# REVIEW ARTICLE
# Molecular aspects of the endocytic pathway

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Observation of the flow of material along the endocytic pathway has lead to the description of the basic architecture of the pathway and provided insight into the relationship between compartments. Significant advances have been made in the study of endocytic transport steps at the molecular level, of which studies of cargo selection, vesicle budding and membrane fusion events comprise the major part. Progress in this area has been driven by two approaches, yeast genetics and *in vitro* or cell-free assays, which reconstitute particular transport steps and allow biochemical manipulation. The complex protein machineries that control vesicle budding and fusion are significantly conserved between the secretory and endocytic pathways such that proteins that regulate particular steps are often part of a larger family of proteins which exercise a conserved function at other locations within the cell. Well characterized examples include vesicle coat proteins, rabs (small GTPases) and soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptors (SNAREs). Intracompartmental pH, lipid composition and cytoskeletal organization have also been identified as important determinants of the orderly flow of material within the endocytic pathway.

Key words: coated vesicles, endocytosis, membrane fusion, rab proteins.

## COMPARTMENTAL BOUNDARIES

Definitions of endosomal compartments tend to emphasize particular attributes including function, morphology and composition. There is a clear overlap between compartments variously described as multivesicular body, prelysosomal compartment and late endosome. Similarly with tubulovesicular endosome, sorting endosome, compartment of uncoupling ligand from receptor (CURL) and early endosome. Early and late endosomes reflect an operational definition of endocytic compartments based on pulse or pulse–chase protocols respectively (see, for example, Anieto et al. [1]). Established marker proteins associated with these compartments are given in Table 1.
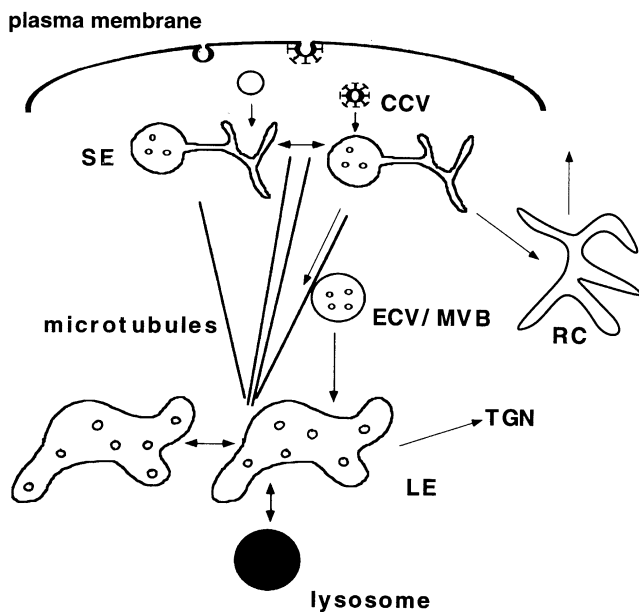
The early endosome is the major sorting station on the endocytic pathway. From this organelle, material can be directed towards the pathway of recycling to the plasma membrane (e.g. transferrin), to later endocytic compartments [epidermal growth factor (EGF) receptor] and to regulated secretory vesicles (glucose transporter GLUT4) [2,3]. Studies on transferrin-receptor recycling have defined a tubular 'recycling endosome' that transferrin receptors enter subsequent to sorting from late-endosome-directed material (Scheme 1) [4,5]. This recycling endosome is typically less acidic (pH 6.4–6.5) than the sorting endosome (pH 6.0) and exhibits a pericentriolar localization [6,7]. The recycling endosome is rich in the small GTPase rab11, whereas rab4 and rab5 have both been ascribed to early

**Table 1    Established marker proteins for endosomal compartments**

| Recycling endosome | Sorting endosome | Late endosome | Lysosomes |
|---|---|---|---|
| Rab11 | Rab5 | Rab7, Rab9 | |
| | EEA1 | ci-M6PR | |
| Transferrin receptor, other recycling receptors | | lgp120, LAMP-1, lysobisphosphatidic acid | |
| | ligands directed to lysosome (e.g. low-density lipoprotein) | | |

**plasma membrane**



**Scheme 1    The endocytic pathway in a non-polarized cell**

Single-headed arrows indicate transport steps, double- headed arrows indicate homotypic fusion events, or, in the case of LE (late endosome) ↔ lysosome, one in which a hybrid organelle may exist transiently. Other abbreviations: SE, sorting endosome; MVB, multivesicular body; RC, recycling compartment.

endosomes on the basis of their distribution and co-localization with transferrin receptor; however, recent work has revealed that the distribution of these proteins only partially overlaps, thereby revealing a further subdivision of compartments [8].

The relationship between early and late endosomes is contro-versial. There are two extreme views: (a) endosome maturation; according to this model, early endosomes are formed *de novo*, presumably from coalesced clathrin-coated vesicles (CCVs). The late endosome is refractory to fusion with incoming material and represents the residue of the early endosomal compartment once recycling to the plasma membrane has been exhausted [9–13]; (b) endosomal carrier vesicles (ECVs); in this model the early endosome represents a 'stable' compartment that is maintained by a balance of incoming and departing material. Carrier vesicles (0.4 $\mu$m spheroids containing internal vesicles) form from early endosomes and subsequently fuse with late endosomes in a microtubule-dependent manner [14]. Carrier vesicles have been operationally defined in baby-hamster kidney (BHK) cells as the compartment that is labelled by a 15 min pulse of horseradish peroxidase (HRP) and 30 min chase in the presence of noco-dazole, a microtubule-depolymerizing drug [15]. These proposed transport vesicles contrast with most vesicles formed within the cell, which are small coated structures. However, other examples of large-vesicle budding exist, such as formation of immature secretory granules from the *trans*-Golgi network (TGN) [16].

Elegant fluorescence-microscopy studies which analyse the distribution of internalized fluid-phase marker place a limitation on the latter model in that they make it clear that marker accumulated in the early endosome is not subsequently trans-ferred to late endosomes in smaller units [10]. A single ECV must therefore carry with it the bulk of fluid of the early endosome from which it derives. Once this is accepted then the difference between the two models is reduced.

Late endosomes represent the point on the endocytic pathway where the cation-independent (ci-) mannose 6-phosphate receptor (M6PR) is most concentrated and from which it is recycled back to the TGN [17]. Degradative enzymes are active in this com-partment, but are more concentrated in lysosomes [18]. It is a puzzling observation that M6PR is found to be concentrated on the internal vesicles of late endosomes, whilst lysosomal mem-brane proteins are on the external membrane [17]. Transfer of material between late endosomes and lysosomes appears to be a direct fusion event which results in a transient hybrid organelle [19]. Lysosomes are also multivesicular, but completely lack M6PR.

