Regulation of Membrane Protein Transport by Ubiquitin and Ubiquitin-Binding Proteins

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■ **Abstract**   Ubiquitin regulates protein transport between membrane compartments by serving as a sorting signal on protein cargo and by controlling the activity of trafficking machinery. Monoubiquitin attached to integral plasma membrane proteins or to associated transport modifiers serves as a regulated signal for internalization into the endocytic pathway. Similarly, monoubiquitin attached to biosynthetic and endocytic membrane proteins is a signal for sorting of cargo into vesicles that bud into the late endosome lumen for delivery into the lysosome. Ubiquitination of *trans*-acting endocytic proteins is also required for transport, and key endocytic proteins are modified by monoubiquitin. Regulatory enzymes of the ubiquitination machinery, ubiquitin ligases, control the timing and specificity of plasma membrane protein downregulation in such diverse biological processes as cell fate specification and neurotransmission. Monoubiquitin signals appended by these ligases are recognized by endocytic proteins carrying ubiquitin-binding motifs, including UBA, UEV, UIM, and CUE domains. The UIM proteins epsins and Hrs are excellent candidates for adaptors that link ubiquitinated cargo to the clathrin-based sorting machinery at appropriate regions of the endosomal or plasma membranes. Other ubiquitin-binding proteins also play crucial roles in cargo transport, although in most cases the role of ubiquitin-binding is not defined. Ubiquitin-binding proteins such as epsins, Hrs, and Vps9 are monoubiquitinated, indicating the general nature of ubiquitin regulation in endocytosis and suggesting new models to explain how recognition of monoubiquitin signals may be regulated.

CONTENTS

INTRODUCTION TO UBIQUITIN AND THE UBIQUITINATION MACHINERY

Ubiquitin is a 76–amino acid protein that has the unusual property of forming a stable chemical bond with other proteins. The ubiquitin C-terminal glycine carboxy group forms an isopeptide bond with the ε-amino group of lysine residues or, less commonly, with the amino group at the N terminus of a substrate protein. The attachment (conjugation) of ubiquitin to a protein can regulate the protein's stability, activity, or location. Ubiquitin modifies proteins involved in many, if not all, cellular functions, and in most cases it appears to act as a signal for recognition by a ubiquitin-binding protein.

Ubiquitin conjugation occurs by the sequential action of three enzymes: a ubiquitin-activating enzyme (E1), a ubiqitin-conjugating enzyme (E2), and a ubiqitin ligase (E3) (reviewed in Hershko & Ciechanover 1998, Weissman 2001). First, the E1 activates and forms a high-energy thiolester bond with ubiquitin. Ubiquitin is then passed to the E2 by transthiolation. The E2, usually in cooperation with an E3, transfers ubiquitin to a lysine side chain of the substrate. E3s specify the timing and substrate selection of ubiquitination reactions. Therefore, ubiquitin ligases are the key regulatory determinants in the ubiquitination reaction, analogous to kinases in phosphorylation reactions. Ubiquitin signals are removed or trimmed by deubiquitinating enzymes.

Proteins can be modified by a single ubiquitin moiety or by polymeric ubiquitin chains, referred to as mono- and polyubiquitination, respectively. At least three lysines within ubiquitin, Lys29, Lys48, and Lys63, are used to form polyubiquitin

chains (Arnason & Ellison 1994), and different types of ubiquitin modification are associated with distinct cellular functions. For example, Lys48-linked polyubiquitin chains regulate numerous nuclear, cytosolic, and ER membrane proteins by targeting these proteins for degradation by the 26S proteasome (Chau et al. 1989, Thrower et al. 2000). Lys63-linked chains regulate the endocytosis of some plasma membrane proteins in addition to a variety of other basic cellular processes, (Fisk & Yaffe 1999, Galan & Haguenauer-Tsapis 1997, Hoege et al. 2002 and references therein). Monoubiquitination also serves as an important regulatory signal (Hicke 2001b, Hoege et al. 2002, Salghetti et al. 2001), and its function is best characterized in the intracellular transport of proteins through the late secretory and endocytic pathways.

In this review we discuss the multiple roles played by ubiquitin in regulating protein transport between membrane compartments. The identification of ubiquitinated substrates, ubiquitin ligases, deubiquitinating enzymes and ubiquitin-binding proteins that are involved in regulating intracellular protein trafficking has been rapid in the past several years. Here, we highlight the importance of ubiquitin-dependent protein sorting in diverse biological processes and illustrate what we currently understand about the molecular mechanisms underlying this phenomenon.

UBIQUITIN SORTING SIGNALS IN THE BIOSYNTHETIC AND ENDOCYTIC PATHWAYS

Proteins and Processes Regulated by Ubiquitin-Dependent Endocytosis

Ubiquitin regulates protein transport by at least two different mechanisms: (*a*) Ubiquitin can modify the activity of the protein transport machinery and (*b*) ubiquitin can serve as a sorting signal attached to transmembrane proteins to direct their movement between different cellular compartments (Figure 1).

The best-characterized functions of ubiquitin in regulating membrane protein transport are in the endocytic pathway. Ubiquitin serves as a signal for the entry of endocytic cargo into vesicles both at the plasma membrane and at the late endosome. Cell surface proteins enter primary endocytic vesicles that bud from the plasma membrane and are delivered to an early endosome. At the early endosome, divergent exit pathways lead to recycling of cargo back to the cell surface or to transport of cargo to a late endosome. Mature late endosomes, also known as multivesicular bodies (MVBs), form when a portion of the late endosomal membrane invaginates and pinches off into the lumen of the organelle, generating interior lumenal vesicles. Fusion of the MVB with the lysosome results in proteolysis of MVB vesicle lipids and proteins (for a review of MVB sorting see Katzmann et al. 2002). Sorting of cargo, such as signaling receptors, into endocytic vesicles at the plasma membrane and into MVB vesicles are crucial steps in downregulation. In

the yeast *Saccharomyces cerevisiae*, ubiquitin appears to be the major, although
not the only (Reggiori & Pelham 2001, Tan et al. 1996), signal to recruit cargo
into vesicles budding from either the plasma membrane or the MVB. In higher
eukaryotes, ubiquitin serves as a sorting signal for proteins that are internalized
and/or sorted into MVB vesicles in a regulated manner.

One large class of proteins that undergo regulated internalization and lysoso-
mal degradation are signal-transducing receptors. These proteins are often con-
stitutively internalized at a slow rate. Ligand binding then stimulates receptor
internalization and degradation as a mechanism to downregulate receptor activity
and signal transduction. For instance, a G protein–coupled receptor (GPCR) in
yeast becomes rapidly ubiquitinated on its cytoplasmic tail in response to bind-
ing pheromone ligand. Ubiquitination of this receptor serves as a signal both to
promote internalization into primary endocytic vesicles budding from the plasma
membrane and to sort the receptor into vesicles budding into the late endosome
for delivery into the lysosome-like vacuole. Some, but not all, mammalian GPCRs
are also ubiquitinated in response to ligand binding (Marchese & Benovic 2001,
Shenoy et al. 2001, Tanowitz & von Zastrow 2002). Unlike the yeast GPCRs,
the known ubiquitinated mammalian GPCRs, the β_2-adrenergic and the CXCR4
chemokine receptors, appear to require ubiquitination only as a sorting signal at
the MVB, not at the plasma membrane. Receptor tyrosine kinases (RTKs) are also
downregulated by ubiquitin-dependent endocytosis that leads to receptor degrada-
tion in the lysosome. In this case, the list of ubiquitinated receptors is extensive and
ubiquitin is used to signal both internalization and sorting into the MVB (Table 1).

Ubiquitin regulates signaling receptors of the immune system in both normal
and infected cells. Several immune receptors and co-stimulatory molecules are
modified by ubiquitin (see Table 1), and for some of these proteins specific com-
ponents of the ubiquitination machinery are known to directly influence trafficking
(Booth et al. 2002, Goto et al. 2003, Naramura et al. 2002). Ubiquitin-dependent
endocytosis has also been co-opted by viruses to downregulate key molecules of
the host immune system as an evasion mechanism. Virus-encoded E3s modify nor-
mally stable plasma membrane proteins, such as major histocompatibility class I
(MHC1) molecules (see Table 1), with a ubiquitin signal that causes rapid internal-
ization and sorting to lysosomes for irreversible inactivation by degradation (e.g.,
Coscoy et al. 2001, Hewitt et al. 2002).

