Annu. Rev. Cell Dev. Biol. 1996. 12:575–625

ENDOCYTOSIS AND MOLECULAR SORTING

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KEY WORDS: clathrin, endosomes, epithelial cell polarity, Golgi complex, lysosomes, receptors

ABSTRACT

Endocytosis in eukaryotic cells is characterized by the continuous and regulated formation of prolific numbers of membrane vesicles at the plasma membrane. These vesicles come in several different varieties, ranging from the actin-dependent formation of phagosomes involved in particle uptake, to smaller clathrin-coated vesicles responsible for the internalization of extracellular fluid and receptor-bound ligands. In general, each of these vesicle types results in the delivery of their contents to lysosomes for degradation. The membrane components of endocytic vesicles, on the other hand, are subject to a series of highly complex and iterative molecular sorting events resulting in their targeting to specific destinations. In recent years, much has been learned about the function of the endocytic pathway and the mechanisms responsible for the molecular sorting of proteins and lipids. This review attempts to integrate these new concepts with long-established views of endocytosis to present a more coherent picture of how the endocytic pathway is organized and how the intracellular transport of internalized membrane components is controlled. Of particular importance are emerging concepts concerning the protein-based signals responsible for molecular sorting and the cytosolic complexes responsible for the decoding of these signals.

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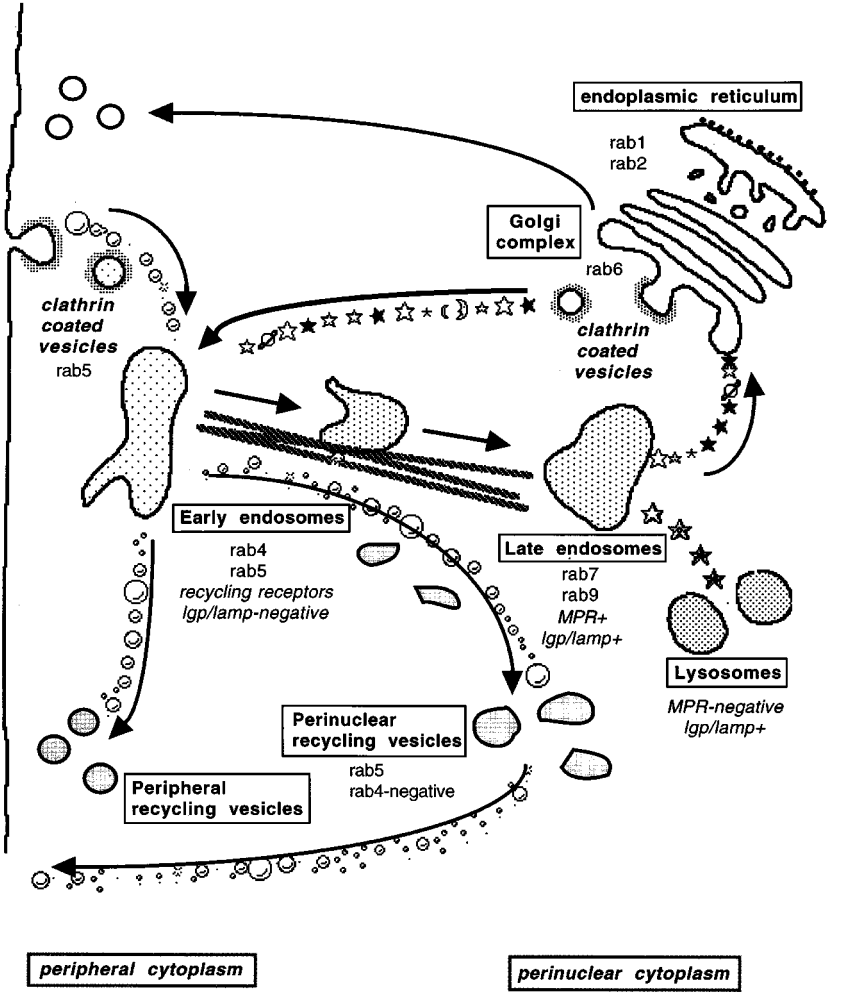
1081-0706/96/1115-0575$08.00

INTRODUCTION

All eukaryotic cells exhibit one or more forms of endocytosis. Their reasons for doing so are as diverse as their individual functions. At a minimum, endocytosis serves to maintain cellular homeostasis by recovering protein and lipid components inserted into the plasma membrane by ongoing secretory activity. More strikingly, endocytosis is essential for organismal homeostasis, controlling an extraordinary array of activities that every cell must exhibit in order to exist as part of a multicellular community. These activities include the transmission of neuronal, metabolic, and proliferative signals; the uptake of many essential nutrients; the regulated interaction with the external world; and the ability to mount an effective defense against invading microorganisms. Paradoxically, many infectious agents mediate their effects only after contriving to be internalized by their intended hosts.

One hundred years ago, Elie Metchnikoff first recognized that material taken up by endocytosis was degraded after encountering an acidic internal environment. During the past decade, the basic organization of the endocytic pathway was elucidated, particularly in the case of the internalization of protein ligands bound to cell surface receptors (Helenius et al 1983, Steinman et al 1983, Kornfeld & Mellman 1989, Trowbridge et al 1993, Gruenberg & Maxfield 1995). Although discussion of detail and semantics continues, there is fundamental agreement on the most important features of the pathway. These features are summarized in Figure 1 (note that review articles have been cited for most work performed before 1990).

Figure 1 Organization of the endocytic pathway in animal cells. The major organelles of the endocytic pathway are illustrated and defined in terms of their kinetic relationships, as well as in terms of diagnostic molecular markers. The markers shown include members of the rab family of ras-like GTPases, cation-independent mannose-6-phosphate receptor (MPR), and members of a protein family enriched in lysosomal membranes (lgp/lamp). Endocytosis is typically initiated by the formation of clathrin-coated vesicles at the plasma membrane, which results in the delivery of receptor-ligand complexes to early endosomes in the peripheral cytoplasm. Here, receptor-ligand complexes dissociate, thus resulting in the return of free receptors to the plasma membrane in recycling vesicles, some of which appear to first migrate on microtubule tracks to the perinuclear cytoplasm before reaching the cell surface. Dissociated ligands and other soluble macromolecules are transferred from early to late endosomes and lysosomes where digestable content is degraded. Clathrin-coated vesicles are also thought to be involved in the transport of selected components (e.g. MPR) from the *trans*-Golgi network of the Golgi complex to early endosomes. See text for details.



Most receptor-ligand complexes accumulate at clathrin-coated pits of the plasma membrane, which bud off to yield clathrin-coated vesicles. The vesicles rapidly lose their coats, which facilitates fusion with early endosomes (EEs), a dynamic array of tubules and vesicles distributed throughout the peripheral and perinuclear cytoplasm. Due to a slightly acidic pH (pH $\approx$ 6.0–6.8) maintained by an ATP-driven proton pump (Al-Awqati 1986, Mellman et al 1986, Forgac 1992), EEs host the dissociation of many ligand-receptor complexes. Free receptors selectively accumulate in the early endosome's tubular extensions, which bud off to yield recycling vesicles (RVs) that transport receptors, directly or indirectly, back to the plasma membrane. Dissociated ligands collect in the vesicular portions of the EEs simply because of their high internal volume relative to the endosome's tubular extensions. The vesicular structures pinch off—or are left behind following the budding of RVs—traverse to the perinuclear cytoplasm on microtubule tracks, and fuse with late endosomes (LEs) and lysosomes. Here, ligands are degraded by the even lower pH (pH $\approx$ 5) and the high concentration of lysosomal enzymes. Recycling from lysosomes occurs relatively slowly, which explains why cells are capable of accumulating large amounts of internalized material. In addition, lysosomal enzymes accumulate in LEs and lysosomes by the same pathway: Clathrin-coated vesicles coming from the *trans*-Golgi network (TGN) also fuse with endosomes, delivering newly synthesized enzymes bound to either of two pH-sensitive mannose-6-phosphate receptors (Kornfeld & Mellman 1989). To a first approximation, clathrin-coated vesicles thus comprise a population of transport vesicles specifically addressed for fusion with endosomes, regardless of whether they originated at the plasma membrane or the TGN.

Like the secretory pathway, the endocytic pathway can be thought of as having functionally and physically distinct compartments (Kornfeld & Mellman 1989). Early endosomes are responsible for the dissociation and sorting of receptor and ligands in an environment that minimizes the risk of damaging receptors intended for reutilization. Late endosomes and lysosomes are responsible for accumulating and digesting exogenous and endogenous macromolecules. Unlike the secretory pathway, however, the signposts for these compartments are less clear. The functions of endocytic organelles do not involve the predictable and easily determined enzymatic activities associated with the synthesis, folding, and posttranslational processing of glycoproteins and lipids. Moreover, the physical appearance of endocytic organelles is more pleiomorphic than is true for secretory organelles, which can often be identified on morphological grounds alone. An additional difficulty comes from the fact that truly prolific quantities of membrane are processed by the endocytic pathway (Steinman et al 1983), possibly obscuring compositional differences between compartments.

Nevertheless, biochemical, functional, and genetic probes for monitoring and manipulating the endocytic pathway and its organelles have begun to appear, which have facilitated the dissection of the mechanisms underlying the internalization and recycling of plasma membrane components. In addition, we are beginning to understand the variations responsible for generating specializations of the endocytic pathway found in different cell types. This review summarizes advances in our understanding of endocytosis since the last comprehensive treatments of the topic (Kornfeld & Mellman 1989, Trowbridge et al 1993). Particular attention is paid to the mechanisms underlying the capacity of endocytic organelles for molecular sorting, i.e. the ability to distinguish and selectively transport components to different destinations. Many of these mechanisms are also shared by the secretory pathway. We also consider the growing body of information concerning the regulation of membrane transport during endocytosis. Because much of what has been learned about membrane fusion has emerged from the study of the secretory pathway, this topic will be treated elsewhere (S Pfeffer, this volume).

CELLS EXHIBIT MULTIPLE TYPES OF ENDOCYTOSIS

It has long been clear that there are at least two routes into the cell, generally classified as phagocytosis (cell eating) and pinocytosis (cell drinking) (Silverstein 1977, Steinman et al 1983). Phagocytosis refers to the internalization of large ($>0.5\ \mu$ diameter) particles that must bind to specific plasma membrane receptors capable of triggering their own uptake, usually by causing the formation of F-actin-driven pseudopods that envelop the bound particle. Pinocytosis, more commonly if somewhat less accurately known as endocytosis, typically refers to the constitutive formation of smaller ($<0.2\ \mu$ diameter) vesicles carrying extracellular fluid and macromolecules specifically or nonspecifically bound to the plasma membrane. These vesicles are usually initiated at clathrin-coated pits (see below). Possible alternatives to clathrin-mediated endocytosis include the involvment of caveolae and/or an actin-based mechanism. To greater or lesser degrees, two or more of these mechanisms co-exist in a single cell type.

Phagocytosis

PHAGOCYTIC CELLS The ability to internalize large particles is most often associated with phagocytic protozoa (*Dictyostelium, Acanthamoeba*) or phagocytic leukocytes of the mammalian immune system (macrophages, neutrophils). Uptake is triggered by binding of opsonized particles to cell surface receptors capable of transducing a phagocytic stimulus to the cytoplasm. This stimulus results in the localized polymerization of actin at the site of particle attachment

and subsequent pseudopod extension that engulfs the bound particle into a cytoplasmic phagosome (Greenberg et al 1990, 1991). In macrophages, the forming pseudopods are directed by sequential ligand-receptor interactions, which yield a vacuole custom fit to the internalized particle (Silverstein et al 1977).

Under most cirumstances, phagosomes rapidly fuse with endosomes and/or lysosomes exposing their contents to hydrolytic enzymes (Kielian & Cohn 1980, Steinman et al 1983, Rabinowitz et al 1992, Desjardins et al 1994). Receptors that mediate phagocytosis in leukocytes include members of the IgG Fc receptor family as well as some integrins (e.g. complement receptor) and lectins (e.g. mannose receptor) (Kielian & Cohn 1980, Mellman et al 1983, Wright & Silverstein 1983, Ezekowitz et al 1991, Isberg et al 1994). It is thought that lectin-like receptors are responsible for triggering phagocytosis of bacteria in protozoa (Cohen et al 1994), but as yet no receptors have not been identified. In mammals, phagocytosis serves as a first line of defense against microorganisms, as well as providing an important component of the humoral immune response by allowing the processing and presentation of bacterial-derived peptides to antigen-specific T lymphocytes (Harding & Geuze 1992, Pfeifer et al 1993). In protozoa, phagocytosis probably serves a nutritional function; *D. discoideum* mutants conditionally defective in phagocytosis starve to death (Cohen et al 1994).

