immuno-EM. Dynamin1 K44A overexpression in MEFs resulted in the abrogation of Golgi delivery of CTxB. Instead, CTxB was found to accumulate in tubular compartments (8). These were not labeled with CTxB at 4°C, suggesting that these structures were not still connected to the plasma membrane, but these may be partially inaccessible to washing steps attributable to diffusion limitations within such a structure. Microinjection of antidynamin2 antibodies or dynamin2 depletion in cells inhibits basal levels of fluid phase uptake, but not stimulated levels (suggesting that this effect of dynamin is not through inhibition of macropinocytosis), and only expression of specific dynamin2 splice variants in dynamin2-depleted cells rescues this phenotype (200). Because CLIC/GEEC endocytosis is associated with so much fluid phase internalization (14, 71) these data suggest that this pathway is dynamn dependent. Furthermore, acute dynamin inhibition abrogates CLIC/GEEC endocytosis, and dynamin is strongly linked biochemically to this process through binding the SH3 domain of GRAF1 (14).

The clathrin-independent pathway responsible for IL2Rβ internalization appears to be dynamin dependent (78), as does caveolar-type endocytosis. Caveolin1 and dynamin form a complex in vivo. An in vitro system using the plasma membranes of lung endothelial cells showed that cytosolic factors and GTP hydrolysis were sufficient to induce the fission of caveolar structures rich in caveolin-1 and

trafficking markers in a dynamin-dependent manner (201). Dynamin has also been found at the neck of caveolae, and GTP hydrolysis by dynamin appears necessary for the pinching off of such structures (202, 203). Dynamin2 may also control the production of single mobile caveolae from the larger, much more static, multicaveolar assemblies. Dynamin2 K44A overexpression results in the accumulation of large multi-invaginated caveolar-type structures and reduces SV40 internalization and infectivity (204), although these observations may be due to pleiotropic effects of mutant dynamin overexpression. CDR internalization appears to be dynamin dependent. Although dynamin K44A can inhibit PDGF-induced macropinocytosis, this may be through modulation of rac localization (205). Dynamin does not appear generally necessary for macropinocytic mechanisms, where the PAK1 substrate CtBP1/BARS has been shown to be important for fission (206). However, whether this protein has direct or indirect effects on the fission process is unclear. Flotillin-associated endocytosis has been shown to be both dynamin dependent and independent (64, 67), whereas arf6-associated endocytosis appears to be dynamin independent, although the Vp22 protein from herpes simplex virus is internalized by an arf6-dependent, dynamin-dependent, clathrin-independent pathway (207).

The use of dominant-negative dynamin constructs to inhibit CME has been well documented. K44A (without any bound nucleotides) and S45N (the putative GDP-locked state) mutants are used and presumably inhibit the function of endogenous dynamin competitively (199). Using an inducible cell line expressing dynamin K44A, it was shown that the long-term internalization of ricin was not blocked by overexpressed levels of this mutant known to significantly abrogate CME, despite a reduction up to at least 15 min after induction (208). The extent of toxin degradation was not significantly different in overexpressing and noninduced cells. However, although the ricin-containing endolysosomal compartments looked similar at the ultrastructural level in both mutant-expressing and control cells, the amount of the toxin reaching the Golgi apparatus, and its toxic effect, was profoundly reduced in mutant-expressing cells. Ricin is ordinarily internalized by tubular carriers, which merge with clathrin-dependent markers soon after internalization, and it may be that transport of ricin from this compartment to the Golgi is dynamin dependent (138, 208). Conversely, an effect similar to that observed for CTxB uptake in the CLIC/GEEC pathway may be responsible or an alternative pathway may be upregulated. Interestingly, increased expression of inducible dynamin K44A by butyric acid treatment potentiated the reduction in toxicity observed in mutant-overexpressing cells (208). This may suggest that different dynamin-dependent pathways are differentially sensitive to the amount of mutant available.

Dynamin1 knockout mice die shortly after birth. It appears that the large increase in clathrin-coated profiles (including those connected to tubular plasma membrane invaginations) at resting state in neurons from these mice occurs predominantly at inhibitory synapses, similar to effects reported for synaptojanin knockout mice (209, 210). In other synapses perhaps, although dynamin1 usually performs vesicle endocytosis, other dynamins may compensate in CME, or clathrin-independent mechanisms that may normally be more silent are upregulated in extent, and only when synapses are strongly stimulated, does the lack of dynamin1 become apparent.

Before the precise function of each dynamin splice variant is known, it is difficult to predict in which particular splice variant background mutants should be produced in order to study specific effects of their overexpression. Dynamin inhibitors show great promise for acute dynamin inhibition (211), and the use of these will allow more simple interrogation of the roles of dynamin and provide novel insight into whether and how dynamin regulates particular endocytic pathways.

THE CONTROL OF ENDOCYTOSIS AND ITS PHYSIOLOGICAL IMPORTANCE

Although much is known about the role of CME, it is unclear why distinct endocytic pathways are required in vivo. This may be due to differential requirements for many parameters, including speed, cell signaling, cargo delivery to specific compartments, and membrane area/lipid turnover. Endocytosis intimately regulates many processes, including nutrient uptake, cell adhesion and migration, signaling, pathogen entry, synaptic transmission, receptor downregulation, antigen presentation, cell polarity, mitosis, growth and differentiation, and drug delivery. We discuss only some intriguing examples here.

The recycling/degradation fate of a receptor is dependent on endocytic mechanisms. Indeed, although clathrin-independent EGFR endocytosis appears to target the receptor to be degraded, EGFR internalized by CME appears to be recycled to the plasma membrane where it can continue to participate in signaling (212). Such observations have dramatic implications for drug discovery, opening up new avenues to modulate cell signaling pathways. For example, EGFR is hyperactive in many cancers, and routing this receptor to clathrin-independent mechanisms, e.g., by CME adaptor function inhibition, should enhance degradation of the receptor and abrogate signaling. The study of endocytic mechanisms also provides a basis for the understanding of drug delivery and will hopefully inspire new techniques to deliver drugs to specific intracellular locations.

The abilities of cargo molecules to associate with adaptor proteins of the CME machinery and their relative affinities for distinct types of membrane lipids (which could, in clathrin-independent endocytic events, assume the role of protein adaptors in CME) will likely ultimately determine whether these proteins are internalized by clathrin-dependent or -independent mechanisms. Likewise, different subtypes of both clathrin-dependent and clathrin-independent endocytosis will be coordinately regulated such that appropriate further trafficking may occur in response to ligation of protein or lipid receptors. The common dependence of clathrin-independent endocytic pathways on cholesterol and tyrosine kinase activity is suggestive of a common type of mechanism underlying these pathways and may provide the basis through which this control can be studied. There is a distinction between what are known as constitutive (where endocytosis continuously occurs in the absence of activation by ligands) and induced forms (where endocytosis is stimulated by ligand binding and downstream events) of endocytosis (34). Clearly these require differential signaling modulation, and receptor clustering, ubiquitination and kinases appear to be important in this; these are well reviewed elsewhere [see e.g., (213)]. Because many ligands undergo both types of internalization, because distinct endocytic pathways can be specifically upregulated in extent, and because there are often many difficulties in distinguishing between these two modes, this issue has not been considered further here, nor has the issue of apicobasal polarity, where distinct endocytic mechanisms operate at distinct poles, because this is also discussed elsewhere (47).