Transporters and channels make up another large class of proteins that undergo
regulated endocytosis mediated by ubiquitin signals (Table 1). In yeast, amino
acid permeases, peptide transporters, and sugar transporters are ubiquitinated and
use ubiquitin as a signal for internalization and/or endosomal sorting (reviewed
in Katzmann et al. 2002, Rotin et al. 2000). In mammalian cells, ion channels are
downregulated by lysosomal degradation that requires prior ubiquitination (Staub
et al. 1997; see Table 1). Although the mammalian sugar transporters GLUT1 and
GLUT4 are not known to be ubiquitinated, they are modified with the ubiquitin-like
protein SUMO (Lalioti et al. 2002 and references therein), and the expression level
of the SUMO conjugating enzyme, Ubc9, appears to affect transporter cell surface

TABLE 1   Selected transmembrane proteins regulated by ubiquitin-dependent sorting

Transmembrane protein	Cis signal(s) for ubiquitination	E3	Cellular function	References
S. cerevisiae—signal transduction/mating				
α-factor receptor (Ste2)	SINNDAKSS; phosphoSer/Thr	Rsp5	GPCR; response to mating pheromone	(Dunn & Hicke 2001b and references therein; Hicke 1999)
α-factor receptor (Ste3)	PEST-like		GPCR; response to mating pheromone	(Roth & Davis 2000 and references therein)
α-factor transporter (Ste6)	DAKTI		Peptide transporter; pheromone biosynthesis	(Kölling & Losko 1997, Losko et al. 2001 and references therein)
S. cerevisiae—nutrient/ion transport				
Uracil permease (Fur4)	PEST-like	Rsp5	Nutrient uptake	(Rotin et al. 2000)
General amino acid permease (Gap1)	PEST-like	Rsp5	Amino acid uptake	(Helliwell et al. 2001, Soetens et al. 2001, Springael & Andre 1998)
Tryptophan permease (Tat2)	N-terminal 31 amino acids	Rsp5	Amino acid uptake	(Beck et al. 1999)
S. cerevisiae—catabolism				
Carboxypeptidase S	PEVKAPR		Vacuolar degradation	(Katzmann et al. 2001)
Polyphosphate endophosphatase (Phm5)	N-terminal cytoplasmic tail (Lys6)		Vacuolar degradation	(Reggiori & Pelham 2001)
Metazoans—regulation of growth, differentiation, and/or metabolism				
Growth hormone receptor (GHR)	UbE motif[a]		Chemokine receptor; signal transduction	(Strous & Govers 1999)
EGFR	PhosphoTyr	Cbl	RTK; signal transduction	(Shtiegman & Yarden 2003)
Macrophage colony-stimulating factor (CSF-1) receptor	PhosphoTyr	Cbl	RTK; hematopoiesis	(Lee et al. 1999, Wilhelmsen et al. 2002)
PDGFR	PhosphoTyr	Cbl	RTK; chemotaxis, development in the nervous system	(Joazeiro et al. 1999, Miyake et al. 1998)
Hepatocyte growth factor receptor (c-Met)	PhosphoTyr	Cbl	RTK; epithelial and endothelial cell motility and morphogenesis	(Hammond et al. 2001, Peschard et al. 2001, Petrelli et al. 2002)
Transforming growth factor β receptor	PPXY motif[a] in Smad7 modifier protein	Smurf1/2	Heterodimeric type I/II RTK; regulation of growth and development	(Ebisawa et al. 2001, Kavsak et al. 2000)
β₂-adrenergic receptor			GPCR; relaxation of smooth muscle cells	(Shenoy et al. 2001)

(Continued)

TABLE 1 (*Continued*)

Transmembrane protein	*Cis* signal(s) for ubiquitination	E3	Cellular function	References
Metazoans—immune response				
Interleukin-2 receptor	PDPSKFFQSL (α-helical)		Cytokine receptor; lymphokine signaling	(Rocca et al. 2001, Yu & Malek 2001)
T cell receptor	Phospho-Tyr; (through Zap-70 Cbl adaptor)	Cbl, Cbl-b	Immune recognition	(Cenciarelli et al. 1992, Naramura et al. 2002, Wang et al. 2001)
CXCR4	SSLKILSKGK		GPCR; chemokine signaling	(Marchese & Benovic 2001)
IgG receptor (Fcγ receptor II)			Immunoglobulin superfamily; phagocytosis of antibody particles	(Booth et al. 2002)
Pre-T cell receptor		Cbl	Immunoglobulin superfamily; T cell development	(Panigada et al. 2002)
B7-2	*Trans*- and juxta-membrane domains, cytosolic lysines	c-MIR	Transmembrane co-stimulatory protein; antigen presentation and T cell activation	(Goto et al. 2003)
Metazoans—neurotransmission				
AMPA glutamate receptors	LGEFLYRSRIEARK		Ligand-gated ion channel; regulation of synaptic strength	(Burbea et al. 2002)
Glycine receptors			Ligand-gated ion channel; postsynaptic inhibition	(Büttner et al. 2001)
Metazoans—cell-cell contacts				
E-cadherin	PhosphoTyr	Hakai	Cell adhesion	(Fujita et al. 2002)
Occludin	PPYP motif	Itch	Cell polarity; permeability barrier	(Traweger et al. 2002)

	Motif/domain	Ligase	Function	References
Metazoans—developmental patterning				
Delta		Neuralized	Transmembrane Notch	(Deblandre et al. 2001, Lai et al. 2001, Pavlopoulos et al. 2001)
Notch		Cbl, Itch	Receptor for Delta; development	(Jehn et al. 2002, Qiu et al. 2000)
Roundabout (Robo)	PPXY motifs[a] in Comm modifier protein	Nedd4	Roundabout receptor family; Axon guidance	(Myat et al. 2002)
Metazoans—ion channels				
Epithelial sodium channel (ENaC)	PPXY motifs	Nedd4, WWP2	Sodium resorption	(Rotin et al. 2000)
ClC-5 chloride channel	PPXY-like motif, PPLPPYTPP	WWP2	Resorption of low molecular weight proteins in kidney	(Schwake et al. 2001)
Metazoans—immune molecules downregulated by viruses				
MHC class I	Trans- and juxta-membrane domains	K3/MIR1; K5/MIR2	Immunoglobulin superfamily; antigen presentation	(e.g., Coscoy et al. 2001, Hewitt et al. 2002)
B7-2	Trans- and juxta-membrane domains	K5/MIR2	Antigen presentation; T cell activation	(Coscoy et al. 2001)
ICAM-1		K5/MIR2	Antigen presentation; T cell activation	(Coscoy & Ganem 2001)
CD4		M153R	TCR-associated protein; T cell signaling	(Mansouri et al. 2003)

[a]This motif mediates ubiquitin-dependent sorting, but does not necessarily promote ubiquitination of the transmembrane protein itself.

activity. Similar to ubiquitin, SUMO may also regulate protein transport, either by serving as a signal (although SUMO cannot serve as an internalization signal in yeast; J. Jemc & L. Hicke, unpublished data), or by competing with ubiquitin for lysine modification sites.

In neurons, ubiquitin has emerged as a major regulatory molecule in many processes, including vesicle-mediated protein transport (Hegde & DiAntonio 2002). The ubiquitination of *Caenorhabditis elegans* glutamate receptors and of mammalian neuronal glycine receptors expressed in *Xenopus* correlates with the level of cell surface receptor activity and lysosomal degradation (Burbea et al. 2002, Büttner et al. 2001). These observations suggest that neurotransmitter receptors are regulated by a ubiquitin endocytic signal. In addition, axon guidance in the developing *Drosophila* nervous system is regulated by ubiquitin-dependent transport of a cell surface receptor called Roundabout (Robo) that receives a signal important during the navigation of extending axons. The discovery of E3s that target the transforming growth factor-β (TGFβ) receptor and components of the Notch signaling pathway for ubiquitination highlights a general role for ubiquitin-regulated endocytosis in development. Control of TGFβ receptor and Notch signaling by ubiquitin-dependent endocytosis are described in more detail in the following sections.

Recent evidence suggests an important role for ubiquitin-dependent endocytosis in cell adhesion and polarity. E-cadherin, a key transmembrane protein of epithelial adherens junctions, undergoes endocytosis under certain conditions (e.g., during mitosis) to allow modulation or remodeling of cell-cell contacts. In a series of events similar to ligand stimulation of growth factor receptors, tyrosine phosphorylation of E-cadherin by Src kinase stimulates its ubiquitination and subsequent internalization (Fujita et al. 2002). Ubiquitin-dependent endocytosis may independently regulate cell polarity by controlling the cell surface level of occludin, a tight junction protein that spans the membrane four times. Occludin interacts with and is ubiquitinated by the ubiquitin ligase Itch (Traweger et al. 2002).

Finally, as in the cytosol and the ER, ubiquitin probably regulates quality control at the plasma membrane and endosome. Mutant or unassembled versions of a plasma membrane protein that is normally stable at the cell surface undergo ubiquitin-dependent internalization (Gong & Chang 2001, Wang & Chang 2002). In the late secretory pathway, the Tul1 ubiquitin ligase has been proposed to modify abnormal proteins with a ubiqitin signal that targets them into MVB vesicles for destruction in the lysosome (Reggiori & Pelham 2002). Thus at each transport step where ubiquitin acts as sorting signal for normal proteins, it might also serve a quality control function, directing abnormal membrane proteins to the lysosome for degradation.

Ubiquitin as an Internalization Signal at the Plasma Membrane

Although the modification of plasma membrane proteins with ubiquitin was demonstrated several decades ago, proof that ubiquitin acts as a regulated internalization signal came from relatively recent studies in yeast (reviewed in Hicke 1999,

Mosesson et al. 2003, Rotin et al. 2000). Initially, investigators observed that GPCRs, permeases, and transporters accumulated as ubiquitinated forms in yeast mutants that were defective at the internalization step of endocytosis. Lysines in cytoplasmic domains of these proteins were found to be crucial for rapid internalization, and mutations in genes encoding the cellular ubiquitination machinery severely inhibited internalization. These observations demonstrated that ubiquitination of plasma membrane cargo is required for internalization.