## INTERNALIZATION

The best-defined internalization pathway is the CCV pathway. CCVs budding from the plasma membrane of mosquito oocytes were the first coated vesicles to be observed by Roth and Porter in 1964 [20]. The properties of these vesicles have been extensively reviewed elsewhere [21–24]. They comprise a clathrin coat linked to cargo-bearing receptors via a heterotetrameric adaptin com-plex (HA2 or AP-2) which is comprised of 100 kDa $\alpha$ and $\beta 2$ adaptins together with smaller $\mu 2$ (50 kDa) and $\sigma 2$ (17 kDa) subunits (Scheme 2) of which $\alpha$-adaptin and $\beta$-adaptin both contain clathrin-binding sites [25,26]. They are distinct from the other major class of CCVs which bud from the TGN and incorporate a different adaptor complex (AP-1). Initial local-ization of AP-2 to the plasma membrane is believed to occur by interaction with a high-affinity receptor, a role which can be fulfilled by synaptotagmin 1 [27]. The further role of cargo receptors in CCV formation remains controversial [28,29].

Signals for incorporation of cargo receptors into plasma-membrane-derived CCVs have been defined based on manipu-lation of protein sequence. The best characterized are the YXXØ motif where Ø represents a bulky hydrophobic amino acid [30]. Such signals are found in both constitutively endocytosed recep-tors (e.g. transferrin receptor) and those such as the EGF receptor, which are only endocytosed following ligand binding. In the latter case ligand binding causes phosphorylation of the receptor and a conformational change that results in exposure of the internalization signal. The $\mu 2$ adaptin subunit has been shown to interact with these tyrosine-based internalization signals [30,31]. This interaction is strengthened when the AP-2 complex is present in clathrin coats, indicating co-operativity between receptor–AP-2 interactions and coat formation [32]. An alterna-tive internalization signal, dileucine, does not interact with the $\mu 2$ chain *in vitro* [30], although there is evidence indicating an interaction with AP-2 [33]. Dileucine motifs can also provide a signal for lysosomal sorting from the TGN, in which instance they have been shown to interact with the $\beta 1$ chain of the TGN localized adaptor complex, AP-1 [34]. An exception to the model of AP-2-mediated recruitment of receptors to CCVs is provided by G-protein-coupled receptors. In this instance the activated proteins are recruited by $\beta$-arrestin, which is believed to function as a specialized adaptor protein as it also binds to clathrin [35,36].

Much information on the formation of endocytic CCVs has been provided by *in vitro* or permeabilized cell assays which reconstitute the sequestration of receptors from the plasma membrane [37,38]. The process is generally agreed to be cytosol- and ATP-dependent with a requirement for multiple GTP-binding proteins [39]. The best characterized GTPase in terms of CCV formation is dynamin [40–42]. The *Drosophila* homologue was originally identified as the temperature-sensitive allele in the
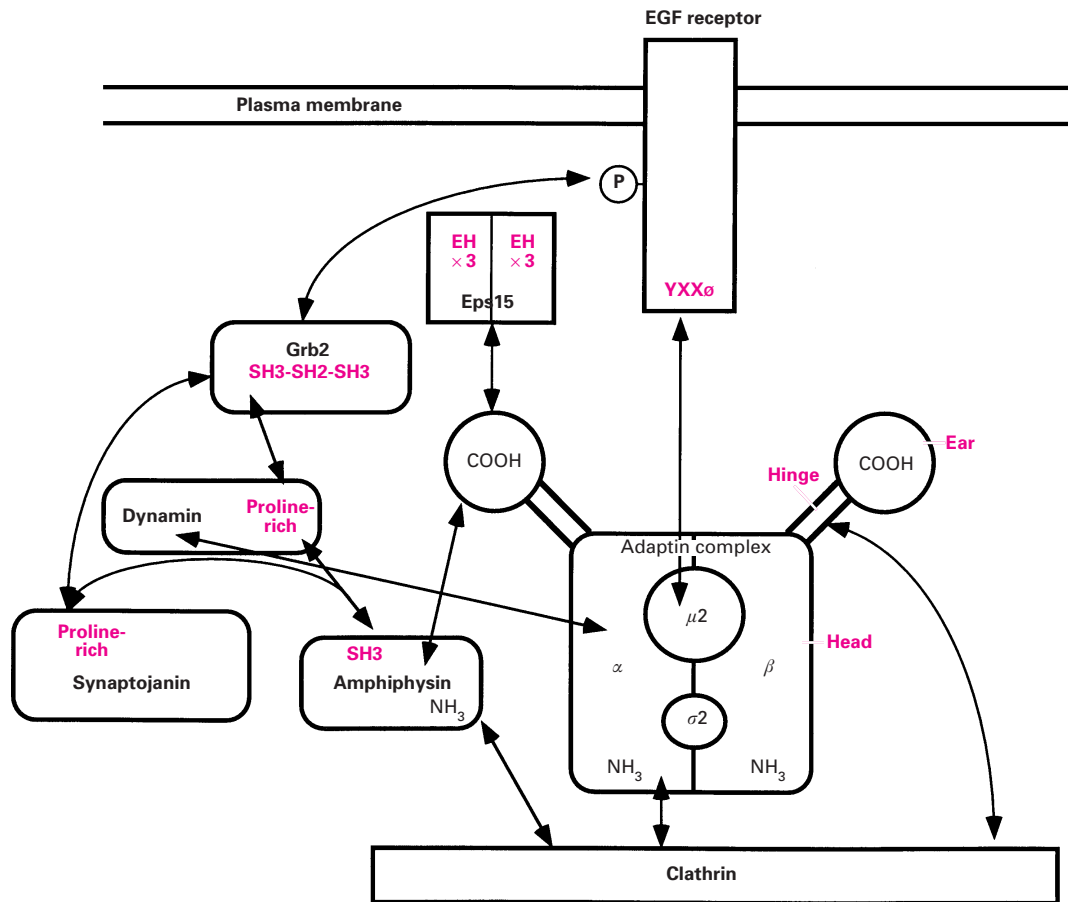
**Scheme 2     Established interactions between proteins implicated in endocytosis of the EGF receptor**

Structural motifs identified within the proteins are indicated in red.

*shibire* mutant fly, which exhibits a defect in synaptic vesicle recycling at the restrictive temperature [43,44]. Subsequent work has shown that dynamin is required for endocytosis in mammalian cells [45–47], most likely at a late stage corresponding to scission of the invaginated clathrin-coated pit [48] (but see Roos and Kelly [49] for discussion of this point).

