Role of Ubiquitylation in Cellular Membrane Transport

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Staub, Olivier, and Daniela Rotin. Role of Ubiquitylation in Cellular Membrane Transport. Physiol Rev 86: 669–707, 2006; doi:10.1152/physrev.00020.2005.—Ubiquitylation of membrane proteins has gained considerable interest in recent years. It has been recognized as a signal that negatively regulates the cell surface expression of many plasma membrane proteins both in yeast and in mammalian cells. Moreover, it is also involved in endoplasmic reticulum-associated degradation of membrane proteins, and it acts as a sorting signal both in the secretory pathway and in endosomes, where it targets proteins into multivesicular bodies in the lumen of vacuoles/lysosomes. In this review we discuss the progress in understanding these processes, achieved during the past several years.

I. INTRODUCTION

In contrast to soluble cytoplasmic proteins, membrane proteins, which are inserted into a lipid bilayer, cannot freely diffuse throughout the cell and therefore have to be targeted to their destination by specific mechanisms. These mechanisms, generally known as membrane transport or protein traffic between different cellular organelles, follow a common scheme, in which protein cargoes are recruited into budding regions in a donor organelle. These regions then bud off as vesicles and move on to a recipient organelle, with which they fuse and to which they deliver the cargo proteins. These mechanisms have been extensively studied and described over the past two decades (142, 182, 272, 459). One of the dominating themes during the last decade, which has considerably changed our view of protein trafficking, was the discovery that ubiquitylation (i.e., the covalent modification with the polypeptide ubiquitin) is playing a major role in the internalization of membrane proteins, but also during intracellular protein traffic. Especially surprising was the finding that not only membrane proteins themselves become modified by ubiquitin and thereby targeted to their destination, but also the proteins of the trafficking machinery are often themselves ubiquitylated, rendering this machinery highly dynamic. The aim of this review is to provide an overview of these new concepts and use salient examples that highlight the importance of ubiquitin in trafficking, endocytosis, and sorting of transmembrane proteins.
II. UBIQUITYLATION

Ubiquitylation (also referred to as ubiquitination or ubiquitylation) is a posttranslational modification that involves the covalent attachment of ubiquitin polypeptides to target proteins. Ubiquitin is a highly conserved polypeptide of 76 amino acids; it contains a diglycine motif at its COOH-terminal end, and it is ligated via this COOH terminus to lysine residues of target proteins (Fig. 1A). This modification was originally described as a signal that could target cellular proteins to rapid degradation by a cytosolic complex, the proteasome (160). It turned out to be a highly regulated system that is important for many cellular functions. Ubiquitylation was later found to regulate numerous other processes in the cell (not just serving as a degradation signal for the proteasome), including protein trafficking. For excellent overviews on ubiquitylation, see References 133, 325, 345. Here, we will only briefly describe the mechanism leading to ubiquitylation. It involves the action of a cascade of enzymes (Fig. 1, B and C). First, E1 or ubiquitin-activating enzyme forms a thioester bond between its catalytic cysteine and ubiquitin, a process that requires ATP hydrolysis. Only a few E1 enzymes exist (1 in yeast, ~10 in humans) that are involved in ubiquitylation in mammalian cells, whereas at least three other E1 enzymes are involved in the conjugation of ubiquitin-like proteins (163, 367, 402). The ubiquitin moiety is then transferred to an E2, a ubiquitin-conjugating or ubiquitin-carrier enzyme, which also forms a thioester bond between its cysteine and ubiquitin. There are 11 E2 enzymes in yeast and at least 100 in human cells. E2 enzymes then act in concert with E3 enzymes, or ubiquitin-protein ligases, which are the enzymes that are responsible for substrate recognition. There are hundreds of E3 enzymes (54 in yeast and ~1,000 in humans) (163) comparable to the number of kinases, another indication that ubiquitylation likely plays an important role in cellular regulation, much like phosphorylation. E3 ligases carry out the important tasks of substrate recognition and transfer (or facilitation of transfer) of ubiquitin onto the substrate, usually on lysine residues. There are two major types of E3 ligases: the RING finger E3s and the HECT E3s.

The RING (really interesting new gene) domain-containing E3s bring the E2 enzyme in close proximity of the target protein, allowing the E2 to directly ubiquitylate the substrate. (428). The RING domain is defined by the consensus CX2CX(CX9–39)CX(C1–3)C/HX2CX14–48CX2C, in which the cysteines and histidines bind Zn2+ (428). This arrangement produces a structure that is referred to as the “cross-brace motif.” This structure is stabilized by the Zn2+ coordination for the first Zn2+ atom by cysteines in positions 1, 2, 5, and 6, and for the second Zn2+ atom by cysteines or histidines in positions 3, 4, 7, and 8. The RING fingers are classified into RING-HC and RING-H2 motifs depending on whether a cysteine or histidine occupies the fifth coordination site, respectively. Structures of RING-HC fingers show two interleaved Zn2+ binding sites, which is different from the tandem arrangement of Zn2+ binding sites characteristic of Zn2+ fingers (119). An example of a RING-containing E3 is Cbl (Fig. 2A), which plays an important role in the ubiquitylation of plasma membrane proteins.

The second class of E3 enzymes involves the HECT (homologous to E6-AP COOH terminal) domain containing ubiquitin-protein ligases, which also directly interact with E2 enzymes (173). However, here ubiquitin is first transferred onto a conserved cysteine in the HECT domain, and it is the HECT E3 enzyme that ubiquitylates the substrate protein. The HECT containing E3 enzymes includes the family of Nedd4 (neuronal precursor cell expressed developmentally downregulated)/Nedd4-like ubiquitin-protein ligases (Fig. 2B), many of which are involved in the ubiquitylation of membrane proteins.

Ubiquitin is usually ligated onto lysine residues of the substrate or of ubiquitin itself, forming di- or polyubiquitin chains in the latter. Hence, proteins can either be monoubiquitylated (one ubiquitin polypeptide on a single lysine), mult ubiquitylated (several lysines modified with just one ubiquitin), diubiquitylated (diubiquitin on substrate lysine), or polyubiquitylated (extended polyubiquitin chain). While cytosolic proteins destined for destruction by the proteasome are polyubiquitylated, during trafficking, membrane proteins and proteins of the trafficking machinery are generally monoubiquitylated (383) or diubiquitylated (124). There are seven lysines in ubiquitin itself (Fig. 1A). Although Lys-48 and Lys-63 are the most frequently utilized residues to form polyubiquitin chains, linkage through the other five lysines has been reported (319). The mechanisms that lead to polyubiquitylation are not very well understood. A novel class of enzymes, the E4 enzymes containing a U-box motif, have been proposed to be involved in this process, but ubiquitin-protein ligases may be involved in the process as well (158, 223, 243), and U-box containing proteins can function also as E3 enzymes (189). Modification with ubiquitin is a reversible process. This is achieved by the action of isopeptidases, or deubiquitylating enzymes. Although there are probably hundreds of such enzymes, deubiquitylation has so far not been extensively studied, but it is likely that this is as important as ubiquitylation itself and also highly regulated (8, 217). There are a number of small polypeptides with homology to ubiquitin, commonly known as ubiquitin-like proteins. Examples include SUMO, Nedd8, ISG15, and FAT10. These ubiquitin-like proteins are targeted to substrate proteins using enzymatic cascades highly similar, but not identical, to the ubiquitylation cascades, including E1, E2, E3, and deubiquitylation enzymes (for a review, see Refs. 367, 402). The following is a brief overview of E3 ubiquitin-protein ligase families particularly pertinent to this review.
A. The Cbl Family of E3 Ligases

Cbl (Casitas B-lineage lymphoma) was first discovered as a viral oncogene in 1989 in a study involving a retrovirus that induces early B-cell lymphomas (234) and was called v-Cbl. Cloning of its murine cellular homolog revealed that v-Cbl encoded only the first 355 amino acids of the murine protein, c-Cbl (39). Two other members of...
FIG. 2. Cbl and Nedd4/Nedd-like families. A: Cbl family: human, *Drosophila* (D-Cbl) and *C. elegans* (SLI-1) Cbl family members are shown. The Cbl proteins are highly conserved in the NH₂-terminal region where they comprise a tyrosine-kinase binding domain (TKB), which is composed of a 4-helix bundle (4H), a calcium binding EF domain, and a variant SH2 domain that is linked via a linker with the RING finger domain. In the COOH-terminal half are a number of proline-rich regions (indicated with orange boxes), a ubiquitin-associated UBA domain, and a leucine zipper. v-Cbl is a retroviral oncogenic form of Cbl lacking the RING finger domain and the rest of the COOH terminus. B: Nedd4/Nedd4-like family members from yeast, worm, fly, and mammalian species are shown. They all contain a C2 domain (which in some cases can be spliced out, dashed lines), WW domains, and a HECT domain. Diagrams are not to scale.
the Cbl family have been identified in mammals, Cbl-b and Cbl-3 (210, 211, 423) (Fig. 2A). These proteins share a tyrosine kinase binding (TKB) domain that includes a variant SH2 domain, a linker, a RING finger domain, and in c-Cbl and Cbl-b, also a COOH-terminal region that contains a proline-rich region and a ubiquitin association (UBA) domain. The oncogenic v-Cbl comprises only the NH2 terminus and lacks the linker region and the RING finger domain (422). It has been shown that the linker region interacts with the RING finger domain, permitting an optimal placement of an E2 enzyme to allow transfer of ubiquitin onto the substrate protein (485). v-Cbl, which lacks the RING finger, is not able to ubiquitylate substrates and acts in a competitive fashion towards c-Cbl. For a detailed review on Cbl proteins, see Reference 422.

B. Family of HECT Ubiquitin-Protein Ligases

This family is part of the HECT domain containing ubiquitin-protein ligases (Fig. 2B). Its best studied member, Nedd4, was identified in a subtractive screen between neuronal precursor cells and adult brain cells (232). There are nine members encoded in the human genome, and all are characterized by the presence of an NH2-terminal C2 (calcium-dependent lipid binding domain) (for a review, see Ref. 351), two to four WW (protein-protein interaction) domains (reviewed in Refs. 354, 405), and a COOH-terminal HECT domain (173) (Fig. 2B). The C2 domain and the WW domains are involved in localization and substrate recognition, respectively (197, 200, 326, 327), whereas the HECT domain is an E3 ubiquitin-protein ligase domain. WW domains of Nedd4 proteins bind a short recognition motif called the PY motif (L/PxY) (200, 202). Nedd4/Nedd4-like proteins are involved in a plethora of functions, including ubiquitylation of membrane proteins or of proteins of the trafficking machinery. Reviews on Nedd4/Nedd4-like proteins can be found in References 154, 179, 354.

C. Ubiquitin Binding Domains

There is a growing list of ubiquitin binding domains and motifs (“ubiquitin receptors”) (Fig. 3). These define the cellular proteins that recognize and interact with ubiquitin and translate ubiquitin signal into a cellular action, such as degradation by the proteasome, internalization involving Eps15, or targeting to the multivesicular body.