The ubiquitin internalization signal acts autonomously. Ubiquitin does not require additional sequences in the cargo protein to function, and the fusion of ubiquitin to the cytoplasmic tail of a normally stable plasma membrane protein induces internalization (Haglund et al. 2003, Nakatsu et al. 2000, Roth & Davis 2000, Shih et al. 2000). The internalization signal in ubiquitin is three-dimensional and does not resemble previously identified linear peptide internalization signals. Instead, the internalization information in ubiquitin maps to two hydrophobic patches on distinct surfaces of the ubiquitin molecule (Shih et al. 2000). One patch surrounds Ile44, an essential residue that is also required for proteasomal targeting. The second hydrophobic patch, surrounding Phe4, is not required for proteasome targeting but is required for yeast cell viability (Sloper-Mould et al. 2001 and references therein).

Conjugation of monoubiquitin to a single lysine residue on a cytoplasmic domain is sufficient for rapid internalization of several proteins in yeast, and monoubiquitin is also a sufficient internalization signal in mammalian cells (Hicke 2001b). For other proteins, specifically a subset of yeast nutrient permeases, maximal internalization rates require the formation of di-ubiquitin chains linked through Lys63 of ubiquitin (e.g., Galan & Haguenauer-Tsapis 1997). At this time it is not known how Lys63-linked ubiquitin chains might further stimulate rapid internalization; however, Lys63-linked di-ubiquitin may simply present tandem internalization signals in a conformation that increases signal efficiency.

As described above, many growth factor receptors undergo ligand-stimulated ubiquitination, and this modification appears to control both internalization and subsequent endosomal sorting (reviewed in Shtiegman & Yarden 2003, Haglund et al. 2003, Mosesson et al. 2003). However, in many cases these receptors appeared to be polyubiquitinated rather than monoubiquitinated. This paradox has now been resolved by careful demonstration that the EGFR is not modified with polyubiquitin chains, but with monoubiquitin appended to multiple tail lysines (Haglund et al. 2003). The presence of multiple monoubiquitin molecules on a receptor tail, rather than a single monoubiquitin, may increase the avidity or diversity of interaction with monoubiquitin-binding components of the endocytic machinery, thereby maximizing internalization rate.

Ubiquitin as an Endosomal Sorting Signal

The endocytic and biosynthetic pathways converge at the MVB where ubiquitination signals the sorting of both endocytic and biosynthetic transmembrane proteins into vesicles destined for delivery into the lysosome/vacuole (Figure 1; reviewed

in Katzmann et al. 2002). Receptors that cannot be ubiquitinated and sorted into lumenal MVB vesicles recycle back to the plasma membrane or remain on the limiting membrane of the organelle, thereby escaping downregulation. Thus the roles of ubiquitin at both the plasma membrane and MVB are crucial for regulation of cell surface protein activity.

The first hint that ubiquitin might play a role in the late endocytic pathway came from studies on the intracellular trafficking of internalized growth factor receptors in mammalian cells. Overexpression of a specific ubiquitin ligase dramatically stimulates the sorting of endosomal EGFR to the lysosome, without affecting the rate of EGFR internalization (Levkowitz et al. 1998). Experiments with the *doa4* yeast mutant, defective in a deubiquitinating enzyme, also suggested that ubiquitin is involved in endosomal sorting. Endocytic cargo that is normally targeted to the vacuole lumen is missorted to the vacuole limiting membrane in a *doa4*Δ mutant, and this effect can be reversed by ubiquitin overexpression (e.g., Losko et al. 2001).

Conclusive evidence that ubiquitin acts as a signal for entry into MVB vesicles came from the study of resident vacuolar proteins that traverse the biosynthetic pathway in yeast (Katzmann et al. 2002). Precursors of some vacuolar enzymes are monoubiquitinated on their cytosolic domains. Inhibiting ubiquitin modification, by mutations either in cargo ubiquitination sites or in enzymes of the ubiquitination machinery, blocks entry of the precursors into MVB vesicles. Fusion of ubiquitin to the cytoplasmic domain of MVB vesicle cargo proteins that lack post-translational ubiquitination sites rescues the ability of the cargo to enter the vacuole lumen (Reggiori & Pelham 2001, Urbanowski & Piper 2001). These observations indicate that ubiquitin is necessary and sufficient to sort multiple cargo proteins into MVB vesicles. Ubiquitination is also required for a variety of mammalian proteins to enter MVB vesicles and be degraded in the lysosome (reviewed in Katzmann et al. 2002, Shtiegman & Yarden 2003) (see Table 1). Ubiquitin acts as a sufficient signal for endosomal sorting in metazoans because fusion of ubiquitin to a protein (the transferrin receptor) that normally recycles back to the plasma membrane after internalization prevents recycling (Raiborg et al. 2002).

The function of ubiquitin as a signal for MVB sorting has been co-opted by a subset of enveloped viruses to promote budding of nascent virions from the cell surface (reviewed in Pornillos et al. 2002b; Figure 1). Although enveloped viruses bud from the plasma membrane, the topology of membrane deformation and scission is equivalent to the inward budding of MVB vesicles into the endosome lumen. Ubiquitination of virus-encoded assembly proteins (Gag proteins) and components of the ubiquitin-dependent sorting machinery are functionally linked to the final step of virus budding from the plasma membrane. Thus viruses manipulate ubiquitin signals in multiple ways to influence trafficking of both host and viral cargo.

Ubiquitin-Dependent Sorting at the *trans*-Golgi Network

Newly synthesized proteins that arrive at the *trans*-Golgi network (TGN) are sorted either to the plasma membrane or to an endosomal compartment en route to the lysosome (Figure 1). Yeast amino acid permeases can take either route depending

on extracellular nutrient availability, and it is now apparent that ubiquitin signals play an important role in this decision (Beck et al. 1999, Helliwell et al. 2001, Soetens et al. 2001). Conditions that promote transport of permeases directly to the vacuole also induce ubiquitination of the permease by the Rsp5 ubiquitin ligase. Regulators of Rsp5, Bul1 and Bul2, are required for conversion of permease to a polyubiquitinated form, suggesting that polyubiquitination is necessary for vacuolar targeting of permeases (Helliwell et al. 2001). Although there is controversy over the role of polyubiquitin versus monoubiquitin in regulating this sorting decision (Soetens et al. 2001), the data clearly indicate that ubiquitin regulates the choice between secretion and routing to the late endocytic pathway at the TGN. It is likely that this sorting mechanism also functions in higher eukaryotes. For example, the cell surface localization of Robo appears to be controlled by regulated diversion of a ubiqitinated Robo complex from the secretory pathway to the lysosome (Keleman et al. 2002).

It is not clear whether ubiquitin-dependent sorting of cargo exiting from the TGN is related to ubiquitin-dependent sorting into MVB vesicles. Permeases destined for the cell surface probably recycle between the late endosome and the TGN before being packaged into secretory vesicles (Helliwell et al. 2001 and references therein). Thus a plasma membrane versus vacuole destination may depend on whether permeases are ubiquitinated, enter MVB vesicles, and are degraded, or whether they remain on the limiting membrane of the MVB and are recycled back to the TGN for another chance at entering vesicles destined for the plasma membrane. If this scenario is correct, the proposed location differences due to mono- versus polyubiquitination may be because the permeases, unlike other MVB cargo, require oligo- or polyubiquitin chain signals to efficiently enter MVB vesicles, as they do for internalization into primary endocytic vesicles at the plasma membrane (Galan & Haguenauer-Tsapis 1997).

REGULATION OF THE TRANSPORT MACHINERY BY UBIQUITINATION

Evidence that Ubiquitin Regulates *trans*-Acting Endocytic Proteins

The conclusion that ubiquitination regulates the activity of the endocytic machinery comes from observations that ubiquitination enzymes are required for the endocytosis of proteins that do not require attachment of a ubiquitin-sorting signal. The growth hormone receptor (GHR) is one well-studied cargo protein that does not use ubiquitin as an internalization signal but requires active ubiquitination machinery for rapid internalization (Strous & Govers 1999, van Kerkhof et al. 2000). Like many signaling receptors, GHR undergoes ligand-stimulated ubiquitination at the cell surface and is then transported through the endocytic pathway to the lysosome for degradation. However, a mutant GHR lacking any ubiquitination sites is internalized normally, even though internalization is blocked by inactivation of

a temperature-sensitive E1 allele. A simple interpretation of these observations is that ubiquitination of endocytic machinery, rather than the cargo, is necessary for GHR internalization (although this does not explain why the receptor becomes ubiquitinated).

Similarly, in yeast, the internalization of receptors that do not need to be post-translationally modified with ubiquitin is still dependent on both ubiquitin-conjugating enzymes and the Rsp5 ubiquitin ligase. Rsp5 is required for the internalization of a receptor that carries an in-frame fusion of ubiquitin to its cytoplasmic tail and for internalization of a fluid-phase endocytic marker (Dunn & Hicke 2001b). Thus Rsp5-dependent ubiquitination of *trans*-acting proteins is necessary for efficient internalization of primary endocytic vesicles. Ubiquitination of proteins that act in the late endocytic pathway is also likely to be important (see below).