Phagocytosis can result in the internalization of huge areas of plasma membrane. Classical experiments from Cohn and colleagues demonstrated that more than 50% of a macrophage's surface area could be involved during the uptake of a phagocytic load (Werb & Cohn 1972, Steinman et al 1983). Most membrane proteins are equally susceptible to internalization, although the receptor mediating the uptake can be selectively internalized and degraded (Mellman et al 1983). Phagosomes thus remain at least partly connected to the endocytic pathway, allowing the recycling of many of the bulk-internalized plasma membrane proteins (Muller et al 1983, Joiner et al 1990). During this time, the phagosome membrane is gradually remodeled and comes to resemble the lysosomal membrane in composition (Desjardins et al 1994).

Although the mechanism of phagocytosis remains incompletely characterized, the plasma membrane receptors themselves play a primary role. In macrophages, Fc receptor-mediated phagocytosis of IgG-coated particles by macrophages is associated with localized tyrosine phosphorylation of a variety of cytoplasmic proteins (Greenberg et al 1993, Greenberg 1995). This reflects the recruitment of cytosolic src-family kinases to the Fc receptor cytoplasmic domain via its consensus tyrosine-containing ITAM motif (Greenberg et al 1994, 1996). Although it is widely believed that macrophages and other phagocytes are highly specialized for the uptake of large particles, in fact,

transfection of Fc receptor cDNAs into normally non-phagocytic cells also can result in phagocytosis (Joiner et al 1990, Hunter et al 1994, Greenberg et al 1996). Moreover, fibroblasts can be made to ingest IgG-coated particles as efficiently as macrophages by ensuring that the appropriate src-family kinases are co-expressed, either as soluble proteins or as cytoplasmic domain fusions to the expressed Fc receptors (Greenberg et al 1996). Thus given the appropriate receptor coupled to the appropriate signaling molecule, phagocytosis is not a property limited to professional phagocytes but makes use of a mechanism common to many or all cell types.

ENTRY OF INFECTIOUS MICROORGANISMS Although some infectious agents preferentially infect macrophages, many bacterial and protozoan parasites long ago discovered that phagocytosis is not a proprietary feature of professional phagocytes. Some bacteria (*Yersinia, Salmonella, Listeria*) synthesize surface proteins that permit bacterial attachment and stimulation of one or more plasma membrane receptors, which stimulate membrane ruffling and subsequent engulfment of the bound bacteria, even by epithelial cells (Bliska et al 1993, Pace et al 1993, Gálan 1994). This type of phagocytosis differs in some respects from that mediated by Fc receptors in leukocytes in that pseudopod extension is essentially undirected, as opposed to being zippered across the particle surface. Thus bacteria such as *Salmonella* may simply generate an enormous local stimulus that causes sufficient membrane ruffling to allow almost the accidental internalization of bound bacteria. Indeed, *Salmonella* entry is also accompanied by a transient increase in the uptake of extracellular fluid, as would be expected for such a mechanism (Francis et al 1993). The nature of the receptors or signals stimulated by such bacteria are not completely characterized. However, there is a critical role for members of the rho protein family as downstream effectors almost certainly required for the reorganization of the actin cytoskeleton (J Gálan, unpublished results). It is interesting that in leukocytes apparently the same rho family proteins play a critical role in assembling the complex of proteins needed to generate the respiratory burst as well as transcriptional activation of genes involved in mediating the inflammatory responses (Bokoch & Knaus 1994).

Although beyond the scope of this review, it should be mentioned that many bacterial and protozoan pathogens have developed strategies to avoid killing by oxidative mechanisms or lysosomal digestion (Falkow et al 1992). Some (e.g. *Listeria, T. cruzi*) escape from newly formed phagosomes by utilizing the acidic internal pH of the phagosome to activate lytic enzymes that rupture the vacuole membrane (Andrews & Portnoy 1994). *T. cruzi* even appears to actively recruit some lysosomes to the site of entry (Tardieux et al 1992). Finally, protozoan parasites such as *Toxoplasma gondii* heavily modify the phagocytic process,

resulting in the formation of vacuoles that are modified such that they are no longer capable of fusing with host cell endosomes and lysosomes (Joiner et al 1990). This mode of entry also avoids triggering a cytotoxic respiratory burst by the phagocyte.

Clathrin-Dependent Endocytosis of Receptors, Ligands, and Extracellular Fluid

The uptake of extracellular fluid and receptor-bound ligands is exhibited to varying extents by all cells. The amounts of membrane and fluid internalized by this ongoing endocytic activity are prolific. Cells such as macrophages and fibroblasts have been estimated to internalize more than 200% of their entire surface area every hour (Steinman et al 1983). The amount of membrane uptake at active presynaptic nerve terminals is probably far greater. Despite the fact that many plasma membrane proteins are subject to endocytosis, most exhibit long half-lives (typically > 24 h) and escape degradation by recycling back to the plasma membrane. Similarly, individual receptors have been estimated to mediate as many as 10 rounds of ligand uptake and recycling each hour yet exhibit long half-lives (Steinman et al 1983). Much has been learned concerning the pathways of endocytosis, and recent work has provided important new insights into the mechanisms and regulation of these events.

THE PRIMARY ROLE OF CLATHRIN IN ENDOCYTOSIS In most animal cell types and under normal conditions, the uptake of receptor-bound ligands and extracellular fluid results mainly from the formation of clathrin-coated vesicles (CCVs). Early evidence in favor of a primary role for clathrin in endocytosis came from quantitative arguments in which the number of CCVs of known diameter formed per minute was measured and used to calculate the volume of fluid they were predicted to contain. Such calculations indicated that a sufficient number of CCVs formed to account for fluid uptake, as determined biochemically (Marsh & Helenius 1980). Indeed, CCV-mediated uptake of virus particles was found to reduce pinocytosis owing to physical displacement of fluid. The primary role of clathrin-coated pits in ligand endocytosis was demonstrated by the correlation between the selective localization of receptor-ligand at coated pits and rapid ligand uptake: Mutant receptors defective in endocytosis were also defective at coated pit localization (Brown & Goldstein 1979).

Recently, additional genetic evidence supports a primary role for clathrin in endocytosis. Deletion of the single gene for the 180-kDa clathrin heavy chain in *Dictyostelium* greatly suppressed the considerable capacity of these cells for fluid pinocytosis, as well as inhibiting the internalization of at least one potential plasma membrane receptor (O'Halloran & Anderson 1992). In *Drosophila, shibire,* a well-known mutation causing temperature-sensitive paralysis, was

found to block endocytosis at nerve endings, as well as in nonneuronal cells, apparently by blocking CCV formation from coated pits that accumulated in abundance at the nonpermissive temperature (Koenig & Ikeda 1989, Poodry 1990). The *shibire* locus encodes dynamin, a GTPase initially believed to be a microtubule motor but more likely to be involved in the physical budding of CCVs from clathrin-coated pits (Chen et al 1991, van der Bliek & Meyerowitz 1991). Expression of mutant alleles of dynamin in mammalian cells in culture produced a similar effect, blocking CCV formation from coated pits and inhibiting receptor-mediated and fluid endocytosis (van der Bliek et al 1993, Damke et al 1994, 1995a). Interestingly, the effect on fluid uptake was transient after shifting to the nonpermissive temperature, suggesting that in the absence of a clathrin-mediated pathway, an alternative form of endocytosis could be induced (see below).

STRUCTURE AND FUNCTION OF CLATHRIN-COATED VESICLES The basic composition and structure of CCVs has long been known (Pearse & Robinson 1990). Although a complete consideration of their properties is beyond the scope of this review, it is necessary to discuss their more important features because CCVs serve as an important paradigm for understanding most transport and sorting events on the endocytic pathway.

CCVs were first identified in and isolated from brain, where they were associated with the endocytosis of presynaptic membrane following synaptic vesicle exocytosis. Their coats form lattices of hexagons and pentagons of the protein clathrin (Pearse 1987). The functional unit of these arrays is the triskelion: Three 180-kDa clathrin heavy chains each complexed with a 30–35 kDa light chain. Isolated triskelions can self-assemble into empty cages in vitro in the absence of ATP or other energy sources, suggesting that they exhibit a favorable ΔG for assembly. As such, clathrin itself may provide the energy required for the formation of CCVs from coated pits, although direct evidence for this appealing possibility is still lacking. Indeed, even the formation of invaginated coated pits on plasma membranes in vitro has been found to require energy (Lin et al 1991, Smythe et al 1992), suggesting that however favorable the ΔG for clathrin assembly, it is not sufficient to mediate the entire process.

Coated vesicles have long been thought to form by progressive invagination of planar, hexagonal clathrin lattices, first imaged in the classical electron micrographs of Heuser and colleagues (Heuser & Evans 1980). Although possible, it remains unclear whether such planar arrays directly mature into CCVs, as opposed to clathrin that may be initially recruited to sites already having some degree of curvature. It is not immediately obvious how such a stucture consisting entirely of triskelion-containing hexagons can reassemble into curved lattices consisting of both hexagons and pentagons. For this to happen,

significant rearrangements of inter-triskelion interactions must occur (Liu et al 1995). Conceivably, such rearrangements may be catalyzed by molecular chaperones that are not stable components of CCVs or may reflect the partial effect of GTPase activators (e.g. GTPγS) on coated pit invagination in vitro (Carter et al 1993).

Clathrin coats also contain a non-clathrin component, a heterotetrameric adaptor complex (Pearse & Robinson 1990). Originally identified as factors that promoted the assembly of clathrin cages in vitro, adaptors are now known to play a critical role in the attachment of clathrin to membranes (Chang et al 1993, Peeler et al 1993, Robinson 1994, Traub et al 1995). Indeed, it is the adaptor complex that must be first recruited to membranes in order to provide the binding site for clathrin. Conceivably, adaptor binding may induce the membrane curvature that facilitates the clathrin-mediated formation of invaginated coated pits. Thus far, two adaptor complexes have been well characterized. AP-1 and AP-2 consist of four related subunits (adaptins), two of $\approx$100-kDa and one of $\approx$50 kDa (designated μ for medium chains) and one of $\approx$20 kDa (designated σ for small chains). The AP-2 complex consists of either of two closely related (90% identical) 100 kDa α chains (α_A, α_C), a 100-kDa β chain, $\mu 2$ and $\sigma 2$. AP-1 contains 100 kDa γ and β' chains, together with $\mu 1$ and $\sigma 1$ subunits. The β and β' subunits are closely related, but all others are distinct ($<$50% similarity). All the 100-kDa subunits do, however, have similar domain structures, with a large head domain, a proline-rich hinge or extension, and a COOH-terminal appendage or ear (Heuser & Keen 1988, Ponnambalam et al 1990, Robinson 1993, Page & Robinson 1995). Together with the medium and small chains, the tetramers are assembled into a rectangular box with the two ears extending from the head domain at adjacent corners by their proline-rich hinges (Figure 2).

In addition to mediating clathrin attachment, adaptors are also known to have a second critical function, i.e. recruiting membrane proteins that selectively localize to clathrin-coated regions. This was first suggested by affinity chromatography experiments (Pearse 1988, Glickman et al 1989, Sorkin & Carpenter 1993, Sorkin et al 1995) and, more recently, using the yeast two-hybrid system (Ohno et al 1995). AP-1 and AP-2 adaptors mediate these activities at distinct intracellular sites, however. Under normal conditions, AP-2 complexes are localized to the plasma membrane coated pits; AP-1 complexes, on the other hand, are largely restricted to clathrin-coated buds of the TGN (Robinson & Pearse 1986, Ahle et al 1988). Thus AP-2 complexes must recognize plasma membrane receptors involved in endocytosis, whereas AP-1 complexes interact with proteins in the TGN that exit the Golgi complex via CCVs. Of these, the 205-kDa cation-independent and 47-kDa cation-dependent receptors

for mannose-6-phosphate are responsible for targeting newly synthesized lysosomal enzymes from the TGN to endosomes (Geuze et al 1984a; Kornfeld & Mellman 1989).

Adaptor complexes must also selectively include membrane proteins that function in the actual targeting of CCVs to endosomes. Such proteins may include members of the v- or t-SNARE families involved in vesicle docking and/or fusion (Rothman 1994). The only v-SNARE identified in the endocytic pathway (cellubrevin) does not function at the level of CCV fusion with EEs(Link et al 1993) but rather at a later step in recycling (Galli et al 1994). That such proteins are rapidly internalized in CCVs is stongly suggested in neurons where the v-SNAREs synaptobrevin/VAMP-1 and -2 appear to be internalized by CCVs following synaptic vesicle release (Maycox et al 1992).