Dynamin localization and activity is most likely determined by a complex set of protein interactions (Scheme 2). As well as binding directly to $\alpha$-adaptin [50], it binds to proteins that contain Src homology 3 (SH3) domains [51] through interactions with its proline rich C-terminus [52,53]. In brain, its major SH3-containing partner has been identified as amphiphysin [54]. The growth-factor-receptor-binding protein Grb2 also binds dynamin via an SH3 domain and activates its GTPase activity synergistically with phosphoinositides [55]. Disruption of EGF-receptor recruitment of Grb2 by microinjection of a peptide corresponding to its SH2 domain, or of a phosphopeptide corresponding to the receptor-binding site, impairs receptor endocytosis [56]. Amphiphysin and Grb2 both also bind to the phosphatidylinositol 5-phosphatase synaptojanin through its proline-rich region [57,58]. Expression of the SH3-binding domain of amphiphysin has been shown to impair constitutive endocytosis, presumably by titrating out the SH3-binding capacity of dynamin [59]. Microinjection of this domain into giant nerve terminals from lamprey has provided stunning pictures of

clathrin-coated buds which accumulate at the plasma membrane following stimulation [60], providing a strong argument for the CCV pathway as a major route for synaptic vesicle recycling.

The yeast gene *END3*, which is required for $\alpha$-factor endocytosis [61] is homologous with the mammalian protein Eps15 that was originally identified as a substrate of EGF-receptor kinase [62]. Eps15 contains three Eps15 homology (EH) domains that can mediate protein–protein interactions [63]. It constitutively associates with AP-2 [64] via the ear domain of $\alpha$-adaptin [65,66], is dimeric in solution and associates with both cytosolic and membrane-bound AP-2 independently of EGF [67]. Immunoelectron microscopy has revealed that Eps15 decorates the edge of forming coated pits and the rim of budding coated vesicles, in contrast with AP-2, which is evenly distributed throughout the clathrin-coated structures [67]. Microinjection of antibodies against Eps15 or a related protein, Eps15R, inhibits internalization of EGF and transferrin [68] and expression of the C-terminal domain competes with endogenous Eps15 to inhibit transferrin and EGF-receptor endocytosis [69]. Benmerah et al. have used a permeabilized-cell assay to demonstrate that Eps15/AP2 interaction is required at an early stage of receptor sequestration [69], whilst Cupers et al. have shown that Eps15 is not a component of fully formed CCVs purified from brain [70]. Furthermore, they have used an *in vitro* clathrin-lattice-assembly assay in which they add clathrin to AP-2 saturated with Eps15;

normal structures are formed, but with a stoichiometry of Eps15 relative to AP-2 much less than one. The authors suggested that Eps15–AP-2 complex can be recruited at the growing edge of a lattice, but that binding of clathrin to the $\beta$-subunit of AP-2 may induce an allosteric conformational change leading to the displacement of Eps15 [70]. The role of Eps15 may be to recruit further proteins to the locale of a nascent vesicle. An Eps15 homologue in yeast, Pan1p, has been shown to interact with yAP180, a clathrin assembly protein, using a yeast two-hybrid screen [71].

Other pathways of endocytosis have been detected which do not involve the formation of CCVs [72]. In general these are characterized by uptake of a fluid-phase marker under conditions where CCV formation does not occur. Cells which express a temperature-sensitive mutant form of dynamin rapidly lose the ability to undergo receptor-mediated and fluid-phase internalization when shifted to the restrictive temperature. However, the ability to endocytose fluid-phase marker is quickly re-established, whilst receptor internalization is not [73]. These alternative pathways are less well characterized in molecular terms; the pinching off of caveolae undoubtedly provides an alternative means of internalization under some conditions [74] and is perhaps most pronounced in endothelial cells. However, it may be a relatively minor pathway in terms of membrane turnover for most cell types. Interestingly, some non-clathrin-dependent endocytic events have recently been shown to require functional dynamin, including the uptake of ricin for transport to the Golgi complex [75] and the internalization of caveolae [76,77].

## VACUOLAR-ATPase-DEPENDENT ENDOSOMAL TRAFFICKING

The progressive acidification of endosomal compartments due to vacuolar H$^+$-ATPase activity is a well-established phenomenon [78], that is crucial to many aspects of the endocytic pathway. The recent availability of specific, membrane-permeable vacuolar ATPase inhibitors bafilomycin and concanamycin [79] has stimulated research on the cellular function of this enzyme. Endocytic CCVs purified from rat liver apparently do not acidify [80], although, in CCVs purified from rat synaptosomes, the proton pump must be active, because concanamycin inhibits ATP-dependent glutamate uptake [81]. Ratiometric measurements of early endosomal pH using endocytosed fluorophores converge at about pH 6.0, whereas late endosomes are at about pH 5.0 and lysosomes are even more acidic [7]. Transferrin, recycling from the early endosome, subsequently experiences a more alkaline environment prior to exocytosis [6,7]. In some types of cells the early endosomal pH is limited by the electrogenic Na$^+$/K$^+$ pump [82,83]. This pump recycles back to the plasma membrane from early compartments, allowing the later compartments to acidify to a greater extent. This is not the case for all cells: transferrin experiences a pH of 5.5 in K562 cells and no influence of the Na$^+$/K$^+$ATPase inhibitor ouabain is observed [84]. Relative permeability to Cl$^-$ could also influence endosomal pH, but studies utilizing the cystic-fibrosis transmembrane conductance regulator chloride channel, which is present and functional in endosomes, have not supported this proposal [85,86].

The acidic environment within endosomes is utilized to dissociate many ligands from their receptors. Altered trafficking of receptors due to pH neutralization can therefore not be used to make a general statement about membrane flow, since the primary perturbation may well be due to changes in the ligand–receptor interaction. As an alternative strategy, fluid-phase markers have been used to investigate the flow of material along the endocytic pathway as a function of vacuolar ATPase activity [87]. In BHK cells, ECV formation is inhibited by bafilomycin

treatment, whilst initial internalization and recycling are essentially unchanged [87]. Early endosomes adopt a more perinuclear, clustered distribution and tubular morphology [87,88]. Similar observations pertaining to ECV formation were reported for HeLa cells in a study that examined the entry of viruses into endosomal fractions, purified by free-flow electrophoresis [89].

It has been postulated that the requirement for a low-pH endosomal lumen reflects the existence of an allosteric transmembrane pH-sensor governing the assembly of the budding apparatus at the cytosolic surface of the early endosome [87]. Consistent with this is the discovery of coatomer subunits associated with endosomes [90,91] and, in particular, the finding that $\beta$-COP (where COP is coatamer protein), which is required for an *in vitro* assay of ECV formation, associates with endosomes in a pH-sensitive manner [91]. ECVs are not particularly enriched for $\beta$-COP, such that coatomer is unlikely to form any regular lattice covering the surface. The role of coatomer is more likely to reflect formation of, or sorting into, smaller coated vesicles coupled to ECV formation. In Hep2 cells it has been reported that there is no effect of bafilomycin on transport from early to late endosomes, but, rather, a block to transfer between late endosomes and lysosomes is evident [92,93]. It is possible that the underlying mechanism to the pH-dependent step in ECV formation in BHK cells and lysosomal transport in Hep2 cells is the same, but that the boundaries between early and late compartments are drawn differently by various cell types.