Specific proteasome inhibitors impair the internalization, endosomal sorting and degradation of the GHR and other mammalian endocytic cargo (e.g., Longva et al. 2002, van Kerkhof et al. 2000, Yu & Malek 2001). Despite the functional requirement for proteasome activity, degradation of the ubiquitinated cargo occurs by lysosomal hydrolysis (Hammond et al. 2001, Strous & Govers 1999). Thus rather than directly degrading the transmembrane protein, the proteasome probably regulates discrete steps of endocytic transport. One role for the proteasome might be to regulate the stability of endocytic proteins. However, the components of the endocytic machinery that are known to undergo ubiquitination are primarily monoubiquitinated (see below). It is possible that a monoubiquitinated endocytic protein that has performed its function is then transiently polyubiquitinated and targeted to the proteasome for obligatory degradation, as has been proposed for activated transcription factors (Salghetti et al. 2001). Alternatively, endocytic factors that are targets of the proteasome have not yet been identified.

Monoubiquitinated Endocytic Proteins

What are the components of the endocytic machinery regulated by ubiquitination? Several specific and general endocytic proteins are known to be modified by ubiquitin, but how ubiquitin regulates their activity or location is not yet understood.

β-arrestin binds to phospholipids, clathrin, and clathrin adaptors and links activated GPCRs to the clathrin-dependent endocytosis machinery. β-arrestin also undergoes rapid and transient ubiquitination after ligand stimulation of β_2-adrenergic receptors (Shenoy & Lefkowitz 2003, Shenoy et al. 2001). The ubiquitin ligase that catalyzes β-arrestin ubiquitination, Mdm2, is required for rapid receptor internalization. Furthermore, translational fusion of ubiquitin to β-arrestin increases receptor internalization rate, suggesting that β-arrestin ubiquitination is important for internalization. Lysine residues in the receptor 'are not required for internalization, consistent with the idea that ubiquitination of β-arrestin, and not of the β_2-adrenergic receptor, promotes rapid endocytosis. One of the functions of β-arrestin ubiquitination appears to be to promote β-arrestin association with a GPCR, because a ubiquitin-β-arrestin fusion, which cannot be deubiquitinated, remains associated and internalizes with the receptor into endosomes. In

addition, β-arrestin illustrates one example of an emerging class of proteins that are modified with a ubiquitin signal but are not the ultimate targets of the ubiquitin-dependent trafficking event. Proteins within this class are not general components of the transport machinery and instead function as ubiquitinated transport modifiers of specific target proteins. Ubiquitinated transport modifiers may facilitate interaction of the modifier-target complex with ubiquitin-binding proteins of the transport machinery to promote target internalization and/or degradation.

A specific ubiquitinated transport modifier that functions in regulating the cell surface activity of Robo during axon guidance in the developing nervous system is the integral membrane protein Commissureless (Comm). Robo is a *Drosophila* cell surface receptor that transmits a repulsive signal during the navigation of extending axons. In axons that are not sensitive to repulsive signals, Robo is synthesized but is rapidly downregulated due to association with Comm. Comm is ubiquitinated and negatively regulates Robo cell surface activity by providing a ubiquitin sorting signal in *trans* to downregulate the Robo/Comm complex. Ubiquitination increases Robo internalization (Myat et al. 2002) but also diverts newly synthesized Robo from the secretory pathway to the lysosome (Keleman et al. 2002) as a mechanism to decrease cell surface activity. Therefore, Robo is another protein for which regulated transport is not controlled by its own ubiquitination state but by its ability to associate with a ubiquitinated transport modifier.

CIN85 (Cbl-interacting protein of 85 kDa) is a general component of the endocytic machinery, first identified as a protein that interacts with Cbl, a ubiquitin ligase that modifies activated growth factor receptors. CIN85 binds stably to endophilins, proteins that regulate clathrin-coated vesicle budding, and CIN85-endophilin binding to Cbl is stimulated by activation of growth factor receptors. Together the CIN85-endophilin-Cbl complex is required for ligand-stimulated receptor internalization, but not receptor ubiquitination (Petrelli et al. 2002, Soubeyran et al. 2002). Association of CIN85 with Cbl and CIN85 ubiquitination may be important for rapid internalization because mutations that ablate the interaction impair ligand-dependent receptor internalization without disrupting Cbl-mediated receptor ubiquitination. CIN85 itself is monoubiquitinated by Cbl in response to EGF treatment of cells (e.g., Haglund et al. 2002) and by the Nedd4 ubiquitin ligase in the absence of EGF, suggesting that monoubiquitin regulates its activity or interaction with binding partners.

Numb is an endocytic protein that binds to Eps15 and the AP-2 clathrin adaptor and negatively regulates Notch during development (Santolini et al. 2000 and references therein). This negative regulation probably occurs by promoting receptor endocytosis because the overexpression of Numb fragments causes a defect in EGF receptor internalization in mammalian cells (Santolini et al. 2000). Another Numb-interacting protein, LNX, is a ubiquitin ligase that modifies Numb and decreases Numb stability (Nie et al. 2002). Proteasome inhibitors reduce Numb degradation, although it is not known whether Numb is degraded in the lysosome or by the proteasome, or whether Numb turnover is important for its endocytic function.

In addition to the proteins mentioned above, several endocytic proteins that carry ubiquitin-binding domains are monoubiquitinated. These include the epsins

and Eps15, which act at the internalization step of endocytosis, and Hrs (yeast Vps27) and Rabex (yeast Vps9), which act in endosomal transport. These proteins and their modification with ubiquitin are discussed further below.

UBIQUITIN LIGASES AND DEUBIQUITINATING ENZYMES THAT REGULATE PROTEIN TRANSPORT

Ubiquitin Ligases

Ubiquitin ligases are classified into two major families (reviewed by Pickart 2001, Weissman 2001). The first family, characterized by the zinc-binding RING (really interesting new gene) finger domain and related domains, promotes ubiquitination by simultaneously binding the substrate and an E2 (Joazeiro & Weissman 2000). The second family, defined by the HECT (homologous to E6-AP carboxy terminus) domain, participates directly in catalysis by forming an obligate thiolester bond with ubiquitin during the ubiquitination reaction (Huibregtse et al. 1995). Because E3s select substrates for modification at the correct time and place, understanding the function of this class of enzymes is critical for understanding events regulated by ubiquitin in vivo. Ubiquitin ligases are emerging as a class of enzymes as important in cellular regulation as kinases.

Many substrate-specific E3s for endocytic cargo proteins have been identified, and the list is growing rapidly (Figure 2). Genetic evidence indicates that many of these E3s are important negative regulators of their membrane substrates. To date, the most extensively characterized ubiquitin ligases that function in endocytosis are HECT domain E3s in the Rsp5/Nedd4 family and the RING finger protein Cbl. Human diseases that result from mutations that inhibit E3-cargo interaction highlight the importance of these ligases and their role in endocytosis. Cbl was originally described as the product of a proto-oncogene, and Nedd4 is intimately connected to the development of Liddle's syndrome, a dominant hereditary form of hypertension.

RSP5/NEDD4 FAMILY OF HECT DOMAIN UBIQUITIN LIGASES   HECT domain ligases share a conserved ∼350 amino acid catalytic domain. An essential cysteine within this domain forms a thiolester bond with ubiquitin during transfer of ubiquitin from an E2 to a substrate. The Rsp5/Nedd4 HECT domain ligases share a highly similar structural organization (Figure 2), and many members of this family are required for or implicated in regulating protein transport (reviewed in Rotin et al. 2000). At the N terminus of these proteins is a C2 domain, a protein module that can bind proteins and lipids, followed by two to four copies of the WW protein-protein interaction domain that binds to proline-rich target sequences or to phosphoserine/threonine (Sudol & Hunter 2000). At the C terminus is the HECT catalytic domain that binds ubiquitin-conjugating enzymes and contains the catalytic cysteine residue. Rsp5 is the sole member of the C2-WW-HECT E3s in

S. cerevisiae. Rsp5 is an essential protein required for ubiquitin-dependent membrane trafficking, in addition to other cellular functions.

Rsp5 appears to be responsible for the ubiquitination of all yeast plasma membrane proteins that use ubiquitin as an internalization signal (reviewed in Rotin et al. 2000), although the mechanism by which Rsp5 recognizes endocytic cargo proteins is an enigma. Structure-function analyses suggest that the WW protein-protein interaction domains are important for cargo protein recognition (Dunn & Hicke 2001a, Gajewska et al. 2001); however, plasma membrane substrates do not contain consensus PPXY motifs predicted to bind Rsp5 WW domains. Ubiquitination of endocytic cargo often requires prior phosphorylation of the cargo's cytoplasmic domain (Hicke 1999, Rotin et al. 2000), but Rsp5 WW domains do not contain the key arginine residue that is important for phosphoserine/threonine recognition by WW domains (Sudol & Hunter 2000). Thus there is not a simple prediction for how Rsp5 would directly bind phosphorylated cargo. Rsp5 may recognize cargo by a novel type of interaction mechanism that has not yet been elucidated. Alternatively, Rsp5 could require an adaptor protein as a link to plasma membrane substrates, or Rsp5 may not be the direct ubiquitin ligase for endocytic cargo.

In addition to its role in ubiquitin-dependent internalization, Rsp5 is required for the sorting of biosynthetic cargo into MVB vesicles (R. Haguenauer-Tsapis, personal communication; D. Katzmann, personal communication; A. Adler, R. Dunn & L. Hicke, unpublished data). Rsp5 is also the ubiquitin ligase that functions together with the Bul1/Bul2 proteins to ubiquitinate and divert secretory cargo from the TGN to the vacuole. Finally, Rsp5 interacts with and ubiquitinates multiple *trans*-acting endocytic proteins (see below), emphasizing its central role as a regulator of protein trafficking in the endosomal system.