SIGNALS FOR SELECTIVE INCLUSION IN CLATHRIN-COATED VESICLES The cytoplasmic domains of many receptors, as well as other membrane proteins that selectively accumulate at plasma membrane coated pits, contain specific

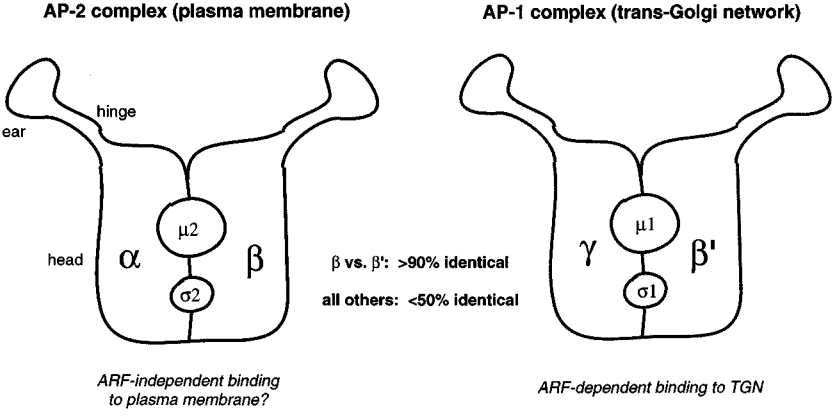


Figure 2 Organization of AP-1 and AP-2 clathrin adaptor complexes. The two known clathrin adaptor complexes (AP-1 and AP-2) are heterotetramers consisting of two ≈ 100-kDa subunits (α, β, β', or γ); two medium chains ($\mu 1$ or AP47; $\mu 2$ or AP50); and two small chains ($\sigma 1$ or AP20; $\sigma 2$ or AP17). The corresponding β and β' subunits are very closely related, whereas all others are related to their counterparts at <50% identity. The complexes consist of a large head domain and a proline-rich hinge region from which extends a COOH-terminal ear. AP-1 binds in an ARF-dependent fashion to the TGN, whereas AP-2 is commonly associated with the plasma membrane, although the ARF (if any) required for its membrane binding is unknown. A third adaptor complex consisting of the proteins β-NAP (≈ 30% identical to α-adaptin) and p47B (≈ 30% identical to the μ subunits) has recently been identified, also possibly associated with the TGN (Simpson et al 1996).

sequence information that facilitates coated pit localization. These signals are degenerate and somewhat variable thus making it difficult to establish precise motifs. Nevertheless, coated pit localization signals usually involve critical aromatic (usually tyrosine) residues placed in a context of one or more amino acids with large hydrophobic side chains (Trowbridge et al 1993) (Figure 3). A second general class of coated pit localization signal, also characterized, involves vicinal leucine residues (or leucine plus another small hydrophobic amino acid) as its most critical feature. Of potential significance is the fact that the di-leucine-based motif seems to occur most commonly among immune receptors expressed in leukocytes (e.g. Fc receptors, CD3, MHC class II-associated invariant chain) (Matter et al 1994, Hunziker & Fumey 1994), whereas the tyrosine-based motifs are far more widely distributed. Although multiple receptors are known to enter the same coated pit, and both tyrosine and di-leucine motifs clearly specify coated pit localization (Miettinen et al 1989, Hunziker et al 1991a, Amigorena et al 1992), whether the two motifs mediate entry into common coated pits, or are decoded by the same adaptor components has not been proven.

The physical structure of coated pit localization signals has been a topic of some interest. Based on an initial screening of crystallographic data of all proteins, the idea first emerged that tyrosine-containing signals adopted a β- or tight-turn conformation exposing the tyrosine on the turn surface (Collawn et al 1990). Evidence favoring this possibility was then provided by two-dimensional NMR analysis of short synthetic peptides, which were found to be capable of adopting such conformations for at least a portion of the time in aqueous solution (Bansal & Gierasch 1992, Eberle et al 1992). Given the limitations of conformational analysis of short peptides in solution, however, much additional information will be required before reaching firm conclusions regarding the actual structure of the coated pit signal. Nor is there any clear indication that the di-leucine motif adopts or participates in a tight-turn conformation. At least one potential coated pit localization domain has been modeled as a nascent helix (Wilde et al 1994).

Additionally, the simple presence of a coated pit localization domain does not ensure that a receptor will localize at clathrin-coated pits. In the case of the major leukocyte Fc receptor FcRII-B, cell type-specific alternative mRNA splicing in B-lymphocytes (FcRII-B1) introduces an in-frame insertion of 47 amino acids at a site membrane-proximal to the di-leucine-type coated pit signal (Miettinen et al 1989). Although the signal itself is not disrupted, its ability to function is inhibited. The effect seems to be one of conformational change from specific intramolecular interactions within the FcRII cytoplasmic tail: The insert does not inhibit coated pit localization of other receptors, nor does its position in the

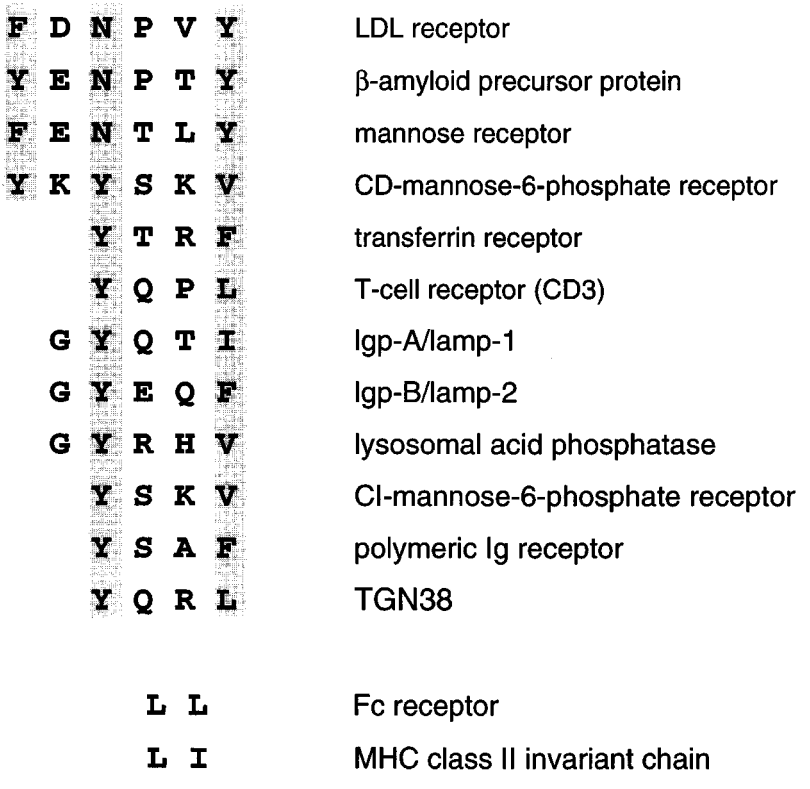


Figure 3 Consensus sequence motifs for localization at clathrin-coated pits. Although degenerate, the short tetra- or hexapeptide tyrosine-containing sequences demonstrated to be necessary and sufficient for programming localization at clathrin-coated pits have some features in common. Typically, this involves a tyrosine (or phenylalanine) residue followed by one or more hydrophobic or aromatic residues. Many receptors make use of tyrosine-independent signals, usually consisting of a di-leucine-type motif (e.g. Fc receptor, invariant chain). At least three of these motifs (LDL receptor, acid phosphatase, transferrin receptor) have been modeled to adopt a tight-turn conformation; others (TGN38, Ii chain) may not be so configured.

FcRII tail matter (Miettinen et al 1992). Another related example comes from CD4, a membrane protein of T cells that forms a complex with the cytoplasmic src-like kinase lck. In cells expressing lck, CD4 internalization is markedly slowed, presumably owing to masking of the CD4 coated pit localization domain by interaction with the kinase (Pelchen et al 1992). Thus it is difficult to predict the internalization phenotype of a protein from its sequence alone.

Coated pit localization signals are not restricted to plasma membrane receptors; they have also been identified on membrane proteins found in intracellular membranes. Most notable are the major membrane glycoproteins of LEs and lysosomes, lgps or lamps, that have short (usually 10–11 residues) cytoplasmic tails containing a conserved glycine-tyrosine sequence followed by a hydrophobic amino acid two residues towards their COOH-termini (Kornfeld & Mellman 1989). In many cells, lgp/lamps reach their destination by transport directly from the TGN to the endocytic pathway, possibly via CCVs (Kornfeld & Mellman 1989). Some missorting of lgp/lamps to the cell surface does occur, particularly at high expression levels, suggesting that the sorting mechanisms in the TGN are saturable. Nevertheless, cell surface appearance of lgp/lamps is followed by their rapid internalization via plasma membrane-derived CCVs. Thus it might be expected that the coated pit localization signal of lgp/lamps can be recognized by both AP-1 and AP-2 adaptor complexes (see below). Alteration of the conserved glycine residue does not inhibit internalization from the plasma membrane but does reduce sorting of newly synthesized lgp/lamps from the TGN directly to endosomes and lysosomes (Harter & Mellman 1992). Thus the glycine residue may be more important for specifying interactions with AP-1 than with AP-2.

Another example is TGN38, a resident protein of the TGN. TGN38 actually recycles continuously between the TGN and the plasma membrane, presumably via EEs and/or LEs (Reaves et al 1992, 1993, Ladinsky & Howell 1993, Rajasekaran et al 1994, Miesenbock & Rothman 1995). Like lgp/lamps, the TGN38 cytoplasmic domain has a tyrosine-containing motif required for TGN localization as well as for rapid internalization (Wilde et al 1992, Humphrey et al 1993). Although the transport step(s) under the control of the coated pit signal are unclear, given its equilibrium distribution, it appears that TGN38 is likely to interact with AP-1 adaptors (see below).

RECOGNITION OF COATED PIT LOCALIZATION SIGNALS Until recently there was no indication as to which adaptor subunit(s) might be involved. Based on a yeast two-hybrid screen using any of several tyrosine-containing motifs as "bait", Bonifacino and colleagues (Ohno et al 1995) found that the 50-kDa μ subunits are at least partly responsible for interacting with coated pit signals. Indeed, the coated pit signals of lgp/lamps and TGN38 could bind to both the

μ1 and μ2 subunits of AP-1 and AP-2 adaptors, respectively. This is consistent with the known or suspected abilities of both proteins to be included in TGN- and plasma membrane-derived CCVs. In contrast, the coated pit signal of the plasma membrane receptor (Tfn receptor) was found to interact with only the AP-2 μ2 subunit. Thus conventional plasma membrane receptors may avoid exiting the Golgi complex via CCVs because of their relative inability to interact with AP-1 adaptors. It seems likely that this inability is not the result of a fundamental difference is the structure of the coated pit signal, but rather because of relatively modest differences in affinity. Probably, only coated pit signals with the strongest affinities for adaptors can be detected in the two-hybrid assay. A remaining unknown is the behavior of the cation-independent mannose-6-phosphate receptor (MPR), the one receptor definitively shown to exit the TGN via CCVs (Geuze et al 1984a, 1985, Kornfeld & Mellman 1989). As different regions of the cation-independent and -dependent MPR cytoplasmic domains have been associated with endocytosis and sorting of lysosomal enzymes from the TGN (Johnson & Kornfeld, 1992a,b), different regions of the receptor's tail may be involved in AP-2 versus AP-1 binding. There is evidence that coated pit localization or adaptor interaction of the cation-independent MRP is regulated by serine phosphorylation (Le Borgne et al 1993, Meresse & Hoflack 1993). Finally, di-leucine motifs have not yet been shown to interact with either the AP-1 or AP-2 μ chains (Ohno et al 1995), suggesting that another adaptor exists or that there are requirements for μ interaction with di-leucine signals that were not properly reproduced in the two-hybrid system.

These results are important in that they confirm a role for direct and specific protein-protein interactions in receptor localization at clathrin-coated pits and, by extension, in other sorting events. It is remarkable that μ1 and μ2, which are $<$50% identical to each other, bind such similar motifs (e.g. lgp/lamps) while simultaneously distinguishing the closely related targeting signal in the transferrin receptor tail.

RECRUITMENT OF ADAPTOR COMPLEXES TO MEMBRANES How are AP-1 and AP-2 complexes recruited to membranes? One possibility is that the binding site is provided by the ligands with which they interact, i.e. receptor tails bearing coated pit localization motifs. Indeed, overexpression of transferrin receptor was reported to increase the extent of clathrin assembly on the plasma membrane, possibly reflecting an increase in AP-2 binding sites (Miller et al 1991). There is also evidence that AP-1 binding to Golgi membranes may require the cytoplasmic domain of the cation-independent mannose-6-phosphate receptor (Le Borgne et al 1993, 1996). It is also possible that recruitment is receptor independent and may be controlled by specialized adaptor-binding proteins or docking proteins (Mahaffey et al 1990). One possible candidate is the synaptic

vesicle component synaptotagmin (Zhang et al 1994). While acting as a calcium sensor during synaptic vesicle exocytosis, synaptotagmin also exhibits a strong affinity in vitro for AP-2 complexes in vitro. Although coated pits do not normally appear at the surface of synaptic vesicles, it is conceivable that synaptotagmin's AP-2 recruitment activity, or the analogous activity of any potential docking protein, is turned off intracellularly and turned on upon insertion into plasma membrane (Wang et al 1993). The phenotype of synaptotagmin mutants in *C. elegans*, which exhibit reduced neuronal endocytosis (Jorgensen et al 1995), supports this possibility. However, knock-outs in mice or *Drosophila* do not have such effects (Neher & Penner 1994). In such cases, this function might be performed by any of the non-neuronal synaptotagmins (Ullrich et al 1994).