Data from work in insulin-stimulated cells may provide a mechanism (albeit perhaps oversimplistic) to explain how and why internalization by distinct endocytic pathways may be endogenously controlled in a physiological process. In unstimulated adipocytes, the GLUT4 glucose transporter is usually internalized by a cholesterol-dependent pathway, with a minority going in via a slow AP2-dependent pathway (owing to the presence of a FQQI intracellular motif). Upon treatment with insulin, uptake through the cholesterol-dependent pathway is inhibited through a broad effect on cholesterol-dependent endocytic mechanisms (214). Consequently, more of the transporter may be presented upon the cell surface to participate in glucose transport (presumably because FQQI motifs cannot be as efficiently recruited into CCPs as can canonical recruitment motifs), despite an increase in transferrin receptor endocytosis occurring in

insulin-treated cells. This may be reversed in vivo by a classical negative feedback mechanism whereby the induced fall in plasma glucose concentrations, through glucose uptake into cells, will result in reduced insulin secretion, whereupon a shift back to a predominantly cholesterol-dependent, AP2-independent type of internalization occurs. This mechanism may act in concert with insulin-stimulated exocytosis of GLUT4-containing vesicles.

It is well-known that receptor tyrosine kinases (such as the EGF, PDGF, and HGF receptors) are capable of undergoing CME, and in the presence of low EGF concentrations, the majority of EGF receptors undergo CME and are not ubiquitinated. At higher levels of EGF, where the receptor is endocytosed via CDR-associated mechanisms, the receptor becomes ubiquitinated and degraded (215), presumably to allow growth factor gradient-sensing capabilities to be restored as well as to avoid a massive response to sustained signaling. EGFR signaling appears to differ depending on microdomain/nonmicrodomain association, as well as on endosomal location, perhaps owing to the relative partitioning of downstream effector molecules [e.g., H-Ras is enriched in lipid microdomains, whereas K-Ras is excluded from these regions (216)]. Thus, the switching of endocytic mechanism might not only influence the fate of the receptor, but also the extent and type of signaling that can ensue after ligation. Surprisingly, clathrin-independent endocytosis of the EGFR appears to be dependent on epsin, eps15, and eps15R, proteins heavily implicated in CME mechanisms (215), and such studies suggest that caution be advised when interpreting results relying on the modulation of these proteins. As with EGFRs, TGFβRs are endocytosed by both clathrin-dependent and cholesterol/caveolin1-dependent pathways. CME of the receptor seems to occur upon association with Smad anchor for receptor activation (SARA) and permits signaling (217), presumably by allowing interaction with downstream signaling components such as Smad proteins. By contrast, endocytosis of this receptor via clathrin-independent pathways appears to enhance receptor degradation (217). Such rerouting appears to be dependent on the interaction of the receptor with the Smad7-Smurf2 E3 ubiquitin ligase (218).

Although the PDGFR induces CDRs at high concentrations of PDGF, it undergoes CME at low concentrations of PDGF. Low concentrations of PDGF induce cell migration, whereas higher doses induce cell proliferation (219). It is likely therefore distinct endosomal membranes are controlling very different signaling pathways downstream of PDGF ligation. The cross talk between signaling and endocytosis has been reviewed elsewhere (220) and is a hot area of research. Endosomal control of cell signaling has been conserved even in flies where physiological Notch signaling requires entry of the receptor into endosomes (221).

Caveolin1 increases the export of, and the total amount of, free cholesterol in cells (222). A role for caveolin1 in the regulation of intracellular lipid homeostasis has been proposed owing to these results and to the finding that it regulates cellular fatty acid uptake (223). Parton & Simons (10) have recently argued how caveolae may function as stores for microdomain-associated lipids and have suggested that communication between lipid droplets (fat-storing organelles) and caveolae might allow the regulation of lipid storage and release within the cell.

Another putative, but elegant, role for the function of caveolin1, and associated caveolae, is in the storage of apparent membrane surface area (i.e., the area of the membrane that would be able to interact with flat surfaces parallel to the major plane of the plasma membrane; this includes the apparent spaces in the main membrane plane formed by, for example, caveolar invaginations) because flattening of infoldings would be expected to increase this area. Some evidence exists that caveolin1 and caveolae may respond to shear stress and cellular stretch (10). Stretch results in the redistribution of caveolin1 (presumably from caveolae) to sites of adhesion (224). Because caveolin1 at adhesion sites is not associated with caveolae, presumably then,

this redistribution is concomitant with caveolar collapse and an increase in apparent surface area. As caveolae can undergo kiss-and-run fusion and fission with and from the plasma membrane (37), this may contribute to the flexibility of such regulation. Such mechanisms may allow for cellular elasticity and counter forces that might otherwise result in the ripping of the plasma membrane. Consistent with this, the shape changes that follow ICAM1-dependent adhesion of neutrophils to lung endothelial cells and accompany hyperpermeability of this site are caveolin1 dependent (225). Intriguingly, caveolae are enriched in cell types that are most exposed to physiological stretch and shear.

Caveolae are extensively implicated in the modulation of signaling events, but whether and how this is coupled to endocytosis is not known. The N-terminal domain of caveolin1 interacts with a variety of signaling molecules, including src family kinases (226), H-Ras (227), and eNOS (228). Caveolin1 also interacts with the kinase domain of the EGFR and negatively regulates its kinase activity (229). G_{α} subunits of heterotrimeric G proteins are enriched in caveolae and interact with caveolin1, an interaction which also appears to negatively regulate their signaling (143). Certainly caveolin1 appears to have many effects on important cellular processes, including the regulation of cell growth and division, mitogen-activated protein kinase signaling, and contact inhibition (10). It also appears to act as a tumor suppressor protein in cultured cells (230), yet caveolin1-null mice have no increased cancer incidence, suggesting that loss of the protein alone is not sufficient to produce malignancies [despite an increase in cell proliferation observed in the mammary glands of these mice (231)]. However, mutations have been found in caveolin1 in breast cancer tissue, and loss of this protein may therefore promote progression to cancer (232, 233). Consistent with this, dysplastic lesions are found more often in caveolin1-null mice than in heterozygotic mice on a breast tumor-prone genetic background (234), and caveolin1-null mice are more prone to developing malignancies from epithelially applied carcinogens (235).

CME and clathrin-independent endocytosis are both stimulated by an increase in cytosolic Ca^{2+} concentrations in pancreatic β-cells, likely as a result of increased exocytosis. Indeed, exocytosis and endocytosis are intimately coupled (166). The clathrin-independent pathway identified here is actin- and dynamin-dependent and is stimulated by higher Ca^{2+} concentrations than the CME pathway. The roles of these distinct pathways remain to be established but are reminiscent of fast clathrin-independent endocytosis at synapses, where CME appears to have a role in slower vesicle retrieval to maintain synaptic area (236). The precise contribution and roles of presynaptic CME and clathrin-independent endocytic pathways are currently under hot debate. The most controversial debate of all is whether kiss-and-run exo-endocytic cycling occurs (237). In this process exocytosed vesicles do not fully collapse into the plasma membrane but transiently fuse, allowing content release before rapid endocytosis, and this process presumably requires less energy than endocytosis after full collapse and has been shown to occur in other cell types (238).

Endocytosis, and membrane trafficking in general, has been intimately linked to the ability of cells to move. In **Supplementary Information**, we discuss links between endocytic mechanisms, cell-matrix adhesion turnover, and cell migration (see also **Supplementary Figure 4**).

ENDOCYTOSIS AND DISEASE

Given the obvious importance of the exo-endocytic cycle, it is not surprising that this and other membrane trafficking pathways are often disrupted in human disease (239, 240), and this link could be much more far reaching than previously anticipated given the variety of processes endocytosis regulates [which now include even sex steroid internalization (241) previously thought to occur via diffusion through the plasma membrane] and the plethora of proteins being identified as endocytic proteins. With the probable vital importance of many core endocytic components and

key adaptors, these proteins are unlikely to be found commonly mutated in human disease or (given the robustness of the network of core components) result in mild phenotypes if mutated (4). Most of the mutations may be found in more specialized accessory proteins or in cargo proteins themselves. For example, the LDL receptor adaptor protein ARH, which links the receptor to clathrin and AP2 and allows it to be recruited to CCPs (242), is mutated in autosomal recessive hypercholesterolemia. Caveolin3 is found mutated in a variety of dominant myopathies and muscular dystrophies.