Nedd4 (neuronally expressed developmentally downregulated 4) is a vertebrate homologue of Rsp5 that also plays multiple roles in protein transport. *Drosophila* Nedd4 ubiquitinates Comm to control Robo localization, and mammalian Nedd4 ubiquitinates plasma membrane cargo and *trans*-acting endocytic proteins. The role of Nedd4 in protein transport was first characterized in the negative regulation of ENaC, the major ion channel that controls salt and fluid resorption in the kidney, colon, and lung. Nedd4 WW domains bind directly to PPXY motifs in the cytosolic domains of channel subunits that are mutated in Liddle's syndrome. ENaC is ubiquitinated by Nedd4, and ubiquitination regulates channel activity and stability at the cell surface (Staub et al. 1996, 1997). Nedd4 also interacts with the serum and glucocorticoid-regulated kinase (SGK). SGK contains two PPXY motifs that bind Nedd4 WW domains, and SGK-dependent Nedd4 phosphorylation inhibits Nedd4-ENaC interaction, thus increasing channel activity at the cell surface (Debonneville et al. 2001, Snyder et al. 2002). The PPXY motifs of SGK are conserved in the yeast SGK homologue Ypk1p, which also plays a role in regulating ubiquitin-dependent internalization (deHart et al. 2002). SGK is itself ubiquitinated (Brickley et al. 2002), although whether this modification is catalyzed by Nedd4 or is important for regulation of endocytosis is unknown.

Other C2-WW-HECT ubiquitin ligases have been implicated in the ubiquitin-dependent downregulation of Notch and receptors of the TGFβ family of peptide hormones. The transmembrane receptor Notch has a number of essential roles in the development of multicellular organisms, including the differentiation of immune cells. Suppressor of deltex and Itch are Nedd4-like E3s expressed in *Drosophila* and mice, respectively, that are implicated as negative regulators of Notch signaling (Cornell et al. 1999, Perry et al. 1998). Itch WW domains bind to the intracellular domain of membrane-tethered Notch, and Itch promotes ubiquitination of Notch in cultured cells (Qiu et al. 2000), although a link between Itch and Notch endocytosis has not been established. Notch signaling may be additionally regulated by interactions with the RING finger ubiquitin ligase Cbl (Jehn et al. 2002).

Smurfs (Smad ubiquitin regulatory factor) are C2-WW-HECT ubiquitin ligases that negatively regulate receptors of the TGFβ family of peptide hormones. Receptor-associated signaling molecules called Smads contain PPXY motifs that mediate interaction with the WW domains of Smurfs (Ebisawa et al. 2001, Kavsak et al. 2000). In transfected cells, co-expression of Smurf and Smad7 induces recruitment of the Smurf/Smad complex to the receptor and ubiquitination of Smad7. The catalytic cysteine residue of Smurf and the PPXY motif of Smad7 are required for degradation of the TGFβ receptor–Smad complex, indicating that ubiquitination is important for TGFβ receptor downregulation. Inhibitors of both the lysosome and proteasome diminish receptor degradation, indicating that ubiquitin-dependent endocytosis is responsible for TGFβ receptor turnover. Smad7 may function as a ubiquitinated transport modifier for the TGFβ receptor, or ubiquitination of the receptor itself may be necessary for downregulation.

RING FINGER UBIQUITIN LIGASES   Ubiquitin ligases that carry a RING finger domain exist as multisubunit complexes, or as monomers with substrate binding information and E3 activity built into the same molecule (Joazeiro & Weissman 2000). Genome sequencing suggests that RING finger E3s may far outnumber their HECT domain counterparts, and the identification of E3 activity in domains related to the RING finger, the PHD (plant homeodomains) and U box (UFD2-homology domain), lengthens the list (e.g., Coscoy et al. 2001, Hatakeyama et al. 2001). The RING finger directly interacts with an E2 and the substrate. RING finger ligases do not function as enzymes per se, but instead activate E2s to modify specific substrates.

Cbl is a RING finger E3 that promotes ubiquitin-dependent endocytosis of growth factor receptors (reviewed in Shtiegman & Yarden 2003). Cbl also negatively regulates immune receptor signaling by ubiquitination of transmembrane and soluble components of immune receptor signaling complexes (Lupher et al. 1999, Naramura et al. 2002, Panigada et al. 2002). Cbl proteins are expressed in metazoan organisms from nematodes to humans. The highly conserved amino terminal half of Cbl contains a phosphotyrosine binding domain (PTB) and a RING finger (Figure 2). The variable C terminus of Cbl contains a proline-rich region that interacts with signaling molecules, and in some cases a ubiquitin-associated (UBA) domain or a leucine zipper (Lupher et al. 1999). Cbl was first identified as

the cellular homologue of v-Cbl, a protein expressed by murine retroviruses that potently induces B cell lymphomas (Langdon et al. 1989). Subsequently, Cbl was characterized genetically as an antagonist of growth factor receptor and immune receptor signaling (e.g., Jongeward et al. 1995).

Biochemical characterization of Cbl function in cell culture studies revealed that Cbl binds to activated receptors and promotes their ubiquitination and degradation (reviewed in Lupher et al. 1999, Shtiegman & Yarden 2003). Cbl-dependent ubiquitination has been functionally linked to internalization of EGFR, colony-stimulating factor-1 (CSF-1) receptor, and hepatocyte growth factor receptor (Met) (Lee et al. 1999, Levkowitz et al. 1998, Petrelli et al. 2002, Waterman et al. 2002). The role of Cbl in endocytic sorting has been most extensively characterized for EGFR. The Cbl PTB domain binds to the EGF-induced phosphotyrosine residue 1045 at the plasma membrane and catalyzes receptor ubiquitination on lysines in the cytoplasmic tail in a RING finger–dependent reaction (de Melker et al. 2001, Levkowitz et al. 1999, Stang et al. 2000). Contrary to initial conclusions that growth factor receptors are polyubiquitinated at the cell surface, EGFR is monoubiquitinated on multiple cytoplasmic tail lysines (Haglund et al. 2003, Mosesson et al. 2003). Mutation of the Cbl docking site impairs EGFR internalization (Waterman et al. 2002). Furthermore, fusion of ubiquitin to a truncated EGFR lacking its cytoplasmic tail restores the ability of the receptor to internalize ^{125}I-EGF (Haglund et al. 2003), suggesting that ubiquitin functions as a ligand-stimulated internalization signal for EGFR. Cbl remains associated with the receptor after it is internalized and plays an additional distinct role in specifying sorting of EGFR to the lysosome by promoting inclusion into MVB vesicles (Levkowitz et al. 1998, Longva et al. 2002). The PTB and RING domains of Cbl are both necessary and sufficient for negative regulation of growth factor–induced signaling (Lill et al. 2000, Miyake et al. 1999, Waterman et al. 1999), linking Cbl-mediated ubiquitination and endocytosis with Cbl function in negative regulation of receptor signaling.

In addition to controlling receptor downregulation, Cbl also appears to mediate the degradation of multiple signaling and endocytic proteins that associate with the EGFR (Ettenberg et al. 2001). These proteins may piggyback into the lysosome with the receptor and be degraded there (Haglund et al. 2002), or they may be polyubiquitinated and degraded by the proteasome. Cbl is negatively regulated by interaction with *trans*-acting negative regulators such as the Sprouty proteins, which bind to the RING finger, displace the E2, and inhibit EGFR ubiquitination and endocytosis (Wong et al. 2002).

Other RING finger E3s regulate the endocytosis of a variety of proteins. Hakai (Japanese word for destruction) promotes the ubiquitination and endocytosis of E-cadherin, the cell-cell adhesion molecule of epithelial cell adherens junctions (Fujita et al. 2002). Hakai is similar to Cbl, with a RING finger, a functional PTB domain, and a C-terminal proline-rich domain. Overexpression of Hakai in epithelial cells disrupts cell-cell contacts and enhances cell motility, suggesting that Hakai is an important physiological regulator of epithelial cell adhesion.

Drosophila Neuralized is an evolutionarily conserved RING finger protein of the Notch signaling pathway. In this pathway, signaling occurs between adjacent

cells by the binding of a transmembrane ligand, Delta, in the signaling cell to a transmembrane receptor, Notch, on the surface of the receiving cell. Cell surface levels of both Notch and Delta are controlled by endocytosis. Furthermore, internalization of Delta, together with the bound extracellular domain of Notch, facilitates proteolysis and release of an intracellular signaling Notch fragment in the receiving cell (Pavlopoulos et al. 2001 and references therein). Delta is ubiquitinated by Neuralized (Deblandre et al. 2001, Lai et al. 2001, Pavlopoulos et al. 2001), and cells lacking Neuralized or expressing a Neuralized mutant lacking the RING domain are unable to ubiquitinate or internalize Delta, suggesting that ubiquitin signals Delta endocytosis. Neuralized and Delta appear to remain associated after endocytosis because the two proteins colocalize in endosomes (Lai et al. 2001). Thus, like Cbl, Neuralized may function to promote both internalization and sorting of cargo into MVB vesicles.