At least in the case of AP-1 recruitment to the TGN, it appears that binding is under the control of the ras-like GTPase Arf (ADP-ribosylation factor). In vitro binding is stimulated by the presence of Arf and the non-hydrolyzable GTP (Stamnes & Rothman 1993, Traub et al 1993). Association of AP-1 with TGN membranes is also inhibited by brefeldin A (BFA), which blocks nucleotide exchange on Arf (Robinson & Kreis 1992). In this sense, AP-1 attachment is similar to the binding of at least two other coat proteins, the COP-I and COP-II complexes associated primarily with the ER and Golgi complex (Orci et al 1993a, Barlowe et al 1994). The role of Arf in the assembly of any coat complex remains unknown but may reflect its ability to activate membrane-associated phospholipase D (Brown et al 1993).

Interestingly, AP-2 binding to the plasma membrane is not stimulated by GTPγS or blocked by BFA (Robinson & Kreis 1992). Although this may suggest that AP-2 does not require Arf for membrane attachment, unlike all other coats studied thus far, it is possible that the plasma membrane contains a stably associated Arf protein, or in some other way provides the equivalent of its activity (constitutively active phospholipase D?). Recent results have shown that Arf6 is uniquely plasma membrane-associated and remains tightly bound even in the presence of BFA (Whitney et al 1995, Cavanagh et al 1996). It is not clear, however, that Arf6 is responsible for AP-2 recruitment and is absent from CCVs. Moreover, treatment of permeabilized cells with GTPγS can cause mislocalization of AP-2 adaptors to intracellular endosomal or lysosomal membranes (Seaman et al 1993). Thus potential AP-2 binding sites on endocytic organelles exist. This follows from the likelihood that adaptor-binding sites (receptors or specialized docking proteins) are internalized during CCV formation and delivered to endosomes. It appears that the activity of these binding sites is regulated in some way; recent results indicate that endosomes may host the formation of clathrin coats, although the adaptor complexes involved

are not clear (Whitney et al 1995, Stoorvogel et al 1996, Takei et al 1996). As discussed below, such coats may be important for endosome function.

Clathrin-Independent Endocytosis

Clathrin-independent mechanisms for endocytosis are also known to exist (Sandvig & van Deurs 1991, Lamaze & Schmid 1995). In most cell types, such mechanisms are best detected only when CCV formation is inhibited genetically or pharmacologically, or following a hormonal stimulus associated with a transient burst of fluid uptake. However, even under normal conditions, some plant or bacterial toxins are internalized in structures devoid of clathrin coats (Hansen et al 1991). Nevertheless, some of these uncoated vesicles deliver their contents to endosomes and lysosomes (Hansen et al 1993), raising the possibility that they were actually clathrin coated but that the coats were simply not visualized.

Several groups discovered various treatments (depletion of cytosolic K^+, growth in hyperosmotic sucrose, or cytosolic acidification) that effectively blocked the proper assembly of plasma membrane clathrin coats (Heuser & Anderson 1989) and/or arrested the internalization coated pit receptors without inhibiting the uptake of markers such as ricin or IL-2 (Davoust et al 1987, Hansen et al 1993, Subtil et al 1994). Because such treatments also incompletely (or inefficiently) inhibited the endocytosis of extracellular fluid, the idea emerged that cells were capable of considerable endocytic activity in the absence of clathrin. Until recently, it remained unclear as to whether clathrin-independent endocytosis was induced by the experimental manipulations used or coexisted with the clathrin-dependent pathway.

ENDOCYTOSIS IN CLATHRIN-DEFICIENT YEAST The first direct genetic evidence in favor of a clathrin-independent pathway of endocytosis came from early experiments in the yeast *Saccharomyces cerevisiae.* Deletion of the single gene for clathrin heavy chain was found to decrease only partially ($\approx 50\%$) the uptake of receptor-bound α-factor or the marker of fluid phase endocytosis lucifer yellow (Payne & Schekman 1985). This finding was initially controversial because clathrin deletion also greatly slowed cell growth and because endocytosis in yeast was only very poorly characterized at the time (Payne et al 1987, Schekman & Payne 1988). The subsequent isolation and characterization of yeast mutants with conditional defects in α-factor uptake has convincingly demonstrated that yeast exhibit a process reminiscent of endocytosis in animal cells (Chvatchko et al 1986, Zanolari et al 1992, Raths et al 1993, Singer et al 1993, Benedetti et al 1994, Hicke & Riezman 1996). This involves the internalization of receptor-bound α-factor, its transient appearance in a population of non-lysosomal vesicles (endosomes), and final delivery to

and degradation in the vacuole (lysosomes). In addition, the intracellular steps of the pathway involve ras-like GTPases (e.g. ypt5, ypt7) that are directly homologous to the rab proteins (rab4, 5, 7, 9) that control the fusion activities of early and LEs in mammalian cells (Schimmoller & Riezman 1993, Singer et al 1995). Thus yeast would appear capable of mediating at least some form of endocytosis in the absence of clathrin or in the presence of conditionally defective clathrin alleles (Seeger & Payne 1992; G Payne, unpublished observation).

What is the mechanism of clathrin-independent endocytosis? Potential insight into this question has been provided by the analysis of genes that control α-factor uptake. Although a coherent picture has yet to emerge, several of these genes have some relationship to the actin cytoskeleton (e.g. actin, fimbrin) (Kubler & Riezman 1993, Benedetti et al 1994). Whether clathrin-dependent uptake or an as yet poorly characterized actin-based system is more important under normal conditions is not clear; nor is it necessarily the case that the two mechanisms work independently of each other. Given other similarities, it is possible that a yeast-like actin-based mechanism plays an underappreciated role in endocytosis in mammalian cells, conceivably accounting for the cell's ability for clathrin-independent uptake. In *Dictyostelium*, where clathrin deletion exerts a more complete effect on endocytosis than in yeast, a role for nonconventional myosins in endocytosis has nevertheless been suggested (Novak et al 1995). Actin-dependence of endocytosis in yeast may also reflect the process of phagocytosis in mammalian cells, which is also strictly actin dependent (see above). On the other hand, it is not at all clear that actin plays a direct role in endocytosis in yeast. Although mutation of type I myosin genes (*Myo3* and *Myo5*) have recently been found to inhibit yeast endocytosis (Gell & Riezman 1996), the effect may be an indirect consequence of a dramatic reorganization of the actin cytoskeleton in these cells (Goodson et al 1996).

ELIMINATION OF CLATHRIN FUNCTION IN MAMMALIAN CELLS DOES NOT ALWAYS BLOCK ENDOCYTOSIS As discussed above, dynamin is a GTPase known from genetic, morphological, and biochemical evidence to provide a critical accessory function in clathrin-dependent endocytosis (De Camilli et al 1995). By complexing around the necks of invaginated coated pits, it may provide the mechanochemical force to accomplish the final step in the budding process (Hinshaw & Schmid 1995, Takei et al 1995) (Figure 4). Expression of mutant dynamin in mammalian cells in culture has been used to inhibit the clathrin-dependent endocytosis of receptor-bound ligands, but also has provided important insight into the clathrin-independent pathway (van der Bliek et al 1993, Damke et al 1994, 1995b). By using a temperature-sensitive mutant allele of human dynamin (predicted from the homologous *Drosophila* sequence), Schmid and colleagues found that shift to the nonpermissive

temperature resulted in the rapid reduction of both receptor-mediated and fluid phase endocytosis (Damke et al 1995b). Within 30–60 min, however, fluid uptake was restored to normal, whereas transferrin uptake remained depressed. These results strongly suggest that animal cells have the capacity to induce an apparent clathrin-independent pathway of endocytosis in response to an inactivation of the clathrin (or dynamin)-dependent pathway. They may also explain why animal cells expressing constitutively defective dynamin mutants, cells subjected to K^+ depletion, or clathrin-deficient yeast exhibit fluid phase endocytosis: The cells have already compensated for the loss of the clathrin-dependent pathway.

The nature of the clathrin-independent pathway of endocytosis is unknown, but its existence, at least upon inactivation of the clathrin pathway, is no longer in doubt. At least two options have been suggested to provide endocytic activity in the absence of clathrin: caveolae and macropinocytosis.

CAVEOLAE The existence of plasma membrane pits, independent of clathrin coats, was initially in endothelial cells and adipocytes, where they were eventually termed caveolae (Peters et al 1985, Anderson 1993a). Subsequently observed at varying frequencies in many other cell types, the cytoplasmic faces of caveolae are distinguished by characteristic striations thought to be formed by an accumulation of the integral membrane protein caveolin (Peters et al 1985, Rothberg et al 1992). They are thought also to occur on internal organelles

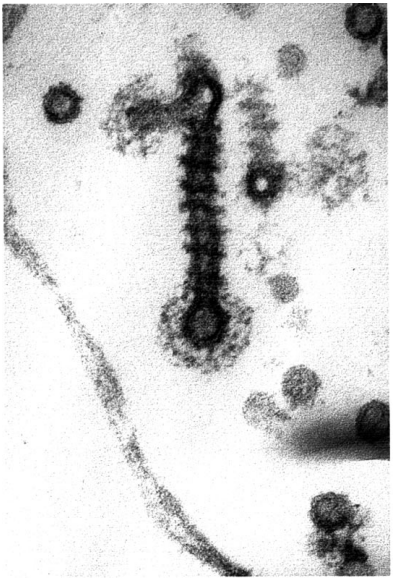


Figure 4 Dynamin is found at the necks of budding clathrin-coated vesicles. Dynamin assembles as regularly spaced rings on the necks of invaginated clathrin-coated pits arrested in the presence of GTPγS at presynaptic nerve endings. Although the precise function of the dynamin array is unknown, it may serve as a mechanochemical GTPase involved in the final budding of coated vesicles from coated pits. Such structures are best observed in synaptosomal preparations, but in somewhat less dramatic fashion are likely to form in nonneuronal cells as well. (Micrograph provided by K Takei & P De Camilli, Yale Univ. Sch. Med.; Takei et al 1995).

(Dupree et al 1993). In endothelium, the formation of caveolae is associated with the endocytic transport of solutes across the cell (transcytosis), as opposed to transport to endosomes and lysosomes, an activity associated with the formation of conventional CCVs (Milici et al 1987). In other cell types, however, it remains unclear the extent to which caveolae mediate fluid uptake or the net internalization of plasma membrane.

Although caveolae have been associated with functions as diverse as endocytosis, polarized sorting in epithelial cells, and signal transduction (Anderson 1993a, Lisanti et al 1994), their actual activities and features have remained somewhat confusing due to difficulties in studying their properties (Parton 1996). They are known to accumulate GPI-anchored membrane proteins such as the folate receptor (Rothberg et al 1992, Ying et al 1992), although even this may occur only after antibody cross-linking (Mayor et al 1994). Uptake of folate via caveolae may not involve endocytosis per se, but rather the budding and immediate reformation of caveolae pits, a process termed potocytosis (Anderson 1993b). Indeed, endocytic uptake of folate via CCV does not appear to result in the productive accumulation of the vitamin (Ritter et al 1995) although folate does reach conventional endosomes after internalization by it physiological receptor (Rijnboutt et al 1996). There is also evidence that caveolin can be transported between the plasma membrane and the secretory pathway and can be induced by certain drug treatments to accumulate in intracellular vesicles (Parton et al 1994, Smart et al 1994, Conrad et al 1995). Nevertheless, it remains highly speculative as to whether caveolae contribute to clathrin-independent fluid phase endocytosis. The existence of viable, caveolin-defective cells (lacking identifiable caveolae) further questions whether they provide an important mechanism for clathrin-independent endocytosis (Fra et al 1994, 1995, Kuliawat et al 1995).

MACROPINOCYTOSIS Cells such as macrophages, dendritic cells, and fibroblasts that can exhibit abundant membrane ruffling activity have a form of fluid phase endocytosis termed macropinocytosis (Steinman & Swanson 1995). This mode of uptake appears to reflect the passive capture of extracellular fluid when the rims of membrane folds fuse back with the plasma membrane. Vesicles or vacuoles formed in this way can be quite large, 1 to 5 μm in diameter. Macromolecules internalized in macropinosomes can be delivered to lysosomes or recycled (Racoosin & Swanson 1992, 1993).