However, alongside mutations in canonical endocytic proteins, it is also possible that several neurodegenerative conditions either produce or are caused by defects in endocytic processes. Much thought on endocytic abnormalities accompanying neurodegeneration has focused on these as downstream consequences of protein aggregation, which could be caused by titration of cellular proteins into, or cytotoxicity of, these aggregations. It is possible, however, that such endocytic defects are more primary in these diseases and that aggregates and other pathological and clinical findings form downstream of endocytic defects, for example, through aggregation and loss of function of the aggregation-prone protein. For example, polyglutamine expansions in ataxin2 can cause spinocerebellar ataxia type 2. Although the normal function of ataxin2 is not clear, it seems to inhibit EGFR internalization and intriguingly binds to endophilin A1 and A3, suggesting an important role for ataxin2 in endocytic regulation (243). Endocytic/retrograde trafficking phenotypes are also early features of Alzheimer's disease and Down's syndrome (244, 245). Furthermore, huntingtin and HAP40 interact and appear to act as an effector complex for rab5 in control of endosome dynamics, reducing their overall motility by favoring the association of such organelles with actin over microtubules (246). Huntingtin interacts with membranes, and this membrane interaction is critical for huntingtin aggregation (247). The toxic Aβ protein associated with plaques in Alzheimer's disease is released from amyloid precursor protein (APP) in an endocytosis-dependent manner, and flotillin2, which promotes cholesterol-dependent clustering of APP on cell surfaces, is necessary for a portion of APP uptake by neurons by CME and Aβ generation (248).

The desmosomal cadherin Dsg3 is a target for autoantibodies in pemphigus vulgaris, and these antibodies induce Dsg3 endocytosis through a clathrin- and dynamin-independent pathway, seemingly resulting in loss of desmosomal adhesion (249). Ordinarily cadherins undergo endocytosis by both CME and clathrin-independent mechanisms, and the endocytic regulation of sites of cell-cell adhesion has been reviewed elsewhere (250). It is important that we study in detail the endogenous mechanisms of endocytosis to ever fully understand how these contribute to health and thereby how their dysregulation contributes to in human disease.

SUMMARY POINTS

1. Endocytosis has many functions. It controls the composition of the plasma membrane and thus controls how cells respond to, and interact with, their environments. For example, endocytosis plays key roles in nutrient uptake, cell signaling, and cell shape changes.
2. There are many distinct endocytic pathways that coexist in mammalian cells. In this review, we outlined ten different pathways. Currently pathways are best defined through their differential dependencies on certain lipids and proteins, including clathrin, caveolin1, flotillin1, GRAF1, kinases, small G proteins, actin, and dynamin. Although some cargoes enter exclusively by one pathway, most cargoes can enter by several pathways.

3. Any endocytic mechanism requires the coordinated action of proteins that are capable of deforming the plasma membrane to produce highly curved endocytic intermediates and proteins that can induce scission of these intermediates from the plasma membrane. Such proteins include ENTH domain-containing proteins, BAR superfamily proteins, arf family small G proteins, proteins that nucleate actin polymerization, and dynamin superfamily proteins. The best understood mechanism is clathrin-mediated endocytosis (CME).
4. Although caveolae have been extensively reported as endocytic intermediates, the contributions of caveolae to endocytosis are uncertain.
5. The molecular and functional dissection of endocytic mechanisms is vital for the understanding of many physiological phenomena and disease processes. Such understanding will allow the rational discovery of novel therapeutic agents.

FUTURE ISSUES

1. How is clathrin-coated pit (CCP) formation coupled to cargo incorporation? What controls CCP size, and how is CCP fission regulated by dynamin? How are the membrane curvature-modulating proteins acting in CME spatiotemporally coordinated?
2. What are the cell biological functions of clathrin-independent endocytic pathways, and how are they controlled? How is cargo recruited to these membranes? Is cargo concentrated by adaptor proteins? What are their coat proteins? How is membrane curvature generated in these pathways? How is the scission of these endocytic membranes mediated?
3. What are the distinct features of CME and clathrin-independent endocytosis in cell signaling and cargo delivery? How is entry of cargoes that can enter by multiple pathways controlled?

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SUPPLEMENTARY INFORMATION AND SUPPLEMENTARY FIGURES FOR MECHANISMS OF ENDOCYTOSIS

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LIPIDS AND ENDOCYTOSIS

As well as providing an appropriate hydrophobic environment for membrane-inserted proteins (via transmembrane domains) or membrane-associated proteins (via GPI anchoring/acylation/myristoylation/prenylation, etc.), membrane lipids play vital recruitment and scaffolding functions for membrane-associated signaling events (via binding of a variety of protein domains, including PH, PX, ENTH, ANTH, BAR, N-BAR, F-BAR, I-BAR, FYVE, and FERM domains). These functions for lipids and another in the modulation of endocytic events (as is reviewed below) may be intricately interconnected because both signaling and other membrane-resident proteins would be expected to modulate endocytic events and vice versa. This has made it difficult to decipher whether certain lipids that are necessary for certain types of endocytic events are simply permissive bystanders (e.g., in terms of being appropriate shapes to sterically permit the architectural changes that occur in budding regions of the plasma membrane) or are indeed active controllers of endocytic events. Certainly, in the case of clathrin-mediated endocytosis (CME), there is clear evidence for the coexistence of both types of modulatory capacity.

In vitro saturated chains of hydrocarbons pack together better than do unsaturated lipids, and this certainly creates some degree of membrane heterogeneity within the plane of a membrane monolayer. The shapes of the volumes occupied by membrane lipids allow them to coordinately pack with other lipid types with “cognate” volume shapes, and this likely permits membrane curvature generation, a prerequisite for any endocytic pathway. However, whether particular lipid components of cellular membranes, at physiological temperatures and diffusion rates, form clusters in which lipid-interacting proteins are enriched remains surprisingly controversial (1). A great deal of evidence supports their existence, but there is extensive debate surrounding the size and nature of such “lipid rafts,” or “membrane microdomains,” and theoretical and recent experimental approaches studying model membranes have shown the existence of liquid-ordered and -disordered, large and nanoscopic, lipid domains (2--5). Providing

that cellular membranes exist with relative lipid concentrations approximating these studied ranges, and it appears that they do, then it is likely that similar membrane heterogeneities will exist in vivo, and indeed, these have been convincingly detected through several approaches (5, 6).

Low-density (floating) membrane fractions after detergent extraction are thought to represent purified liquid-ordered phases of the plasma membrane (membrane microdomains). These detergent-resistant membrane isolates are enriched in many cellular components including GPI-anchored proteins, cholesterol, lactoceramide, glycosphingolipids (GSLs) such as Gb3 and Gb5, flotillins, caveolin1, small and heterotrimeric G proteins, and doubly acylated members of the src family of tyrosine kinases (7, 8). However, the method of isolation of these lipids by Triton extraction involves such extreme perturbations that these methods appear not to be sufficient to reliably identify bona fide proteins and lipids that are endogenously associated with membrane microdomains. Addition of Triton has been shown to artifactually produce microdomains and induce the redistribution of proteins into such structures (9). Despite these limitations, it has been elucidated that clathrin-independent endocytic structures are critically dependent on cholesterol and specific sphingolipids (SLs), which are proposed to be enriched in microdomains of the plasma membrane. However, a recent study that has isolated lipid rafts at 37°C, and neutral pH suggests that cholesterol may not even be enriched in these domains (10), potentially necessitating reinterpretation of many findings using cholesterol modulation.