Another E3 that acts on Delta, Mind bomb, was identified in zebrafish as a RING finger protein essential for efficient activation of Notch signaling (Itoh et al. 2003). Although unrelated to Neuralized, Mind bomb also modifies Delta's cytoplasmic domain with ubiquitin and promotes Delta internalization. Mind bomb is required in the signaling cell to activate Notch. The simplest explanation of these observations is that Mind bomb ubiquitinates Delta in the signaling cell. Delta is then internalized via a ubiquitin signal, thereby facilitating Notch signaling. Endocytosis of Notch itself is also likely to be controlled by ubiquitin signals, an unusual case in which both receptor and ligand are subject to downregulation by ubiquitin-dependent endocytosis.

Several of the HECT and RING E3s discussed above act both at the plasma membrane and in sorting at the endosome. In yeast, additional proteins that are known or thought to function in endosomal sorting carry RING fingers, and some have demonstrated ubiquitin ligase activity. Tul1 is a transmembrane ubiquitin ligase and resident Golgi protein that ubiquitinates acid hydrolase precursors destined for the vacuole lumen (Reggiori & Pelham 2002). Pib1 (phosphatidylinositol-3-phosphate-binding) is a bona fide E3 that is localized to endosomes (Shin et al. 2001), although its role there has not been established. Vps8, Pep3, and Pep5, all RING finger proteins, are required for transporting proteins to the vacuole from the biosynthetic and endocytic pathways. Mammalian homologues of some of these proteins also have zinc/RING fingers; however, ubiquitin ligase activity for the yeast or mammalian proteins remains to be demonstrated. The plethora of RING and HECT proteins with known functions or location at the Golgi or endosomal compartments suggests that additional ways by which ubiquitin regulates transport between organelles of the secretory and endocytic pathways remain to be discovered.

An important question that remains to be answered is how the type of ubiquitin modification that occurs on a substrate is specified. This question is important for ubiquitin-dependent protein transport because changes in the extent of ubiquitination (mono versus poly) on both soluble and membrane proteins can alter their fate. Rsp5/Nedd4 and Cbl E3s can catalyze both monoubiquitination and polyubiquitination. Therefore, this choice must be regulated by factors other than

the identity of the E3. The type of modification may depend on whether the E3 is stably or transiently associated with a substrate because stable association may allow sufficient time for extension of a polyubiquitin chain (Di Fiore et al. 2003). For Rsp5, regulatory Bul proteins cause the ligase to extend a polyubiquitin chain on substrates that are monoubiquitinated in the absence of Bul activity, perhaps by extending the length of time a substrate is bound to Rsp5. Regulation of monoubiquitination might also occur by deubiquitination enzymes that trim back extended chains or by capping proteins that bind to a monoubiquitin-conjugated substrate to prevent chain extension.

Deubiquitinating Enzymes

Ubiquitination is a reversible process, and eukaryotic genomes encode a large number of putative and confirmed deubiquitinating enzymes (DUBs) that hydrolyze ubiquitin-protein isopeptide bonds. Because DUBs remove ubiquitin signals, they are analogous to phosphatases and are likely to be crucial regulatory proteins. Systematic mutagenesis of yeast DUBs has suggested that these enzymes are functionally redundant (Amerik et al. 2000a), and few specific substrates of DUBs have been identified. However, a *Drosophila* DUB known as Fat facets has been shown to regulate endocytosis by deubiquitinating epsin, a component of the clathrin-based endocytosis machinery (Chen et al. 2002 and references therein). *fat facets* mutants were identified in a screen for mutations that affect eye development, and genetic analyses indicated that the function of Fat facets in cell fate specification is linked to endocytosis. A dominant enhancer of *fat facets*, *liquid facets*, encodes *Drosophila* epsin. Subsequent biochemical experiments demonstrated that epsin and Fat facets physically interact, and ubiquitin-modified epsin is detected only when Fat facets activity is impaired. These studies demonstrate a substrate-specific function for a DUB and strongly suggest that epsins are *trans*-acting components of the endocytic machinery regulated by ubiquitination (see below).

Doa4 is a yeast DUB that recycles ubiquitin from proteolytic intermediates. A screen for suppressors of *doa4* identified several genes encoding vacuolar protein sorting (Vps) proteins involved in MVB vesicle formation. Furthermore, Doa4 localizes to a late endosomal compartment in a *vps* mutant defective in MVB vesicle formation (Amerik et al. 2000b), and ubiquitin-modified cargo destined for the vacuole accumulates in Doa4-deficient cells (Dupré & Haguenauer-Tsapis 2001, Katzmann et al. 2001, Losko et al. 2001). Together, these data indicate that a major cellular activity for this DUB is to remove ubiquitin from transmembrane proteins prior to their transport into the vacuole lumen.

UBIQUITIN-BINDING ENDOCYTIC PROTEINS

Modular Monoubiquitin-Binding Domains

One way cells interpret and transmit the information conferred by monoubiquitin signals is through proteins that bind ubiquitin noncovalently. Several distinct

sequence motifs are known to bind directly to monoubiquitin (reviewed in Di Fiore et al. 2003, Schnell & Hicke 2003). Here we discuss proteins carrying UBA (<u>ub</u>iquitin-<u>a</u>ssociated), UIM (<u>ub</u>iquitin-<u>i</u>nteracting <u>m</u>otif), UEV (ubiquitin conjugating enzyme <u>E</u>2 <u>v</u>ariant), CUE or NZF (<u>N</u>pl4 <u>z</u>inc <u>f</u>inger) domains that participate in membrane protein trafficking (Table 2). These domains bind directly to the Ile44 surface on ubiquitin with weak affinity; however, affinity probably increases when ubiquitin is recognized together with another signal (Garrus et al. 2001) or when the ubiquitin-binding protein is part of a multimeric complex (Katzmann et al. 2001, VanDemark et al. 2001).

Candidate Adaptors for Ubiquitin-Sorting Signals at the Plasma Membrane and the Endosome

Proteins that carry UIM or UBA domains act in endocytic vesicle budding from the cell surface. Epsins (<u>Eps</u>15-<u>in</u>teracting proteins) and Eps15 (<u>e</u>pidermal growth factor receptor <u>s</u>ubstrate <u>15</u>) are proteins that function in the assembly of clathrin-coated primary endocytic vesicles at the plasma membrane (Benmerah et al. 1998, Chen et al. 1998, Tebar et al. 1996). Epsins contain an N-terminal ENTH phospholipid-binding domain, two copies of the UIM, and clathrin/AP-2 interaction motifs. Eps15 binds to epsins and harbors three UIMs, two at its C terminus. The yeast homologue of Eps15, Ede1, carries a single UBA domain at the C terminus instead of two UIMs (Figure 3). The UIMs of epsins and Eps15 and the UBA domain of Ede1 bind directly to ubiquitin (Aguilar et al. 2003, Polo et al. 2002, Shih et al. 2002). In yeast, Ede1 and the UIMs of epsins play redundant roles in receptor internalization, suggesting that epsin ubiquitin-binding is important for entry of cargo into budding plasma membrane vesicles (Shih et al. 2002).

The ubiquitin internalization signal is unlike linear peptide tyrosine- or dileucine-based internalization signals, sequences that promote internalization by binding to clathrin adaptors. Consistent with this difference, classical clathrin adaptor complexes are not required for the internalization of ubiquitinated receptors (Huang et al. 1999, Nesterov et al. 1999). However, epsins share characteristics of clathrin adaptors, such as phospholipid- and clathrin-binding, and they directly bind monoubiquitin through residues that are part of the ubiquitin internalization signal (Shih et al. 2002). Thus they are excellent candidates for adaptors to link ubiquitinated cargo to the clathrin-based endocytic machinery (De Camilli et al. 2002, Hicke 2001a, Wendland 2002), although this idea awaits experimental proof (Figure 3).

The ubiquitin-binding proteins Hrs/Vps27 and STAM/Hse1 associate with early and late endosomal membranes and are required for the transport of endocytic cargo to lysosomes (Bilodeau et al. 2002, Lohi & Lehto 2001, Raiborg et al. 2002 and references therein). Both Hrs/Vps27 and STAM/Hse1 carry one or two UIM domains that bind ubiquitin directly (e.g., Bache et al. 2003, Bilodeau et al. 2002, Raiborg et al. 2002, Shih et al. 2002). Like epsins and Eps15 at the plasma membrane, Hrs/Vps27 and STAM/Hse1 bind to each other at the endosome (e.g., Bache et al. 2003, Bilodeau et al. 2002) and the UIM domains of these proteins are

TABLE 2   Modular monoubiquitin-binding domains

Domain name	Length (a.a.)	K_d (monoUb)	Structural features	Transport membrane proteins	References
UBA (ubiquitin-associated)	~45		Three-helix bundle with hydrophobic interaction surface	Cbl, Ede1	(Hofmann & Bucher 1996, Mueller & Feigon 2002, Shih et al. 2002)
UIM (ubiquitin-interacting motif)	~20	200–300 μM	Short α-helix	Epsin, Eps15, Hrs, Vps27, STAM, Hse1	(Hofmann & Falquet 2001, Raiborg et al. 2002, Shekhtman & Cowburn 2002)
UEV (ubiquitin-E2-variant)	~145	500 μM	Sequence and structural homology with E2s; lacks catalytic cysteine	Tsg101, Vps23	(Katzmann et al. 2001, Pornillos et al. 2002a)
CUE (homology to Cue1 protein)	42–43	20–160 μM	Three-helix bundle with hydrophobic interaction surface; similar to UBA	Vps9, Rabex	(Kang et al. 2003, Ponting 2000, Prag et al. 2003, Shih et al. 2003)
NZF (Npl4 zinc finger)	~35	100–200 μM	Four antiparallel β-strands; zinc finger	Npl4, Vps36	(Meyer et al. 2002, Wang et al. 2003)

important for sorting of endocytic cargo to the lysosome/vacuole (Bilodeau et al. 2002, Raiborg et al. 2002, Shih et al. 2002). The molecular architecture of Hrs/Vps27 and epsins is similar, with a notable but practical difference in lipid-binding preference. The epsin ENTH domain binds to phosphatidylinositol-4,5-bisphosphate, a lipid enriched at the plasma membrane, whereas the Hrs/Vps27 FYVE domain binds phosphatidylinositol-3-phosphate, a lipid enriched in the endosomal membrane. Similar to epsin's binding, Hrs binds clathrin (Raiborg et al. 2001). Thus these proteins are likely to function by analogous mechanisms at distinct sites in the endocytic pathway that are marked by different phosphoinositides.