Macropinosome formation is clearly independent of clathrin, although whether it is responsible for endocytosis observed in cells whose clathrin pathway has been inactivated is not clear. However, there are a number of intriguing features of macropinocytosis that link this activity with both phagocytosis and

the actin cytoskeleton. For instance, stimulation of fibroblasts with polypeptide hormones such as epidermal growth factor (EGF) causes a transient burst of macropinocytic activity (Haigler et al 1979). A very similar series of events occurs concomitant with the binding and phagocytosis of pathogenic bacteria, particularly *Salmonella* (Pace et al 1993, Alpuche et al 1994). In fact, it seems likely that *Salmonella* entry itself reflects a localized stimulation of membrane ruffling activity in a fashion intimately dependent on actin and the regulation of actin function by GTPases of the rho family (J Gálan, unpublished results). Indeed, a link between signal transduction, membrane ruffling, and rho GTPases has been well established by microinjection experiments (Ridley et al 1992). Further emphasizing the actin dependence of such events is the observation that melanoma cells deficient in actin binding protein (ABP-1) exhibit defects in constitutive membrane ruffling/macropinocytic activity (Cunningham et al 1992).

MOLECULAR SORTING IN ENDOSOMES

Although the selective inclusion of receptors at plasma membrane coated pits is the initial sorting event during endocytosis, no less important are the sorting events that occur in endosomes. Indeed, endosomes are little more than sorting stations whose minimal tasks involve the discharge of internalized ligands from their receptors and the subsequent transfer of ligands to lysosomes and receptors back to the plasma membrane. Recently, it has become clear that endosomes mediate even more sophisticated sorting events, playing an important role in the targeting of membrane proteins to their correct plasma membrane domains in polarized epithelial cells, to a variety of specialized endosome-derived recycling or secretory vesicles, and to the TGN. The mechanisms responsible for these activities remain incompletely understood and are currently the subject of intense interest. A number of important principles have emerged regarding endosome function, however; these are considered below.

Organization and Biogenesis of Endosomes

As illustrated in Figure 1, four classes of endocytic organelles are typically distinguished based largely on their relative kinetics of labeling by endocytic tracers: EEs, LEs, recycling vesicles, and lysosomes (Kornfeld & Mellman 1989). The precise relationship among these structures has yet to be determined, and, in fact, may never be because of the great plasticity and dynamics of the system. It is also almost impossible to recognize these structures on the basis of morphology or position in the cytoplasm alone. Nevertheless, such operational distinctions have proved useful because they reflect important functional specializations in the endocytic pathway and have facilitated much recent

analysis. Several biochemical markers of endosomes populations and lysosomes have been identified, allowing these distinctions to be made in more precise terms than is possible using only functional criteria. Among the most important of these are rab proteins: rab4, rab5, and rab11 are associated with EEs; rab7 and rab9 are LE specific (Chavrier et al 1990, van der Sluijs et al 1991, Simons & Zerial 1993, Nuoffer & Balch 1994, Pfeffer 1994). In addition, although recycling plasma membrane receptors are restricted to EEs and recycling vesicles, LEs contain MPR (which recycles predominantly to the TGN) (Kornfeld & Mellman 1989). Both LEs and lysosomes are highly enriched in distinctive, highly glycosylated and conserved lysosomal membrane glycoproteins (lgps, lamps, etc), which are generally depleted from EEs and the plasma membrane (Kornfeld & Mellman 1989).

It is important to emphasize that the organelle populations of the endocytic pathway are intimately interconnected by continuous endocytic and recycling activity. Indeed, much of their existence is a reflection of this activity. Given estimates that the entire cell surface is internalized at least once or twice per hour, the entire endosomal network also must turn over with similar kinetics because the total surface area of endosomes is probably far less than that of the plasma membrane (Steinman et al 1976, Griffiths et al 1989). Indeed, transient cessation of endocytosis in cells expressing a *ts* dynamin mutant is accompanied by a transient disappearance of endosomes (Damke et al 1995a), indicating that much of the bulk membrane area of endosomes is in transient residence, derived by internalization from the plasma membrane (Mellman et al 1980). Finally, the number and appearance of lysosomes can be drastically altered by growth of cells in high concentrations of poorly digestible solutes (Cohn 1966, Cohn et al 1966), which suggests that their existence is regulated by the amount and nature of internalized material. Although it is expected that endosomes and lysosomes have unique membrane components responsible for their special activities, attempting to conceptualize them as pre-existing or stable structures is a difficult and possibly useless task. Nor is it necessary to solve this issue in order to study endosome function or the biochemical mechansims responsible for these functions.

EARLY ENDOSOMES EEs are operationally defined as the first structures labeled by incoming endocytic vesicles (typically CCVs) (Helenius et al 1983). In simple tissue culture cells, EEs represent a dynamic network of tubules and vesicles dispersed throughout the cytoplasm; occasionally, the network has the appearance of a tubular reticulum, particularly in absorptive epithelial cells (Geuze et al 1983, Marsh et al 1986, Hopkins et al 1990, Tooze & Hollinshead

1991, Wilson et al 1991). The primary function of EEs is sorting, and they may represent a common site for all sorting events in the endocytic pathway (see below). For those receptors engaged in rapid recycling, EEs host the initial dissociation of receptor-ligand complexes. Their internal pH is only slightly acidic, usually ranging between pH 6.3–6.8 (Yamashiro et al 1984, Kornfeld & Mellman 1989). It has proved difficult to measure EE pH using fluorescence pH measurements of intact cells because transit through EEs is very rapid (2–3 min), which does not allow for a sufficiently selective accumulation of fluorescent reporter for in vivo measurements. However, the use of enveloped viruses as intracellular pH probes demonstrates that viruses with fusion thresholds of pH 6.3 or higher can penetrate into cells from EEs, whereas those requiring more acidic pH for fusion must await delivery to LEs or lysosomes (Schmid et al 1989). Moreover, this pH range is sufficient to result in the dissociation of ligands such as low density lipoprotein (LDL) (Kornfeld & Mellman 1989).

EEs next engage in the physical separation or sorting of vacant receptors from dissociated ligands, as well as other macromolecules internalized in the fluid phase. Accordingly, EEs have been referred to as sorting endosomes (Mayor et al 1993). The primary mechanism of this event may be geometric: Due to their greater fractional surface area, receptors (and other membrane proteins) accumulate in the EE tubular extensions, whereas due to their greater fractional volume, soluble contents accumulate in vesicular regions. The tubular elements would then pinch off or serve as sites for budding, forming carriers involved in receptor recycling, and the larger vesicular elements would proceed to LEs and lysosomes. Although the recycling carriers would contain some internal fluid that would be returned to the extracellular space, much of it would be retained within the cell.

This general model was well supported by early immunocytochemical evidence (Geuze et al 1983, 1984b). These data also demonstrated that at least some specificity must govern the process because the apparent concentration of receptors in the membrane of EE tubular regions was many fold higher than in the membrane of the vacuolar regions with which they are continuous (Geuze et al 1987). The ability of receptors to concentrate in tubular extensions may reflect a generalized physical property of the membranes involved or the existence of specific signals that direct recycling. There is now considerable evidence in favor of the latter possibility (see below).

RECYCLING VESICLES Recycling vesicles (RVs) are thought to arise from the tubular extensions of EEs, although it is unclear whether they are formed by the pinching off of entire tubules or by the production of buds in a manner

analogous to the formation of CCVs (Figure 1). Although some RVs may fuse directly with the plasma membrane, it is clear that at least some translocate to the perinuclear cytoplasm on microtubule tracks where they accumulate as a distinctive population of vesicles and tubules closely apposed to the microtubule organizing center (MTOC) (Hopkins 1983, Hopkins & Trowbridge 1983, Yamashiro et al 1984). These perinuclear recycling vesicles (PNRVs) appear to contain an intracellular pool of recycling receptors, suggesting that there is a rate-limiting step in receptor recycling from PNRVs to the plasma membrane. In the case of transferrin receptor (Tfn-R), up to 75% of the total cell receptor is found intracellularly, much of which in PNRV (van der Sluijs et al 1992). Transit through peripheral EEs requires only 2–3 min, while transit through PNRVs can take 5–10 min (Schmid et al 1988, Daro et al 1996). However, the fraction of receptors that recycle directly from EEs to the plasma membrane versus transit through PNRV is unknown; nor is the function of these structures clear. At least in polarized cells, transport of RVs to the MTOC may reflect the necessity to accumulate vesicles at a site from which physical access to multiple plasma membrane domains can be maximized (Hughson & Hopkins 1990, Hunziker et al 1990, Apodaca et al 1994, Barroso & Sztul 1994, Knight et al 1995).

RVs, particularly those in the MTOC region, have been assumed to represent a distinct compartment, i.e. a population of membrane vesicles functionally and biochemically distinct from all other vesicle populations (Gruenberg & Maxfield 1995). Although evidence for this view is scant, it is based on the observations that RVs are post-sorting and generally do not contain markers destined for lysosomes, that they may have a less acidic pH than EEs, and that they have a characteristic distribution in the cytoplasm (Yamashiro et al 1984). Video microscopy suggests that they also selectively but transiently accumulate ligands such as cross-linked Tfn (Ghosh & Maxfield 1995, Marsh et al 1995); in the absence of biochemical markers for RVs, however, it is impossible to be certain if this is the case.

Very recently, evidence has emerged that some of the RVs in the perinuclear region are not just functionally, but also biochemically distinct from EEs. Although rab4 and rab5 are GTPases characteristic of EEs, rab4 appears to be depleted from PNRVs (Daro et al 1996) (Figure 5). They may also be enriched in a different endosomal rab protein, rab11 (M Zerial, manuscript submitted). It is premature to conclude that these structures comprise a distinct compartment, but these findings do suggest that the membrane of PNRVs is not identical to the EE membrane: i.e. there is specificity in the process that gives rise to RV formation. Nevertheless, much remains to be learned about these structures. They may yet prove to be simple transport vesicles in transit to the plasma membrane

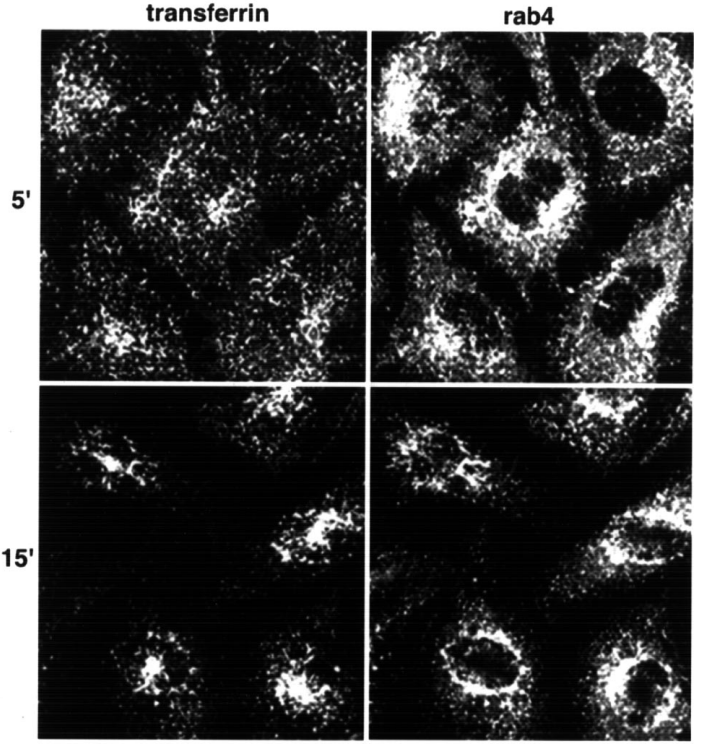


Figure 5 The pathway of receptor recycling consists of at least two distinct vesicle populations: early endosomes and recycling vesicles. CHO cells expressing human transferrin receptor and rab4 were allowed to internalize FITC-labeled transferrin for 5 or 15 min, then processed for immunofluoresence. While initially found in peripheral early endosomes that are strongly positive for rab4, at the 15 min point, a significant fraction of not yet recycled, intracellular transferrin is found in a cluster of vesicles directly over the microtubule organizing center in the perinuclear region of the cell (*lower left panel*). These perinuclear vesicles, commonly referred to as recycling vesicles, are relatively depleted in rab4 and, at least by this criterion, are biochemically distinct from the more peripheral early endosomes.

as opposed to a distinct population or compartment with its own functional attributes. Such evidence in favor of a compartment is the recent observation that some sorting activity occurs in RVs, at least as defined by the retrograde movement of fluorescent Tfn from perinuclear vesicles back to the cell periphery (Ghosh & Maxfield 1995). However, this evidence must be tempered by the likelihood that the endosomal vesicles concentrated in the MTOC region represent a biochemically and functionally heterogeneous array of structures, which makes their study difficult in the absence of distinct markers. Nor is it clear whether PNRVs fuse directly with the plasma membrane, or give rise to yet another transport vesicle population that completes the recycling circuit.