SLs and cholesterol in the outer leaflet appear to be necessary for the formation of membrane microdomains. SLs consist of a sphingosine backbone linked to a fatty acid chain. The different types of headgroups present in such molecules divide SLs into three main structural classes: ceramides (the simplest subfamily, which contains only the CH_2OH headgroup of sphingosine); sphingomyelins (SMs, which have phosphorylcholine or phosphorylethanolamine headgroups); and GSLs (which have sugar moieties as their headgroups and can be further subclassified as cerebrosidal or gangliosidal depending on the type of such glycosylation). Large headgroups of SLs (relative to their heavily saturated chains of hydrocarbons) result in spaces that are likely filled by cholesterol moieties, which interact intimately with SLs.

Whether saturated/unsaturated lipid tail packing, in addition to GSL-cholesterol interactions in liquid-ordered regions of the outer membrane monolayer, affects the

packing of lipids in the inner leaflet is unknown, but some evidence suggests that this may well occur (11). SLs, found on the extracellular leaflet of the lipid bilayer, may be able to interdigitate with components of the inner leaflet. Here, cholesterol may fill the spaces created by such interdigitation, thereby allowing transbilayer communication through cholesterol clustering. Transmembrane domains have been shown to interact closely with membrane lipids (12), and because the plasma membrane in vivo has a high concentration of such domains, these likely play prominent roles in the creation and stabilization of membrane microdomains. Patching of membrane lipids and proteins by addition of multivalent ligands, such as antibodies and bacterial exotoxins, likely also creates and stabilizes membrane microdomains. Once created, the affinity of endogenous proteins for microdomain-associated lipids and proteins (over other lipids and proteins) will determine their abilities to cluster in microdomains. If this hypothesis is true, then many subtypes of microdomain will likely form.

CURVATURE AND ENDOCYTOSIS

Changes in membrane shape are required for membrane trafficking events, and known ways in which membranes can be deformed are schematized in **Figure 2** and Supplementary Figure 2. The crystallization of the membrane curvature sensing/stabilizing/generating BAR domain (13) has led to a greater understanding of how proteins with such domains may be mechanistically involved in such membrane shape changes. This BAR module consists of two all α-helical coiled-coil monomers that dimerize in a roughly antiparallel manner. The angle at which these domains dimerize, coupled with kinks in some helices, creates the characteristic banana shape of the dimeric BAR module (**Figure 2** and Supplementary Figure 2). This BAR module binds membranes via electrostatic interactions between positively charged residues concentrated on its concave face and negatively charged lipid headgroups (13). In addition to this cognate charge recognition, BAR domains can distinguish cellular membranes on the basis of their curvatures. The nature of the concave face of the BAR module, which preferentially binds membranes with curvatures that most precisely fit its intrinsic molecular curvature, allows this to be achieved. The membrane-binding interface is highly curved in each of the BAR modules whose structures have been

crystallized to date, and these proteins bind with highest affinity to highly curved lipid membranes.

The structures of other coiled-coil domains with lower homology to BAR domains have since been solved (14--16). The F-BAR (a BAR domain with an FCH homology region or FCH-BAR) module has a much larger dimerization interface, and this, coupled with distinct kink angles in the helices of each monomer, creates a concave lipid-binding face with a much lower intrinsic curvature than that observed in BAR modules (14, 15). This finding is consistent with observations that it binds flatter membranes with greater affinity than do BAR domains. Another weakly homologous module is created by IRSp53-MIM homology domain (IMD) dimerization (16). Here, dimerization creates a lenticular (or zeppelin-shaped) dimeric module, which binds lipids on a modestly convex surface. This module appears to bind to membranes curved in the opposite direction to those bound by BAR domains, and thus, we call this the inverse BAR (I-BAR) domain (17). It is possible that a complete spectrum of coiled-coil domains exist that dimerize into functional modules for the detection of the whole gamut of membrane curvatures that exists in vivo.

Curvature sensing by proteins would be predicted to be important for the delivery of effector domains to distinct subcellular locations. Consistent with this, many BAR, F-BAR, and IMD domain-containing proteins are multidomain proteins and comprise a wide variety of effector functions. Many of these proteins have domains involved in the regulation of small G proteins of the arf and rho families. Many also contain protein-protein interaction components, such as SH3 or PDZ domains and ankyrin repeats. The ability to interact with other proteins might recruit these interactors to specifically curved membrane regions. Conversely, such domains may allow for greater specificity in the differential recruitment of coiled-coil domain-containing proteins to cellular subcompartments. Because there are many similarly curved regions of the cell, such recruitment signals likely allow discrimination through “coincidence detection.” For example, some BAR domain-containing proteins contain other lipid-binding domains, such as PX or PH domains, and this would be expected to enhance membrane-binding specificity. Furthermore, although the ultimate functions of membrane curvature sensing by these proteins is not in question, such coiled-coil domains likely also function as general dimerization interfaces, increasing the avidity with which effector and protein-protein interaction domains found in such proteins can function.

BAR, F-BAR, and IMD domains have all been shown to be capable of deforming lipid membranes in vitro and thus also function as membrane curvature generators (13, 14, 17). BAR modules are capable of deforming large, roughly spherical liposomes into very highly curved tubular membranes with a narrow range of diameters similar to that predicted by superimposition of circular arcs onto the concave structure of each module (Supplementary Figure 2). This suggests that BAR modules are found on these tubular structures with their long axes roughly perpendicular to the long axis of the tubule. F-BAR domains are also capable of producing tubular membranes, and these are of highly variable diameter up to approximately 130 nm, suggesting that the orientation of these modules on membranes is more flexible (14), and these form helical coats on membrane tubules (18). IMD domains are capable of deforming membranes into short tubules with a more rigid diameter of around 80 nm, and these tubules were shown to be invaginations of larger membrane structures (17), in contrast to the long evaginated tubules induced by BAR and F-BAR modules (13, 14).

A subfamily of BAR domain-containing proteins contains an N-terminal amphipathic helix, which folds upon membrane binding (Supplementary Figure 2) (19). Such "N-BAR modules" likely function as a unit, with efficient membrane deformation provided by the N-terminal amphipathic helix with the subsequent membrane curvature produced, being further promoted, and stabilized by the canonical BAR module. In addition, an additional amphipathic helix, found at the apex of the concave face of the Nadrin/Endophilin subfamilies of N-BAR modules, is likely to contribute to membrane curvature generation (19).

Proteins that bind membranes with specific curvatures (which have been induced either through spontaneous fluctuation, the specific accumulation of particular lipids, or through membrane curvature generation by other cellular proteins), and especially those that do so with high affinity, will promote the maintenance of that membrane curvature. In so doing, these proteins can passively allow the stabilization and growth of specific membrane curvatures. This is distinct from active curvature generation whereby these modules, upon binding membranes that are not optimally curved to fit the domain, change the shape of membranes such that the highest affinity interaction between membrane and protein occurs. The capacities to actively generate and passively maintain membrane curvature likely coexist and should be considered as part of a spectrum of domain activity. These properties cannot be effectively distinguished using conventional

analytical membrane deformation techniques. However, it is likely that modules with additional mechanisms to produce membrane curvature changes, and those that efficiently and robustly generate curvature, lie at one end of this spectrum, whereas others, which are less potent in producing membrane deformation in vitro and lack additional modular (curvature-inducing) components, lie at the other end. Distinguishing between passive and active membrane deformation is important to identify primary and secondary effectors of membrane deformation and precise mechanisms of membrane deformation in vivo.