## COAT PROTEINS ON THE ENDOCYTIC PATHWAY

Recent work has identified coat proteins on early endosomes. Most strikingly, Stoorvogel and colleagues have used immuno-electron microscopy to observe clathrin-coated buds decorating the tubular elements of endosomes in A431 cells [94]. These buds were distinguished from TGN-derived and plasma-membrane-derived vesicles by virtue of lack of $\alpha$- or $\gamma$-adaptin labelling and by their size. Endosome-derived CCVs are otherwise small (60 nm compared with 100 nm diameter). The clathrin-coated buds disappear in the presence of brefeldin A, concomitant with a decrease in the recycling rate of transferrin receptor. However, the function of these vesicles remains unclear, the authors being unable to detect selective enrichment of transferrin receptor or of M6PR in the buds [94].

A further adaptin-like complex, AP3, has been identified that can be bound to endosomes [95,96]. Although most initial data supports a role for this complex on the Golgi-to-lysosomal pathway, it has also been implicated in the budding of small synaptic-like vesicles from the early endosome of PC12 cells, a neuroendocrine cell line [97]. TIP47 may represent a component of a new kind of coat involved in endosome-to-Golgi trafficking [98]. It was identified on the basis of interaction with the cytoplasmic domain of ci-M6PR and has been shown to localize to late endosomes and bind both forms of M6PR. Depletion of TIP47 inhibits an *in vitro* assay of M6PR late-endosome-to-Golgi transport [98].

A second class of coated vesicles, COP-coated vesicles, have been identified and purified by Rothman from mammalian cells [99] and by Scheckman and colleagues from yeast [100]. These are coated with stoichiometric amounts of coatomer subunits (COP proteins) which can be observed under the electron microscope. Assembly of these coats requires the action of a small GTPase, ADP-ribosylation factor (ARF) 1 [99], and is inhibited by the fungal metabolite brefeldin A by virtue of its action on an ARF nucleotide exchange factor [101,102]. Until recently it was held that components of COP-coated vesicles only acted upon the secretory pathway.

The ability to purify endosomes has allowed the identification of associated COP proteins. In particular, it is critically important to be able to separate them from organelles of the biosynthetic pathway, which are themselves rich in these proteins. Mellman and colleagues have used free-flow electrophoresis to identify endosome associated coatomer subunits [90]. By quantitative Western blotting they were able to show that the coatomer subunits $\alpha$-, $\beta$-, $\beta'$- and $\epsilon$-COP bound to endosomal membranes at 10–35 % of the levels for an equivalent amount of endoplasmic reticulum (ER)/Golgi membranes and $\zeta$-COP bound even more efficiently (> 50 %). In contrast, binding of $\gamma$- and $\delta$-COPs to endosomes was not detected, despite the fact that these bind to ER/Golgi. Similar results were obtained by Aniento et al. with respect to $\beta$-,$\gamma$- and $\epsilon$-COP using gradient purified fractions [91]. In both studies a limited amount of immunoelectron-microscopic data has been provided to support these conclusions.

The ldlF mutant cell line bears a temperature-sensitive defect in $\epsilon$-COP [103]. Two groups have used this cell line to demonstrate a role for this protein on the endocytic pathway [104,105]. The major defect appears to be in the transfer of bulk-phase marker from early to late endosomes [104]. In fact, according to Gu et al. [104], the phenotype at the restrictive temperature is very similar to that observed following vacuolar ATPase inhibition by bafilomycin treatment. Incubation of endosomes with cytosol prepared from ldlF cells incubated at 34 °C or 40 °C (the restrictive temperature, which leads to time-dependent loss of $\epsilon$-COP) has shown that association of $\beta$-, $\beta'$- and $\zeta$-COP with endosomes occurs normally in a pH and guanosine 5'-[$\gamma$-thio]triphosphate (GTP[S])-sensitive fashion, but that $\alpha$-COP recruitment is inhibited [104].

To summarize this work it appears that recruitment to early endosomes of a minimal coatomer comprising $\beta$-, $\beta'$- and $\zeta$-COP requires an acidic endosomal lumen. It is the recruitment step that is pH-sensitive, as collapse of the pH gradient does not lead to dissociation of COPs. The recruitment is also GTP[S]-sensitive [90,91] and brefeldin A-sensitive [90], implying a role for an ARF protein, as is the case with coatomer recruitment to biosynthetic membranes. Recruitment promotes the production of endosomal carrier vesicles by a process that requires both $\beta$ and $\epsilon$-COP. The point of interaction with the postulated transmembrane pH sensor is conceivably at the level of the ARF protein. In this respect, it is noteworthy that acidification-dependent association of ARF to crude membranes prepared from pancreatic acinar cells has been previously reported [106].

Currently, six mammalian ARF proteins and several structurally related ARF-like proteins have been cloned [107,108] that have differing subcellular locations [109]. Whilst ARF1 is localized to the Golgi complex and has been shown to influence recruitment of coatomer and of $\gamma$-adaptin [110,111], ARF6 is localized to the plasma membrane and to endosomes [112,113]. Accordingly, the influence of ARF6 on the endocytic pathway has been studied in some detail, although other members of the family may be equally important. In distinction to ARF1, the subcellular distribution of ARF6 is insensitive to brefeldin [113]. An ARF6 GTPase-defective mutant [Q67L (Gln$^{67}$ → Leu)] localizes to the plasma membrane, whilst a mutant defective in GTP binding [T27N (Thr$^{27}$ → Asn)] localizes to endosomes. Electron-microscopic studies revealed morphological changes associated with both ARF6 mutants; expression of Q67L leads to the appearance of actin-rich vaginations at the plasma membrane, whereas T27N leads to the accumulation of coated (non-clathrin) vesicles [113]. The effects and distribution of ARF6 vary between cell types; in Chinese-hamster ovary (CHO) cells the T27N mutant co-localizes with the transferrin receptor and exerts effects on transferrin receptor recycling, whilst the Q67L mutant

inhibits transferrin uptake [112]. Prolonged internalization of HRP leads to labelling of a small proportion of ARF6-labelled vesicles in the pericentriolar region of the cell [114]. In HeLa cells, no effects on transferrin-receptor cycling are observed, although the Q67L stimulates localized fluid-phase uptake. In these cells the T27N mutant does not co-localize with internalized transferrin receptor, but demarcates a distinct tubular endosomal compartment which contains plasma membrane markers that lack internalization signals for the CCV pathway [115]. These authors propose that this novel endocytic compartment may represent a means by which the cell redistributes plasma membrane for purposes such as motility.