LATE ENDOSOMES AND LYSOSOMES The transfer of material from EEs to LEs involves the dissociation of vacuolar elements from the EE network, and their subsequent migration on microtubules to the perinuclear cytoplasm where they fuse with LEs (Figure 1). Dissociation of the vacuolar elements, sometimes called carrier vesicles (Gruenberg et al 1989, Aniento et al 1993, Clague et al 1994), reflects either an active budding process or, more simply, the removal of tubular elements by the continuous formation of RVs. In either event, formation of the carrier vesicles seems to be accompanied by an increase in the number of internal vesicles they contain. These are derived from invaginations of the limiting endosomal membrane (Cohn et al 1966, McKanna et al 1979). Carrier vesicles and subsequent structures thus take on the general appearance of classical multivesicular bodies (Felder et al 1990, Hopkins et al 1990, Hopkins 1994).

Late endosomes (LEs) are defined as vesicular structures that accumulate and concentrate internalized contents after their passage through EEs. They often have abundant internal membranes and are enriched in lgp/lamps, rab7, rab9, and the cation-independent MPR (Griffiths et al 1988, Geuze et al 1989, Kornfeld & Mellman 1989, Chavrier et al 1991, Lombardi et al 1993, Simons & Zerial 1993, Feng et al 1995). Although still of low density by Percoll gradient centrifugation, LEs also contain hydrolytically active lysosomal hydrolases and likely initiate the degradative process. These lysosome-like characteristics sometimes lead to LEs being referred to as pre-lysosomes or the pre-lysosomal compartment.

The relationship between LEs and lysosomes is dynamic and thus not easily defined. By convention, lysosomes are distinguished from LEs by virtue of the lysosome's higher density on Percoll, the absence of MPR, and the operational feature of being the final site of accumulation of internalized macromolecules (Helenius et al 1983, Kornfeld & Mellman 1989). Because lysosomes almost

certainly receive endocytosed material by fusion with LEs (or carrier vesicles), it is useful to view their life history as more of a cycle. As summarized in Figure 6, incoming LEs are active lysosomes that progressively degrade their contents, provide for the recycling of surviving receptors (e.g. MPR), and ultimately increase in density as digestible membrane and content are processed, catabolic products released, and the remaining non-digestible material (lysosomal hydrolases, certain membrane and lipid components) is concentrated. At this stage, they become resting lysosomes, which will again be activated upon fusion with another carrier vesicle or LE. This scheme is consistent with observations that even after extended periods it is impossible to completely chase tracers such as colloidal gold from immunocytochemically defined LEs to lysosomes (Griffiths et al 1988). Such a result suggests the existence of an equilibrium, rather than unidirectional transfer, between LEs and lysosomes.

Specificity of Receptor Sorting in Endosomes

It is well known that most internalized membrane recycles from early stations in the endocytic pathway; the "deeper" into the pathway a marker traverses, the less likely it is to recycle (Besterman et al 1981). The fact that plasma membrane receptors such as the Tfn receptor are found only in EEs and RVs demonstrates that recycling receptors are removed from the pathway at the level of EEs (Stoorvogel et al 1987, Schmid et al 1988, Kornfeld & Mellman 1989, Ghosh et al 1994). How selective is receptor sorting into RVs? The fact that receptors such as those for polypeptide hormones and antibody molecules are often targeted from EEs to LEs and degraded along with their ligands shows that EEs must be able to sort membrane proteins. Early on, it was demonstrated that the stable cross-linking of receptors otherwise capable of rapid recycling was sufficient to prevent their return to the cell surface and instead target them to lysosomes (Mellman & Plutner 1984, Mellman et al 1984, Neutra et al 1985). Phosphorylation-induced changes in oligomeric structure may achieve the same end for tyrosine kinase-containing growth factor receptors subject to down regulation during ligand uptake (Felder et al 1990, Sorkin et al 1992, Cadena et al 1994). Some membrane proteins such as P-selectin and EGF receptor also may contain cytoplasmic domain signals that specify their transport to lysosomes (Green et al 1994, Opresko et al 1995). Accordingly, the view emerged initially that recycling occurred by default, with any membrane protein able to return via RVs to the cell surface unless it was prevented from doing so, for example, by ligand-induced aggregation.

The possibility that receptor recycling occurs in a signal-independent default mode was supported by several considerations. For example, the return of fluorescent sphingolipid analogues from endosomes to the plasma membrane occurs with kinetics indistinguishable from the kinetics of Tfn receptor

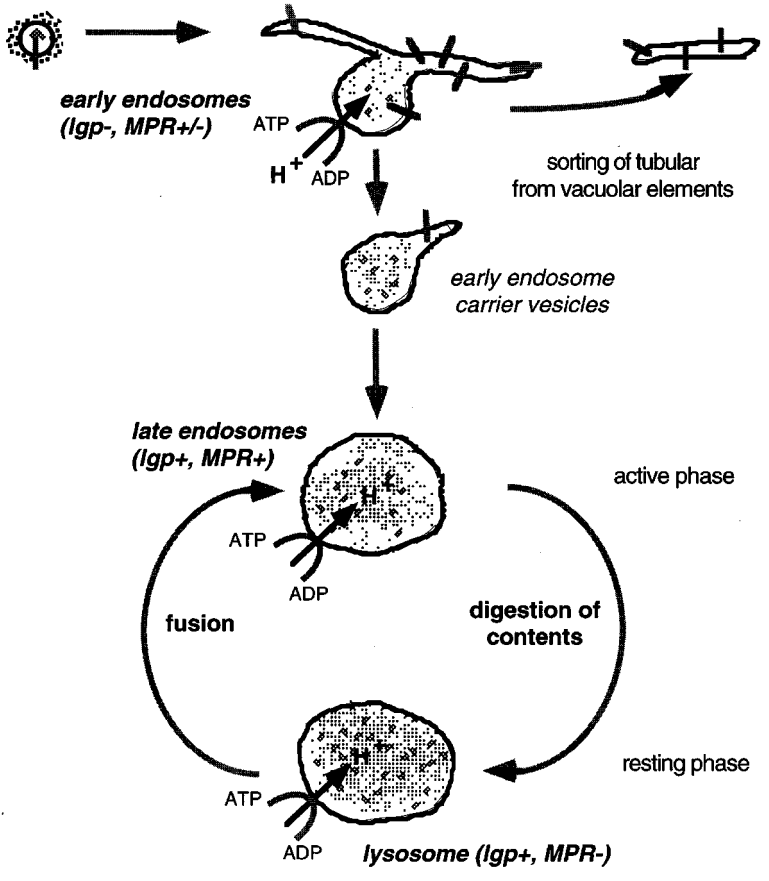


Figure 6 The lysosome cycle. This diagram illustrates a proposed relationship between late endosomes and lysosomes that suggests the two compartments exist in a dynamic equilibrium with each other. After sorting in early endosomes, vacuolar portions of the early endosome network pinch off to yield vesicles (endosome carrier vesicles) containing content destined for lysosomal digestion as well as some receptors (e.g. mannose-6-phosphate receptor, MPR) that may not have been recycled. The carrier vesicles fuse with late endosomes or lysosomes, defined as hydrolase-rich, lgp/lamp-positive structures that, respectively, do or do not contain MPR. The product of this fusion is by definition always a late endosome. As remaining MPR escape by recycling (presumably to the TGN) and as the hydrolases degrade digestible membrane and content, the late endosomes are gradually transformed into lysosomes. By this view, lysosomes consist largely of non-digestible or poorly digestible proteins, lipids, and carbohydrates and thus are hydrolytically inactive due to an absence of substrate. The lysosomes are cyclically converted into late endosomes upon fusion with an incoming early endosome carrier vesicle or with a late endosome. (Model developed by A Helenius & I Mellman, Yale Univ. Sch. Med.)

recycling (Mayor et al 1993). Thus the transport of bulk membrane lipids would appear to occur as rapidly as possible. In addition, relatively rapid recycling was observed for a mutant Tfn receptor lacking a cytoplasmic domain, suggesting that receptors simply followed the pathway defined by membrane lipids (Marsh et al 1995). On the other hand, sphingolipids can engage in lateral hydrogen bonding to form defined membrane domains that themselves are able to sort membrane proteins (Fiedler et al 1993, 1994). Thus kinetic arguments alone cannot be used to establish a case for or against specificity.

TARGETING SIGNALS CONTROL RECEPTOR RECYCLING IN POLARIZED EPITHELIAL CELLS In polarized epithelial cells, receptors internalized at one plasma membrane domain typically recycle back to that domain. This has been clearly demonstrated in Madin-Darby canine kidney (MDCK) cells in which Tfn receptor and LDL receptor, both proteins restricted to the basolateral surface, are continuously internalized and recycled basolaterally with a high degree of fidelity (Mellman et al 1993b). Transport across the cell to the apical domain (transcytosis) is possible as well, although this normally occurs only in the case of specialized transcytotic receptors such as the polymeric Ig receptor (Mostov 1994). It is obvious from the study of epithelial cells that receptor recycling cannot be a default process, but must be governed by one or more targeting signals.

The nature of targeting determinants for polarized transport in epithelial cells have been elucidated recently in some detail. By analyzing the transport of membrane proteins from the TGN to the apical or basolateral domains of MDCK cells, it is now clear that polarity is controlled primarily by discrete determinants found in the cytoplasmic domains of most or all proteins destined for the basolateral surface (Mellman et al 1993a, Matter & Mellman 1994, Mellman 1996). Although degenerate, many sequence motifs responsible for basolateral targeting bear at least a superficial resemblance to coated pit localization signals (Hunziker et al 1991a; Matter et al 1992, 1993, 1994, Prill et al 1993, Rajasekaran et al 1994, Monlauzeur et al 1995). Basolateral signals often depend on critical tyrosine residues for activity, some of which are also shared by a colinear coated pit signal. The tyrosine-dependent signals may also require an adjacent residue as well as a region of three acidic residues, features not required for coated pit localization. A well-characterized example of such signals is provided by the LDL receptor, which contains two distinct tyrosine-dependent determinants (Figure 7). Further emphasizing a possible relationship between basolateral targeting and coated pit determinants is the fact that some receptors (e.g. Fc receptor) are sorted to the basolateral surface by the exact same di-leucine motif that is responsible for rapid endocytosis (Hunziker & Fumey 1994, Matter et al 1994).

The signals are both necessary and sufficient for targeting to the basolateral surface from the TGN. In many cases, they also seem to be hierarchically arranged with a second, cryptic signal for apical targeting. Elimination of the basolateral signal by mutagenesis often results in efficient apical transport, rather than a random distribution on both plasma membrane domains. Thus in the absence of a functional basolateral signal, many proteins may be selectively delivered to the apical surface, which suggests the existence of a second signal for apical transport from the TGN. Although the nature of the apical signal is not known, it is apparently found in the lumenal domain of membrane proteins and may actually be provided by N-linked sugars (Scheiffele et al 1995). Conceivably, a carbohydrate determinant interacts at low affinity with a lectin that facilitates inclusion into nascent apical transport vesicles in the TGN. It is also possible that such interactions can lead to the incorporation of apical proteins into glycolipid rafts serving as differentiated microdomains that concentrate lipid and protein components destined for the apical surface (Fiedler et al 1994).

A striking and important feature of this system is its generality. First, the signals that control basolateral versus apical trasport are exceedingly broadly distributed, present on membrane proteins (e.g. LDL receptor, Tfn receptor) whose expression is not limited to polarized cells. Second, the same signals appear to control polarity in a variety of epithelial cell types and perhaps in non-epithelial polarized cells such as neurons (Dotti & Simons 1990, Dotti et al 1991). Finally, evidence from LDL receptor, Fc receptor, and pIg receptor has demonstrated that the targeting of receptors from endosomes back to the

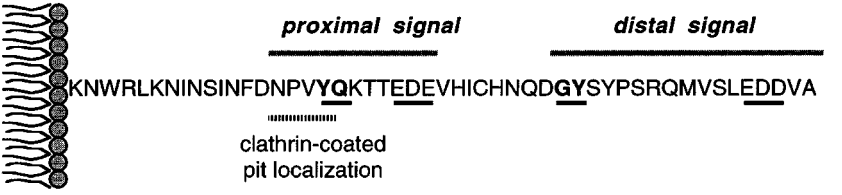


Figure 7 Signals for basolateral sorting in the LDL receptor cytoplasmic domain. The LDL receptor contains two independently acting targeting determinants capable of directing transport from endosomes and the TGN to the basolateral surface of polarized MDCK cells. The membrane proximal determinant shares a dependency on a critical tyrosine residue that is also essential for clathrin-coated pit localization, although no other features of the coated pit signal (minimally, NPXY) are required for optimal basolateral targeting. Removal of the proline residue increases the targeting efficiency of this signal (Matter et al 1993). The membrane distal signal is also tyrosine dependent, but cannot facilitate endocytosis. Both the proximal and distal signals depend on acidic residues downstream from the two tyrosines. Residues identified thus far as being essential for the activity of both signals are shown by solid underlines.

basolateral surface of MDCK cells is ensured by the same signals that are utilized in the TGN (Matter et al 1993, Aroeti & Mostov 1994) (Figure 8). In the case of LDL receptor, for example, partial inactivation of it basolateral targeting signals results in inefficient sorting of receptors internalized at the basolateral surface, causing increasing amounts of basolateral receptor to recycle to the apical surface (transcytosis). This mechanism is probably the basis for the physiological process of transcytosis exhibited by pIg receptor. Here, binding of multimeric ligand (e.g. dimeric IgA) partially inactivates the receptor's constitutively active basolateral targeting signal, which results in its inclusion in RVs targeted to the apical plasma membrane. It seems likely that these pIg receptor-containing transcytotic vesicles (TCVs) form as a consequence of a sorting event in basolateral EEs, although some sorting may also occur in apical EEs (Apodaca et al 1994).