Multiple observations support the idea that Hrs/Vps27 acts as a clathrin adaptor to sort ubiquitinated cargo at the endosome. Ubiquitin conjugates colocalize with Hrs and clathrin at discrete subdomains of the endosomal membrane, and Hrs physically associates with an internalized receptor fused to ubiquitin. Furthermore, a conserved UIM residue in Hrs is important for sorting of a ubiquitin-receptor chimeric protein (Raiborg et al. 2002). In yeast, Vps27 is generally required for transport of cargo to the vacuole and for the formation of MVB vesicles. However, deletion of the Vps27 UIM domains inhibits transport only of ubiquitinated cargo and does not affect the formation of vesicles (Bilodeau et al. 2002). This observation strongly suggests that the Vps27 UIMs directly bind to a ubiquitin-sorting signal on cargo proteins. Hence, Hrs/Vps27 is likely to serve as an adaptor to link ubiquitinated cargo to clathrin for sorting at the endosome (Figure 3).

Other Ubiquitin-Binding Proteins that Control Endosomal Transport

Ubiquitin-binding proteins are also present in proteins that act at the MVB to promote vesicle budding into the lumen. Three protein complexes, termed ESCRT-I, ESCRT-II, and ESCRT-III (endosomal sorting complex required for transport), are recruited sequentially to the MVB membrane to function in vesicle budding (Katzmann et al. 2002). One component of ESCRT-I is Tsg101 (tumor susceptibility gene 101; yeast Vps23) (Bishop et al. 2002, Katzmann et al. 2001). Vps23 carries a ubiquitin-binding UEV domain that is required for MVB sorting and for the interaction of ESCRT-I with ubiquitin in vitro and with ubiquitinated MVB cargo in vivo (Katzmann et al. 2001). In mammalian cells, Tsg101 also carries an UEV domain, and functional inactivation of this protein inhibits sorting of activated EGF receptors to the lysosome (e.g., Bishop et al. 2002). These observations suggest that the Tsg101 component of the ESCRT-I complex binds ubiquitinated cargo for sorting into MVB vesicles.

How do the ubiquitin-binding proteins Hrs and Tsg101 act in concert to promote transport of cargo from endosomes to the lysosome? Perhaps the simplest explanation is that the two proteins act sequentially. Hrs probably binds ubiquitinated cargo at the early endosome, recruiting cargo into clathrin-coated regions of the membrane to prevent recycling back to the plasma membrane. Hrs may remain bound to cargo during transport to a late endocytic compartment where cargo is transferred to ESCRT-I via direct binding to Tsg101. This model suggests that

ubiquitin serves two distinct post-internalization functions: (*a*) as a signal for Hrs- and clathrin-dependent recruitment into specific regions of the early endosome to prevent recycling and (*b*) as a signal that subsequently interacts with Tsg101 to promote entry into vesicles at the MVB.

The idea that Tsg101 and Hrs act cooperatively in cargo transport is supported by mechanisms described to promote the budding of enveloped viruses. The cellular MVB budding machinery unwittingly aids the budding of enveloped viruses from the plasma membrane by recognizing viral signals that probably mimic the function of Hrs (reviewed in Pornillos et al. 2002b). Retroviral Gag proteins can carry multiple types of budding motifs, one of which is the peptide motif PT/SAP. Tsg101 recognizes this motif, and ubiquitination of Gag carrying PTAP dramatically increases its affinity for Tsg101. Budding of viruses bearing PTAP requires functional Tsg101 and the mammalian Vps proteins that act downstream of Tsg101, including ESCRT complexes. Hrs, like Gag, is ubiquitinated and carries a PSAP sequence, suggesting that it may interact with Tsg101 in a similar manner. A second type of viral budding motif is PPXY, a motif that recruits ubiquitin ligases of the Nedd4 family. Budding of viruses bearing this motif also requires ubiquitination and downstream Vps proteins (but not Tsg101), suggesting an intriguing, but poorly understood, link between Nedd4 proteins and the MVB-sorting machinery.

In the cell, ubiquitinated cargo may be transferred from Tsg101 to the ESCRT-II complex because this complex contains Vps36, a protein with a NZF domain that directly binds ubiquitin (Meyer et al. 2002). Release from ESCRT-II into a nascent bud would then occur by deubiquitination of cargo by Doa4. Instead of, or in addition to, binding ubiquitinated cargo, it is possible that ubiquitin-binding domains of Vps27, Vps23, or Vps36 and their homologues are important for interaction with ubiquitin conjugated to the sorting machinery as a means of regulating protein-protein interactions.

One endocytic ubiquitin-binding protein with a function not linked to vesicle budding and cargo recruitment is Vps9 (Davies et al. 2003, Donaldson et al. 2003, Shih et al. 2003). Vps9, the yeast homologue of Rabex, is a guanine nucleotide exchange factor that promotes exchange of GDP for GTP on Vps21, the yeast homologue of Rab5. Both Vps9 and Vps21 are required for the transport of proteins from the biosynthetic and endocytic pathways into the vacuole and are thought to control vesicle fusion mediated by SNAREs (Hama et al. 1999 and references therein). At the terminus of Vps9 is a CUE domain, conserved in Rabex, that binds to the I1e44 patch on ubiquitin. Deletion of the CUE domain from Vps9 does not affect the transport of most endocytic or biosynthetic cargo. This observation prompted the hypothesis that the CUE domain is a negative regulator of Vps9 activity. Removal of the Vps9 CUE domain suppresses the transport defect of a receptor-ubiquitin chimera carrying a ubiquitin Ile44A mutation (Donaldson et al. 2003), consistent with a model in which binding of the CUE domain to ubiquitinated cargo alleviates CUE inhibition of Vps9 catalytic activity. Alternatively, the CUE domain in Vps9 may be required only for the transport of specific cargo (Davies et al. 2003).

Monoubiquitin-Binding Domains and Intramolecular Monoubiquitination

An intriguing twist to the story of endocytic ubiquitin-binding proteins is the finding that UIM and CUE domain proteins are monoubiquitinated by a mechanism that depends on their UIM or CUE domains, an event referred to as intramolecular monoubiquitination. Proteins known to be monoubiquitinated in this way include the epsins, Eps15, Hrs, and Vps9 (e.g., Davies et al. 2003, Klapisz et al. 2002, Oldham et al. 2002, Polo et al. 2002, Shih et al. 2003). Ubiquitin attachment to these proteins appears to occur outside the ubiquitin-binding domain (Klapisz et al. 2002, Oldham et al. 2002) and is catalyzed by HECT domain ligases (e.g., Davies et al. 2003, Polo et al. 2002, Shih et al. 2003), consistent with the unusual possibility that the UIM/CUE domains may cause intramolecular ubiquitination by recruiting a ubiquitin-HECT E3 thiolester intermediate (Polo et al. 2002). Although the endocytic CUE and UIM proteins are ubiquitinated, they are not obvious targets for proteasome-mediated degradation discussed above. Epsins, for instance, are stable proteins (Oldham et al. 2002; L. Hicke, unpublished data).

Why are monoubiquitin-binding and intramolecular ubiquitination coupled in these proteins? Monoubiquitinated proteins that also bind ubiquitin may interact to form a network of proteins that is assembled by temporally and spatially regulated ubiquitin-protein interactions (Figure 4*a*). This potential ubiquitin-mediated network has been compared with signaling networks propagated by phosphotyrosine and phosphoserine/phosphothreonine interactions with modular domains such as SH2 and 14-3-3 motifs in signal transduction proteins (Di Fiore et al. 2003). For instance, epsins may interact with ubiquitinated cargo and become ubiquitinated to enhance interaction with another endocytic ubiquitin-binding protein (e.g., Eps15) or to regulate binding to the membrane or clathrin. Epsins, Eps15, and Hrs all are phosphorylated in addition to being monoubiquitinated, suggesting that regulation of endocytic proteins is likely to occur via multiple post-translational modifications that may be interrelated.