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**FIG. 3.** Examples of ubiquitin binding motifs/domains (in color) present in proteins regulating trafficking, endocytosis, and sorting of cellular cargo. Only key domains/motifs (in addition to the ubiquitin binding domains) are shown. Figure is not to scale. CB, clathrin binding motif. See text for other definitions.
1. UIM motif

The UIM (ubiquitin-interacting motif) was first described as a peptide sequence consisting of a highly conserved Ac-Ac-Ac-Ac-Φ-X-X-Ala-X-X-Ser-X-X-Ac, where Φ represents a large hydrophobic residue (typically Leu) and Ac represents an acidic residue (Glu, Asp) (115, 168). This motif was originally found in the ubiquitin-binding region of the Rpn11 proteasome subunit and shown to bind to polyubiquitin (121, 482). The motif was subsequently identified in many other proteins (50, 168, 328), often in combination with other protein-protein interaction motifs. Many of these proteins are either part of the ubiquitin system or play a role in protein trafficking (see below). These also include two different deubiquitylating enzymes [KIAA1594 (USP37) and USP25], an F-box protein (Ufo1), a HECT-type ubiquitin-protein ligase from plants (Up11), but no E1 or E2 enzyme. UIM containing proteins involved in trafficking include the following (Fig. 3): 1) the Eps15 subfamily of EH proteins (219, 329), 2) the Ent1/2-epsin subfamily of ENTH proteins (381), 3) the FYVE domain proteins including Vps27/Hrs (381), and 4) the SH3-containing proteins STAM and HBP (168). Eps15 and its ortholog Ede1 in Saccharomyces cerevisiae comprise two to three EH (Epsin15 homology) domains (110), which bind preferentially to the NPF motifs of epsin/Ent1/2 (4, 316, 329, 359). A number of proteins containing UIM motifs are ubiquitylated, including Hrs/Hgs (205, 265), Eps15 (219), epsins (308, 329), and likely STAM. Often, the UIM domain is required for the ubiquitylation of the protein that harbors it and appears to recruit the relevant ubiquitin-protein ligase (205, 219, 308, 329). The significance of this dual presence of ubiquitin moiety and UIM motif on the same proteins is not clear but may facilitate the formation of complexes with other proteins that contain ubiquitin-binding motifs/domains. The UIM motif by itself does not contain any lysine residues and hence is not directly modified by ubiquitin. It may recruit ubiquitin-protein ligases, for example, via ubiquitin linked by thioester to HECT-type ubiquitin-protein ligases. After transfer of the ubiquitin onto the protein, the HECT E3 enzyme is released. Alternatively, an E3 enzyme may be recruited by the UIM motif, promoting ubiquitylation. The presence of the UIM motif and monoubiquitin on the same protein may also lead to intra- or intermolecular interactions. Intramolecular interactions may hide internal lysines on ubiquitin and thereby interfere with polyubiquitylation of the protein. As ubiquitylation usually is a regulated process and also reversible, this likely suggests that there are dynamic networks of interacting proteins that are controlled by the ubiquitin system (88).

2. Cue domain

The Cue domain was originally identified by database searches and proposed to be scaffolding domain for ubiquitylated proteins (27, 331). It is present in the Cue protein, which binds ubiquitin; is associated with the endoplasmic reticulum (ER) membrane; and recruits the E2 enzyme Ubc7 to the ER membrane (34). The Cue domain is also present in Vps9, a guanidine nucleotide exchange factor involved in yeast endocytosis (73, 91, 335, 382); in Tollip, an intermediate in interleukin-1 signaling; in AMFR, a cytokine receptor involved in tumor cell motility and metastasis; and in AUP1, a ubiquitins protein involved in integrin signaling (331). Like the UIM motif, Cue binds to both monoubiquitin and polyubiquitin and promotes ubiquitylation of Vps9 (73, 201, 382). The structure of a Cue domain associated with monoubiquitin has been solved (201, 335). The contact sites between the Cue domain and ubiquitin are conserved hydrophobic residues in the latter, including Leu-8, Ile-44, and Val-70. These same amino acids were shown previously to be important for receptor endocytosis (30, 389), and in vitro binding assays illustrated a requirement of this hydrophobic patch for interaction with both the UIM and Cue domains (381, 382). The crystal structure of the Vps9-Cue domain bound to monoubiquitin also shows that the contact surface covers Lys-48, which may represent an occlusion mechanism for inhibiting polyubiquitin formation (201). The cue domain shares architecture very similar to the UBA domain (see below) (307), which also has a hydrophobic contact points with ubiquitin. It is likely that the Cue domain and the UBA domain bind ubiquitin in a similar fashion.

3. UBA domain

The UBA (ubiquitin associated) domain was also originally identified by bioinformatics means in proteins that were known to interact with ubiquitin (167). It is present in many proteins involved in the ubiquitylation cascade such as E2, E3, and deubiquitylating enzymes. Of interest for this review is c-Cbl, the E3 ligase involved in ubiquitin-dependent downregulation of many receptor tyrosine kinases, as well as Ede1p (the yeast homolog of Eps15) (340), which contain a UBA domain. The three-dimensional structure of the UBA domain has been solved and shown to be a compact three-helix bundle and to bind to ubiquitin in a very similar manner as does the Cue domain (87, 307, 463). It is likely able to replace functionally the UIM domain, as mammalian Eps15 contains two UIM domains at the COOH terminus, while the yeast homolog has a UBA domain instead.

4. UEV domain

The UEV (ubiquitin E2 variant) domain is homologous to the catalytic UBC (ubiquitin-conjugating) domain of E2 enzymes involved in the conjugation of ubiquitin to target proteins, but it does not contain the catalytic cysteine essential for E2 activity (226, 332). This domain is
found in a number of different proteins. Of interest, it is included in the NH$_2$ terminus of Tsg101 (Vps23), one of the proteins involved in the multivesicular body (MVB) pathway, i.e., for sorting ubiquitylated cargo to the lumen of the lysosomes (see below). In this context, Tsg101-UEV:ubiquitin interactions have been shown to be essential for the MVB pathway (36, 206, 255). The structure of the Tsg101-UEV domain complexed to ubiquitin has been solved (410). The UEV domain has essentially the same structure as in the unbound form, which resembles the canonical E2 enzymes and adopts the typical α/β-fold, but has an additional NH$_2$-terminal helix and lacks two COOH-terminal helices (333).

5. GAT domain

The GAT (GGA and Tom1 homologous) domain is a ubiquitin binding module that plays a role in protein trafficking. It was originally described as a domain present in GGA (Golgi localized γ ear containing ARF binding) proteins (see below) (81), and later described in a number of proteins shown to participate in intracellular trafficking and sorting. The GAT domain is composed of an all α-helices fold that comprises two subdomains. An NH$_2$-terminal “hook” that can bind ARF, and a COOH-terminal triple α-helix bundle, that can interact with Rabaptin 5 and ubiquitin (67, 336, 369, 378, 379, 409, 469, 486, 487) (see below).

6. NZF domain

The NZF domain is another domain that binds ubiquitin and is present on proteins that are involved in intracellular protein trafficking. Its name is derived from NP14 zinc finger (or RANBP2/Nup358 zinc finger) (17, 80, 280, 296). NP14 NZF forms a compact module composed of four anti-parallel β-strands linked by three ordered loops. A single Zn$^{2+}$ is coordinated by four conserved cysteines from the first and the third loop, which form two rubredoxin knuckles. It binds weakly to free ubiquitin, using a conserved threonine-phenylalanine dipeptide that interacts with Ile-44 (5, 448). The NZF domain has been identified in Vps36, which plays a role in sorting cargoes into the MVB pathway (see below) (5, 17). However, the NZF domain is not present in mammalian Vps36, where ubiquitin binding appears to be achieved via a novel domain, the Glue domain.

7. Glue domain

The Glue (Gram-like ubiquitin binding in Eap45) domain was identified in Eap45, the mammalian ortholog of Vps36. As is the case for NZF in yeast Vps36, this domain is situated at the NH$_2$ terminus of Eap45. It binds to ubiquitin in the micromolar range, comparable to the other ubiquitin binding domains. The GLUE domain shares similarities in its primary and predicted secondary structures to phosphoinositide-binding GRAM and PH domains. Accordingly, Eap45 binds to a subset of 3-phosphoinositides, suggesting that ubiquitin recognition could be coordinated with phosphoinositide binding (388).

Interestingly, while the various ubiquitin binding domains described above adopt different folds/conformation from each other, they all appear to bind the same hydrophobic surface patch of ubiquitin that includes I44 (Fig. 1A) (163).

III. UBIQUITIN AND INTRACELLULAR MEMBRANE TRANSPORT IN YEAST

A. Yeast as a Model System to Study Protein Transport

For more than two decades, the yeast cell model played a leading role in the elucidation of molecular mechanisms involved in protein trafficking, initiated by the classical studies of Schekman and collaborators (303), involving the screening for a large numbers of secretion (Sec) mutants. Studying trafficking in yeast has been very useful due to the powerful combination of genetics with biochemistry and cell biology afforded in this eukaryote (207), and the conservation of many of the relevant pathways in higher organisms. Ubiquitylation of membrane proteins plays a role at different stages of their lifespan: 1) during and after biosynthesis in the ER, when quality control and ER-associated degradation (ERAD) take place; 2) in the trans-Golgi Network (TGN) for sorting of proteins to different cellular destinations; 3) at the plasma membrane for internalization of plasma membrane proteins; and 4) sorting and translocation to MVBs in the late endosome. Two classes of yeast plasma membrane proteins have been extensively studied with respect to ubiquitylation: permeases/transport proteins and G protein-coupled seven-transmembrane receptor (GPCR). The following section summarizes the knowledge on the involved pathways, including the implicated organelles, and the role of ubiquitylation of the above mentioned classes of proteins.

B. Ubiquitin and Endocytosis in Yeast

1. Organelles in yeast and mammalian cells involved in protein traffic

Protein trafficking in the cell refers to protein transport between various organelles or subcellular compartments. Most of these organelles (i.e., nuclei, ER, Golgi, lysosomes, mitochondria, endosomes, peroxisomes) are well characterized. However, it is less appreciated that the organelles involved in the endocytic and lysosomal pathway are highly dynamic (reviewed by Maxfield and
McGraw, Ref. 272). Endocytosis (the internalization of membrane proteins and soluble extracellular proteins) is achieved by various mechanisms, including clathrin- and non-clathrin-mediated endocytosis, caveolae formation, and pinocytosis. The best studied is clathrin-coated endocytosis (291). Because of its dynamic nature, it has been difficult to clearly define organelles within the endocytic pathway. An “organelle,” such as the “sorting endosome,” often matures into another type of organelle. “ Resident” proteins of an organelle are constantly escaping from their organelle and are then retrieved by specific retrieval mechanisms. Hence, it is difficult to define such an organelle just by the presence of certain marker proteins, or by morphological features. It is therefore necessary to use several characteristics (composition, morphology, and function) to define them.

The organelles of the endocytotic pathway have been originally divided into early and late endosomes, whereby early endosomes are defined as the organelles that can be directly reached by vesicles derived from the plasma membrane, and from which direct recycling to the plasma membrane still occurs (272), whereas such processes are no longer possible from the late endosome. Early endosomes are subdivided into sorting endosomes and endocytic recycling compartment (ERC). The sorting endosomes are formed by internalized vesicles. They have a half-life of ~5–10 min. Proteins in the sorting endosome can travel to three different destinations: 1) to the plasma membrane, which is the default pathway for transmembrane proteins; 2) to the ERC; and 3) to the late endosome (by maturing of the endosome, and consequently the default pathway for soluble proteins and specific targeting pathway for transmembrane proteins). This specific targeting of membrane proteins to the late endosome is achieved in many cases through their ubiquitylation.

The late endosome can be distinguished morphologically from sorting endosomes by their more spherical appearance compared with the more tubular sorting endosomes. Late endosomes tend also to be located closer to the nucleus. Moreover, parts of the late endosomes have a multivesicular appearance. The elegant cell biological and genetic studies that led to the characterization of the multivesicular (MVB) pathway are described below. Late endosomes containing MVBs fuse then with their outer, limiting membrane with the lysosome (or vacuole in yeast), thereby releasing the MVBs into the lysosomal lumen, where they are dissociated and their cargos degraded (207).

2. The yeast plasma membrane transport proteins

This group of plasma membrane proteins includes two proteins of the ABC transporter family, namely, Ste6, which secretes the yeast mating pheromone a-factor, and Pdr5, involved in multiple drug resistance in yeast (104), as well as the family of transporters of the MFS (multifacilitators superfamily; often referred to as permeases), i.e., proteins that have a central hydrophobic core of 10–12 transmembrane domains, flanked by cytoplasmic NH2 and COOH termini (13, 127). Two permeases of this family have been particularly well studied: the general amino acid permease Gap1 and the uracil permease Fur4. A mammalian member of the ABC transporter family, cystic fibrosis transmembrane conductance regulator (CFTR), has also been extensively studied in yeast, especially with respect to ER-associated degradation.

