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REVIEW

The Roles of Ubiquitin and Lipids in Protein Sorting along the Endocytic Pathway

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ABSTRACT. **After cell surface receptors are internalized for endocytosis, they are accurately sorted in endosomes. Some are recycled to the plasma membrane and others are downregulated by delivery to lysosomes. Evidence is rapidly accumulating that ubiquitination of cargo proteins acts as a sorting signal during endocytosis. Sorting devices that recognize ubiquitin are distributed to various compartments, probably acting in a concerted manner. Cholesterol is enriched in the plasma membrane and endosomes, and is involved in protein sorting by forming microdomains called lipid rafts. Ubiquitin and cholesterol hold the key to control the endocytic sorting, and they are likely acting cooperatively.**

Key words: protein sorting/endocytosis/ubiquitin/sterol

Endocytosis mediates the trafficking from the plasma membrane to lysosomes. Endosomes are the relay stations along this pathway, where the sorting of proteins and lipids takes place. Cell surface receptors follow the endocytic pathway where they are eventually degraded by lysosomal proteases. In some cases, however, the receptors are recycled from endosomes to the plasma membrane. The sorting function in endosomes thus controls the amount of receptors exposed to the cell surface, which is important to maintain normal cell growth. If the growth factor receptors fail to be eliminated from the plasma membrane after the ligand stimulation, the prolonged signaling will lead to cell transformation. The endocytic sorting of receptor tyrosine kinases (RTKs) has been extensively studied from this viewpoint. Recently, a major breakthrough has been made that ubiquitination of cargo proteins acts as a sorting signal in the endocytic pathway. Although ubiquitination has been known as a degradation signal by the proteasome, the ubiquitinated cargo proteins seem to be transported to lysosomes without proteasomal degradation. At present, ubiquitin is known to act as a sorting signal at three steps: internalization from the plasma membrane, multivesicular body (MVB) sorting in endosomes and Golgi-to-endosome trafficking (Fig. 1).

In addition to ubiquitin, certain lipids such as cholesterol and phosphatidylinositol 3-phosphate (PI(3)P), play roles in protein sorting. These lipids are preferentially localized to post-Golgi compartments. On the cytoplasmic side of the endosomal membrane, PI(3)P marks the site where the sorting machinery is assembled. Cholesterol regulates protein sorting by forming membrane microdomains called lipid rafts.

In this review, recent findings will be introduced to address how ubiquitin and lipids regulate protein sorting.

Internalization from the plasma membrane

The yeast mating pheromone receptor Ste2p undergoes ubiquitination upon ligand binding, and this is required for subsequent internalization from the plasma membrane (Hicke and Riezman, 1996). In the case of proteasomal degradation, Lys^{48}-linked polyubiquitin (at least four ubiquitin chains) need to be appended to the target protein (Deveraux *et al.*, 1994). In contrast, monoubiquitin is sufficient for endocytic internalization, because Ste2p is efficiently internalized under conditions where polyubiquitin formation is suppressed (Terrell *et al.*, 1998). Similarly, monoubiquitination of sugar transporters triggers their internalization (Lucero *et al.*, 2000; Horak and Wolf, 2001). Another yeast plasma membrane protein, uracil permease Fur4p, is

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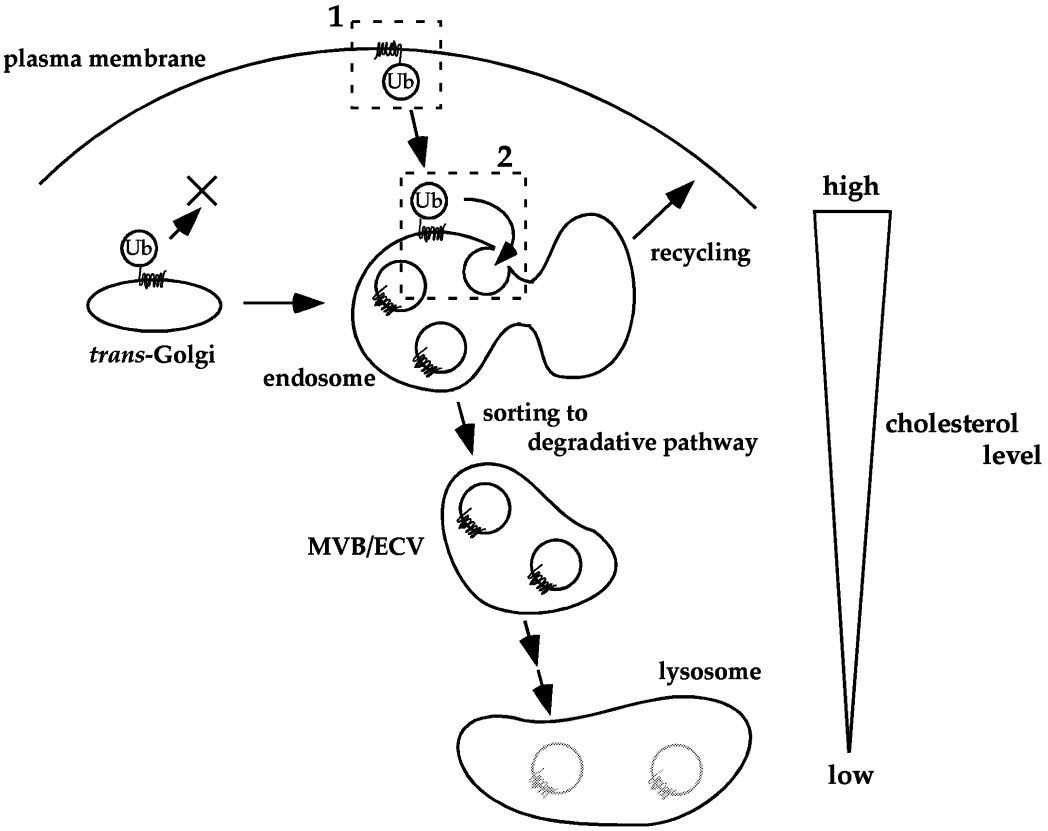
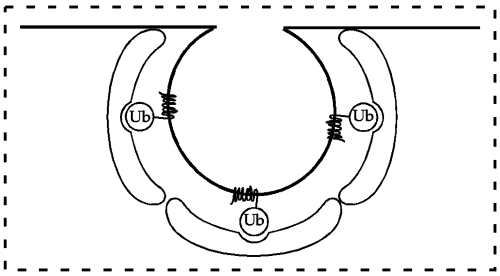
Abbreviations: AAA, ATPase associated with a variety of cellular activities; CPS, carboxypeptidase S; ECV, endosomal carrier vesicle; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ENTH, epsin N-terminal homology; ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body; PI(3)P, phosphatidylinositol 3-phosphate; PVC, prevacuolar compartment; RTK, receptor tyrosine kinase; TSG, tumor susceptibility gene; UEV, ubiquitin E2 variant; UIM, ubiquitin-interacting motif; Vps, vacuolar protein sorting.