Another reason that monoubiquitin binding and ubiquitination are coupled may be that conjugated ubiquitin acts as an intramolecular negative regulator of monoubiquitin-binding domains. Binding of ubiquitin attached to a neighboring lysine would protect a UIM or CUE domain from occupation by the free ubiquitin present at high concentration in the cytosol (Figure 4*b*). Regulated deubiquitination of the ubiquitin-binding protein would then expose a ubiquitin-binding domain to its cellular binding partner at the appropriate time and location. This model, although speculative, is consistent with genetic data from *Drosophila* indicating that the deubiquitination of epsins is required to activate endocytosis. Association of a ubiquitin-binding domain with a ubiquitin conjugated in *cis* might also be a mechanism for preventing polyubiquitin chain extension (Polo et al. 2002). This idea is supported by nuclear magnetic resonance data suggesting that ubiquitin Lys48, a major site of polyubiquitin formation, may be occluded during interaction of monoubiquitin with a UIM or CUE domain (Kang et al. 2003, Shekhtman & Cowburn 2002).

CONCLUDING REMARKS

Understanding how ubiquitin regulates transport through the biosynthetic and endocytic pathways is critical for a mechanistic description of the events that regulate the activity of cell surface proteins. The role of ubiquitin as a *cis* signal for membrane protein transport is well established. The next step will be to understand how ubiquitin-binding proteins recognize ubiquitin-sorting signals and coordinate cargo recruitment with vesicle budding. A second important task is to define the mechanisms by which ubiquitination regulates the activity of the protein transport machinery. More generally, we need to know how the addition of a single ubiquitin to a substrate is regulated and how monoubiquitin-binding domains are regulated to avoid domain occupancy by free monoubiquitin. Answers to these questions will provide paradigms for the regulation of other basic cellular processes by monoubiquitin signals.

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NOTE ADDED IN PROOF

After this review was written, Mizuno et al. (2003) reported that the VHS domain of Stam binds to mono- and polyubiquitin. In Stam, the VHS and VIM domains appear to act synergistically to bind ubiquitinated proteins.

Mizuno E, et al. 2003. Stam proteins bind ubiquitinated proteins on the early endosome via the VHS domain and ubiquitin-interacting motif. *Mol. Biol.* In press
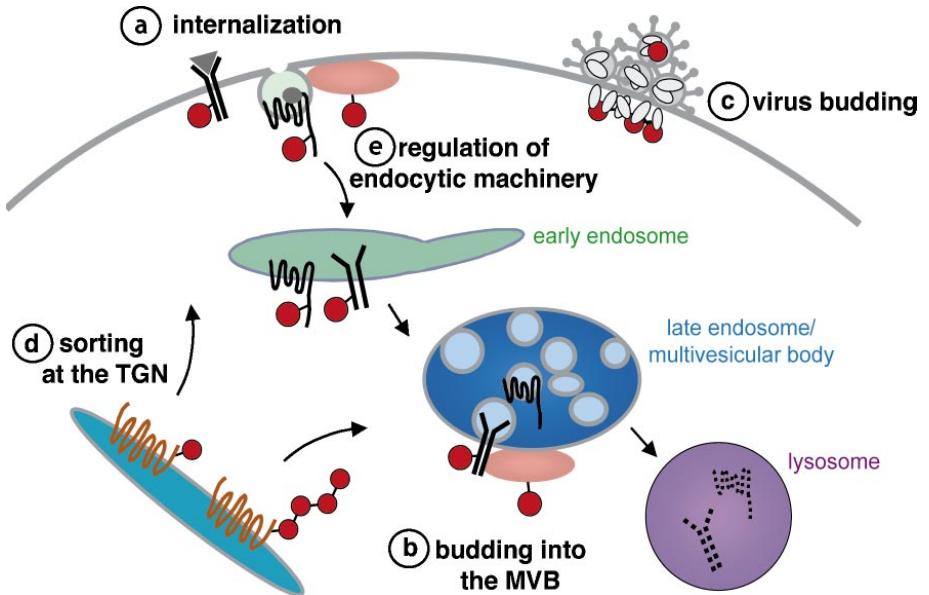
Figure 1 Regulation of protein transport by ubiquitin signals. (*a*) Monoubiquitin and Lys63-linked di-ubiquitin chains serve as regulated internalization signals that can be appended to a plasma membrane protein to trigger entry into primary endocytic vesicles budding from the plasma membrane. (*b*) Monoubiquitin serves as a signal for the entry of transmembrane proteins into MVB vesicles. (*c*) Ubiquitin, ubiquitin-binding proteins, and ubiquitin ligases are important for the budding of enveloped viruses. (*d*) (Poly)ubiquitination regulates the sorting of proteins at the *trans*-Golgi network to the lysosome/vacuole. (*e*) Ubiquitin modifies and regulates components of the endocytic machinery.
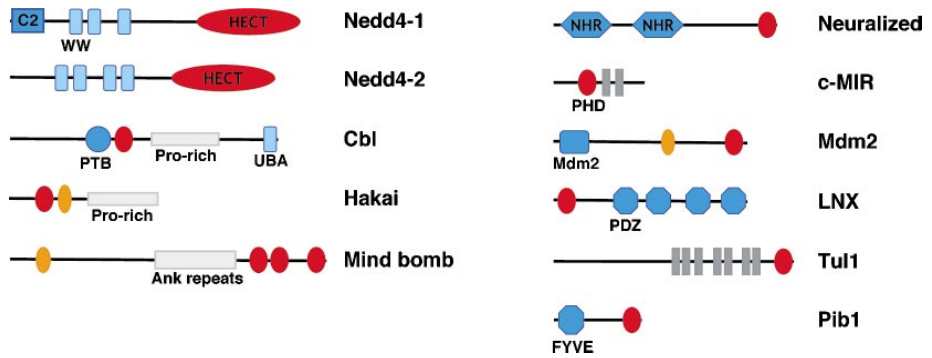
Figure 2 Ubiquitin ligases important for protein transport. Nedd4-1 and Nedd4-2 HECT domain E3s: Rsp5 (*S. cerevisiae*), Suppressor of deltex (*Drosophila*), Itch, Smurf1, and Smurf2 are similar in size and domain structure to Nedd4-1. The number of WW domains in these proteins varies from two to four. RING finger E3s: red ovals indicate RING finger, or PHD zinc finger (labeled) catalytic domains. Grey vertical bars indicate predicted transmembrane domains. Orange ovals represent zinc fingers that are not RING fingers or PHD domains.
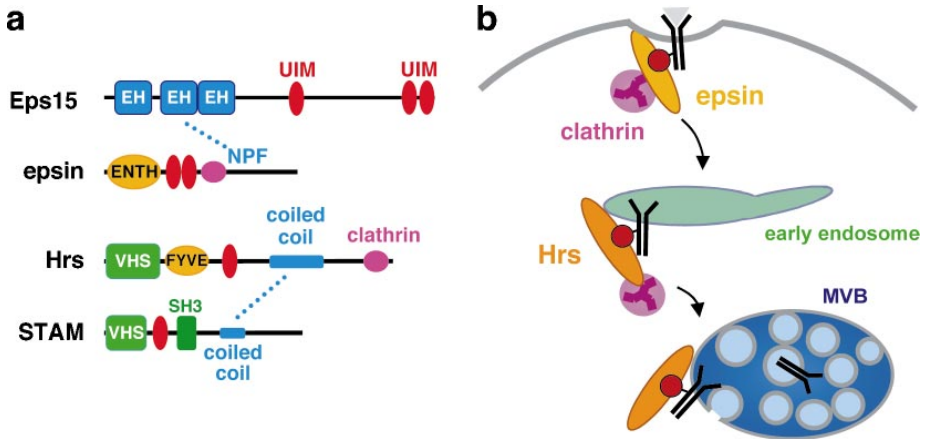
Figure 3 Candidate adaptors for ubiquitin sorting signals. (*a*) Ubiquitin-binding epsin-Eps15 and Hrs-STAM complexes act at the plasma membrane and endosome, respectively. The epsin ENTH domain binds PI-4,5-P$_2$ and the Hrs FYVE domain binds PI-3-P. EH (Eps15 homology) and VHS (Vps27-Hrs-STAM) domains are indicated. A dashed line indicates an interaction between Eps15 EH domains and epsin NPF motifs, or between the coiled-coil domains of Hrs and STAM. (*b*) Epsin-Eps15 and Hrs-STAM complexes may link ubiquitinated cargo to the clathrin vesicle budding machinery at domains of a membrane marked by specific phosphatidylinositols.
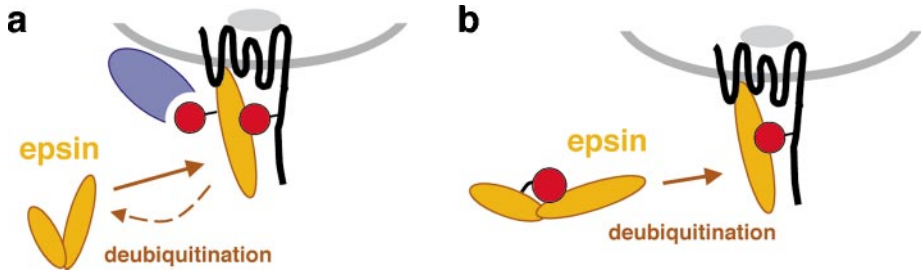
Figure 4 Models for regulation of endocytic monoubiquitin-binding proteins by intramolecular monoubiquitination. (*a*) Ubiquitin, together with phosphorylation, may provide a signal that coordinates a network of interactions that must be regulated temporally and spatially to assemble a budding vesicle. (*b*) Ubiquitin-binding and monoubiquitination may be coupled in the same protein to regulate the exposure of a ubiquitin-binding domain to its specific binding partner. These models are not mutually exclusive.

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