3. The ABC transport protein Ste6

The first evidence of a role for ubiquitin in the control of cell surface expression of a plasma membrane protein came from work on Ste6, an ABC transport protein required for secretion of the mating pheromone a-factor (231, 277). In this original work it was shown that Ste6 accumulates in endocytosis defective mutants as a ubiquitylated protein, suggesting that ubiquitylation of Ste6 occurs on the way to or at the plasma membrane (224). In support of a role of the ubiquitin system in the regulation of Ste6 was the finding that its rapid turnover was attenuated in ubc4ubc5 mutants. Ubiquitylation of Ste6 is mediated by the region linking the two halves of the molecule, referred to as D-box (destabilization box). The D-box is composed of an upstream region containing mostly acidic residues (A-box) and a downstream region with basic residues (B-box). The A-box contains a motif, “DAKTI,” which closely resembles the DAKSS motif in the Ste2 receptor (225, 352) (see below). Deletion of the A-box leads to the accumulation of Ste6 at the plasma membrane, whereas wild-type Ste6 is mostly intracellular. A deubiquitylating enzyme, Ubp1, plays a role either in internalization or in the recycling of Ste6 to the plasma membrane, as its overexpression causes accumulation of Ste6 at the cell surface. However, Ste6 does not seem to be the direct target of Ubp1, since its ubiquitylation level is not affected by Ubp1 (364). Ste6 targeting into the lumen of the late endosome or vacuole (MVB pathway) for degradation requires the action of Doa4, another deubiquitylating enzyme also known as Npi2/Ubp4/Ssv7, as Ste6 accumulates in the vacuole in doa4 mutants (250, 253). However, it is known that Doa4 is required for efficient ubiquitin homeostasis, because free ubiquitin levels are reduced significantly in doa4 mutants (397, 411). Indeed, overexpression of ubiquitin restores targeting of Ste6 to the MVB pathway and accompanied degradation, suggesting that lack of free ubiquitin was the cause of the impaired MVB targeting (253). It is not yet clear which degradation systems are involved in Ste6 turnover, as its turnover is not only slowed down in the vacuolar protease mutant pep4 (224, 251), but also in mutants defective in the chymotrypsin-like activity of the protea-
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Some (pre1–1/pre2–1 double mutant) (251). Whether this means a cooperative activity of vacuolar proteases and the proteasome at the level of the vacuole remains to be determined. Alternatively, the observed impairment of Ste6 turnover may be due to degradation of misfolded Ste6 proteins in the ER, which requires proteasome activity. Another protein of the ABC transport family in yeast is Pdr5, which has also been shown to be regulated by ubiquitylation (103).

4. Yeast permeases

Remarkably, the expression, activity, and turnover of yeast permeases is highly regulated and allows a yeast cell to adapt to rapidly changing extracellular milieu conditions and nutritional availability (171). For most of these proteins the regulation controls the steady-state expression at the cell surface. Indeed, there has been only one case so far of a yeast plasma membrane protein whose activity is not controlled by protein trafficking, namely, the copper-transport protein (Ctrl), a three-transmembrane domain spanning protein which undergoes copper-induced proteolysis at the plasma membrane (310). Most of the other permeases are regulated at least in part by the ubiquitin system (43, 99, 161, 354). The emphasis of this review focuses on recently developed concepts with respect to molecular mechanisms involved in ubiquitylation-dependent membrane protein transport, using the Gap1 and Fur4 permeases as examples.

A) The General Amino Acid Permease, Gap1. Evidence that ubiquitylation may be involved in the control of internalization of permeases came originally from genetic studies. It had been known for a long time that yeast cells control precisely the uptake of organic nutrients. This control is exerted by a process referred to as "catabolite inactivation," during which sugar or amino acid transporters are rapidly downregulated. This is achieved by the addition of either glucose, the favored carbon source of yeast, or by the addition of NH4+, which is preferred as a nitrogen source over amino acids. The latter, nitrogen catabolite inactivation, had been studied for years and led to the identification of yeast mutants that were deficient in the inactivation of amino acid uptake (referred to as npi1, npi2, and npi3) (140, 141, 460). In this context it was shown that Gap1 was affected by these mutants and was not downregulated. Molecular characterization of these mutants showed that the defects were in enzymes involved in ubiquitylation, namely, npi1 in the gene encoding the HECT domain containing ubiquitin-protein ligase Rsp5 (Fig. 2B) (155) and npi2 in the gene encoding the deubiquitylation enzyme Doa4 (397). Indeed, NH4+ stimulates ubiquitylation and endocytosis of Gap1 and subsequent degradation in the vacuole. In npi1 mutants (expressing 10% of Rsp5), ubiquitylation is impaired, Gap1 is not internalized, and its turnover is attenuated (155, 395). Moreover, ubiquitylation and downregulation of Gap1 is dependent on enzymatic activity of Rsp5 (396). The same authors also found that NH4+ stimulates the modification of Gap1 via lysine-63 linked ubiquitin chains, although blocking the formation of K63-linked chain does not completely inhibit NH4+-induced internalization of Gap1 (397).

Analysis of the second npi mutant, npi2, bearing a mutation in a gene encoding Doa4 revealed that Gap1 is regulated by deubiquitylation (397). Similar to Ste6, overexpression of ubiquitin in npi2 cells restored free ubiquitin levels and proper ubiquitylation, internalization, and degradation of Gap1, suggesting that Doa4 regulates Gap1 indirectly via impairing the generation of free ubiquitin levels. Gap1 is also regulated by another deubiquitylating enzyme, Dot4, which appears to stimulate Gap1 activity, as suggested by its loss of function in Δdot4 cells (195). Whether Dot4 is acting via direct deubiquitylation of Gap1 or via another substrate remains to be demonstrated.

A third npi mutant (npi3) was identified in nitrogen-induced catabolite inactivation. NPI3 (corresponding to the BRO1 gene, see below) is required for NH4+-induced downregulation and efficient ubiquitylation of Gap1 (398) and appears to be important for efficient sorting of Gap1 into the vacuole (see below). Sorting of Gap1 is also controlled by the Npr1 kinase (433), which stabilizes Gap1 at the plasma membrane. Inactivation of Npr1 activity causes direct targeting to the vacuolar pathway, rapid internalization, and increased ubiquitylation of Gap1, suggesting that Npr1 counteracts the ubiquitylation of Gap1 by Rsp5 (79).

B) The Uracil Permease, Fur4. The uracil permease Fur4 was the second permease for which an Npi1/Rsp5-dependent inactivation was demonstrated (155). Although this permease undergoes constitutive endocytosis, this endocytosis is increased under stress conditions, such as elevated temperature, nutrient starvation, and inhibition of protein translation (125, 444), or in the presence of excess of uracil (132). As a result, Fur4 enters the endocytic pathway and is degraded in the vacuole. Like Gap1, Fur4 becomes ubiquitylated in a Rsp5-dependent manner, and the ubiquitylated species accumulate at the plasma membrane in cells deficient in the internalization step of endocytosis (125, 286). In npi1 mutant cells, Fur4 accumulates at the nonpermissive temperature at the plasma membrane, and the protein is stabilized (125). This suggests that ubiquitylation is necessary for internalization of the permease. Moreover, Fur4 is not degraded by the proteasome but is stabilized in cells deficient in vacuolar proteases (125). Further evidence for a role of ubiquitylation in the internalization of Fur4 comes from experiments showing that cells lacking Doa4 are also defective in uracil permease ubiquitylation and internalization. This phenotype can be corrected by overproducing ubiquitin, suggesting that inactivation of Doa4 leads to a lack of free ubiquitin available for ubiquitylation (124). This system
allows studying the type of ubiquitylation that is important for Fur4 endocytosis. Accordingly, overexpression of ubiquitin mutants that carry Lys to Arg mutations at Lys-29 and Lys-48 restore Fur4 ubiquitylation, whereas a ubiquitin mutated at Lys-63 does not, suggesting that Fur4 is ubiquitylated by ubiquitin chains extended through Lys-63. Similar to Gap1, when polyubiquitylation is blocked, the Fur4 permease still undergoes endocytosis, but at a reduced rate. This suggests that monoubiquitylation is sufficient to induce permease endocytosis, but that Lys-63-linked ubiquitin chains appear to augment this process (124).

C) OTHER PERMEASES. The maltose transporter is another permease whose internalization is induced by nitrogen starvation if a fermentable carbon source is present and for which this internalization depends on Rsp5 and Doa4 (256, 278). The impaired internalization in doa4 mutant can be restored by overexpression of ubiquitin, indicating that it is mostly the low levels of ubiquitin that interfere with internalization of this transporter (257). Moreover, monoubiquitylation appears to be sufficient as an internalization signal.

The tryptophan permease Tat2 is internalized in a manner dependent on high pressure and requiring the activity of Rsp5, the Rsp5-associated proteins Bul1 and Bul2 (1), and the vacuolar sorting protein Vps27 (295). Similar to Fur4 and Ste6, the internalization and degradation of Tat2 also depends on Doa4; however, in this case, two other deubiquitylating enzymes, Ubp6 and Ubp14, are also required (283). Overexpression of ubiquitin does not rescue the defect of any of these DUBs, indicating that they are not only involved in ubiquitin recycling, but also have other specific effects. Ubp6 is a protein that is associated with the base of the regulatory unit of the proteasome and may be involved in the removal of ubiquitin from substrates, and appears to be important for the recycling of ubiquitin (9, 239). Ubp14 is involved in the disassembly of free polyubiquitin chains, which correlates with ubiquitin-dependent defects of the proteasome (7). It is therefore likely that these enzymes play a specific role in high-pressure-dependent internalization and degradation. Ubiquitylated Tat2p may be recognized by the endosomal machinery involved in translocation into the MVBs, in the course of which Doa4 may play a role in deubiquitylation of the enzyme. Ubp6 and Ubp14 may play a role here as well, in conjunction with the proteasome. It will be necessary to establish if these deubiquitylating enzymes are directly acting on Tat2p (283).

5. The pheromone receptor Ste2

The α-factor receptor Ste2 is a GPCR that binds the mating pheromone α-factor. Upon ligand binding it activates a signal-transduction pathway and undergoes rapid internalization and degradation in the vacuole. α-Factor-dependent stimulation triggers the rapid appearance of ubiquitylated Ste2 species, which accumulate at the plasma membrane in endocytosis-deficient ent1Δ cells (162). The ubiquitylation and accompanied endocytosis of Ste2 depends on the E2 enzymes Ubc1, Ubc4, and Ubc5 and on the E3 enzyme Rsp5 (95, 162). Ste2, carrying a deletion of two-thirds of its cytoplasmic COOH-terminal tail, still internalizes in an α-factor-dependent manner (352). This truncated receptor contains a short sequence of nine amino acids, SINNDAKSS, which plays a critical role in internalization. K337, within the SINNDAKS motif, is essential for ubiquitylation of the truncated receptor, as are the three serines in this sequence (162). Phosphorylation of the three serines in response to receptor stimulation is a prerequisite for receptor ubiquitylation (164). This is similar to the phosphorylation of the PEST sequence in Fur4 that is required for its ubiquitylation (263).

Moreover, as is the case for Fur4, it appears that casein kinase I is the kinase that phosphorylates the receptor (164, 262). Monoubiquitylation was found to be sufficient for internalization of Ste2, since overexpression of ubiquitin with all its lysines mutated to arginines is sufficient to efficiently internalize Ste2 upon α-factor stimulation, as was fusion of a lysine-less ubiquitin moiety to the (lysine-less) cytoplasmic tail of Ste2 (421). With the use of this model it was demonstrated that a hydrophobic patch on the surface of ubiquitin, comprising Phe-4 and particularly Ile-44, serves as signal for internalization (383, 389).