1. Recognition of ubiquitinated cargoes by clathrin-coated pits



2. Ubiquitin as a sorting signal to the multivesicular body

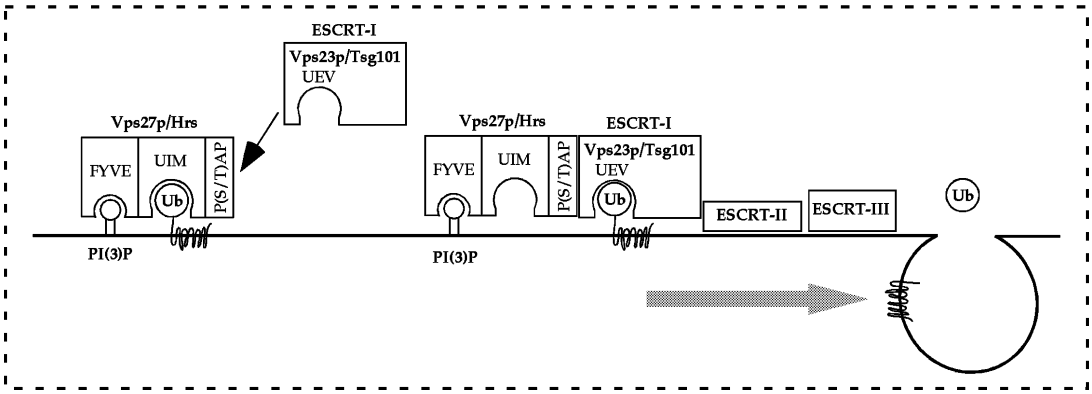


Fig. 1.

conjugated with Lys^{63}-linked ubiquitin chains (Galan and Haguenauer-Tsapis, 1997). Inhibition of ubiquitin chain extension through Lys^{63} reduces the rate of endocytosis. Similarly, the yeast general amino acid permease Gap1p is conjugated with Lys^{63}-linked ubiquitin chains, which is required for a maximal rate of endocytosis (Springael *et al.*, 1999; Soetens *et al.*, 2001). In both cases, the Lys^{63}-linked ubiquitin chains are not essential for the internalization although they do accelerate the rate of endocytic trafficking. The molecular mechanisms underlying the recognition of Lys^{63}-linked ubiquitin chains along the endocytic pathway remain to be resolved, but the current concept is that monoubiquitination of cargo proteins is sufficient to trigger internalization.

The ubiquitin-dependent internalization appears to be conserved between yeast and mammalian cells. The important finding came from mammalian cells that the endocytic machinery recognizes monoubiquitin. Invaginating vesicles from the plasma membrane are coated by the clathrin protein complex. Eps15 binds the clathrin adaptor complex AP-2 and therefore is a component of the clathrin coated pit. Polo *et al.* (2002) have found that eps15 also binds monoubiquitin. The binding is mediated by a conserved motif found in eps15, called UIM (ubiquitin-interacting motif). It is plausible that eps15 links monoubiquitinated cargoes to the clathrin coat, thereby enabling the cargo loading onto clathrin coated vesicles. UIMs are found in other proteins that regulate endocytosis. In addition to binding monoubiquitin, these proteins themselves are monoubiquitinated (Polo *et al.*, 2002). Thus, UIM-containing proteins may assemble into a large oligomeric complex, by interacting with each other and with monoubiquitinated cargoes. UIM-monoubiquitin binding may prevent the elongation of monoubiquitin to a polyubiquitin chain, thus allowing monoubiquitin to remain as a recognition signal. Yeast studies also suggest that the ubiquitination of endocytic machinery is important in internalization. The ubiquitin ligase Rsp5p, the relative of mammalian Nedd4, mediates the ubiquitination of Ste2p. Fusion of ubiquitin in-frame to the cytoplasmic tail of Ste2p bypasses the requirement that Ste2p itself is ubiquitinated upon internalization (Terrell *et al.*, 1998). However, the Ste2p-Ub chimera is internalized inefficiently in the *rsp5* mutant (Dunn and Hicke, 2001). This suggests that Rsp5p ubiquitinates not only the cargo proteins but also the components of the endocytic machinery. Consistent with this, eps15 is ubiquitinated by Nedd4 (Polo *et al.*, 2002).