## MEMBRANE FUSION ON THE ENDOCYTIC PATHWAY

*In vitro* assays that reconstitute fusion between operationally defined endosomal compartments have been constructed [116–118]. Elegant studies by Aniento et al. examined the fusion predilections of operationally defined early endosomes, ECVs and late endosomes. They found that early endosomal and late endosomal fractions undergo efficient homotypic fusion, but do not undergo heterotypic fusion, i.e early with late endosomes. ECVs do not undergo homotypic fusion or fuse with early endosomes; rather they fuse with late endosomes in a micro-tubule-dependent manner which also requires cytoplasmic dynein [1,119]. Each of these fusion events is blocked by an antibody (2C8) to the ubiquitous fusion regulator NSF, and there is partial rescue of an *N*-ethylmaleimide block by application of NSF together with SNAPs [120]. The involvement of NSF and SNAPs in multiple fusion events on both the secretory and the endocytic pathway implies that other components provide the specificity for intracellular fusion events [99]. This specificity is generally accepted to be conferred, at least in part, by SNARE molecules, which have distinct subcellular distributions. In the best-studied case of synaptic-vesicle fusion the relevant SNAREs are synapto-brevin (v-SNARE), syntaxin 1 and SNAP-25 (t-SNARES) [121]. According to the original terminology a v-SNARE represents a SNARE predominantly associated with donor vesicles, whilst a t-SNARE represents a SNARE predominantly associated with target membrane [121]. v-SNAREs interact with t-SNAREs, such that a major function of SNAP/NSF is to disassemble this complex. Many homologues of the original SNAREs have now been identified, of which a few have been localized to endosomal structures [122,123]. Cellubrevin localizes to elements of the early endosomal system [124] and regulates the consumption of recycling vesicles in permeabilized CHO cells [125] without apparently influencing homotypic early endosome fusion [126]. Syntaxin 7 has been localized to early endosomes [127], and Tlg1p in *Saccharomyces cerevisiae* has been localized to a putative early endosomal compartment [128].

There is an emerging consensus that the requirement for NSF/SNAP in many fusion assays can occur prior to docking of vesicles [129–132]. This has been most elegantly demonstrated in an *in vitro* assay system that reconstitutes the homotypic fusion between vacuolar membranes of the yeast *S. cerevisiae* [133]. This assay bears many similarities to *in vitro* assays of endosome fusion, including a requirement for the NSF/SNAP homologues sec18/sec17 and a small GTPase of the rab family, ypt7, which is most closely related to rab7, a marker of late endosomes [134]. Sec18 acts to release sec17 from the membranes in an ATP-dependent manner, such that sec17 can be removed from the system prior to combination of fractions. In this system the sole requirement for sec17/sec18 has been unambiguously shown to be during a preincubation stage prior to combination of vacuolar membranes [132]. Ypt7 acts at a later point on the fusion
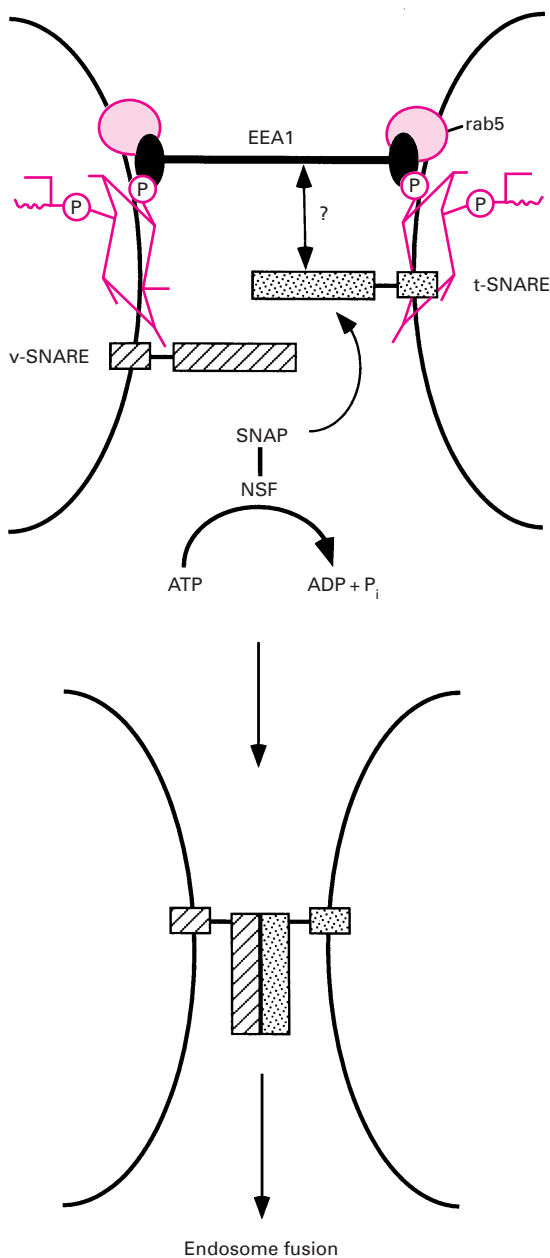
**Scheme 3   Homotypic early endosome fusion (speculative)**

Initial docking is mediated by the 'velcro factor' EEA1, by virtue of a dual interaction of its C-terminus with PI3P and rab5 and of its N-terminus with rab5. EEA1 may dimerize, owing to the extensive coiled-coil domain possibly allowing interaction with PI3P at both membranes. This coiled-coil domain could also interact with a syntaxin homologue (t-SNARE). Other 'velcro factors', such as rabaptin-5, may substitute for EEA1or mediate fusion between subtly different endosome populations. SNAP recruits NSF to the t-SNARE, which then stimulates NSF hydrolysis of ATP, leaving the t-SNARE in an altered state which allows interaction with a v-SNARE on a partner vesicle in a parallel configuration [237], resulting in closer membrane apposition and possible membrane deformation, leading to fusion.

pathway that requires co-incubation of fractions. Wild-type vacuoles contain both a t-SNARE (Vam3p) and a v-SNARE (Nyv1p), but vacuoles can be prepared from strains lacking one or other of the SNAREs. Hence a requirement for cognate SNARE interaction has been shown for fusion: vacuoles bearing only a t-SNARE will fuse with vacuoles bearing only a v-

SNARE, whilst vacuoles containing neither SNARE cannot fuse with those containing both [135]. The v-SNAREs and t-SNARES on individual vacuoles exist predominantly in a complex, and the role of sec17/sec18 is to disassemble this complex such that the SNAREs can participate in docking. However, for fusion of vesicles containing only one SNARE 'priming' with sec17/sec18 is still necessary, but only for t-SNARE-containing vesicles [136].