Ste2 has been used as a model for studying the molecular mechanisms of internalization of ubiquitylated proteins. It has been demonstrated that proteins involved in endocytosis contain ubiquitin binding domains or motifs, such as the UIM, UBA, or CUE motifs/domains described above. When systematically mutating proteins containing such domains, several of them were found to display endocytosis defects (381). Yeast strains that were mutated in EDE1, VPS27, ENT1, and ENT2 had endocytosis defects. Ent1 and Ent2 have UIM domains and are homologs of the mammalian epsins, required for the internalization step of endocytosis (59). Ede1 is a UBA-containing protein that functions in fluid-phase endocytosis and receptor-mediated endocytosis. It is the homolog of mammalian Ep15, which contains a UIM motif, and is a component of the mammalian endocytosis machinery (31). All these proteins bind monoubiquitin in vitro. The binding depends on the UIM motif (Vps27), involves Ile-44 of ubiquitin, and in vitro assays show that UIM of Ent1 and Vps27 bind directly to ubiquitin (381). Ede1, Ent1, and Ent2 play a role in internalization of α-factor. In ent1Δent2Δ cells, carrying an allele with a temperature-sensitive mutation in ent1p (ent1ts), the internalization of α-factor is severely impaired at the nonpermissive temperature. Cells expressing ent1ΔUIM have an internalization defect that is milder compared with ent1ts cells. In mammalian cells, epsins and Ep15 have been shown to
interact via a NPF motif and the EH (epsin homology) domain (60). These domains are conserved in yeast, making it likely that the complex is conserved as well. It was therefore speculated that the Edel1 UBA domain interacts with ubiquitin and that this may be sufficient to promote internalization. Indeed, ent1ΔΔent2ΔΔede1ΔΔ cells carrying ent1ΔΔUIM are defective in α-factor internalization, indicating that Edel1 overcomes the defect of ent1ΔΔUIM.

Internalization of the α-factor receptor and fluid-phase endocytosis depends also on the yeast proteins Rvs167 and Sla1 (28, 292). Rvs167 is the yeast homolog of endophilin/amphiphysin, and Sla1 of CIN85 (Cbl interacting protein 85) (416), proteins that are both known to be involved in mammalian receptor internalization (see below). Both Rvs167 and Sla1 bind to the ubiquitin-protein ligase Rsp5, and Rvs167 becomes monoubiquitylated on Lys-481, which is situated in the COOH-terminal SH3 domain of Rvs167 (400). Monoubiquitylation within the SH3 domain may control protein-protein interaction but is not required for endocytosis or general function of Rvs167.

C. Ubiquitin-Dependent Protein Traffic in Yeast

In recent years it has become evident that ubiquitin plays also an essential role in intracellular protein trafficking, in protein targeting either from the Golgi or from the plasma membrane via the endosomal system to the MVBs in the vacuolar lumen. Again, much of the current understanding was first elucidated in yeast and subsequently in mammalian cells.

1. Role of ubiquitin in Golgi to vacuole transport

A) PERMEASES. Ubiquitylation is not only important for the internalization of plasma membrane proteins, but it also plays a role in their sorting. Helliwell et al. (158) showed that the general amino acid permease Gap1 becomes either monoubiquitylated or polyubiquitylated and that both types of modifications depend on Rsp5. Two Rsp5-associated proteins, Bul1 and Bul2, are required for polyubiquitylation (158). Bul1 and Bul2 associate via their PY motifs with the WW domains of Rsp5 (472, 473) and promote polyubiquitylation of Gap1, thereby targeting Gap1 into the vacuolar pathway. When Bul1p and Bul2p are overexpressed, Gap1 is sorted to the vacuole independently of the nitrogen source, whereas deletion of these proteins leads to the constitutive expression at the cell surface and diminution of polyubiquitylation of Gap1. These data therefore suggest that Bul1p/Bul2p can act as an E4 enzyme, promoting polyubiquitylation of Gap1, and consequently targeting it to the vacuole (158). These data differ from the ones of Soetens et al. (392), who observe only polyubiquitylation of Gap1 in the presence of NH₄⁺ and could not demonstrate an essential role of polyubiquitylation in sorting into the vacuolar pathway. Further experiments will be required to clarify the role of polyubiquitylation and monoubiquitylation in the control of Gap1 activity.

Like Gap1, the uracil permease Fur4 can be sorted directly from the biosynthetic pathway into the vacuolar MVB pathway. The signal for such sorting seems to be uracil, which triggers subsequent ubiquitylation and targeting of this permease into the lumen of the vacuole, where it is degraded (40).

B) GGA AND GOLGI TO ENDOSOME TRANSPORT. GGAs [Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor (ARF)-binding proteins] are a family of monomeric adaptor proteins involved in membrane trafficking from the TGN to endosomes and lysosomes (42). They are ubiquitously expressed, and they are able to bind to clathrin in an ARF-dependent manner. GGAs are composed of a VHS (Vps27, Hrs, Stam) domain, a GAT [GGAs and Tom1 (target of myb1)] domain, and an unstructured hinge region that connects to the COOH-terminal GAE (γ-adaptin ear) domain (42) (Fig. 3). Such proteins mediate the sorting of mannose-6-phosphate receptors between the TGN and the endosomes. Recently, these proteins have been shown to bind to ubiquitin via the GAT domain (336, 369, 379), which binds the Ile-44 surface patch on ubiquitin. The residues of this patch are also necessary for the binding to Tsg101, the mammalian homolog of Vps23 (see below) (336). Knockdown of GGA3 by RNA interference (RNAi) leads to the accumulation of the mannose-6-phosphate receptors and of epidermal growth factor (EGF) receptor within large endosomes, likely the class E compartment (336). As mentioned above, Gap1 is sorted directly from the TGN to the vacuole when cells are grown on a good nitrogen source (158, 392), and this depends on polyubiquitylation of Gap1 (158). This sorting of Gap1 (or of Ste6) requires GGA2, which has been shown to interact with ubiquitin via its GAT domain. In Δgga2 cells, or in cells expressing gga2 lacking a GAT domain, Gap1 is targeted to the plasma membrane, even in the presence of a good nitrogen source (369).

2. Ubiquitin and targeting to the vacuole

Once plasma membrane proteins are internalized they are either recycled back to the plasma membrane or they are targeted to the endosomal system, which coordinates protein transport from both the endocytic and the biosynthetic pathways (143). A critical step in plasma membrane protein downregulation occurs in the sorting endosome, where MVBs are generated (111, 143). MVBs were described over 50 years ago (207 and references therein). They consist of a limiting membrane and up to several hundreds of internal vesicles of 40–90 nm in diameter. These are endosomal transport intermediates, which later mature or fuse with the late endosome compartment and subsequently with the lysosome/vacuole.
They are generated when the limiting membrane in the sorting endosome invaginates and buds off toward the lumen of the organelle. During this process a subset of membrane proteins of the limiting membrane is sorted into these invaginating vesicles. Fusion of the organelle with late endosome, and ultimately the lysosome/vacuole, results in the delivery of the internal vesicles, along with the associated cargo, to the lumen of the lysosome/vacuole allowing their degradation by hydrolytic enzymes (123). Proteins that remain at the limiting membrane of endosomes remain also at the limiting membrane of lysosomes. This process, referred to as the MVB sorting process, serves different functions. Transmembrane proteins in the intraluminal membranes are susceptible to lysosomal degradation, whereas proteins in the limiting membrane are not, as they are exposed to the proteolytic environment only with their small luminal side, which often is proteolytically resistant due to extensive glycosylation. Second, the MVB may represent a storage compartment for proteins that are destined for secretion into the extracellular space. Third, signaling from the limiting membrane is in principle still possible, where this is precluded from receptors in the intraluminal membranes. We are now just beginning to understand the molecular mechanisms underlying protein sorting into the MVB pathway. This involves ubiquitin modification and recognition of the different ubiquitin binding domains/motifs. Importantly, these mechanisms are highly conserved between yeast and mammalian cells.

A) SORTING TO THE MVB PATHWAY. As indicated above, both cell biological studies in mammalian cells and genetic investigations in yeast have been used to characterize the MVB pathway. Emr and collaborators (26) have identified so-called Vps (vacuolar protein sorting) mutants, which are defective in vacuolar sorting (26). More than 50 corresponding proteins have been found to function at different stages of protein sorting to the vacuole; 17 of them are required for targeting to the MVB pathway, and loss of function of these proteins leads to the formation of a malformed, very large late endosome/MVB compartment (349).

Many proteins that are sorted into the MVB are monoubiquitylated, whereas others, such as the Gap1 permease, are polyubiquitylated (158). This includes both newly synthesized hydrolytic enzymes, which are targeted via the TGN to the endosomes and ultimately to the lysosome/vacuole, but also the plasma membrane proteins described above that are internalized via ubiquitin-independent endocytosis. Continuous ubiquitylation is necessary and sufficient to target these proteins to the endosomal/lysosomal lumen, as demonstrated by EGF receptor, which is recycled if its ubiquitylation is not maintained (252) (see below), or by fusion of a ubiquitin polypeptide to a transmembrane protein (346). However, Reggiori et al. (346) also demonstrated that not all proteins require ubiquitylation to enter the MVB pathway.

B) RECEIVERS RECOGNIZING UBIQUITYLATED CARGOES. There have been three ubiquitin receptors described on endosomal membranes that recognize ubiquitylated cargo, namely, the Vps27/HseI (35, 381); the ESCRT-I (Fig. 4), which is composed of the three proteins Vps23, Vps28, and Vps37 (21, 38, 206); and the ESCRT-II complex comprising the proteins Vps22, Vps25, and Vps36 (17). The three complexes appear to bind monoubiquitylated cargoes via known ubiquitin interacting motifs or domains, namely, the UIM motif in Vps27/HseI (35), the UBC-like UEV domain in ESCRT-I (206), and the NZF domain in Vps36 (5). These binding motifs or domains have relatively low affinity for ubiquitin (115, 128, 333, 340, 374, 381, 412) but often appear in multiple copies on a protein, which likely lead to increased avidity. Different ubiquitin binding proteins may also cooperate in binding to ubiquitylated proteins, as suggested for Vps27 and Vps23 (20, 36, 208), which appear to interact via a PSDP motif at the COOH terminus of Vps27. This PSDP motif resembles the PT/SAP motif in the HIV Gag protein, which is involved in binding to Tsg101 (the Vps23 mammalian homolog), and thereby recruiting the ESCRT machinery (128) (see below).

C) PROTEIN COMPLEXES INVOLVED IN TARGETING TO THE MVB PATHWAY. Genetic evidence suggests that Vps27/HseI acts upstream of ESCRT-I (207). Lipids appear to play an important role in binding Vps27 via its FYVE (Fabl, YOTB, Vac1, and early endosomal antigen 1) domain to phosphatidylinositol-3-phosphate (PtdIns-3P)-enriched endosomal membranes (132, 208, 399). The lipid that is generated by phosphorylation of PtdIns by a class III PtdIns-3P kinase is found both on the limiting and intraluminal membranes (131). Further support for a role of phospholipids comes from the demonstration that the Fab1 PtdIns-3P 5-kinase is essential for the MVB pathway (305). Once Vsp27, which most likely is constitutively associated with HseI, is attached to the endosomal membrane (35), it recruits Vsp23 and the other components of the ESCRT-I complex (36, 208). This likely enables the two complexes to interact with the ubiquitylated cargo via the UIM motif in Vps27 and HseI and the UEV domain on Vsp23.