In mammalian cells, RTKs are ubiquitinated and subsequently endocytosed. In the case of epidermal growth factor receptor (EGFR), Cbl was identified as the ubiquitin ligase (Levkowitz *et al.*, 1998). Following EGF stimulation, Cbl ubiquitinates EGFR for downregulation. Although the Cbl-mediated ubiquitination is considered to trigger the internalization (for review see Oved and Yarden, 2002), some results indicate that it is rather required for a later step, that is, the sorting from endosomes to lysosomes. Using $Cbl^{-/-}$ cells, Duan *et al.* (2003) have indicated that EGFR is internalized at a normal rate in the absence of Cbl. In this case, EGFR appeared to reach endosomes but its degradation was inhibited. The oncogenic viral form of Cbl (v-Cbl) is composed of the N-terminal 355 amino acids and lacks the ring finger domain required for ubiquitin ligase activity. Levkowitz *et al.* (1998) have shown that expression of v-Cbl does not affect the internalization of EGFR but promotes its recycling to the cell surface. These results strongly suggest that the ubiquitination of EGFR by Cbl is required for endosome-to-lysosome transport, rather than internalization from the plasma membrane.

Multivesicular body sorting in endosomes

In electron microscopy, endosomes are often observed as multivesicular structures with internal membranes called multivesicular bodies (MVBs), the formation of which is initiated by inward budding of the limiting membrane. Subsequently, vesicles are formed and released to the organelle lumen. Some cargo proteins are sorted to the invaginating vesicles, while others are retained on the limiting membrane. It has been known that accurate sorting to the MVB is required for receptor downregulation, control of signaling pathways, antigen presentation and so on (for review see Katzmann *et al.*, 2002). Recent yeast studies shed light on the molecular mechanisms of MVB formation. It turns out that ubiquitin acts as a sorting signal to the MVB.

Yeast *vps* (vacuolar protein sorting) mutants were identified as being defective in protein sorting to the vacuole, the yeast counterpart of lysosomes. Based on the morphology of the vacuole, the mutants were classified into 6 groups

Fig. 1. Model for the ubiquitin-dependent protein sorting in post-Golgi trafficking. It is known that ubiquitinated cargo proteins are internalized from the plasma membrane, sorted to the interior of the MVB, and diverted from the Golgi to the vacuole/lysosome targeting pathway. Upon internalization, clathrin-coated pits recognize the monoubiquitin that is appended to cargoes. In mammalian cells, formation of the MVB occurs in early endosomes. MVB is also referred to as endosomal carrier vesicle (ECV). In yeast, the MVB is formed in the PVC, the equivalent of late endosomes. In both cases, however, the components that recognize and sort ubiquitinated cargoes, such as Vps27p/Hrs and ESCRT complexes, are conserved. Considering that Hrs and ubiquitinated proteins are colocalized to the limiting regions of endosomes (Raiborg *et al.*, 2002), formation of the MVB appears to occur in distinct domains from where cargoes are recycled to the plasma membrane. Lipids are also important in these sorting processes. PI(3)P is enriched in endosomes and appears to act as a platform for assembly of the sorting machinery. Cholesterol is enriched in early endocytic organelles, but not in late ones. Consistent with this biased distribution, cargoes associated with the cholesterol-rich lipid rafts do not enter the degradative pathway in general.

(Raymond *et al.*, 1992). Of these, the class E *vps* mutants exhibited exaggerated prevacuolar compartments (PVCs). The PVCs in yeast correspond to late endosomes in mammalian cells, and the exaggerated PVC in these mutants is called the class E compartment. Odorizzi *et al.* (1998) showed that MVB sorting of a cargo protein is impaired in all of the class E *vps* mutants. In electron microscopy, intralumenal vesicles are not observed in the class E compartment. Thus, the class E Vps proteins are required for the formation of MVBs. The Vps proteins that appear in the following all belong to class E.

Katzmann *et al.* (2001) indicated that ubiquitination of cargo proteins is sufficient for their MVB sorting. Endocytosed cargoes are ubiquitinated upon internalization, and the ubiquitin is likely to be recognized as a MVB sorting signal in endosomes. Biosynthetic MVB cargoes such as the vacuolar hydrolase carboxypeptidase S (CPS) are directed from the Golgi to endosomes, and then to the vacuole. Ubiquitination of the cytoplasmic domain of the CPS precursor occurs after exit from the Golgi, and prior to reaching endosomes (Katzmann *et al.*, 2001). In both types of cargoes, they are in ubiquitinated status when transported to endosomes, where the sorting to invaginating vesicles occurs. The sorting is initiated by recognition of ubiquitin by the ESCRT-I (endosomal sorting complex required for transport) composed of Vps23p, Vps28p and Vps37p. Of these, Vps23p is responsible for binding to ubiquitin (Katzmann *et al.*, 2001). Vps23p has a conserved domain that is found in ubiquitin E2-conjugating enzymes. Unlike E2 enzymes that catalyze the ubiquitin transfer, however, the active site cysteine residue is missing in Vps23p. This domain, called UEV (ubiquitin E2 variant), would simply mediate the binding to ubiquitin. Once ubiquitinated cargoes are captured by Vps23p, ESCRT-I somehow activates the downstream sorting complex ESCRT-II (composed of Vps22p, Vps25p and Vps36p), which in turn recruits ESCRT-III (composed of Vps2p, Vps20p, Vps24p and Snf7p) onto the endosomal membrane (Babst *et al.*, 2002a and 2002b). ESCRT-III, whose subunits exist abundantly, is considered to be the core of MVB formation that sorts and concentrates the cargoes to the sites where vesicle invagination is to occur. Before entry into the lumen, ubiquitin is removed from the cargoes and the resultant free ubiquitin molecules are recycled (Katzmann *et al.*, 2001). This is carried out by the deubiquitinating enzyme Doa4p, which is localized to the class E compartment in a Vps24p-dependent manner (Amerik *et al.*, 2000). In the *doa4* mutant, ubiquitin is considerably degraded by vacuolar proteinases (Swaminathan *et al.*, 1999), suggesting that the cargoes and the conjugated ubiquitin are simultaneously eliminated in the organelle lumen. Thus, it seems that the deubiquitination is not essential for MVB sorting. This is consistent with the findings that the cargoes fused in-frame to ubiquitin are efficiently sorted to the MVB (Urbanowski and Piper, 2001). It should be noted that ubiquitin-independent MVB sorting does exist (Reggiori and Pelham, 2001), but its precise molecular mechanism remains an open question.