The most widely studied fusion event on the endocytic pathway of mammalian cells is the homotypic fusion between early endosomes (Scheme 3). Using a cell-free assay system, a requirement for NSF and $\alpha$-SNAP has been demonstrated [137,138], as well as for an early-endosome-specific factor, the small GTPase rab5 [139]. The involvement of other GTP-binding proteins has also been reported [140,141]. In this system, too, NSF mediates release of $\alpha$-SNAP from endosomal membranes [142] and the assay becomes independent of NSF at an early time point [143]. A number of other regulators of early endosome fusion have been identified: inhibitory factors include mitotic cytosol [144], okadaic acid [145] and phosphoinositide 3-kinase (PI-3K) inhibitors (see below) [146–148]. Additionally phospholipase D activity has been shown to exert a tonic stimulation of endosome fusion [149]. Biwersi et al. have used an elegant fluorescence-microscopic assay of endosome fusion within 3T3 fibroblasts to demonstrate a stimulation of endosome fusion due to the chloride channel activity of the cystic fibrosis transmembrane conductance regulator [150].

The role of rab5 has received particular attention. A mutant defective in GTP hydrolysis (Q69L) is stimulatory to fusion, whilst a mutant defective in GTP binding [S34N (Ser[34] → Asn) is inhibitory [151]. A mutant has also been made that binds XTP rather than GTP [D136N (Asp[136] → Asn)], which when added to the fusion incubation renders the assay dependent on the presence of XTP [152,153]. As this mutant hydrolyses XTP at essentially the same rate as wild-type rab5 hydrolyses GTP, it allows the isolation of the nucleotide cycle of rab5 in an endosome fusion assay, which represents a complex biological mixture containing many other GTP-binding proteins. Rybin et al. found that, under conditions where fusion could not occur (in the absence of cytosol), the measured rate of XTP hydrolysis was the same as under fusion-promoting conditions. This has been taken to show that nucleotide hydrolysis is not coupled to fusion [154], an assumption that is invalid if only a small percentage of the rab5 molecules that are hydrolysing are actually regulating a fusion event. If this is so, it would lead to the same result whatever the relationship between fusion and nucleotide hydrolysis, since the vast majority of rab5 molecules would be hydrolysing nucleotide irrespective of fusion events. One cannot measure total ATP turnover in a butterfly and conclude that it flaps its wings independently of ATP hydrolysis! Uncoupling of docking/fusion from nucleotide hydrolysis is much better demonstrated by fusion in the presence of non-hydrolysable analogue (e.g. XTP[S]) [152,153].

An extensive effort to identify proteins that interact with rab5 has been undertaken. Rabaptin-5 was identified using a yeast two-hybrid screen [155], whilst rabex-5 was found to interact with rab5 using an overlay technique [156]. Rabex-5 is homologous with Vps9p, a yeast protein implicated in endocytic trafficking [157]. It is complexed with rabaptin-5 and acts as a rab5 nucleotide-exchange factor [156]. This complex is absolutely required for endosome fusion; rabaptin 5 alone is not sufficient. Rabaptin-5 interacts with rab5 at its C-terminus, but intriguingly can also interact with GTP-bound rab4 at a distinct N-terminal site [158]. This encourages the notion that rabaptin-5 can act as a molecular linker between rab4 and rab5, possibly even mediating interaction between distinct compartments.

Rabaptin-5 is an extended molecule that exists as a homodimer in the cytosol [158]. Dimerization depends on the presence of an extensive coiled-coil sequence [158] that has homology with myosin heavy chain and to early endosomal autoantigen 1 (EEA1; see below). A further effector protein of rab5 has been identified by two-hybrid screening, namely rabaptin-5$\beta$ [159]. It is a 62 kDa coiled-coil protein that shares 42 % sequence identity with rabaptin-5 and which can form a distinct complex with rabex-5. This complex also co-operates in endosome fusion [159].

Inhibition of early endosome fusion due to PI-3K inhibitors, such as wortmannin, can be reversed by addition of the constitutively active rab5 [Q79L (Gln$^{79}$ → Leu)] [147]. This led to the proposal that PI-3K activity was required upstream of rab5 activation for endosome fusion [147]. However, it has now been shown that the amount of rab5 (Q79L) required to overcome wortmannin inhibition is far in excess of that which could have been prevented from activation in the first place [153]. Inhibition of rab5 activation cannot therefore be the primary cause of wortmannin inhibition of endosome fusion. Intriguingly, a specific marker of early endosomes, EEA1 [160], dissociates from membranes following wortmannin treatment and has recently been shown to bind phosphatidylinositol 3-phosphate (PI3P) through a double zinc-finger domain, termed a FYVE domain after the initial letters of the first four identified proteins containing this domain [161–163]. EEA1 is involved in early endosome fusion [164,165] such that the dissociation of this protein induced by wortmannin, is sufficient to explain the observed inhibition of endosome fusion. Moreover, EEA1 has two binding sites for the GTP form of rab5 at its N- and C-terminus [165]. It is likely that a co-operative interaction between PI3P and rab5 is responsible for EEA1 localization to early endosomes, such that a large excess of rab5 may be able to overcome the requirement for PI3P by Mass Action [147,153,165].

EEA1 is a 162 kDa protein comprising an extended coiled-coil region flanked by two zinc-binding domains [160]. By virtue of this lengthy coiled-coil domain it shows homology with myosin heavy chain, rabaptin-5 and to p115, a protein implicated in Golgi transport [166]. The C-terminal 134 amino acids, which include a zinc finger of the FYVE class, are sufficient to localize it to early endosomes [167]. This domain is found in the yeast proteins VAC1/Pep7p [168] and Vps18p/Pep3p [169], both of which are required for vacuolar sorting. VPS45 and and PEP12 represent allele-specific high-copy suppressors of a PEP7 mutant strain [170]. As these belong respectively to the Sec1p (which interacts with t-SNAREs) [171] and syntaxin family of proteins, it is likely that PEP7 is involved in SNARE complex function at some level.

There are now three identified effectors of rab5, EEA1, rabaptin-5 and rabaptin-5$\beta$, all of which have been implicated in endosome fusion to some degree. One possible explanation for this is that, although fusion assays are referred to as homotypic by virtue of an operational definition of labelled compartments, this may mask the fact that the fusion signal is a sum of fusion events, of which some may be vectorial in character. These different effectors may allow greater promiscuity of fusion partners for rab5-containing endosomes. Different cell types or assay configurations may favour a subset of possible fusion events.

## RAB FUNCTION ON THE ENDOCYTIC PATHWAY

The most incisive studies of rab protein function to date have been reported for Ypt1p-dependent consumption of yeast-ER-derived COPII vesicles by fusion with the Golgi. In this instance

it has been proposed that Ypt1p regulates the association of Uso1p with membranes and that this protein then takes part in a SNARE-independent 'tethering' of fusion partners [172]. Ypt1p may then also play a role in regulating SNARE complex assembly by a transient interaction with a t-SNARE [173]. Uso1p is the yeast homologue of p115 and is likewise homologous with EEA1 and rabaptin-5 within an extended coiled-coil region. It could be that these proteins represent a family of 'velcro factors' with a common function prior to SNARE complex assembly [172,174].