Two other complexes downstream of ESCRT-I, namely, ESCRT-II and ESCRT-III, participate in protein sorting (Fig. 4). ESCRT-II is a ~155-kDa soluble complex composed of three proteins Vps22, Vps25, and Vps36 (17). The crystal structure of the core structure of the ESCRT-II complex was solved (165, 420, 457) and shown to comprise a quaternary complex of one Vps22, one Vps36, and two Vps25 molecules. A similar complex has also been described in mammalian cells (196). It forms a three-lobed or Y-shaped complex, with one Vps25 molecule that forms the stem and the other forming one arm. The second arm is composed of a sandwich comprising Vps22 and the COOH terminus of Vps36. Although the proteins have no homology to each other, they all form a pair of winged helices that together build the core of the com-
complex. Such a structural block has been found previously in transcription factors. At the tip of the Vps36/Vps22 arm protrudes the NH₂-terminal coiled-coil domain of Vps22. It was proposed that this NH₂-terminal coiled-coil domain could interact with the coiled-coil region of Vps20 (in the ESCRT-III complex). However, Teo et al. (420) showed by binding assays that although Vps20 is involved in ESCRT-II/ESCRT-III interaction, the coiled-coil region of Vps22 is not necessary. Rather it is Vps25 that interacts with ESCRT-III Vps20 (420, 480). The coiled-coil domain may be involved in interaction with ESCRT-I, as suggested by complementary two-hybrid analysis (49), or play a role in membrane binding. An NH₂-terminal ubiquitin binding NZF domain from Vps36 (not crystallized in either complex) is attached to this arm through a flexible linker (165). The domain may be involved in the transfer of ubiquitylated cargo from ESCRT-I to ESCRT-II. Indeed, mutations in the NZF domains of Vps36 lead to a class E compartment phenotype and to abrogation of CPS import into the endosomal lumen (5). However, the mammalian orthologs of Vps36 do not contain a NZF domain but interact via a novel ubiquitin binding domain, Glue, with ubiquitin (388). The protein complex transiently associates with endosomal membrane; this association may be promoted via direct interaction of the two Vps36 NZF domains with the ubiquitylated cargo, via interaction between ESCRT-I and ESCRT-II (49), or a combination of both.

ESCRT-II is important for the recruitment of ESCRT-III to the endosomal membrane; ESCRT-III is a large complex (Fig. 4) composed of four small proteins each containing a coiled-coil domain and an NH₂-terminal basic region, whereas the COOH terminus is acidic (16). The four proteins are Vps20p, Snf7p, Vps2p, and Vs24p. In the cytosol they exist as soluble monomers. At the membrane they exist as two subcomplexes with different functions. Vps20 and Snf7 are required for the localization to the membrane and recruit Vps2/Vps24. The membrane localization of the complex is facilitated by myristoylation of Vps20. As outlined above, the complex is likely recruited by interaction between Vps25 (ESCRT-II) and Vps20. Snf7/Vps20 are required for membrane localization of Vps2/Vps24. These proteins apparently are the docking sites for Vps4, Bro1, and Doa4 (10).

D) VPS4. Vps4 is a member of the AAA (ATPase associated with a variety of cellular activities) family of ATPases, which are involved in a number of different cellular functions such as membrane fusion (Sec18/NSF, cdc48/p97), protein degradation (Yta10–12, proteasome subunits), and chaperone-like activities (Yta10–12, Fsh) (for a review, see Ref. 68). The mammalian homolog of Vps4 is SKD1 (362). Vps4 has been shown to be essential for sorting of a number of proteins into the endosomal lumen (17–19, 113, 385, 483). Vps4 appears to be important for the release of the ESCRT complexes from the endosomal membrane, by yet unknown mechanisms (208). By analogy to Sec18/NSF, which disassembles coiled-coil complexes of SNARE proteins on membrane surfaces, it is possible that Vps4 also disassembles interactions between coiled-coil regions in the ESCRT complexes (16).

E) DOA4. The deubiquitylating enzyme Doa4 is involved in the internalization/degradation of a number of proteins. As mentioned above, it is identical to Npi2, a protein essential for nitrogen catabolite inactivation of the Gap1 permease. Doa4 plays an essential role in ubiquitin homeostasis, but it has also a more specific role in endosomal targeting of the uracil permease Fur4. In pep4 cells, which lack vacuolar protease activity, nonubiquitylated Fur4 accumulates in the vacuole. In contrast, pep4 cells lacking Doa4 accumulate ubiquitylated Fur4, suggesting that Doa4 deubiquitylates Fur4 before entering into the vacuole (98). The data from Dupré et al. also suggest that numerous membrane proteins have to be deubiquitylated by Doa4 before the entry into the vacuole, as indicated by the accumulation of ubiquitylated membrane proteins in Doa4ΔPep4Δ cells. This is further supported by findings that proteins involved in vacuolar import can act as suppressors of Doa4 cells (10). On the other hand, it has been shown that fusion of a ubiquitin-moiety on the transmembrane protein Phm5p bypasses the requirement of Doa4 activity for sorting into MVBs (346).

F) BRO1. Bro1 (BCK1-like resistance to osmotic shock) was originally identified in a study showing that a bro1 mutation compromises the viability of cells that are mutant for several components of the protein kinase C/mitogen-activated protein (MAP) kinase signaling pathway, including the MEK kinase Bck1 (301). The BRO1 gene was also isolated as suppressor of mutations (syyl and ptr3) that decrease the capacity of a cell to uptake amino acids (117). Moreover, Bro1 is identical to Npi3, which is required for NH₄⁺-induced downregulation and efficient ubiquitylation of Gap1 (388). Bro1/Npi3 is also the homolog of Vps31, involved in MVB sorting (305). In vps31 cells, both CPS and Ste2 are missorted to the vacuolar membrane or the class E compartment (258, 305, 306). Snf7 is necessary for recruitment of Bro1 to the class E compartment, but Vps24 is not required (306). Bro1 contains a coiled-coil (CC) region. This region is required for proper sorting of CPS into the lumen (258), but it is not necessary for targeting Bro1 to the endosomes. Bro1 interacts with Doa4, preferentially when associated with a membrane fraction. However, this interaction is not via the coiled-coil domain. Overexpression of Doa4 can suppress the sorting defect of either Bro1ΔCC or Bro1Δ1 cells (with respect to the missorting of CPY), but can only partially suppress the sorting defect of CPS in Bro1Δ cells. Also, Doa4 suppresses the deubiquitylation defect of CPS in Bro1ΔCC cells. In addition, Doa4 localization to the endosomes requires Bro1, and the ESCRT-III protein Snf7, suggesting that Bro1 recruits Doa4 to the plasma membrane (258).
Bro1 has a mouse homolog, Aip1, which is localized both to the cytoplasm and cell membranes and interacts physically with Alg2, a calcium binding protein involved in cell apoptosis (281, 443). Interestingly, Aip1 also interacts with SETA/CIN85, an SH3-containing adapter shown to bind c-Cbl, which is involved in ubiquitylation and intracellular trafficking of receptor tyrosine kinase (see below). Moreover, it binds also to the CHMP4 proteins,
the homologs of Snf7. Aip1 may thus be part of a protein complex involved in receptor ubiquitylation and/or trafficking, and it is involved in the MVB pathway.

IV. MAMMALIAN MEMBRANE PROTEINS AND UBIQUITYLATION

In recent years, a large number of studies have described the regulation of mammalian transmembrane proteins by the ubiquitin system. Key examples, representing various families of such proteins, are provided here.

A. Ion Channels

The regulation of ion channels by the ubiquitin system was described earlier for CFTR, where its processing in the ER was shown to be regulated by ubiquitylation and by ERAD (187, 453). Perhaps the best studied ion channel in which ubiquitylation plays a key role in regulating its cell surface stability is the epithelial Na\(^+\) channel (ENaC).

1. Regulation of ENaC by Nedd4 proteins

ENaC, comprised of three subunits (\(\alpha\beta\gamma\)) (53, 54), is responsible for salt and fluid absorption in several epithelia including those in the kidney, colon, and lung. Each of the channel subunits contains a PY motif (PPxY) at its cytoplasmic COOH terminus (363), and deletion or mutation of the PY motif in \(\beta\)- or \(\gamma\)-ENaC causes Liddle’s syndrome (149, 384), a hereditary hypertension caused by increased abundance and activity of ENaC at the plasma membrane (114). The same PY motifs were identified as binding partners for Nedd4 proteins (403). As depicted in Figure 2B, Nedd4 family members comprise a C2 domain responsible for membrane binding and subcellular localization (327, 449), three or four WW domains that bind PY motifs (LPPxY) in target proteins (61, 200, 202), and a ubiquitin ligase HECT domain (173). Subsequent studies have demonstrated that Nedd4–2 (closely related to Nedd4–1) can suppress ENaC activity by controlling cell surface stability of the channel (Fig. 5) and that Liddle syndrome mutations impair the ability of Nedd4–2 to downregulate ENaC (3, 153, 197). In accord, cell surface stability of ENaC was shown to be regulated by ubiquitylation (404), and it was demonstrated that Nedd4–2 acts in concert with the E2 enzyme UBE2E3 (78). The ability of Nedd4–2 (but not most Nedd4–1 proteins) to suppress channel activity is dependent on the presence of a high-affinity WW domain (WW3) found in all Nedd4–2 but only in some Nedd4–1 proteins (159); those few WW3-containing Nedd4–1 proteins can also suppress ENaC activity (159), especially when lacking their C2 domain (198, 390). Nedd4–2 uniquely possesses phosphorylation sites for the serum and glucocorticoid-regulated kinase, Sgk (an Akt/PKB relative)(456), not present in Nedd4–1. Sgk is upregulated by aldosterone, a mineralocorticoid hormone controlling ENaC, and ENaC function is enhanced by Sgk (62, 299). Recent work has demonstrated that Sgk phosphorylates Nedd4–2 (77, 391) and that this phosphorylation generates a binding site for 14–3–3 proteins (33, 174). Consequently, this reduces the ability of Nedd4–2 to suppress ENaC, resulting in enhancement of ENaC stability and function (Fig. 5). Moreover, it was demonstrated that aldosterone enhances phosphorylation of Nedd4–2 in renal epithelial cells (116). However, aldosterone and Sgk can upregulate ENaC by mechanisms that are independent of Nedd4–2 (6, 15, 72, 85, 380), suggesting that Nedd4–2 likely contributes, but is not obligatory, to the aldosterone-Sgk-ENaC pathway.

2. Regulation of other ion channels by Nedd4 proteins

In addition to regulating ENaC, Nedd4 family members have been shown to regulate stability of other ion channels. For example, the chloride channel CIC-5, which is mutated in Dent’s disease (proteinurea and kidney stones), is an endosomal channel that regulates endocytosis of TM proteins, and it also contains a PY motif at its cytosolic COOH terminus (366). This PY motif is required for binding to the Nedd4 family members Nedd4–2 and possibly WWP2, and for the regulation of endocytosis and function of CIC-5 itself (172, 366). The kidney Cl\(^-\) channels CIC-Ka and CIC-Kb require an associated transmembrane \(\beta\)-subunit, barttin, for maximal channel activity (108). Mutations in barttin cause a form of Bartter’s syndrome (type IV) that leads to congenital deafness and renal failure. Interestingly, barttin contains a PY motif at
its intracellular COOH terminus, and its mutation leads to enhanced Cl⁻ channel activity (108, 188), analogous to the gain-of-function mutations in the PY motif of β-ENaC seen in Liddle's syndrome. A recent report suggests that the PY motif of barttin may be regulated by Nedd4–2 (107). The voltage-gated Na⁺ channel Naᵥ1.5 plays a critical role in induction and propagation of action potentials in nerves and cardiomyocytes, and mutations in this channel are linked to a variety of diseases, including long QT syndrome, Brugada syndrome, conduction disorders, and sudden infant death syndrome. Naᵥ1.5 as well as the isoforms Naᵥ1.2 and 1.3 each possesses a PY motif that is able to bind Nedd4 family members, and Nedd4–2 functionally downregulates these channels (2, 355, 432).

3. Regulation of cell surface CFTR by ubiquitylation

CFTR is a chloride channel encoded by the gene mutated in cystic fibrosis (CF), an autosomal recessive disease in which lack of CFTR function at the apical surface of epithelial cells lining the airways leads to the morbidity and mortality associated with CF. This is due to altered hydration of airway epithelia and persistent lung infections (350). CFTR is a large glycoprotein, comprised of two transmembrane domains (each containing 6 transmembrane helices), two cytoplasmic nucleotide binding domains (NBDs), and a regulatory (R) region. The most common mutation in Caucasian CF patients is deletion of F508 (CFTRΔF508), located at the first NBD of CFTR. CFTRΔF508 is a temperature-sensitive folding mutant, causing its premature degradation by the ER-associated degradation (ERAD) machinery. Because of this defect, CFTR is one of the best studied ERAD substrates, and the molecular mechanisms involved have been extensively studied (274).