After completion of a series of sorting events, the Vps protein complexes are disassembled and released into the cytosol. This is dependent on the ATPase activity of Vps4, which belongs to the AAA (ATPase associated with a variety of cellular activities) family of ATPases (Babst *et al.*, 1998). At a steady state, components of the ESCRT complexes are mainly localized to the cytosol, probably as a result of shuttling between the cytosol and the endosomal membrane. Either deletion of *VPS4* or expression of mutant Vps4p^{E233Q}, defective in ATP hydrolysis, causes the accumulation of the ESCRT complexes to the class E compartment (Babst *et al.*, 1998, 2002a, 2002b).

More recent studies have placed the function of Vps27p upstream of ESCRT-I (Katzmann *et al.*, 2003). Vps27p is one of the class E Vps proteins but is not involved in the ESCRT complexes. It associates with the endosomal membrane via its FYVE domain, a well known motif that binds PI(3)P (Stenmark and Aasland, 1999). Because PI(3)P is rich in endosomes (Gillooly *et al.*, 2000), this lipid is likely to provide the platform where the MVB sorting is initiated. Vps27p also possesses UIM, and binds ubiquitin *in vitro* (Bilodeau *et al.*, 2002; Shih *et al.*, 2002) and an ubiquitinated cargo *in vivo* (Katzmann *et al.*, 2003). It is conjectured that Vps27p recruits ESCRT-I onto the endosomal membrane through its binding to Vps23p. The PSDP and PTVP motifs of Vps27p, similar to the conserved P(S/T)AP tetrapeptide motif (see below), are likely to mediate the binding to Vps23p, where ubiquitinated cargoes would first be bound to Vps27p and then transferred to Vps23p.

In addition to PI(3)P, PI(3,5)P_2 is required for protein sorting to MVBs. This is based on the observation that PI(3)P 5-kinase Fab1p is essential for sorting of CPS to the organelle lumen (Odorizzi *et al.*, 1998). A yeast epsin-like protein Ent3p has just been identified as a PI(3,5)P_2 effector (Friant *et al.*, 2003). It has been known that epsins bind specifically to PI(4,5)P_2 through their ENTH (epsin N-terminal homology) domains, and that they are involved in the internalization of clathrin-mediated endocytosis. The functions of Ent3p, however, appear to be different from conventional epsins (Friant *et al.*, 2003). First, the ENTH domain of Ent3p preferentially binds to PI(3,5)P_2. Second, Ent3p is not required for endocytic internalization. Ent3p is instead localized to endosomes and is essential for protein sorting to the MVB. The exact mode of interaction between Ent3p and ESCRT complexes, however, remains an open question.

ESCRT-dependent sorting to the MVB seems to be highly conserved in mammalian cells, based on the fact that functional homologues of the class E Vps proteins have been identified. The mammalian tumor susceptibility gene *TSG101* encodes the homologue of Vps23p. Tsg101 binds ubiquitin, probably through its conserved UEV domain (Bishop *et al.*, 2002). In *tsg101* mutant cells, defects in endosomal trafficking are observed. Notably, endocytosed

EGFR is not sorted to lysosomes but is recycled back to the plasma membrane (Babst *et al.*, 2000). Failure in MVB sorting results in retention of endocytosed receptors on the endosomal limiting membrane, and appears to further result in receptor recycling to the plasma membrane (Felder *et al.*, 1990). This causes prolonged receptor signaling which plausibly explains the tumor susceptibility of *tsg101* cells. Like the physical interaction between Vps23p and Vps27p, Tsg101 binds Hrs, the homologue of Vps27p (Bache *et al.*, 2003; Lu *et al.*, 2003). Also in this case, the PSAP motif of Hrs is important for the binding. Hrs binds ubiquitin through its UIM and is involved in the sorting of ubiquitinated cargoes (Raiborg *et al.*, 2002). This is likely due to its ability to recruit the ESCRT subunit Tsg101 to early endosomes (Bache *et al.*, 2003). The Vps4p homologues have been identified in mouse and human (Bishop and Woodman, 2000; Yoshimori *et al.*, 2000). Overexpression of their ATPase-deficient mutant proteins results in accumulation of the class E Vps proteins to exaggerated endosomal structures, and dominantly inhibits endosomal trafficking (Katoh *et al.*, 2003).