Aside from the well-characterized role of rab5 in promoting early endosome fusion, a number of other aspects of the endosomal system have been shown to be dependent on rab function. Rab5 regulates the rate of clathrin-dependent endocytosis at the plasma membrane [175]. Recently, an *in vitro* assay of CCV formation has revealed that rab5 is an essential cytosolic factor [176]. It is an attractive notion that a budding vesicle has some sort of 'quality-control' mechanism which ensures that the proteins required for vesicle consumption are included before budding takes place. In this case rab proteins which regulate vesicle-fusion events will also promote vesicle-budding steps.

Rab7 has been shown to be a positive regulator of homotypic fusion between late endosomes of HeLa cells [177], and on the basis of antibody inhibition, early endosomes from the cellular slime mould *Dictyostelium discoideum* [178]. It has also been shown to enhance the rate of transport between early and late endosomes in a variety of cell types [179–182]. rab9 regulates the rate of recycling of the ci-M6PR from late endosomes to the TGN [183]. Rab4 and Rab11 have been implicated in recycling endosomal material to the plasma membrane. Rab4 appears to be a regulator of fast transferrin recycling [184]. It is present on early sorting endosomes, but absent from pericentriolar recycling endosomes [185]. Rab11 localizes to a pericentriolar sub-population of transferrin-labelled endosomes (recycling endosomes) in CHO and BHK cells and seems to govern transport to this compartment, from which recycling is delayed relative to rab4-dependent recycling from the sorting endosome [186].

## PI-3K ACTIVITY ON THE ENDOCYTIC PATHWAY

Studies of the endocytosis of the platelet-derived-growth-factor (PDGF) receptor, following ligand binding, revealed a coupling to PI-3K activation. By immunofluorescence, the receptor distribution was observed to be altered by treatment with the PI-3K inhibitor wortmannin or by mutation of the binding site on the receptor for the p85 adaptor subunit of the canonical p110 PI-3K catalytic subunit. In both cases internalized receptor remained confined to peripheral vesicles compared with the perinuclear destination in control cells [187]. It was shown that the initial internalization rate of this receptor is unchanged, but that subsequent transport to late endosomes is inhibited [188]. This provides a very interesting example of the coupling of receptor trafficking with a signal-transduction pathway promoted by that receptor. It has been established for EGF receptor that receptor trafficking can influence signal-transduction pathways [189]; the reciprocal effect may prove to be equally important.

Initial steps of fluid-phase endocytosis have been reported to be sensitive to wortmannin [147,190–193]. There is now some reason to believe that wortmannin inhibits non-clathrin-dependent uptake, but that clathrin-dependent internalization is in most cases insensitive. This has been addressed by Sato et al. [191] using the human fibrosarcoma cell line HT-1080; they found profound inhibition of constitutive and stimulated fluid-phase uptake by wortmannin, but transferrin-receptor internalization and recycling was not changed. Using differently sized

fluid-phase markers, Araki et al. have provided evidence that macropinocytosis in macrophages is preferentially inhibited by wortmannin [192]. Unaltered [194] or even increased rates of transferrin receptor internalization into Trvb-1 and K562 cells following wortmannin treatment [148,195] have also been found by other groups. However, in direct contrast with the results that Martys et al. [195] obtained with the same cell line (Trvb-1), Li et al. [147] reported an inhibition of transferrin-receptor internalization. Interestingly, the interaction between AP-2 and tyrosine-based internalization signals (YXXø) has been shown to be enhanced by PtdIns(3,4,5)$P_3$ or PtdIns(3,4)$P_2$ [32]. Several studies have shown that the rate of transferrin-receptor recycling is inhibited by wortmannin treatment [148,194,195].

A variety of morphological changes in endosomes induced by wortmannin have been described. Most striking is the appearance of phase lucent swollen vesicles [196] that contain ci-M6PR. A redistribution of the late endosome/lysosomal marker lgp120 to swollen vesicles devoid of acid hydrolases and distinct from those occupied by M6PR is also observed [197]. Using fluorescence microscopy, Shpetner et al. have detailed a distinct tubularization of transferrin-receptor-containing compartments reminiscent of the effects of brefeldin A [198] .

Among membrane traffic pathways involving the phosphoinositide system, the transport of vacuolar enzymes from the TGN to the yeast vacuole was the first in which a PI-3K activity was implicated. This was based on the abnormal secretion of carboxypeptidase Y (CPY) in a S. cerevisiae strain mutated for a gene encoding Vps34p, subsequently identified as a yeast PI-3K [199–201]. In the wild-type strain, CPY is routed to the vacuolar compartment. On the basis of wortmannin-sensitivity, a role for a PI-3K was reported in mammalian cells for the ci-M6PR-mediated transport of the lysosomal protease cathepsin D [196,202]. This pathway is analogous to the TGN–vacuole pathway in yeast. In these studies pro-cathepsin D was observed to be secreted into the extracellular medium instead of being delivered to the early endosomal compartment, and then processed during its transport toward the lysosome. The results are most simply explained by a block to a transport step in the ci-M6PR cycle, but exactly where the primary block occurs is still controversial. In K562 cells, following 20 min of wortmannin treatment, M6PR is greatly diminished in isolated CCV fractions without a concomitant decrease in γ-adaptin levels [203]; furthermore recycling of receptor from endosomes to the TGN is not influenced by wortmannin [204]. These observations are most consistent with an inhibition to sorting of the ci-M6PR rather than vesicle formation *per se*. However, a recent study using a different fractionation protocol has observed a parallel decrease in cation-dependent M6PR and γ-adaptin in vesicle fractions following wortmannin treatment, suggesting that vesicle formation may indeed be inhibited [205]. Kundra and Kornfeld, using mouse L-cells, detected a retardation in ci-M6PR recycling from endosomes to the TGN [206] and suggested that this will lead to the depletion of receptor from the TGN and consequently to a defect in cathepsin sorting. According to the configuration of their recycling assay this result could also be obtained if transfer from early to late endosomes is retarded. Interestingly, a screen to isolate yeast mutants defective in endocytosis, identified a Vps34 allele as *end12*, which showed defective characteristics consistent with a block in transport from a pre-vacuolar compartment to the vacuole [207]. To resolve this issue satisfactorily it will be necessary to undertake a quantitative analysis with immunocytochemical techniques of the distribution of M6PR before and after wortmannin treatment.