Using maneuvers that allow trafficking of some ΔF508-CFTR to the plasma membrane (e.g., low temperature, chemical chaperones), it was recently found that while endocytosed wild-type (WT)-CFTR is recycled back to the plasma membrane, mutant CFTR is ubiquitylated and diverted to the MVB pathway for lysosomal degradation, resulting in a shorter half-life of the mutant channel at the cell surface (129, 373). Alternatively, it was recently
proposed that ΔF508-CFTR at the plasma membrane has a higher internalization rate (413). The E3 implicated in CFTR (WT or mutant) ubiquitylation is so far unknown.

4. Regulation of other ion channels by the ubiquitin or Sumo systems

Recent reports have revealed a role for the ubiquitin or SUMO systems in regulating K+ channels. ROMK1 is expressed in the kidney and is responsible for K+ secretion and recycling, needed for proper function of the Na+-Cl−-K+ cotransporter. Mutations in ROMK channels cause type II Bartter’s syndrome. ROMK1 was recently shown to be monoubiquitylated (by as yet an unknown E3) on a single Lys residue, which regulates cell surface levels of the channel (246). Interestingly, the “silent” leak K+ channel, K2P1, was recently demonstrated to be regulated by sumoylation at the plasma membrane. The channel is silenced by Sumo modification by Ubc9 and is activated by the SUMO protease SENP1, thus regulating channel activity (341).

B. Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) play a pivotal role in regulating many cellular processes, including cell proliferation and malignant transformation; hence, their regulation is critical. The regulation of their internalization and sorting by the ubiquitin system has received much attention recently and will be described here.

That the ubiquitin system is involved in RTKs function was proposed earlier by the observation that ligand-induced ubiquitylation of the PDGFR plays a role in negative regulation of its signaling (287) and that the EGF receptor (EGFR) becomes ubiquitylated upon ligand binding, an effect requiring its kinase activity (126). Much of our understanding of the regulation of endocytosis/sorting of RTKs by ubiquitylation comes from studies on the EGFR.

1. Regulation of EGFR endocytosis: a key role for Cbl

Earlier work from the late 1980s/early 1990s has demonstrated that inactive (kinase-dead) EGFR has an altered endocytic route; instead of traveling to the MVBs/lysosome for degradation (as seen with the wild-type receptor), it is recycled to the cell surface (111, 169, 170). This suggested that tyrosine phosphorylation of the receptor or associated proteins are required for its sorting. Recent work has implicated the involvement of the ubiquitin ligase c-Cbl (Fig. 2A) in this sorting event (191, 241, 344, 423, 455). c-Cbl is a negative regulator of RTKs, including the EGFR, PDGFR, CSF-1R, Flk1/KDR, VEGFR-1, Met, and others (100, 184, 192, 222, 237, 241, 284, 314, 322), and aberrant c-Cbl lacking the RING finger or bearing mutations/deletions in the linker region separating the RING and SH2 domains is oncogenic (and previously found associated with lymphomas) (234, 422), likely due to their inability to downregulate these receptors (242, 422). Likewise, mutations in the Met or the CSF-1 receptors that abrogate c-Cbl binding are transforming (25, 261, 322, 348). c-Cbl binds to activated (tyrosine phosphorylated) EGFR following ligand binding either directly (on Tyr-1045) via its TKB domain or indirectly via formation of a complex with Grb2 (by binding to the Grb2 SH3 domain), which in turn binds the EGFR on another phosphorylated tyrosine (454) (Fig. 6); the latter could explain the earlier observation demonstrating that Grb2 is involved in EGFR endocytosis (452). Binding of c-Cbl to activated RTK leads to monoubiquitylation of the receptor on multiple lysines (146, 290) mediated via the RING finger domain of c-Cbl (191, 241). It appears that while c-Cbl binding to the receptor and receptor ubiquitylation controls its sorting into MVB and lysosomal degradation (242), the recruitment of c-Cbl to the receptor via Grb2 followed by receptor ubiquitylation is involved in the initial entry step of the receptor into clathrin-coated pits (190, 401). Internalization of monoubiquitylated EGFR is facilitated by binding of ubiquitin (attached to the receptor) to the UIM domains of Eps15 or epsin (308, 329), two proteins that bind AP-2 and are involved in clathrin-mediated endocytosis (59). Such UIM binding likely blocks further polyubiquitylation of the receptor (374), preventing its degradation by the proteasome. However, two recent reports suggest that while the EGFR can internalize into clathrin-coated pits, ubiquitylated EGFR (following exposure to high doses of EGF) is targeted to caveolae-mediated internalization, a process that requires binding to epsin (or eps15) (58, 386). This is similar to internalization of the transforming growth factor (TGF)-β receptor, which also utilizes both clathrin- and caveolae-mediated endocytosis; clathrin-mediated endocytosis promotes continuous TGF-β receptor signaling, while caveolae-mediated internalization targets the receptor for degradation (89). Upon binding ubiquitin, Eps15, epsin, as well as Hrs (which is involved in EGFR sorting) themselves become ubiquitylated by Nedd4 proteins/family members (205, 219, 265, 329, 340). This allows amplification of the ubiquitin network and protein complex formation, such as the Hrs/STAM1/2/Eps15 complex found in clathrin-containing endosomes (20). In addition to interaction of ubiquitylated EGFR with UIM-containing proteins in early and recycling endosomes, the ubiquitylated receptor also binds the UEV domain of Tsg101 in late endosomes/MVBs, where such interaction is critical for sorting the receptor for degradation in the lysosome (252). Tsg101 is itself multimoubiquitylated by the RING finger E3 ligase Tal, a step important for cargo sorting (11). As seen in yeast, lysosomal sorting of proteins, including the EGFR, also requires the action of the
FIG. 6. Endocytosis and sorting of the epidermal growth factor (EGF) receptor. A: proteins involved in early steps of EGFR endocytosis. Upon EGF binding and autophosphorylation of the receptor on Tyr residues, c-Cbl is recruited to the phosphorylated receptor (directly, or via Grb2, as shown), leading to Cbl-mediated ubiquitylation and recruitment of endocytic proteins such as epsin or Eps15 [which bind ubiquitin (Ub) via their UIM motif] to the ubiquitylated receptor. c-Cbl also binds the CIN85/endophilin (end) complex, which promotes curvature alterations of the plasma membrane to enhance endocytosis. B: steps of EGFR endocytosis: activated, Tyr-phosphorylated EGFR recruits c-Cbl (orange), which ubiquitylates the receptor, promoting the recruitment of UIM-containing proteins (epsin/Eps15), as well as CIN85/end (red square). The internalized receptor in the early endosome, still ubiquitylated, recruits Hrs, STAM, or Vps9 (green square), which contain Ub binding domains/motifs. These then facilitate sorting of the ubiquitylated receptor to late endosomes/MVBs. Deubiquitylated receptor recycles back to the plasma membrane via recycling endosomes. At the MVBs, Hrs and several other proteins involved in cargo sorting (tsg101, ESCRT complexes, Vps4; purple square), some of which also contain Ub binding domains/motifs, target the ubiquitylated receptor into the lumen (vesicles) of the MVBs, from where it will be targeted to and destroyed upon arrival to the lysosome. Ubiquitin is released from the receptor before entry into the MVBs by a deubiquitylating enzyme, possibly AMSH or UBPY. Nedd4 proteins may be involved in ubiquitylation of some of these endocytosis/sorting proteins (e.g., Hrs, epsin, Cbl).
AAA-ATPase Vps4/SKD-1, since a dominant negative Vps4/SKD-1 inhibits EGFR lysosomal degradation (481).

c-Cbl-mediated internalization of the EGFR and other RTKs is not solely dependent on receptor ubiquitylation by c-Cbl. Upon receptor activation, c-Cbl also binds, via its Pro/Arg-rich COOH terminus, to the SH3 domains of the adaptor protein CIN85. CIN85 is constitutively associated with endophilins, which are regulatory components of clathrin-coated pits (324, 393, 414). Thus c-Cbl is involved in at least two pathways that promote RTK endocytosis (Fig. 6A).

The c-Cbl-mediated downregulation of the EGFR can also be augmented by proteins that stimulate the process. For example, LRIG1 (similar in architecture to Drosophila kkkon-1, a negative regulator of the fly EGFR) (130) is a transmembrane protein that interacts with the EGFR and recruits c-Cbl to the receptor, resulting in enhanced EGFR ubiquitylation and sorting for degradation (145).

In addition, c-Cbl also interacts with inhibitory proteins that promote RTKs signaling by impairing c-Cbl function and hence receptor internalization/degradation. For example, Sts-1 or -2 (TULA) proteins, which contain UBA, SH3, and dimerization phosphoglycerase mutase domains, bind to c-Cbl via their SH3 domain upon receptor activation and also bind monoubiquitylated RTKs (e.g., EGFR, PDGFR) via their UBA domain (112, 227). Much like Eps15/Epsin, Sts-1/2 also become monoubiquitylated, which could potentially serve as binding sites to other ubiquitin binding proteins, allowing protein complex formation. Another family of c-Cbl interacting proteins is represented by Sprouty-1 and -2. These were originally identified as ligand-dependent inhibitors of RTK signaling in flies (55, 347) but were later shown to provide both negative and positive cues for RTK signaling in mammalian cells (90, 102, 356, 464); the NH2 terminus of Sprouty-1 or -2 sequesters c-Cbl, thus inhibiting receptor ubiquitylation and degradation and promoting signaling and mitogenesis. In contrast, the COOH terminus, which binds phospholipids and mediates membrane localization, inhibits the MAPK pathway. An additional level of regulation is exerted by c-Cbl-mediated ubiquitylation of Sprouty-2 following EGFR or FGFR activation and Tyr phosphorylation of Sprouty-2, leading to its degradation by the proteasome and resumption of Erk activation (148). Thus Sprouty proteins fine-tune c-Cbl controlled RTK signaling. Sequestration of c-Cbl is also carried out by activated Cdc42, attenuating EGFR degradation and promoting cellular transformation (467). Cbl proteins (c-Cbl, Cbl-b, and Cbl-3) themselves are subject to proteasomal degradation following ubiquitylation by the HECT family members Nedd4 and Itch (AIP4), reversing its negative effect on EGFR signaling (280). Additional proteins that oppose Cbl function are deubiquitylating enzymes. It has been shown that both AMSH or UBPY can deubiquitylate ubiquitylated cargo in endosomes (275, 285) (Fig. 6B). Such deubiquitylation reverses the fate of ubiquitylated proteins such as the EGFR, promoting its recycling instead of lysosomal targeting and degradation. Interestingly, both AMSH and UBPY are STAM binding proteins, binding to the SH3 domain of STAM, suggesting similar function of the two enzymes.

Collectively, the above studies demonstrate a key role for the ubiquitin system, especially the ubiquitin ligase c-Cbl, in regulating RTK endocytosis, degradation, and function. However, regulation of RTK stability/endocytosis is not restricted to Cbl family members only, as exemplified by the recent demonstration of Nedd4-mediated ubiquitylation and internalization of the IGF-1R following recruitment of Nedd4 to the receptor via the interactions with the adapter protein Grb10 (438).