Studies in mammalian cells provide additional insights that are not yet obtained from yeast. For example, clathrin is implicated in endosomal sorting. Raiborg *et al.* (2001) have found that Hrs binds clathrin heavy chain through the C-terminal 'clathrin box', a conserved domain found in clathrin-binding proteins. Hrs recruits clathrin to the early endosomal microdomains, which are distinct from those marked by another early endosomal protein EEA1 (Raiborg *et al.*, 2002). Interestingly, ubiquitinated proteins in early endosomes are colocalized with clathrin and Hrs, suggesting that the clathrin- and Hrs-containing microdomains represent the sites where the sorting of ubiquitinated proteins occurs. In addition to clathrin, COP-I is associated with the endosomal membrane (Whitney *et al.*, 1995). COP-I is required for the biogenesis of multivesicular endosomes (Aniento *et al.*, 1996; Gu *et al.*, 1997), and one of the COP-I subunits, β-COP, binds the cytoplasmic domain of a cargo destined for lysosomes (Piguet *et al.*, 1999). Whether clathrin and COP-I act cooperatively or independently in the endosomal transport remains to be elucidated. MVBs are considered as transport intermediates from early to late endosomes (for review see Gu and Gruenberg, 1999). It would seem that the membrane invagination occurs first, and then the multivesicular regions bud off from early endosomes. Annexin II is involved in the formation of MVBs probably by controlling the budding process (Mayran *et al.*, 2003). Cholesterol in the early endosomal membrane may provide platforms for the lysosomal degradation pathway, because annexin II tightly binds cholesterol. It is likely that cholesterol is also involved in the later steps of maturation and fusion of MVBs. This is based on the observation that MVBs are accumulated in a Chinese hamster ovary cell mutant, called LEX2, which shows reduced levels of cellular cholesterol (Miwako *et al.*, 2001). In addition, LEX2 is defective in protein sorting. Cation-independent mannose 6-phosphate receptor is not recycled to the Golgi but accumulated within MVBs (Miwako *et al.*, 2001). Cholesterol may be required for keeping the recycling cargoes on the endosomal limiting membrane.

The MVB vesicle budding toward the organelle lumen is topologically the same as the budding of enveloped viruses from the plasma membrane. Strikingly, a collection of recent studies has demonstrated that the class E Vps proteins are hired by viruses (for review see Amara and Littman, 2003). HIV Gag protein is required for release of virus particles from cells. Gag is synthesized as a precursor, which is myristoylated at the N-terminus and thereby targeted to the membrane. During the budding process, Gag is cleaved to produce four proteins. Of these, the peptide derived from the C-terminus of the precursor, called p6, is required for completion of the budding. It has been known that mutations in the p6 domain arrest the budding at a very late stage. That is, the budding particles fail to be released from the plasma membrane. It turns out that the p6 domain binds Tsg101, and either depletion of Tsg101 or expression of dominant negative Vps4p mutants inhibits HIV release from cells (Garrus *et al.*, 2001); this strongly suggests that p6 recruits the class E Vps machinery for virus budding. For the binding between p6 and TSG101, the PTAP motif of p6 and the UEV domain of Tsg101 are crucial (Garrus *et al.*, 2001). The PTAP sequence in p6 has been known to be indispensable for the viral release, and it is now clear that the conserved P(S/T)AP tetrapeptide in Hrs is also responsible for the binding to Tsg101. When fused to Gag lacking the PTAP motif, Hrs can substitute the viral release function in its PSAP-dependent manner (Pornillos *et al.*, 2003). Interestingly, the Gag PTAP peptide binds the Tsg101 UEV domain with higher affinity than does the Hrs PSAP peptide (Pornillos *et al.*, 2003), suggesting that the HIV Gag snatches the ESCRT complexes from Hrs.

Golgi-to-endosome trafficking

In addition to the endocytic pathway, ubiquitin acts as a sorting signal late in the secretory pathway. Yeast amino acid permeases are delivered to the plasma membrane in a regulated manner, which is specified by their ubiquitinated status (Helliwell *et al.*, 2001; Umebayashi and Nakano, 2003). Intracellular transport of Gap1p is controlled by the nitrogen source in the medium (Roberg *et al.*, 1997). Gap1p is sorted to the plasma membrane on poor nitrogen sources (e.g. urea) and to the vacuole on rich nitrogen sources (e.g. glutamate). The sorting seems to take place in the *trans*-Golgi. Helliwell *et al.* (2001) have suggested that Gap1p is diverted from the Golgi to the vacuole by overexpression of either *BUL1* or *BUL2*. Conversely, deletion of *BUL1* and *BUL2* results in efficient plasma membrane localization of Gap1p. Bul1p and Bul2p are homologous proteins that are components of the Rsp5 ubiquitin ligase complex

(Yashiroda *et al.*, 1996 and 1998) and may be involved in the elongation of polyubiquitin chains (Helliwell *et al.*, 2001). Polyubiquitinated Gap1p is detected in wild-type cells but not in Δ*bul1* Δ*bul2* cells, raising the possibility that polyubiquitin appended to Gap1p by the Rsp5p complex acts as a sorting signal from the Golgi to the vacuole. Soetens *et al.* (2001) have mapped the ubiquitin acceptor sites of Gap1p to two lysine residues in its N-terminal cytoplasmic tail, and indicated that the Bul1p- and Bul2p-dependent ubiquitination is also required for the endocytosis of Gap1p after it has reached the plasma membrane.