Because BAR domains are capable of sensing and driving membrane curvature changes in vitro, this might provide mechanistic insight into how such changes are actively managed in vivo. Overexpressed BAR domains in vivo were shown to bind to tubular networks, and the introduction of mutations that abolished membrane-binding and -tubulating abilities in vitro led to cytoplasmic protein localization (13). Overexpression of BAR domains from different proteins led to distinct patterns of localization, suggesting differential recruitment of the effector functions of such domains. This work supported the idea that BAR domains are functional in effecting membrane curvature changes in vivo and opened up the possibility that distinct BAR domain-containing proteins regulate specific membrane trafficking steps. A wealth of studies has since demonstrated specific roles for BAR domain-containing proteins in vivo (20). The largest bodies of literature exist for amphiphysins, endophilins, sorting nexins, and F-BAR proteins. Because these are reviewed elsewhere (20--24), specific examples are dealt with only briefly here.

Amphiphysin1 is an N-BAR domain-containing, brain-enriched, but rather ubiquitously expressed, protein, which interacts with dynamin1 (25), the endocytic lipid phosphatase synaptojanin1 (26), as well as clathrin and AP2 (27). It localizes to clathrin-coated pits (CCPs) and is essential for CME to proceed under various conditions (28, 29), and it appears that it functions by membrane deformation, dynamin recruitment, and the creation of membrane-cytoskeleton linkages. By contrast, the amphiphysin homolog in *Drosophila melanogaster* (D-Amph) does not appear to be involved in CME because there is no biochemical or cell biological link to this process. Instead, D-Amph is found on T-tubular membranes in striated muscle, and mutants have a severely disorganized T-tubular/sarcoplasmic reticulum system (30). An isoform of amphiphysin2 was also shown to have a similar role in stabilizing the T-tubular network

in mammalian muscle cells (31). Amphiphysin1 appears to have additional roles in phagocytosis and the stabilization of tubulobulbar complexes in Sertoli cells and, along with dynamin2, is necessary for efficient spermatid release (32).

Endophilin proteins have a domain structure similar to amphiphysins (in that they have an N-terminal N-BAR domain followed by a C-terminal SH3 domain). EndophilinA proteins also bind to dynamin (33) and synaptojanin (34) and have been implicated in synaptic vesicle endocytosis (35). Current data are strongly suggestive of a role for endophilinA1 in CME, but there is no biochemical link that specifically ties the protein to this process. However, endophilinA1 has been found at CCPs and has been proposed to function in late stages of CME by recruiting a synaptojanin1 isoform and dynamin (36). Recently, F-BAR domain-containing proteins, including FBP17, have also been localized to CCPs, where they appear to have important roles during CME (15) and where they may coordinate actin polymerization. Clathrin-dependent and -independent endocytic functions have also been ascribed to the F-BAR domain-containing proteins syndapins, and syndapin stimulates bulk endocytosis after strong stimulation in lamprey synapses (37, 38). Furthermore, the BAR domain-containing protein SNX9 localizes to CCPs and is necessary for CME, but a recent study has shown that this protein can also localize to glycosylphosphatidylinositol (GPI)-linked protein-positive tubular membranes and to CDRs, where it appears necessary for their function (39). In addition to binding dynamin, SNX9 can also bind to and stimulate N-WASP and Arp2/3-associated actin polymerization. Indeed, actin regulation appears to be a feature of many BAR superfamily proteins, as is linkage to dynamins. Indeed, large G proteins of the dynamin superfamily, including the classical dynamins, and EHDs are also capable of membrane deformation (see Supplementary Figure 3). Dynamins use this to promote scission of budding vesicles (as discussed in the printed text). Perhaps EHDs (40), which are already heavily implicated in membrane trafficking events, have roles similar to dynamins in other budding events.

CELL ADHESION, MIGRATION AND ENDOCYTOSIS

A recent study has intriguingly suggested that a large proportion of plasma membrane lipid microdomains are regulated by the formation of sites of cellular adhesion to the surrounding matrix. At these sites, integrin clustering by matrix ligation triggers the formation of a large protein complex, which mechanically couples the matrix to the actin

cytoskeleton (so-called focal complexes/adhesions). When these sites are disassembled, a large portion of ordered plasma membrane is lost (41). Furthermore, upon deligation from matrix, cells are induced to undergo endocytosis in a partially caveolin1-dependent manner. The determination that Tyr14 phosphorylation of caveolin1 is necessary for at least some of this membrane order is consistent with results that have shown its ability to bind specific lipids and that this phosphorylation appears to be required for deligation-induced microdomain endocytosis (41, 42). C-src phosphorylates caveolin1 on Tyr14 (43), and this phosphorylation has been indirectly linked to the ability of caveolae to undergo internalization. Interestingly, if SLs are added exogenously to cells, c-src becomes activated, and this is concomitant with caveolin1 and dynamin phosphorylation (44). Caveolin1 pY14 appears to localize to focal adhesions, although there are some doubts as to the specificity of the antibody recognizing this form of the protein because it appears to recognize paxillin in immunofluorescent analysis (45). Great care should therefore be taken in interpreting results from experiments that use this antibody.

Other studies suggest a link between clathrin-independent endocytosis and adhesion. Caveolin1 binds integrins and appears to be necessary for integrin signaling (46). Furthermore, GSL receptors for several bacterial exotoxins (such as GM1 and Gb3) are enriched in focal adhesions (41). A nonnatural GSL stereoisomer, which is not plasma membrane microdomain resident, inhibits caveolar-type endocytosis as well as β1-integrin signaling (47). Moreover, the depletion of β1-integrin results in a decrease in albumin and LacCer endocytosis (47). Turnover of extracellular matrix occurs by endocytic mechanisms, and endocytosis of both β1-integrin and fibronectin is caveolin1 dependent (48). The exposure of cells to SV40, as well as increasing the mobility of caveolin1-positive structures (both going toward and away from the cell surface), results in the loss of focal adhesions and actin stress fibers (49). Focal complexes and adhesions are continually remodeled and turned over. Adhesion mechanisms may direct the formation of clustered lipid domains and may subsequently, through the mechanisms already described, direct the endocytosis of specific ligands from specific regions of the plasma membrane. Interestingly, a screen for kinases involved in clathrin-independent endocytosis demonstrated many positives that are specifically involved in the regulation of cell adhesion (50).

An arf GTPase-activating protein (arfGAP) domain-containing protein specific for arf6, GIT1, has been shown to promote focal adhesion downregulation in a complex together with PAK2 and βPIX, a guanine nucleotide exchange factor (GEF) for rac1 (51). GIT1 has been implicated in trafficking between the plasma membrane and endosomes and appears to act as a scaffold for ERK activation at focal adhesions (52, 53). ERK has also been suggested to regulate the formation of tubular trafficking membranes (54). Another arfGAP protein, PKL, binds the focal adhesion component paxillin as well as PIX and the kinase PAK (55), which is necessary for macropinocytosis (56). Arf6- and EHD1- associated recycling to the plasma membrane has been observed for cargoes taken up by arf6-dependent endocytosis (57). Interestingly, if cells are deligated and replated, an arf6-dependent recycling compartment recycles CTxB (a marker for liquid-ordered lipids) back to the plasma membrane, where it appears to allow rac1 activation and cell spreading to ensue (58). This provides exciting support for a role of the exo-endocytic cycle in adhesion regulation.