C. T-Cell Receptor Signaling: A Role for Cbl Proteins

Positive and negative regulation of immunoreceptors by the ubiquitin system has been described in detail in excellent recent reviews (e.g., Refs. 32, 94, 249, 439). Here we focus on one well-studied example, the T-cell receptor (TCR). Unstimulated TCR is constitutively internalized and recycled back to the plasma membrane, whereas TCR stimulated by peptide-MHC is rapidly endocytosed and degraded (23, 248, 430). Earlier work of Weissman and colleagues (56, 57) has demonstrated that TCR engagement leads to ubiquitylation of its CD3ξ and δ chains, an effect dependent on Tyr phosphorylation of the receptor. Subsequent work of several groups has documented a key role for Cbl proteins (Cbl and Cbl-b), as well as other E3 ligases such as Grail and Itch, in regulating TCR signaling and stability (reviewed in Refs. 94, 249, 439). c-Cbl and Cbl-b are tyrosine-phosphorylated upon TCR stimulation. They interact with various downstream effectors via their variant SH2 domain (e.g., with the kinases Syk and Zap70), their Tyr phosphorylated sites, or their Pro-rich region [e.g., with PI3K, Vav, Crk, Grb2, SLP-76, phospholipase C (PLC)-γ]. It is generally agreed that Cbl proteins negatively regulate TCR function, and much insight into the mechanisms involved has been gained from studies of knockout (KO) mice lacking c-Cbl, Cbl-b, or both.

c-Cbl KO mice exhibit TCR-induced thymocyte proliferation and increased cell number in spleen, thymus, and lymph nodes (293); increased ZAP-70 phosphorylation and signaling (without increasing its protein stability), SLP-76, and LAT; and increased cell surface expression of the TCR (293, 298). In accord, ZAP-70-associated c-Cbl was demonstrated to promote ubiquitylation of the TCR (451). Together, these observations suggest that increased cell surface stability of the TCR due to impaired ubiquitylation in the absence of c-Cbl may be responsible for the enhanced signaling downstream of the receptor,
causing T-cell hyperplasia. Despite sequence similarities between c-Cbl and Cbl-b, the function of the two proteins in the immune system is not the same. Unlike c-Cbl, Cbl-b KO mice show normal immune system (and otherwise) development. However, they develop spontaneous autoimmune disease, with abnormal activation of mature T cells (22, 64). Remarkably, these cells exhibit TCR activation and IL-2 production in the absence of CD28 costimulation. This CD28-independent activation of the TCR was not associated with increased levels of TCR or tyrosine phosphorylation of ZAP-70, but, instead, with increased activation of Vav-1 (a guanine nucleotide exchange factor for Rho). Accordingly, Cbl-b deficiency can partially rescue the hyporesponsiveness of Vav-1-deficient T cells (229). A c-Cbl/Cbl-b double KO mouse is embryonic lethal (297). However, conditional knockout of c-Cbl in T cells of Cbl-b KO mice (i.e., double-KO T cells) resulted in severe autoimmunity, with defective downregulation of the TCR following ligand engagement, caused by reduced trafficking of the internalized TCR to the lysosome and hence diminished TCR degradation (297). These results provide very strong genetic evidence that TCR endocytosis and sorting for lysosomal degradation mediated by Cbl family members is necessary to terminate TCR signals and function.

TCR engagement in the absence of costimulation via CD28 leads to anergy, a state of unresponsiveness to antigen, which is important to prevent autoimmune response. Transcriptional induction of expression of 3 E3 ligases, Cbl-b, GRAIL, and Itch, as well as tsg101, has been observed following anergy stimulation (12, 74, 157, 259). GRAIL is a transmembrane RING finger E3 ligase that resides in lysosomes and belongs to the Goliath family of fly and mammalian proteins. GRAIL expression is induced in T cells following anergic stimulation, and it leads to inhibition of IL-2 production by stimulated T cells, anergic state leads to disintegration of the immunological synapse, a process that is associated with degradation of PLC-γ1 and PKC-θ. In accord, in Cbl-b- or Itch-deficient T cells, synapse disruption is attenuated, an effect correlated with increased stability of these (and likely other) signaling proteins (74, 94, 156).

D. Cytokine Receptors

Ligand binding to cytokine receptors leads to activation of their associated Janus kinases (JAKs), which Tyr-phosphorylate the receptors, creating binding sites for downstream signaling proteins, including STATs and SOCS/Cis (175). SOCS/Cis proteins bind elongin BC and are part of an E3 ligase complex, allowing regulation of stability of both the receptors and JAKs, with which SOCS proteins interact (reviewed in Ref. 216).

1. The growth hormone receptor

The growth hormone receptor (GHR) is a type I cytokine receptor that upon ligand (GH) binding recruits JAK2 and becomes tyrosine-phosphorylated by this tyrosine kinase, resulting in activation of downstream signaling molecules (e.g., STAT5) regulating cellular metabolism, growth, and other functions. The ligand-bound GHR is internalized via clathrin-coated vesicles and transported to the lysosome. Its endocytic journey is regulated by the ubiquitin-proteasome system (139, 408, 435). The intracellular COOH terminus of GHR contains a ubiquitin-dependent endocytic (UbE) motif (DSWVEFIELD) that is required for receptor ubiquitylation and internalization, but direct ubiquitylation of the COOH terminus itself (which contains 19 Lys residues) is not required for this internalization (137, 435). Both the initial internalization step and subsequent transport to the lysosome are inhibited by proteasome inhibitors, suggesting the involvement of the proteasome (434, 435). Regulation of endocytosis of the GHR is more complicated, however, as truncated receptor lacking a segment of its COOH terminus (but still containing the UbE motif) can internalize independent of the ubiquitin system, utilizing instead a di-Leu motif that becomes functional upon such truncation (138). This truncated receptor (and its bound GH) nevertheless requires the ubiquitin-proteasome system for sorting into lysosomes (436). It is possible that the COOH terminus of GHR is the target for proteasomal degradation, and only the truncated GHR is recognized by the endocytic machinery, or alternatively, that the COOH terminus binds a protein that is targeted for proteasomal degradation before endocytosis (435). A search for such protein(s) has recently identified the TPR-containing protein SGT as a binding partner for the UbE motif of GHR (361). It is not known, however, if and how SGT may regulate receptor endocytosis or degradation. The GHR and its associated JAK2 were demonstrated to interact with select members of the SOCS/Cis family, leading to proteasome-dependent decrease in stability of the receptor/JAK2/SOCS complex and thus attenuation of GHR signaling (93, 342, 343).

2. Other cytokine receptors

In addition to the GHR, other type I cytokine receptors have been shown to be regulated by the ubiquitin
system. For example, the erythropoietin receptor (EpoR) and its downstream signaling molecules, or the interleukin (IL)-2 and IL-9 receptors, were shown to be regulated by the proteasome (478). Although the rate of EpoR internalization is not affected, receptor recycling is enhanced in the presence of proteasome inhibitors, suggesting the proteasome is involved in EpoR sorting (441). Moreover, downstream effectors of EpoR and other cytokine receptors are regulated by ubiquitylation as well. Both JAK2 and STAT5 are subject to regulation by the ubiquitin-proteasome system, often by recruitment of SOCS proteins to JAK2, and degradation of JAK2 and STAT5 (216, 429, 440, 465). As seen for the GHR, SOCS/Cis proteins can form a complex with activated cytokine receptors such as the EpoR, leading to ubiquitylation of the complex and degradation by the proteasome, thus contributing to receptor inactivation (440). Another EpoR binding protein is the U-box E3 ligase RUL, which ubiquitylates the receptor, and is required for EpoR mitogenic signaling (120), by as yet unknown mechanism(s). In an analogous fashion to the involvement of SOCS/elongin BC/E3 complex in the regulation of stability of the EpoR, GHR, and other cytokine receptors, recent studies suggest that the prolactin receptor is regulated by the SCF complex via SCFpTyrCPR (244).

Thus most cytokine receptors studied to date and their downstream signaling molecules appear to be regulated by the ubiquitin-proteasome system, often involving E3s of the SOCS/elongin BC or SCF complexes.

E. The TGF-β Receptor Pathway

TGF-β receptor family members are comprised of type I (TβRI) and type II (TβRII) Ser/Thr kinase receptors, where ligand-bound TβRII phosphorylates and activates TβRI, leading to signal propagation via Smad proteins. Receptor Smads [Smads 2, 3 for TGF-β and 1, 5, 8 for bone morphogenesis protein (BMP) receptors] are phosphorylated by activated TβRI, then associate with a common Smad (Smad4) and together move to the nucleus to regulate transcription of target genes. Inhibitory Smads (Smads 6, 7) terminate the signal (14, 270, 279). Ubiquitylation plays a key role in regulating signal termination by the TGF-β receptor superfamily, and key players are the Smurf3 ubiquitin ligases, which are Nedd4 family members (Fig. 2B). Receptor Smads contain PY motifs that bind the WW domains of Smurf proteins. Smurf1 binds to and leads to the degradation of Smad1, thus inhibiting BMP-mediated signaling (488). It was also shown to degrade the TβRI via interactions with the inhibitory Smad7 (101). However, a recent report has demonstrated that Smurf1 KO mice possess normal levels of BMP receptors and several Smads, including Smad1 (470), suggesting these Smads may not be the physiological targets for Smurf1, or that Smurf2 (or other E3 ligases) compensates for the loss of Smurf1. Instead, Smurf1 was shown to target MEKK2 for degradation, altering bone homeostasis (470). Smurf2 has been reported to target Smad1 (484), Smad2 (247), or, instead, the TGF-β receptor itself following an association with Smad7 (209). As indicated above, endocytosis of the TGF-β receptors via caveoli has been suggested to induce receptor degradation by an association with the Smad7-Smurf2 complex in Rafts (89). Thus receptor signaling is attenuated either by destruction of the regulatory Smads or by destruction of the receptor itself following an association of the Smurf proteins with an inhibitory Smad that serves an adapter function to bridge between the E3 ubiquitin ligase and the receptor. Such bridging has also been reported for Smad2-Smurf2 or Smad2/3-APC mediated degradation of the TGB-β repressor SnoN (45, 407, 447). Recent studies have suggested that in addition to regulating TβRII signaling, Smurf1 also participates in TGF-β-induced regulation of cell polarity and epithelial-to-mesenchymal (EMT) transition (313, 450). This is achieved by an association of Par6 (a member of the Cdc42-PKC-ζ-Par3 and other polarity complexes) with TβRII, phosphorylation of Par6 by the receptor, which leads to Par6-Smurf1 interactions and Smurf1-mediated targeting of the RhoA for degradation, resulting in loss of tight junctions.

Because the Smurf proteins are Nedd4 family members, it is perhaps not surprising that other Nedd4 relatives have been recently demonstrated to regulate TGF-β receptor signaling. Itch was found to ubiquitylate and regulate the association of Smad2 with the receptor and Smad2 phosphorylation (24), and Tial/WWP1 was reported to constitutively associate with Smad7 and target both the TβRI and Smad2 for degradation, thus inhibiting receptor signaling (371). Other types of E3 ligases have been also documented to regulate TGF-β receptor signaling, including the RING protein ROC1 that degrades Smad3 (122), and Jab1 and Ectodermin, which ubiquitylate and degrade Smad4 (96, 446).

F. GPCRs

The GPCR family is the largest receptor family known in mammals, but the role of the ubiquitin system in regulating its stability and trafficking is only beginning to be uncovered.

1. The β2-adrenergic receptor

One of the most extensively studied GPCR is the β2-adrenergic receptor (β2-AR). Upon agonist binding, the β2-AR is phosphorylated by GRK2, leading to recruitment of β-arrestin to the phosphorylated receptor and uncoupling of the receptor from G protein activation (a process

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called desensitization). Work from the Lefkowitz group (377) has demonstrated that such agonist binding and β-arrestin recruitment lead to ubiquitylation of both the receptor and β-arrestin. This polyubiquitylation of β-arrestin is carried out by the RING finger E3 Mdm2, which is bound to β-arrestin. β-Arrestin ubiquitylation is required for receptor internalization, but not for receptor degradation, whereas receptor ubiquitylation (by as yet an unidentified E3) is not needed for internalization but is required for its sorting to the lysosomes and degradation (377). Thus β-arrestin, previously demonstrated to be involved in GPCR endocytosis via clathrin-coated pits (134, 238), provides an adaptor role for β2-AR ubiquitylation and internalization, ultimately controlling receptor fate.

GPCRs are categorized into two classes: class A of rapidly recycling (e.g., β2-AR) receptors and class B of slowly recycling receptors (e.g., the vasopressin V2R, or the angiotensin AT1aR). While agonist stimulation of β2-AR leads to a transient ubiquitylation of β-arrestin, its dissociation from the receptor and rapid receptor recycling, agonist stimulation of the V2R or AT1aR leads to a longer association of with β-arrestin, stable β-arrestin ubiquitylation, internalization of β-arrestin with the receptor, and very slow receptor recycling (328, 375, 376). Thus ubiquitylation of β-arrestin regulates rates and routes of endocytosis and recycling of GPCRs.