Ubiquitin moiety required for sorting

That monoubiquitination diverts the destination of cargo suggests the existence of a transport signal in the amino acid sequence of ubiquitin. By transplanting the peptide 36–44 of ubiquitin (IPPDQQRLI) to the cytoplasmic domain of a cargo lacking internalization signals, Nakatsu *et al.* (2000) showed that the Leu^{43}-Ile^{44} sequence of ubiquitin acts as a di-leucine motif, a well characterized endocytosis signal in mammalian cells (Letourneur and Klausner, 1992). Using a similar approach, Shih *et al.* (2000) showed that the ubiquitin sequence $D^{39}QQRLI^{44}$ does not allow endocytosis in yeast. This apparent discrepancy may be reconciled by the fact that there is no report in yeast that a di-leucine motif works as an endocytosis signal. It should be noted that both reports point to the importance of the Leu^{43}-Ile^{44} sequence. Substitution of these residues to alanine retarded the ability of ubiquitin to trigger internalization. It is still an open question whether Leu^{43}-Ile^{44} is recognized as a di-leucine motif or Leu^{43} simply affects the folding of ubiquitin as Shih *et al.* (2000) argued. In any cases, it is likely that ubiquitin itself is recognized as a sorting determinant, rather than the possibility that ubiquitination induces a conformational change of cargo to be recognized by the sorting machinery.

Compared with monoubiquitin, much less is known about the roles of polyubiquitin in post-Golgi trafficking. Ubiquitinated forms of various cargo proteins have been detected, however, whether they are polyubiquitinated or monoubiquitinated at multiple lysine residues has been largely unknown. Recently, it has turned out that the high molecular weight forms of EGFR, which were long considered to be polyubiquitinated, results from multiple monoubiquitination (Haglund *et al.*, 2003). It is plausible that different UIM-containing proteins such as Eps15 and Hrs bind different monoubiquitinated sites of EGFR to achieve the progressive sorting in the endocytic pathway. Characterization of the ubiquitinated status of other cargoes will be important for further understanding the endocytic sorting mechanisms.

Investigation into the binding properties between ubiquitin and ubiquitin-binding motifs will also be important. Although the UIM-containing proteins appear to predominantly recognize monoubiquitin during the endocytic sorting processes, the UIM of the proteasome subunit S5a (Rpn10) binds polyubiquitin, not monoubiquitin (van Nocker *et al.*, 1996). The reason for this difference is unclear, but the target of the UIM is not restricted to monoubiquitin. Recently, another ubiquitin-binding motif, called the CUE domain, has been identified. This domain is found in yeast Vps9p, a guanine nucleotide exchange factor for the Rab5 homologue Vps21p, and binds both mono- and polyubiquitin *in vitro* (Davies *et al.*, 2003; Donaldson *et al.*, 2003; Shih *et al.*, 2003). Like the UIM, the CUE domain is also involved in ubiquitination of Vps9p itself. The structure of the CUE domain has been determined (Kang *et al.*, 2003; Prag *et al.*, 2003). Notably, the latter report has indicated that the CUE domain can dimerize with each other and provided insights into the differential recognition of mono- and polyubiquitin. Two ubiquitin binding surfaces are observed on opposite faces of the CUE monomer. Therefore, the binding of the CUE monomer to monoubiquitin is mediated by only one of these two surfaces. Upon dimerization, however, the CUE domain can bind monoubiquitin through either surface. Based on these findings, it is likely that the dimer is preferentially bound to monoubiquitin and the monomer to polyubiquitin. The binding specificity may be controlled by the dimerization state of the CUE domain.

Lipid raft and protein sorting

Lipid rafts are assemblies of sphingolipids and cholesterol that form liquid-ordered microdomains in the lipid bilayer (for review see Simons and Ikonen, 1997). A specific set of proteins including GPI-anchored proteins, di-acylated proteins, and transmembrane proteins such as influenza virus hemagglutinin are preferentially packed into lipid rafts. The distinct feature of lipid rafts is the resistance to detergent extraction, typically by Triton X-100. Some raft proteins, however, are relatively sensitive to the Triton X-100 extraction while they are resistant to other detergents such as CHAPS (Fiedler *et al.*, 1993). The choice of detergent would depend on the raft protein of interest. Recently, comprehensive analysis to isolate lipid rafts with different detergents has been carried out (Schuck *et al.*, 2003). One of the roles of lipid rafts is to serve as a platform in protein sorting. In polarized epithelial cells, sorting occurs in the *trans*-Golgi and lipid rafts are responsible for transport to the apical plasma membrane. When lipid rafts are disrupted by cholesterol depletion, hemagglutinin becomes extracted by Triton X-100 and concomitantly missorted to the basolateral membrane (Keller and Simons, 1998).

The structure of the major sterol in yeast, ergosterol, is slightly different from cholesterol, but it also assembles with sphingolipids to form detergent-insoluble lipid rafts (Bagnat *et al.*, 2000). Like in mammalian cells, lipid rafts in yeast are involved in protein sorting. During the biosynthetic transport, the plasma membrane ATPase Pma1p is partitioned into lipid rafts in the Golgi (Bagnat *et al.*, 2001).

The raft-association is required for the subsequent plasma membrane targeting, because Pma1p is missorted to the vacuole in the sphingolipid-deficient *lcb1* mutant (Bagnat *et al.*, 2001). It is likely that yeast lipid rafts are responsible for the transport from the Golgi to the plasma membrane. Non-raft domains would be targeted to the vacuolar sorting pathway in yeast. It is also reported that raft-association of Pma1p is required for the stability in the plasma membrane, preventing internalization and subsequent transport to the vacuole (Gong and Chang, 2001; Wang and Chang, 2002).