The discovery that microtubule-associated focal adhesion disassembly was dependent upon dynamin opened the exciting possibility that focal adhesion disassembly may occur via a membrane trafficking pathway. Dynamin2 siRNA treatment resulted in a large increase in the size and number of focal adhesions (59). Many of these were found at the centrobasal surface, usually a region of the cell relatively deficient in focal adhesions. Inhibition of disassembly was also observed by dominant-negative dynamin2 K44E overexpression, and this protein localized in part to focal adhesions. This inhibition was dependent upon the integrity of the proline-rich domain of dynamin2. Endogenous dynamin was also found at focal adhesions. By TIR-FM, corrals of dynamin2 encircling focal adhesion kinase (FAK)-positive regions of focal adhesions were observed as well as punctate colocalization of these proteins at such sites. During the washout phase after nocodazole treatment, an increase in colocalization was observed. Dynamin localization to focal adhesions was shown to be dependent on FAK, which (along with pTyr397 FAK) coimmunoprecipitated with dynamin. Dynamin was also found necessary for normal cell migration, as determined by a wound-healing assay. Because dynamin is found at adhesion sites, which are closely coupled to the plasma membrane, it may control endocytic traffic from these sites, although this putative mechanism has not been explored. It is possible that this occurs through integrin

endocytosis, which would abolish signals for adhesion site assembly, concomitant with dissolution of the site. This would also explain the piecemeal disassembly of these sites, which has been observed (59). Integrin puncta have been found in migrating cells, and integrin endocytosis and recycling is known to occur in these cells (60). Endocytosis of integrins, or other adhesion receptors, would allow irreversible disassembly of adhesion sites. There is, however, no such direct link suggested as yet. Focal adhesion disassembly by these means would also explain the phenomenon of deligation-induced microdomain endocytosis discussed previously and suggests that endocytosis is concomitant with adhesion site disassembly rather than something that occurs through permissive means after disassembly.

Cell migration requires the intricate coordination of membrane trafficking, focal adhesion turnover, and cytoskeletal changes. However, the role of membrane trafficking in migrating cells, and how this coordination occurs, has been hotly debated (61--66). Focal adhesions at the rear of migrating cells must be disassembled in order to allow the cell to continue migrating. The potential links between endocytosis and adhesion site regulation offer a novel means by which this can be achieved, and endocytosis at disassembling adhesion sites would also be capable of providing membranes from the rear that might then be delivered to the leading edge of the cell. In mammalian cells, the greatest body of evidence is consistent with a role for adhesion receptor endocytosis from the rear of a migrating cell and its eventual recycling to the leading edge to take part in further adhesion events. Membrane trafficking is absolutely required for cell migration in *Dictyostelium discoideum* although net exocytosis to the leading edge does not appear to occur in these cells (67).

Clathrin-mediated endocytosis in migrating MDCK cells has been shown by TIR-FM to be enriched toward the leading edges of cells and so is unlikely to provide either endocytic regulation of adhesion sites at the rear or the required directionality of membrane traffic to the leading edge (68). However, in T lymphocytes, clathrin and AP2 are enriched at the uropod, and CME is necessary for chemotaxis in these cells (69). Rac1 (which is critical for cell migration) appears to become activated on early endosomes, and CME allows its activation here (70). Active rac1 is then recycled to the plasma membrane, and activation of actin-based cell migratory mechanisms ensues.

Although integrins are capable of undergoing clathrin-mediated endocytosis at the leading edge (71), they have also been shown to be internalized via clathrin-independent routes (72), and GPI-linked proteins, many of which are involved in cell adhesion, enter

via the CLIC/GEEC pathway (73--75). Such endocytic processes may thereby allow adhesion site disassembly to be coupled to cell migration. Rho family small G proteins are known to be necessary for the formation of clathrin-independent endocytic structures as well as master regulators of cytoskeletal changes. This strongly suggests that these proteins play central (integrating) roles in the coordination of cell migration. How the cytoskeleton is regulated by small G proteins is well understood (76), but how this can be integrated with membrane trafficking is unknown.

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Legends for Supplemental Figures

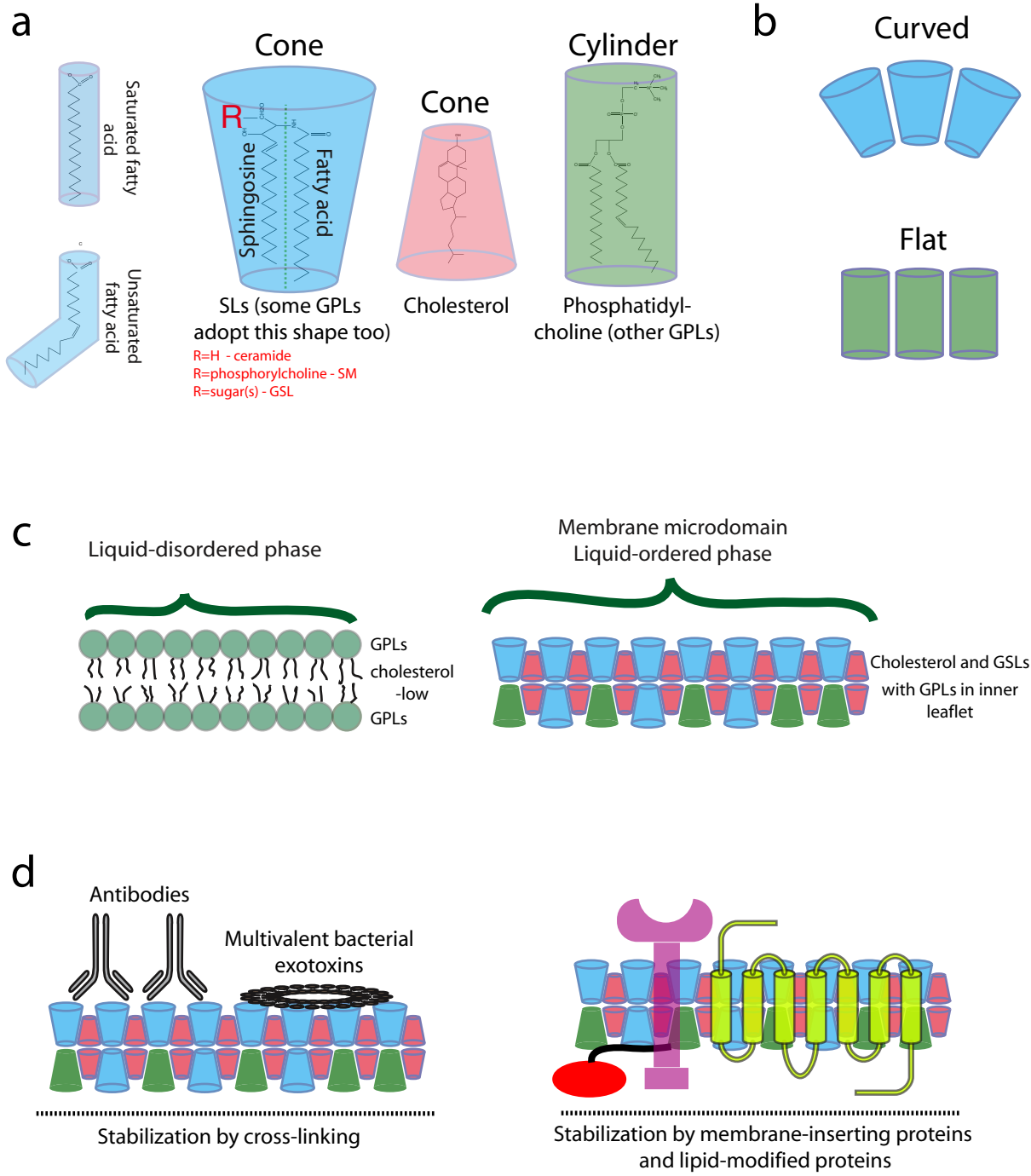
Supplementary Figure 1 The possible nature of plasma membrane lipids and microdomains. (*a*) Schematic diagram depicting the shape volumes that may be occupied by certain plasma membrane lipid types. (*b*) Schematic diagram depicting how the packing of cone-shaped (*above*) and cylinder-shaped (*below*) lipids may determine the curvature of a membrane monolayer or how lipids with certain shapes may cluster at sites of particular membrane curvature. (*c*) Schematic diagrams depicting putative liquid-disordered and liquid-ordered phases of the plasma membrane. (*d*) Schematic diagrams depicting how liquid-ordered phases of the plasma membrane might be stabilized by multivalent ligands and endogenous lipid-associated or -inserting proteins. Abbreviations: SL, sphingolipid; GSL, glycosphingolipid; GPL, glycerophospholipid; SM, sphingomyelin.