Receptor recycling is thus clearly signal dependent, at least in polarized cells. Remarkably, in hepatocytes, endosomes appear to be the sole sorting site for both internalized and newly synthesized membrane proteins. In these cells, apical and basolateral proteins are not sorted in the TGN but rather are transported together to the basolateral surface from which they are internalized and sorted in endosomes to their sites of final residence (Bartles et al 1987).

SIGNAL-MEDIATED ENDOSOMAL SORTING IN NONPOLARIZED CELLS To what extent might sorting signals play a role in endosome function in non-epithelial cells? To consider this question,the extent to which any cell type is truly nonpolarized should be addressed. Most of the differentiated cells in all complex multicellular organisms are indeed polarized morphologically or functionally, at least at certain stages. Even "spherical" T-lymphocytes are known to polarize during antigen recognition (Poo et al 1988, Stowers et al 1995). Combined with the fact that the sorting signals identified as controlling polarity in epithelial cells are widely distributed on proteins expressed in all cell types, the idea has emerged that perhaps all cells possess cognate apical and basolateral pathways that underlie their ability to adopt polarized phenotypes (Mellman et al 1993a, Matter & Mellman 1994). At least with respect to sorting of apical and basolateral protein in the TGN, there is now some evidence, albeit indirect, to support this possibility (Fiedler & Simons 1995, Müsch et al 1996).

It is thus reasonable to expect that the basolateral targeting signals found in many receptors (e.g. Tfn, LDL receptors) play a role in endosomal sorting and recycling in fibroblasts and other single cells. The existence of such sorting signals need not contribute to the kinetics or efficiency of recycling in simple cells. Even if cognate recycling and transcytotic pathways were to exist, they would mediate transport to a common plasma membrane domain. As discussed above, signals or alterations in conformation may be required for targeting receptors

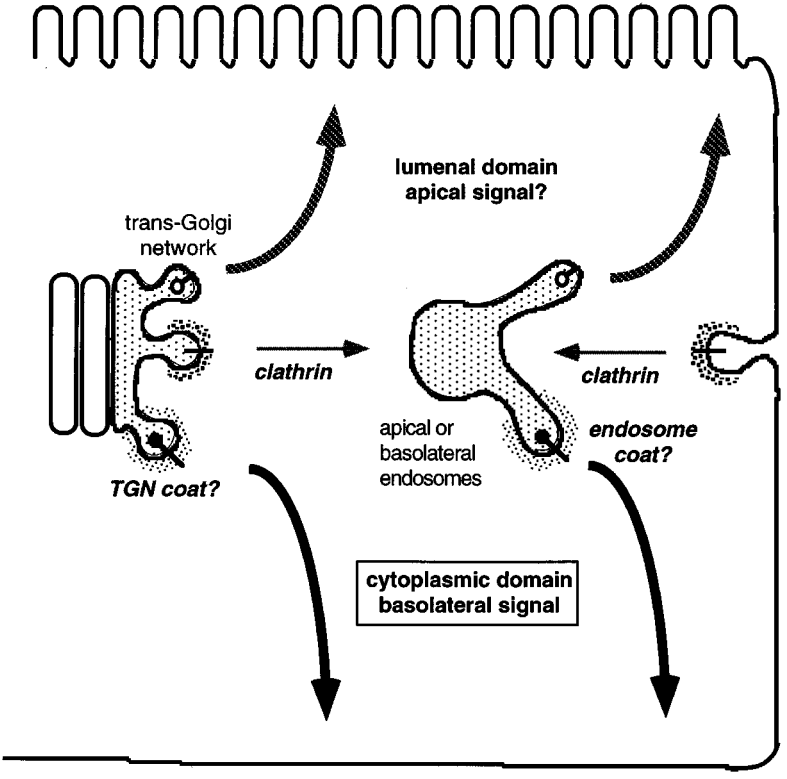


Figure 8 Endosomes and the TGN use common signals for polarized sorting in epithelial cells. Endosomes and the TGN of polarized epithelial cells are capable of decoding a common set of signals that specify transport to the basolateral plasma membrane. This ensures that both newly synthesized proteins and proteins internalized by endocytosis are targeted to their appropriate plasma membrane domains and suggests that biosynthetic and endocytic organelles may use common or at least related mechanisms for polarized sorting. Because the targeting signals are contained entirely within a basolateral protein's cytoplasmic domain, it is presumed that they must interact with one or more adaptor-like complexes recruited from the cytosol.

from early to late endosomes. However, the fact that recycling receptors concentrate in the early endosome's tubular extensions (Geuze et al 1987) implies that they are laterally sorted, which suggests the interaction of a sorting signal with an endosome-associated adaptor complex. Alternatively, it is possible that the recycling tubules represent a differentiated lipid domain (e.g. glycolipid raft) capable of concentrating receptors in an adaptor-independent fashion. In either event, exit from EEs via recycling tubules represents the route taken by the majority of membrane components internalized during endocytosis. Therefore, unless diverted to LEs, internalized membrane proteins would be expected to recycle regardless of whether they possessed a signal or even cytoplasmic domains.

ENDOSOMAL SORTING AND THE FORMATION OF SPECIALIZED RECYCLING VESICLES The ability of endosomes to mediate signal-dependent sorting is further illustrated by their capacity to bud off specialized transport vesicles in addition to the RVs that mediate constitutive receptor recycling. Best characterized is the formation of synaptic vesicles (SVs) from endosomes in neurons and neuroendocrine cells (Kelly 1993, Mundigl & De Camilli 1994). During synaptic transmission, pre-existing SVs undergo regulated exocytosis and fuse with the presynaptic plasma membrane. Vesicle membrane components are then recovered by endocytosis (largely via CCVs) and probably delivered to endosomes, which allows for the budding of newly formed SVs. In neuroendocrine cells, it is clear that the endosomes giving rise to SVs are probably EEs because they contain constitutively recycling receptors such as Tfn receptor (Clift-O'Grady et al 1990, Cameron et al 1991). Such receptors are largely excluded from the nascent SVs, and vice versa. Inclusion of SV components such as the membrane protein synaptobrevin (or VAMP) appears to be controlled by a cytoplasmic domain-sorting determinant, distinct in sequence from synaptobrevin's coated pit localization signal and not obviously related to signals involved in other sorting events (Grote et al 1995). Conceivably, cognate basolateral targeting determinants in the cytoplasmic domains of many constitutively reycling receptors help increase the specificity of SV formation by selectively including such receptors in RVs.

In addition to the formation of SVs in neuronal cells and TCVs in epithelial cells, other examples of specialized post-endosomal recycling vesicles are likely to include insulin-regulated Glut-4 vesicles of adipocytes (Herman et al 1994, Martin et al 1996) and MHC class II-containing vesicles (CIIV) in B-lymphocytes (Amigorena et al 1994, Tulp et al 1994, Wolf & Ploegh 1995). The glucose transporter Glut-4 is well known to be recruited to the plasma membrane from an intracellular pool found in a distinct endosome-like vesicle population in response to insulin stimulation of adipocytes or muscle cells.

Following its endocytosis, sequestration of Glut-4 into this vesicle population is thought to require specific signals found in one or more of the cytoplasmic regions of this polytypic membrane protein (Piper et al 1993, Verhey & Birnbaum 1994, Verhey et al 1993, 1995).

Less is known regarding the entry of MHC class II molecules in to CIIV. Current evidence suggests that newly synthesized MHC class II complexes bound to the class II-associated invariant (Ii) chain are targeted from the TGN to endosomes where Ii chain is proteolytically cleaved. Appearance of class II in CIIV only occurs following Ii cleavage, which suggests that this event requires a determinant to facilitate its transport (Amigorena et al 1995). Although the precise site where these events occur remains to be determined, the fact that CIIV, like Glut-4 vesicles, also contain markers of the receptor reycling pathway suggests that they arise from EEs.

Another potential example of endosomal sorting is provided by TGN38, a membrane protein of unknown function that is enriched in TGN membranes. Rather than being a static TGN marker, TGN38 recycles continuously between the TGN and the plasma membrane, presumably via EEs (Reaves et al 1993, Chapman & Munro 1994). Unlike recycling receptors, however, TGN38 is selectively concentrated in the TGN. Although the TGN38 cytoplasmic domain is known to contain an active coated pit localization domain (with affinity for both AP-1 and AP-2 adaptors) (Wilde et al 1994, Ohno et al 1995), the signal responsible for its transport to and/or retention in the TGN remains unknown. Nor it is definitively established that a putative signal for TGN targeting is decoded in endosomes. Yet, this is the most reasonable possibility, given the fact that TGN38 internalized from the plasma membrane rapidly reaches the TGN, which implies, by definition, an endosomal intermediate (Reaves & Banting 1992). Moreover, treatment of cells with the phosphatidylinositol-3-kinase (PI-3 kinase) inhibitor wortmannin or V-ATPase inhibitors causes missorting of TGN38, which results in its accumulation in endosomes (Chapman & Munro 1994, Reaves & Banting 1994).

Much remains to be learned about the mechanism of endosomal sorting and the extent to which endosomes serve as intermediates in the formation of specialized vesicles involved in transport either to the cell surface or to the Golgi complex. It even remains possible that some or all biosynthetic traffic from the TGN to the plasma membrane passes through endosomes en route. However, what is abundantly clear is that EEs must be capable of serving as sorting platforms responsible for generating a variety of cell type-specific post-endosomal vesicles in addition to vesicles involved in constitutive receptor recycling (Figure 9). As such, endosomes must be capable of decoding the multiple signals likely to specify each of these transport events.

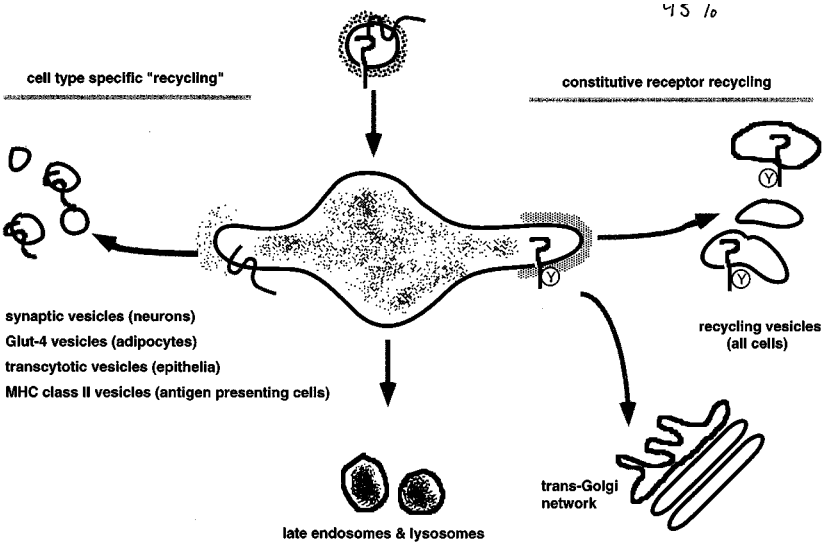


Figure 9 Endosomes serve as a platform that can host multiple sorting events simultaneously. In addition to facilitating the sorting of recycling receptors from dissociated ligands, it is becoming increasingly clear that early endosomes serve as a common sorting site for a variety of transport steps in both specialized and nonspecialized cells. Thus the same early endosomes that give rise to transport vesicles responsible for the return of receptors back to the plasma membrane likely generate a variety of cell type-specific recycling vesicles: e.g. synaptic vesicles in neurons or neuroendocrine cells, Glut-4 vesicles in adipocytes, apically directed transcytotic vesicles in epithelial cells, and possibly class II-containing vesicles in antigen-presenting cells. Early endosomes may also directly give rise to vesicles that mediate transport of Golgi complex proteins (e.g. TGN38) from the cell surface back to the TGN. Because constitutive recycling of receptors such as LDL receptor and transferrin receptor in epithelial cells is restricted to the basolateral surface, it seems likely that inclusion on this pathway involves sorting via specific basolateral targeting determinants (Y). It is possible that such signals help restrict such receptors from entering cell type-specific vesicles even in non-epithelial cells. Thus, cognate apical and basolateral pathways may exist even in cells that are not morphologically polarized in this fashion.