The advantage of yeast in studying sterol-dependent protein sorting is that ergosterol biosynthetic pathway has been almost completely understood (for review see Daum *et al.*, 1998). A number of *ERG* gene products are engaged in ergosterol biosynthesis, and it can be blocked at any particular step by using the *erg* mutants. Riezman's group has shown multiple functions of sterol in the endocytic pathway. Their studies began with the finding that the *END11* gene, which is required for the internalization step of endocytosis, is allelic with *ERG2* (Munn *et al.*, 1999). *ERG2* is one of the five genes (*ERG2-6*) involved in the last five steps of ergosterol biosynthesis. Single or double mutations in these steps result in accumulation of various intermediates that are closely related to the final product ergosterol (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002). Because none of the five genes is essential for viability, these intermediates should be able to replace ergosterol to a certain degree. However, they found that endocytosis is impaired in some of the mutants. For example, the ligand-dependent internalization of Ste2p was most severely inhibited in *erg2 erg6* and *erg3 erg6* double mutants (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002). More detailed analysis has uncovered that phosphorylation of Ste2p, which precedes the ubiquitination (Hicke *et al.*, 1998), is abrogated in these *erg* mutants (Heese-Peck *et al.*, 2002). The phosphorylation and internalization, however, was normal in the *erg3* single mutant (Heese-Peck *et al.*, 2002). Thus, the inhibition of internalization is dependent on the structures of the ergosterol intermediates. Similarly, post-internalization steps were impaired to various degrees among these mutants (Heese-Peck *et al.*, 2002). Although the evidence has not been obtained at present that the ergosterol intermediates impair the endocytic trafficking by altering the architecture and function of lipid rafts, it is plausible that lipid rafts with different structures of sterol (and sphingolipids) are localized to different organelles. If so, the mutation in each of the *ERG* gene would affect the function of a particular type of lipid raft, leading to the various phenotypes among the *erg* mutants.

Possible cooperation of ubiquitin and sterol in protein sorting

We have been studying the intracellular transport of the yeast high-affinity tryptophan permease Tat2p, and recently suggested that ubiquitin and sterol, the key players in post-Golgi protein sorting, act in a concerted manner (Umebayashi and Nakano, 2003). Tat2p undergoes regulated sorting, which depends on the tryptophan concentration in the medium (Fig. 2A). Tat2p is sorted to late endosomes and subsequently to the vacuole at high tryptophan, and to the plasma membrane at low tryptophan. We have followed the route of Tat2p by using mutants defective in post-Golgi trafficking, and suggested that the sorting of Tat2p takes place in early endosomes, rather than the *trans*-Golgi. That is, newly synthesized Tat2p is once delivered from the *trans*-Golgi to early endosomes, and there the tryptophan-dependent sorting occurs. Polyubiquitinated forms of Tat2p are detected under the condition where it is accumulated in the vacuole, indicating that polyubiquitin is appended to Tat2p during the transport to the vacuole. The polyubiquitination of Tat2p is inhibited by deletion of *BUL1* and, strikingly, Tat2p is redirected to the plasma membrane when the Δ*bul1* cells are grown at high tryptophan. These results strongly suggest that the tryptophan-dependent sorting stems from the Rsp5p complex-dependent ubiquitination of Tat2p, and that polyubiquitin acts as a vacuolar sorting signal. Ubiquitin-dependent sorting of Tat2p to the vacuole also occurs upon nutrient starvation (Beck *et al.*, 1999). Thus, ubiquitin controls the sorting of yeast amino acid permeases, Gap1p and Tat2p, to change their localization in a nutrient-dependent manner.

It has been known that uptake of tryptophan from the medium is greatly reduced in the *erg6* mutant (Gaber *et al.*, 1989). We have shown that this is due to missorting of Tat2p to the vacuole. Even when *erg6* cells are grown at low tryptophan, Tat2p is targeted to the vacuole for degradation. In addition, Tat2p is missorted to the MVB pathway. These defects are suppressed by deletion of *BUL1*, suggesting that inappropriate ubiquitination of Tat2p is accountable for the missorting in the *erg6* mutant. This seems to be the case because ubiquitin conjugation in *erg6* cells occurs at extra lysine residues of Tat2p that do not receive ubiquitin in wild-type cells.

Why is Tat2p inappropriately ubiquitinated by the subtle change of sterol structure? We have shown that association with lipid rafts is required for Tat2p to be targeted to the plasma membrane, which implies that lipid rafts control the ubiquitination of Tat2p. It is known that lipid rafts in mammalian cells exist in rich concentrations in the plasma membrane and recycling endosomes, but not in late endosomes and lysosomes (for review see Simons and Gruenberg, 2000). Consistent with the skewed distribution of lipid rafts, Tat2p is sorted to the plasma membrane in association with lipid rafts, and to the vacuole when lipid rafts are disrupted in the *erg13* mutant. The *ERG13* gene encodes HMG-CoA synthase that catalyzes the initial step of ergosterol biosynthesis. The reduced uptake of tryptophan in *erg13* is suppressed when the polyubiquitination of Tat2p is inhibited by deletion of *BUL1*, indicating that raft-association and polyubiquitination have counteracting effects in the sorting