Supplementary Figure 2 Ways to deform cell membranes and how BAR and N-BAR domains achieve this. (*a*) Diagram illustrating several methods by which membranes may be deformed into more highly curved structures. (*b*) Schematic diagrams depicting how dimeric BAR and N-BAR modules interact with membranes. The D-Amphiphysin BAR domain structure (38) in ribbon representation is used as a backbone. Note the intrinsic curvature of this module and the interaction of its concave face with lipid membranes. (*left*) A BAR module is shown binding weakly to relatively flat membranes and strongly to highly curved membranes. In so doing, it can sense and stabilize membrane curvature. (*right*) An N-BAR module is depicted binding more strongly to flatter membranes than the BAR module (owing to lower off rates from the membrane). It folds an N-terminal amphipathic helix, which then inserts into the membrane, allowing anchorage. This insertion also actively generates membrane curvature, and this curvature is then promoted and stabilized by the canonical BAR module.

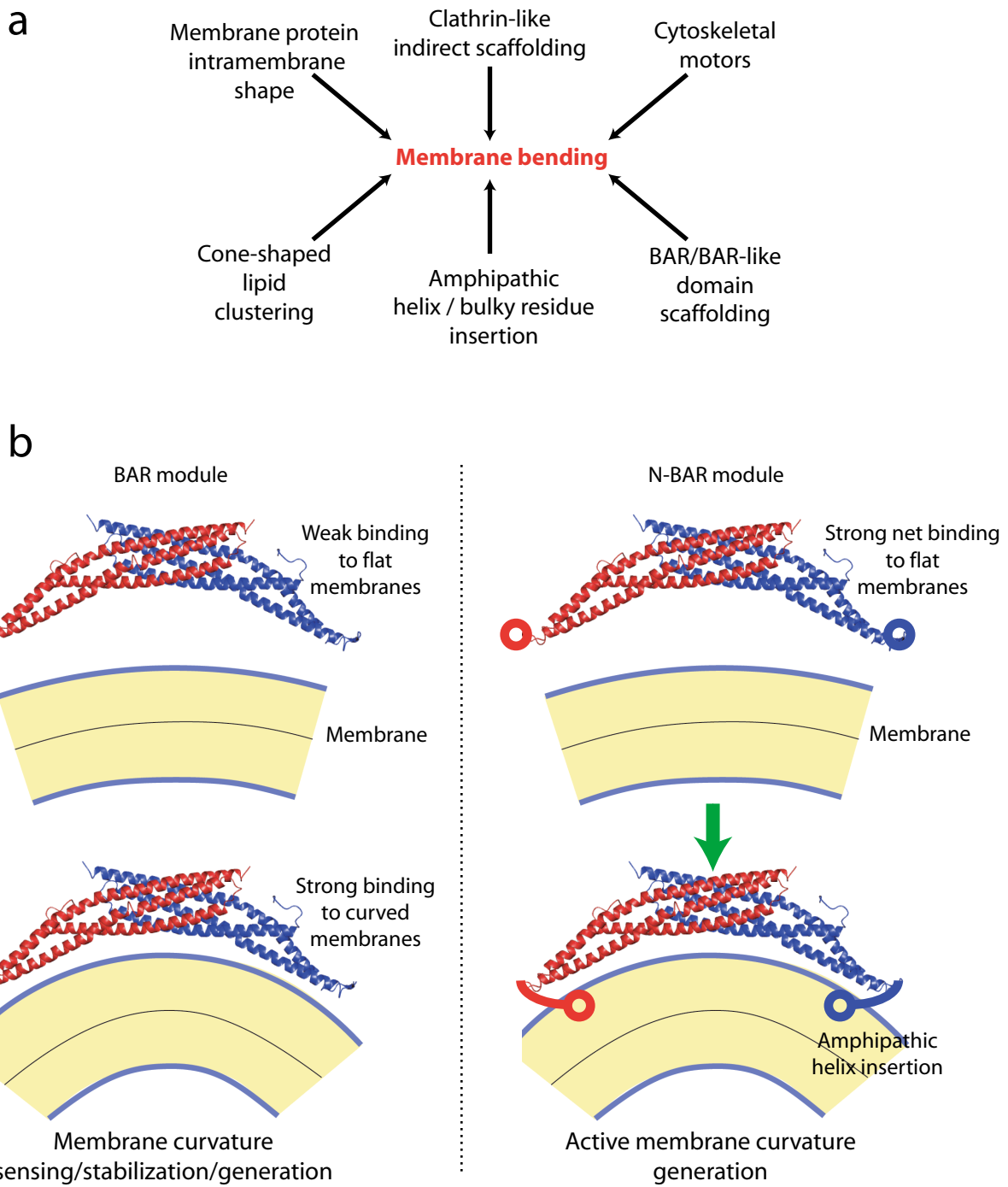
Supplementary Figure 3 Dynamin and membrane fission. (*a*) Schematic diagram depicting the mechanism of action of dynamin in the scission of a budding membrane. The numbers shown refer to the steps in the membrane fission diagram shown in panel (*b*) First, in 1, dynamin is recruited to the neck of the budding vesicle, where it oligomerizes and is GTP bound. Upon GTP hydrolysis by dynamin, in 2, the membranes of the neck are brought closer together, which reduces the energy barrier to their fusion. The inset shows a tubulated liposome with loosely bound spirals of dynamin visible. This change in the pitch of the dynamin spiral is thought to exert pulling and twisting forces on the membranes at the neck and brings these membranes into close apposition. Then membrane fusion occurs, in 3+4, and an internalized vesicle is produced. (*b*) Schematic diagram depicting the putative intermediates in membrane fission. A cross section of the neck of a budding vesicle is depicted. The membranes of the neck, in 1, are brought into close apposition, in 2, by the action of dynamin or a related protein. This reduces the activation energy required for formation of the hemifusion intermediate where the proximal bilayers mix in 3. The outer monolayers then mix, and a fusion pore is produced, in 4, which then widens in three dimensions to produce full membrane fission in 5.