The existence of specific sorting mechanisms not withstanding, it is important to note that the efficiency of sorting in endosomes need not be and probably is not 100% . For example, at least half of the dIgA internalized via pIg receptors in epithelial cells is recycled back to the basolateral plasma membrane rather than being transcytosed to the apical surface (Hunziker et al 1991, Mostov, 1994). Basolateral proteins ectopically inserted into the apical plasma membrane can be recovered following endocytosis and a second round of sorting in endosomes (Matter et al 1993). The system can compensate for such "errors" for the simple reason that it is cyclical: If endosomes fail to correctly sort a receptor in the first

cycle, the receptor's re-internalization will afford a second and third chance to achieve the desired targeting result. Accordingly, even slight changes in recycling efficiency might profoundly influence the equilibrium distribution of a given receptor because the system is inherently iterative over time. This strategy ensures both fidelity and flexibility, but also complicates the functional analysis of endosomal sorting signals because their effectiveness may not require total efficiency at each round of endocytosis.

TRANSPORT OF MANNOSE-6-PHOSPHATE RECEPTORS The transport of newly synthesized lysosomal enzymes to lysosomes is largely dependent on the activity of receptors for mannose-6-phosphate (MPR) (Kornfeld & Mellman 1989). Two receptors are known: a large (205-kDa) cation-independent receptor and a small (47-kDa) receptor that bind MPR-containing enzymes in a cation-dependent fashion (Kornfeld 1992). Both receptors bind their ligands in the Golgi complex and exit the TGN via AP-1-containing CCVs. After delivery to endosomes, the enzymes are discharged in a pH-dependent fashion and accumulate in lysosomes. The receptors, on the other hand, return to the Golgi complex with only a small fraction of the receptor reaching the plasma membrane. Based on the fact that the MPRs are at equilibrium concentrated in LEs, the idea emerged that MPRs enter and exit the endocytic pathway at the level of LEs (Griffiths et al 1988, Geuze et al 1989). More recently, however, Ludwig et al demonstrated that a significant fraction of MPR-bound enzyme was delivered to EEs (Ludwig et al 1991). This is an appealing possibility because it suggests that EEs represent a common point of entry, as well as a common sorting site, on both the endocytic and biosynthetic pathways. Because the pH required for lysosomal enzyme from MPRs is somewhat more acidic than for ligand dissociation from plasma membrane receptors (Kornfeld 1992), it is possible that the enzyme-MPR complex must reach LEs to complete enzyme discharge. If this were the case, then LEs would also be capable of sorting and vesicle budding activities in a fashion directly analogous to EEs.

The rationale behind the existence of two distinct receptors remains somewhat unresolved. Clearly, both receptors function in lysosomal enzyme targeting because cells from mice lacking either receptor show defects in enzyme retention. Conceivably, the receptors are specific for different populations of enzymes (differing by posttranslational modification or protein type) (Koster et al 1993, Ludwig et al 1993, 1994, Pohlmann et al 1995).

Mechanisms of Endosome Sorting: Role of Coat Proteins

Endosomes, particularly EEs, are clearly involved in multiple sorting and vesicle transport events in both polarized and nonpolarized cells. Thus far, we can distinguish at least four general classes of events: formation of constitutive

recycling vesicles; the formation of specialized post-endosomal vesicles (SVs, TCVs, Glut-4 vesicles, CIIV); the production of vesicles destined for the TGN; and the production of vesicular carriers reponsible for transport to LEs. Given that some or all of these sorting/transport events are governed by targeting determinants on the cytoplasmic domains of the proteins being transported, it seems likely that cytosolic adaptors, either known or novel, play a role in endosomes directly analogous to that played by clathrin adaptors in the TGN or at the plama membranes. The observation that many sorting signals, particularly those involved in polarized transport in epithelial cells, bear some sequence relationship to coated pit signals further strengthens the prediction. Although clathrin was found to be associated with endosomes many years ago (Louvard et al 1983), the functional significance of this finding remains unknown.

ENDOSOMES CONTAIN FUNCTIONAL COP-I AND CLATHRIN COATS The first, albeit indirect, evidence in support of a role for coat proteins in endosome function came from experiments using the macrocyclic antibiotic BFA, an agent previously shown to block transport between the ER and the Golgi complex by interfering with the assembly of coatomer-containing coat complexes (Donaldson et al 1990, Orci et al 1991). Treatment of cells with BFA was found to cause a dramatic morphological alteration of endosomes, inducing extensive tubule formation analogous to the drug's initial effect on Golgi membranes (Hunziker et al 1991b, Lippincott-Schwartz et al 1991, Wood et al 1991). Although BFA did not block endocytosis or recycling per se, it did interfere with at least one endosomal sorting activity, selectively inhibiting the pIg receptor-mediated transcytosis of dIgA from the basolateral to the apical domain of MDCK cells (Hunziker et al 1991b). This effect was mediated at the level of EEs. BFA may also interfere with proper sorting between EEs and the TGN, because it also appears to induce some level of physical continuity between the two compartments (Wood et al 1991, Reaves et al 1993).

Although BFA's mode of action is not entirely clear, it does interfere with the recruitment of Arf proteins to membranes, apparently by blocking GDP-GTP exchange required for the stable attachment of soluble GDP-Arfs (Donaldson et al 1992, Helms & Rothman 1992). As such, the inhibitory effects of BFA might directly or indirectly reflect a defect in the assembly of any Arf-dependent coat complex. By assaying the ability of isolated endosomes to bind cytosolic proteins in a fashion stimulated by GTPγS and blocked by BFA, a number of proteins were found to bind specifically to endosomal membranes. Surprisingly, perhaps, most were members of the COP-I coat complex more commonly associated with transport between the ER and the Golgi, as well as through the Golgi stack (Whitney et al 1995). Coatomer, the cytosolic precursor of

COP-I coats, is thought to consist of seven subunits: α, β, β', δ, γ, ϵ, and ζ (Orci et al 1993b). Interestingly, endosomes appeared to bind only a coatomer subcomplex lacking δ and γ COP. By immuno-EM, coatomer components were found together with EE vacuolar elements, as well as small Tfn-containing vesicles. It appears that this association is of some functional importance. Microinjection of antibodies to β-COP, known to block ER to Golgi transport, also inhibited endosome function by blocking the low pH-dependent penetration of vesicular stomatitis virus (VSV) from endosomes (Whitney et al 1995). Because VSV appears to require pH $<$6.0 for efficient fusion, this result suggests that β-COP is required either for acidification or, more likely, for interference with the transit of the virus from EEs to LEs. Using an in vitro system designed to reconstitute carrier vesicle formation from EEs, evidence was also obtained that β-COP in fact may be required for this step (Aniento et al 1996).

Although the precise role of COP-I in mediating endosome function remains to be determined, its likely involvement is further suggested by the phenotype of a CHO cell mutant (*ldl-f*) found to bear a temperature-sensitive defect in ϵ-COP, one of the COP-I subunits found to bind to endosomes in vitro. In addition to the expected block in transport between the ER and the Golgi complex, *ldl-f* cells exhibit a temperature-sensitive defect in maintaining LDL receptors on the plasma membrane (Guo et al 1994, Hobbie et al 1994). Biochemical analysis suggests that the receptors are rapidly degraded at the nonpermissive temperature. To be consistent with suggestions from in vitro experiments, the absence of functional COP-I in these cells may interefere with the normal removal of lysosomal enzymes from EEs, thus exposing LDL receptors to a degradative environment.

Also, there is increasing evidence that endosomes are associated with clathrin-containing coats. Recent results have clearly demonstrated the presence of clathrin buds forming from EEs in fibroblasts as well as in neurons (Whitney et al 1995, Stoorvogel et al 1996, Takei et al 1996). These coated buds appear to contain Tfn receptor, suggesting a role in recycling (Stoorvogel et al 1996). Interestingly, however, the buds contain neither γ or α adaptin, suggesting that they may consist of a novel type of adaptor complex (Stoorvogel et al 1996). In permeabilized cells treated with GTPγS, however, AP-2 adaptors are found to accumulate on endosomal vesicles (Seaman et al 1993). A function for endosomal clathrin or adaptors has yet to be demonstrated.

REGULATION OF COAT PROTEIN BINDING It is possible that endosomal sorting activities are mediated entirely by known coat protein complexes whose recruitment to endosomes may be regulated in some way. Because none of the known Arf proteins exhibits any clear selectivity for endosomes, they are unlikely to be the mediators of such specificity (Cavenaugh et al 1996).

Interestingly, however, in cells overexpressing dominant-negative mutants of the plasma membrane Arf protein Arf6, endosome-like membranes accumulate that are associated with an unidentified cytosolic coat (D'Souza et al 1995, Peters et al 1995).

In the case of COP-I assembly, the fact that endosomes appear to recruit only a subcomplex of coatomer subunits suggests that different subcomplexes exist for attachment to distinct organelles. Although COP-I is known to interact via its α subunit with proteins containing the KKXX-type ER retrieval motif, it is probable that non-KKXX-containing proteins also play a role in coatomer recruitment (Schroder et al 1995). Coatomer subcomplexes do exist, although they have yet to be shown to have specific targets (Cosson & Letourneur 1994, Lowe & Kreis 1995). Phosphorylation of coatomer subunits may also play a role in regulating COP-I binding. Although both β- and δ-COP are known to be phosphorylated, the functional significance of this modification remains unclear (Sheff et al 1996).

Clearly, other factors may regulate the creation or masking of recruitment sites for known coat proteins. Some interesting possibilities include regulation by lipid mediators normally associated with signal transduction mechanisms. Although beyond the scope of this review, it should be pointed out that yeast mutants with defects in sorting of vacuole proteins from the Golgi complex to the vacuole have a well-characterized defect in a PI-3 kinase activity (vps34) (Schu et al 1993). Based on sensitivity to the selective inhibitor of PI-3 kinase wortmannin, transport of lysosomal components, as well as of TGN38, may also be directly or indirectly regulated by alterations in PI-3 kinase activity (Brown et al 1995, Davidson 1995, Shepherd et al 1996).

Various other proteins involved in phosphorylation-dephosphorylation events of both proteins and lipids have been associated with important clathrin-coated pit function. Specifically, it is clear that dynamin physically interacts with a variety of linker proteins and phosphatases (e.g. Grb2, amphiphysin, synaptojanin) closely implicated in signal transduction (McPherson et al 1994, 1996, David et al 1996). Of particular interest is the protein eps15, a substrate for EGF receptor kinase that is associated with the AP-2 clathrin adaptor complex (Benmerah et al 1995). A related protein (End3) also appears to be involved in endocytosis in yeast (Benedetti et al 1994). The precise significance of these interactions remains to be determined. However, these observations appear to presage the existence of an entirely new layer of complexity underlying the regulation of endocytosis and receptor function. These topics have recently been the subject of an excellent review (De Camilli et al 1996).

ALTERNATIVE COAT PROTEINS An alternative possibility is that endosomal sorting, and perhaps even certain sorting events in the TGN (such as basolateral

targeting) whose mechanism has yet to be defined, is mediated by novel coat proteins or complexes. Neuronal proteins distantly related (<30% identity) to β-adaptin (β-NAP) and to the medium chains (p47A, p47B) have been cloned and sequenced (Pevsner et al 1994, Newman et al 1995). These proteins have recently been found to form a complex that can be recruited to TGN-like membranes in permeabilized cells (Simpson et al 1996). Because nonneuronal versions of these proteins may also exist, they could be interesting candidates for alternative coat proteins involved in sorting activities. Examination of the developing human cDNA database has revealed an array of similar adaptor homologues exhibiting varying degrees of identity to known adaptor subunits ($\approx$30% identity, 50–60% identity, 75–80% identity). Thus far at least one of these, a γ-adaptin homologue, appears to localize to Golgi membranes (D Lewin et al, unpublished observation).

Although functions for any of the alternative adaptor proteins have yet to be defined, they are attractive candidates for several reasons, not the least of which is the remarkable, perhaps superficial, similarity that exists among the various targeting signals thus far identified. As discussed above, targeting of membrane proteins in polarized epithelial cells often depends on tyrosine-containing or clathrin-coated pit-like signals. Even KKXX-containing signals, normally associated with the binding of COP-I complexes, have been found under some circumstances to mediate rapid endocytosis from the plasma membrane (Kappeler et al 1994, Itin et al 1995). Such similarities may be serendipitous but also may suggest that the adaptors decoding them are fundamentally similar to the authentic adaptors found in clathrin-coated vesicles. At the same time, one must also be cognizant of the possibility that the paradigm defined by the formation of clathrin-coated vesicles is not applicable to all sorting events. Whereas during coated vesicle formation, sorting and budding appear to be effectively and kinetically linked, this need not always be the case. Sorting might occur prior to vesicle budding, a possibility consistent with the pre-existing concentration of recycling receptors in endosomal tubules (Geuze et al 1987) or the existence of morphologically distinct domains in a continuous TGN (Ladinsky et al 1994). In either event, the identification and eventual functional characterization of the protein components involved in sorting, both in endosomes and the TGN, will finally provide the key to understand these organelles in precise biochemical terms.

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Volume 12, 1996

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