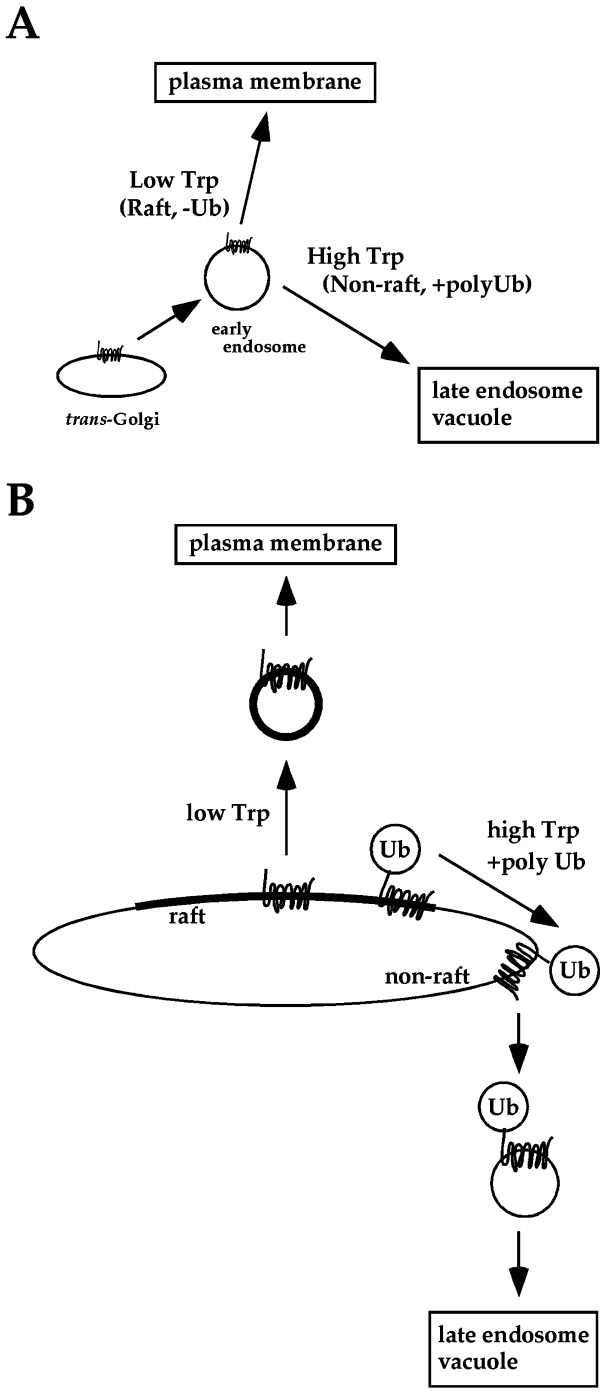


Fig. 2.

of Tat2p. Polyubiquitinated Tat2p is probably diverted from the raft-dependent plasma membrane targeting pathway to the non-raft pathway to late endosomes. Lafont and Simons (2001) have shown that the ubiquitin ligase Nedd4 is partitioned into lipid rafts. Interestingly, the yeast Nedd4 homologue Rsp5p is partially resistant to detergent extraction (Wang *et al.*, 2001), implying that polyubiquitination of Tat2p by the Rsp5p-Bul1p complex can occur in lipid rafts. Sorting receptors that bind polyubiquitin and divert cargo proteins to the non-raft membrane domains may exist. According to this concept, polyubiquitin may act as a signal for exclusion of cargo proteins from lipid rafts (Fig. 2B). It is likely that the properties of lipid rafts are altered by the ergosterol intermediates accumulated in *erg6*. Although the detergent-insolubility of GPI-anchored proteins indicates that lipid rafts are preserved in the *erg6* mutant (Sievi *et al.*, 2001; Umebayashi and Nakano, 2003), inappropriate ubiquitination would occur in such altered lipid rafts. Alternatively, polyubiquitination of Tat2p may occur in non-raft domains. Tat2p may not be able to associate with the lipid rafts of the *erg6* mutant, being mostly partitioned into non-raft domains where it is polyubiquitinated. It should be noted that another plasma membrane protein Fus1p, which is required for cell fusion during the mating process, is largely excluded from lipid rafts and mislocalized to the vacuole in the *erg6* mutant (Bagnat and Simons, 2002). This behavior of Fus1p is similar to that of Tat2p, and supports the view that a subset of plasma membrane proteins is missorted in *erg6* due to impaired raft association and inappropriate ubiquitination.

Conclusion and future perspectives

Owing to the extensive studies done to date, the molecular mechanisms that underlie the recognition and sorting of ubiquitinated cargo proteins are now fairly well understood. On the other hand, less is known about the regulation of ubiquitination, including how many ubiquitin ligases are involved in membrane trafficking. Because ubiquitination drastically affects the fate of cargo proteins in the traffick-

Fig. 2. Possible coordination of sterol and ubiquitin in protein sorting. A: The yeast tryptophan permease Tat2p is subject to regulated sorting, which is likely to occur in early endosomes (Umebayashi and Nakano, 2003). When a high concentration of tryptophan is included in the medium, Tat2p is sorted to late endosomes and the vacuole. At low tryptophan, in contrast, Tat2p is targeted to the plasma membrane. The plasma membrane targeting of Tat2p is dependent on its association with lipid rafts. On the other hand, polyubiquitination of Tat2p acts as a sorting signal to the vacuole. B: The counteracting effects of lipid rafts and polyubiquitination raises the possibility that polyubiquitinated Tat2p is excluded from lipid rafts. Tat2p may be polyubiquitinated in lipid rafts, captured by a sorting receptor that recognizes polyubiquitin, and removed from the rafts to be targeted to the vacuole.

ing pathway, it should be under stringent control. Upon ligand binding and nutrient shift, receptors and permeases may undergo conformational changes that enable the recognition by ubiquitin ligases. Alternatively, the activity and/or localization of ubiquitin ligases may be regulated. Considering that lipids are also involved in protein sorting, the environment in lipid bilayer may affect the processes of cargo ubiquitination. For example, lipid rafts may affect the conformation of cargoes such as oligomerization status, and/or the functions of ubiquitin ligases. Future research will proceed from the viewpoint of clarifying the coordinated actions of proteins and lipids.

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