Supplementary Figure 4 Adhesion and endocytosis. (*a*) Schematic diagram depicting the basal location of cell-matrix adhesions (focal adhesions) (*green*) and their connections to actin stress fibers (*orange*) and the extracellular matrix (*blue*) in cells in culture. (*b*) Schematic diagram depicting putative steps in deligation-induced microdomain endocytosis. Panel 1 shows a close up of a mature focal adhesion shown in panel *a*. Upon deligation from matrix (panel 2), the focal adhesion disassembles, releasing the adhesion-associated microdomain lipids (and possibly caveolin1 pTyr14; panel 3). The release of these lipids may allow caveolar-type endocytosis, or other endocytic mechanisms to proceed (panel 4). These

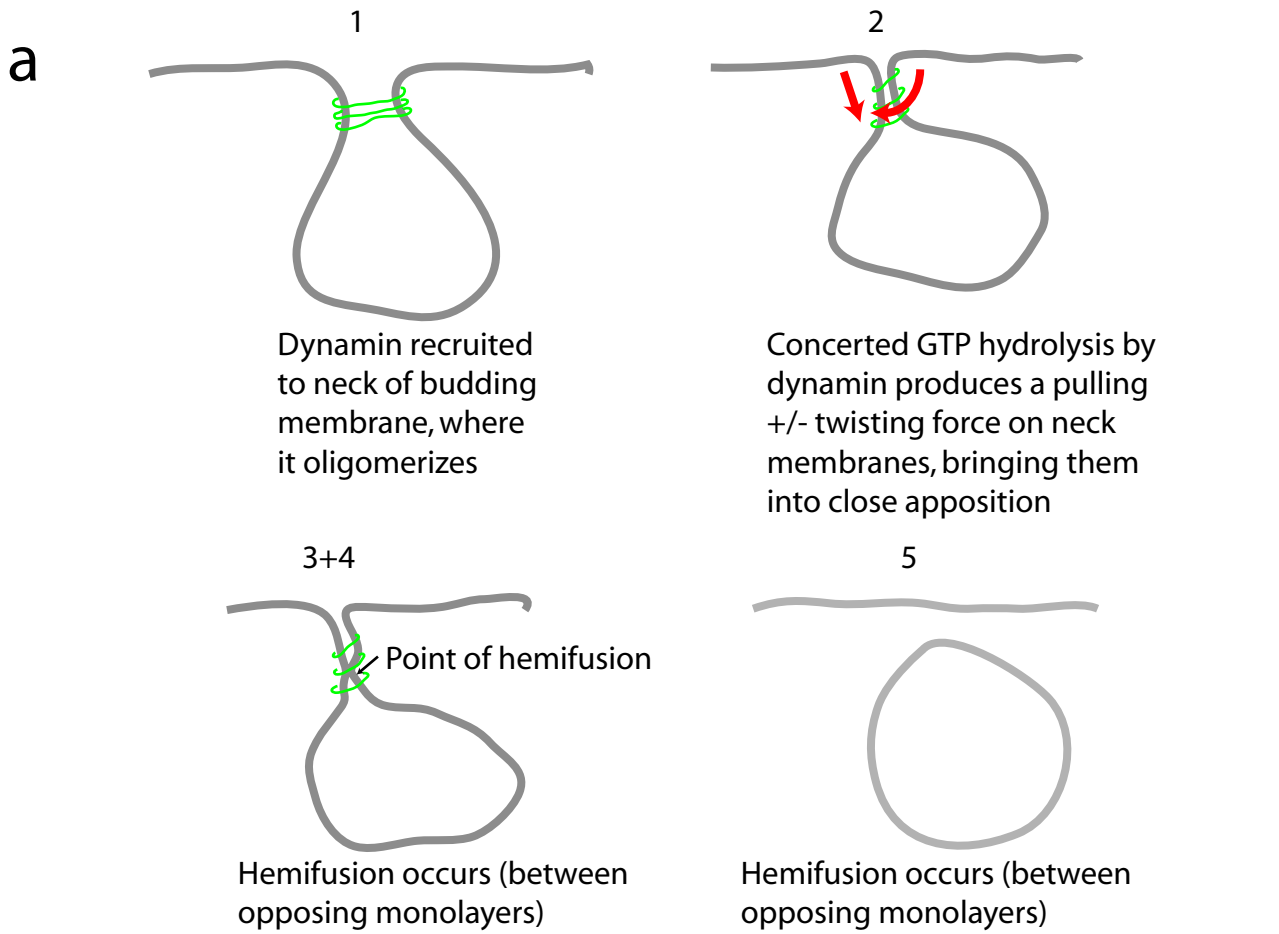
mechanisms might also allow the endocytosis of intergrins or other adhesion proteins, which would allow dominant focal adhesion disassembly in the absence of global deligation, such as may occur physiologically during focal adhesion remodeling and cell migration.



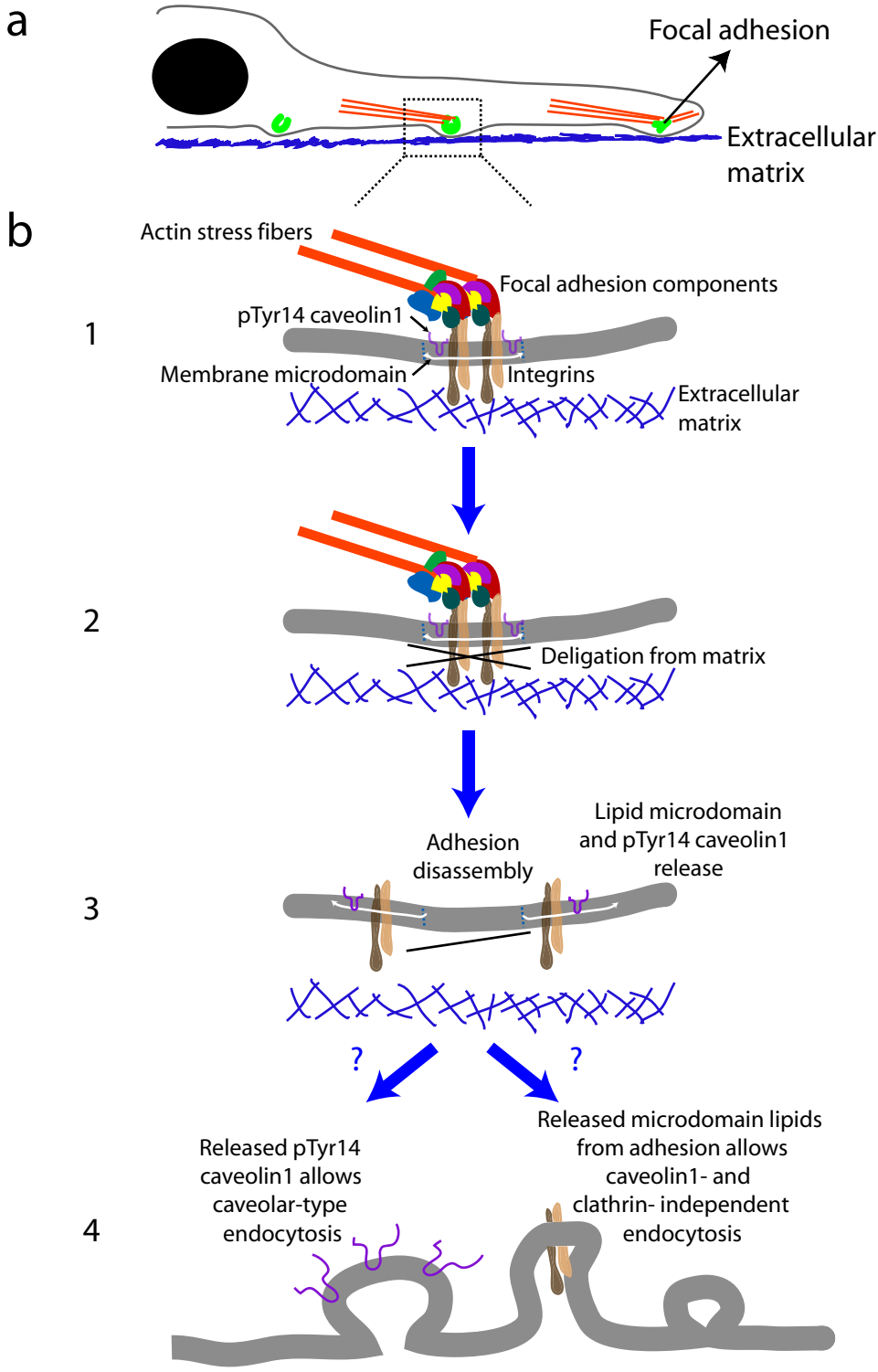
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4