The PI-3Ks, 3-phosphoinositide species and effector proteins involved in these processes are unclear. Vps34p can only use phosphatidylinositol as a substrate, whilst the p85/p110 enzyme, which couples to PDGF receptor, does not discriminate between phosphoinositides *in vitro*, but seems to preferentially utilize PtdIns(4,5)$P_2$ *in vivo*. A mammalian PI-3K has been cloned [200,208] that represents the best candidate for a role in endosomal trafficking; it shares relatively high homology with Vps34, utilizes phosphatidylinositol as its sole substrate and is sensitive to wortmannin. Downstream effects of PI-3K activity on nucleotide-exchange rates for small GTP-binding proteins have been described, in particular rab4 [209] and ARF [210], and on the activity of protein kinases, for example protein kinases B and C [211,212]. As discussed above, EEA1 and other FYVE-domain-containing proteins, such as hrs and Vps27p, represent likely effectors of PtdIns3$P$ production [162,163,213], which may influence endocytic events other than fusion.

## ROLE OF THE CYTOSKELETON

Studies with mutant strains have established that actin is required for the initial internalization step of α-factor in S. cerevisiae, but is not necessary for transport from intermediate endosomes to the vacuole [214]. This internalization step also requires, Myo5p a type I unconventional myosin which is regulated by interaction with calmodulin [215,216]. The requirement for actin in receptor-mediated endocytosis of mammalian cells has been controversial. Treatment with the actin-depolymerizing drug cytochalasin D has provided ambiguous results. In Madin–Darby canine kidney (MDCK) cells it was shown to have a selective effect on endocytosis at the apical, but not the basolateral, surface [217]. The strongest evidence for a general actin involvement has been provided by Lamaze et al. [218], using an assay of transferrin receptor sequestration in perforated A431 cells. Thymosin β4 and DNAse I which both sequester actin monomers inhibited receptor sequestration, whilst crucially their effects could be neutralized by addition of actin monomers [218]. This is consistent with the same groups demonstration of a role for the small GTPases rho and rac, both of which regulate the actin cytoskeleton, in receptor sequestration [219]. A study by Durrbach et al. has also suggested a role for actin in the initial uptake of receptors via clathrin-coated pits in a mouse hepatoma cell line. In addition they have shown that actin enhanced delivery to the degradative compartment, downstream of a microtubule requirement [220].

The dynamics of early endosomal compartments and microtubules are to some extent coupled. Tubular elements of endosomes overlap with microtubules [88,221]; videos of early endosomal compartments show them constantly extending tubular projections to be consumed by fusion or retracted. The tips of these tubular projections provide the site of budding of CCVs [94]. Observation of endocytic vesicles *in vitro* by video microscopy has demonstrated their saltatory movement along isolated microtubules with a preference for movement in the (−) direction [222], in accordance with a centripetal motion within cells. Expression of a constitutively active mutant of the small GTPase rhoD leads to a loss of early endosome motility [223]. Although this protein also causes a reorganization of the actin cytoskeleton, there is as yet, no evidence supporting the view that this is responsible for the effects upon endosomes. Transport from early to late endosomes is microtubule-dependent; treatment with the microtubule-depolymerizing drug nocodazole leads to the accumulation of ECVs in BHK cells and MDCK cells [1,119]. *In vitro* studies by Aniento and colleagues have shown that fusion of ECVs with late endosomes is promoted by the microtubule-stabilizing drug taxol and that the assay is also dependent on the + to − end-directed motor dynein [1]. In contrast the homotypic

fusion *in vitro* between early or late endosomes is microtubule-independent. Sorting endosomes purified from hepatocytes bind to polymerized microtubules *in vitro* in the absence of ATP [221,224,225]. When 5 mM ATP was added to preparations of microtubule-bound vesicles, ligand (asialoorosomucoid)-containing vesicles were released, whilst receptor-containing vesicles remained bound to the microtubules [224]. Those authors proposed the interesting hypothesis that receptor-containing elements are immobilized on microtubules, whilst ligand-containing vesicles can move along microtubules away from the receptor domains by virtue of a motor molecule (e.g. dynein) such that fission ultimately occurs.

## LIPIDS AND ENDOCYTOSIS

As with the secretory pathway, the role and flow of lipids on the endocytic pathway is poorly characterized. PtdIns species are clearly important at several stages of the endocytic pathway (see above). Cholesterol is required for maintaining the structure of caveolae [226] and for receptor-mediated endocytosis via CCVs (K. Sandvig, personal communication). Application of ceramide to cells has also been shown to inhibit endocytosis [227]. One very important issue is whether budding vesicles contain a random or a specialized sample of donor membrane lipid, as lipid-domain formation has been postulated to play a role in protein sorting. Chen et al. added sphingomyelin analogues labelled with 5-(5,7-dimethylboron dipyrromethene difluoride)-pentan-1-oic acid to human skin fibroblasts and monitored the spectral properties of internalized fluorophore after very short internalization pulses. A spectral shift from green to red indicates concentration of the fluorophore. After short internalization pulses (7 s), two populations of vesicles are present (green and red) with red vesicles derived from a green plasma membrane concentrated at the periphery of the cell and co-localizing with internalized low-density lipoprotein [228]. A preliminary report has provided some indications that lipid chain length may be related to sorting on the endocytic pathway. Internalized 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine ($C_{16}$diI) progresses to the late endosomal compartment whilst 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanide ($C_{12}$diI) is directed towards the recycling compartment [229,230].

Recently Kobyashi and colleagues identified a late endosomal antigen as an unusual lipid, lysobisphosphatidic acid, concentrated on the internal vesicles of late endosomes and lysosomes [231,232]. A monoclonal antibody (6C4) against this lipid perturbs both the structure and function of late endosomes [231]. Recycling of M6PR (which also decorates the internal vesicles of late endosomes [17]) back to the TGN is inhibited by the internalized antibody. Kobyashi et al. have also shown that anti-sera from patients suffering from anti-phospholipid syndrome mimic the effects of 6C4 on M6PR distribution, a finding that may account for some aspects of the aetiology of the disease [231].

## CONCLUDING REMARKS

In this review I have summarized the available data under each subheading. It is striking that even the most developed areas are incompletely understood. Clearly the outstanding problems will ensure an active research field for many years.

Other related issues will certainly receive further attention. Some pathogens are known to modify endosomal traffic and compartments to their own requirements [233]. Once the molecular targets have been identified, these pathogens may become useful tools for basic research. The relationship between the endocytic pathway and presentation of antigen by MHC class II

molecules constitutes a flourishing field. Of special interest are dendritic cells, for which maturation, corresponding to migration from the periphery to lymphoid organs, is accompanied by a decrease in endocytic activity and a dramatic alteration in the transport of class II molecules [234]. Endocytosis in neurons, particularly from nerve terminal to cell body, awaits further characterization. Studies of long-term facilitation in *Aplysia* (marine snail) have indicated that clathrin-mediated endocytosis of adhesion molecules may constitute an early step in the learning-related growth of synaptic connections [235,236]. Finally, the dialectical relationship between signal transduction and endocytic traffic [189] is likely to become a major theme in the coming years. One can speculate that some perturbations of endocytic trafficking may be found to be associated with cellular transformation.

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