2. Mu and delta opioid receptors

Internalization of activated mu and delta opioid receptors (MOR and DOR) employs β-arrestins (as seen for the β2-AR). However, subsequent regulation of lysosomal sorting and degradation differ between MOR and DOR. While internalized MOR is not downregulated, activated DOR is targeted for lysosomal degradation. This appears to be mediated by the protein GASP (GPCR associated sorting protein), that specifically binds to DOR, but not MOR, and targets DOR to the lysosome (458). Although newly synthesized DOR that are retained in the ER are ubiquitylated following retrotranslocation to the cytosol, and are degraded by the proteasome (323), GASP appears to bind to the nonubiquitylated endocytosed DOR (458). Despite ubiquitylation-independent internalization and sorting (418), DOR does utilize Vps4/SKD-1 and Hrs (but not Tsg101) for entry into lysosomes and lysosomal degradation (166).

3. CXCR4 receptor

Lysosomal sorting of the chemokine receptor CXCR4, a coreceptor for the HIV virus, is also regulated by ubiquitylation, mediated by the Nedd4 family member AIP4 (Itch) (264, 265). Agonist stimulation leads to receptor internalization, which is dependent on a di-Leu motif and conserved Ser residues, but not on receptor ubiquitylation (264). In contrast, following endocytosis, receptor sorting to the lysosome and degradation is dependent on ubiquitylation by AIP4, which also ubiquitylates Hrs. Much like the EGFR, Hrs and Vps4/SKD-1 appear to be involved in sorting CXCR4 to the lysosomes. Vps4/SKD-1 associates with the AIP4/ubiquitylated receptor/Hrs complex, and its action coincides with the activity of as yet an unidentified deubiquitylation enzyme that deubiquitylates the receptor and Hrs before receptor entry into the endosomal lumen (265).

4. Other GPCRs

Regulation of internalization or sorting of other GPCRs by ubiquitylation has been documented as well. For example, ubiquitylation-mediated lysosomal degradation of the somatostatin receptor sst3, but not of other sst receptors, was recently reported, demonstrating that recycling and degradation of the various sst receptors are differentially regulated (426). As indicated above, agonist stimulation of the vasopressin V2 receptors leads to ubiquitylation of β-arrestin and receptor ubiquitylation that results in its accelerated degradation (266, 376). While the V2 receptor is rapidly degraded following internalization, its relative, the V1a receptor, is recycled to the plasma membrane (48), an effect dependent on the rate of receptor dissociation from β-arrestin (320). Likewise, ligand-induced activation of the platelet activating factor (PAF) receptor is associated with receptor ubiquitylation, endocytosis, and lysosomal and proteasomal degradation (97).

G. Regulation of Transmembrane Proteins by Ubiquitin in the Nervous System

Much attention has been paid to the role of ubiquitylation in the nervous system in recent years, particularly the role it plays in regulating neuronal development, neurodegenerative diseases, and synapse growth and function. Since these have been reviewed extensively recently (e.g., Refs. 46, 65, 69, 75, 86, 106, 353, 479), we focus here in more detail on the regulation of select transmembrane proteins by ubiquitin in the nervous system.

1. Ubiquitin and neurological diseases

Several neurodegenerative diseases, in particular Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease (HD), and amyotrophic lateral sclerosis (ALS), have been found to be associated with accumulation of aberrant misfolded proteins in neurons in the brain leading to neuronal death, often associated with malfunctioning of the ubiquitin proteasome system (UPS). Here we focus on one example, that of PD.

2. PD

PD is associated with loss of dopaminergic neurons in the substantia nigra and accumulation of proteinaceous...
inclusions called Lewy bodies, which include α-synuclein as a major component (394), as well as lipids and other proteins, including ubiquitin. Dominant mutations in α-synuclein are associated with accumulation of Lewy bodies (reviewed in Ref. 69), as are sporadic cases of PD. In general, familial forms of PD are associated with mutations in either of several genes, such as α-synuclein (230, 330, 387), ubiquitin COOH-terminal hydrolase (UCH-L1) (240), DJ-1 (44, 147, 339), and Parkin (218, 417). Parkin (Park2) is a RING finger E3 ubiquitin ligase containing Ubl domain, two RING fingers, and an IBR region between them (178). Although mutations in Parkin itself that cause neuronal degeneration are usually not associated with the accumulation of Lewy bodies (69), it was postulated that accumulation of undegraded Parkin substrates in dopaminergic neurons may contribute to neurodegeneration. Among these substrates is the orphan GPCR Pael-R (415, 471). Pael-R is highly expressed in dopaminergic neurons of the substantia nigra. When expressed in cells, Pael-R accumulates as an unfolded protein in the ER, leading to neurotoxicity. Unfolded Pael-R is normally ubiquitylated by Parkin and degraded, but in Parkin mutants, as seen in autosomal recessive juvenile Parkinson (AR-JP) patients, unfolded Pael-R accumulates, leading to selective death of dopaminergic neurons. Parkin appears to collaborate with two chaperones, Hsp70 and CHIP (itself a U-box containing E3 ligase), in regulating ubiquitylation and degradation of Pael-R (415). In addition, Parkin binds directly to the proteasome (via its Ubl domain), as well as to several other substrates, such as the septins CDCrel-1/2, expanded polyglutamine proteins responsible for PolyQ disease, cyclin E, and others (reviewed in Ref. 70, 415).

3. Regulation of plasma membrane proteins by ubiquitylation in the nervous system

Several studies, often using model organisms, have described the role of ubiquitin in the regulation of plasma membrane proteins in the nervous system. Examples of the ones studied more extensively are described below.

A) GLUTAMATE RECEPTORS. The ubiquitin system has been demonstrated to regulate the stability of numerous presynaptic and postsynaptic proteins. At the presynaptic region, it regulates synaptic development, synapse size, vesicular recycling, and neurotransmitter release. Postsynaptically, the ubiquitin system regulates glutamate receptor endocytosis/recycling, spine size, synaptic plasticity, and signal transduction (reviewed in Ref. 479). The neurotransmitter glutamate, released from the presynaptic active zone, is sensed by glutamate receptor at the postsynaptic membrane. Glutamate receptors (GluRs) play an important role in regulating synaptic strength, implicated in the modulation of long-term potentiation (LTP) and long-term depression (LTD) (reviewed in Ref. 427). A wave of recent papers has described the role of the ubiquitin system in regulating endocytosis of the ionotropic GluRs, in particular the AMPA receptors. Agonist (AMPA or NMDA) treatment of cultured hippocampal neurons was shown to induce rapid internalization of GluRs, an effect dependent on the proteasome (317). This internalization was proposed to be mediated by ubiquitylation/degradation of intermediary proteins associated with the postsynaptic density (PSD), such as PSD-95 (or possibly Shank, GKAP, or AKA-P79/150), and not of the receptor itself (37, 66, 105). The scaffold protein PSD-95 is tethered to the postsynaptic membrane, and it is associated with the NMDA receptor complex. Thus ubiquitination and degradation of PSD-95, likely by Mdm2 (66), appear to destabilize the receptor at the cell surface and trigger receptor internalization. However, in C. elegans, internalization of the AMPA (non-NMDA) receptor GluRI (GLR-1) was recently shown to involve direct ubiquitination of the receptor in vivo (51). It also involves the AP180 clathrin adaptor protein. In both C. elegans and Drosophila, the E3 anaphase promoting complex (APC) appears to negatively regulate synapse development and activity (193, 437). Flies lacking APC exhibit enlarged neuromuscular synapses, likely involving increased levels of the synaptic protein Liprin-α (which is a target for APC ubiquitylation), as well as increased postsynaptic levels of the glutamate receptor GluRII in the muscle (437). Likewise, in worms, temperature-sensitive mutation of APC leads to increased cell surface expression of Glr-1 in the ventral nerve cord, and to locomotion defects (193). Similar defects are also seen in Lin23 mutant worms (92); Lin23 is an E3 ligase that belongs to the SCF complex, similar to β-TrCP, and appears to regulate levels of BAR-1, the β-catenin homolog in worms (92). Thus levels of Glr-1 are indirectly regulated via levels of β-catenin, and thus the Wnt signaling pathway. In addition, the N1R subunit of the NMDA receptor was recently demonstrated to be regulated by direct ubiquitylation of its ectodomain by the F-box protein Fbx2, which recognizes high-mannose N-glycosylated proteins; this ubiquitylation takes place during receptor processing at the ER (203).

Much like the ionotropic glutamate receptors, recent work suggests that metabotropic glutamate receptors (mGluRs) are also subject to regulation by ubiquitylation. For example, group I mGluRs bind to and are regulated by the RING finger E3 ligase Siah1 (180, 289). This could have implications to processes regulated by mGluRs, such as neural plasticity and synapse development and elimination.

Collectively, regulation of glutamate receptors by the ubiquitin system has major implications to synapse development, maintenance, and function in neurotransmitter release.

B) NOTCH SIGNALING. Notch signaling is implicated in the development of numerous organs, but here we focus pri-
An intracellular protein that interacts with Notch and negatively regulates its function is Numb (183, 221). Numb (along with other proteins) plays an important role in controlling asymmetric cell division (220). In Drosophila, sensory organ precursor (SOP) cells give rise to type IIA that then divides into Socket and Hair cells, and type IIB that divides into Sheath and Neuron cells (220, 254). The distribution of Numb in the SOP cell determines the fate of its descendent cells (i.e., numb mutants generate only type IIA while Numb overexpression yields only type IIB cells). Earlier work using genetic tools to inhibit proteasome function in Drosophila revealed the dependence of Notch signaling on functional proteasome (368). Mutations in two of the proteasome subunits (\(\beta_2\) and \(\beta_6\)) leading to reduction of proteasome activity caused Hair to Sheath and Neuron to Sheath fate alterations, stabilized intracellular Notch, and phenocopied elevated Notch signaling (368). Mammalian Numb has been detected in endosomes and associates with components of the clathrin machinery (AP-2), suggesting it plays a role in regulating endocytosis of transmembrane proteins (360), potentially of Notch as well. The stability of Numb itself is regulated by the RING-finger containing E3 ligase, LNX (302). LNX is a vertebrate protein (containing 3 PDZ domains in addition to its RING finger) that binds to the PTB domain of Numb (84), and its ubiquitylation and degradation of Numb can enhance Notch signaling (302). Another negative regulator of Numb described earlier is Mdm2, also a RING finger-containing E3 ligase (better known for its regulation of p53 and \(\beta\)-arrestin), that upon binding to Numb leads to nuclear translocation of the latter and reduction of its cellular levels (194).

A positive intracellular regulator of Notch is Deltex (271), a RING finger-containing protein. In flies, the mutant deltex displays genetic interactions with Notch signaling (468) and is suppressed by the Suppressor of deltex \([Su(dx)]\) mutant (118). \(Su(dx)\) (and its mammalian ortholog Itch) is an HECT domain-containing E3 ligase belonging to the Nedd4 family of proteins (Fig. 2B) and is a negative regulator of Notch signaling (71, 118, 273). Itch/\(Su(dx)\) was reported to directly bind, via its WW domains, to a FY motif present in the intracellular domain of Notch, and to ubiquitylate it (338, 358), although another report suggests this interaction is mediated via Numb (276). Two recent papers have demonstrated that \(Su(dx)\) or dNedd4 interact with Notch at the plasma membrane and regulate its endocytosis, either by controlling its ligand-independent internalization (358) or by subsequent sorting of Notch at the early endosomes en route to the MVBS (461). In addition, Sakata et al. (358) have demonstrated that Deltex is also a target for dNedd4-mediated degradation and proposed that dNedd4 antagonizes Notch signaling by promoting the degradation of both Notch and Deltex.

Mice mutant for the Itch locus develop immunological disorders and scratching (321), and although Notch is involved in regulating the immune system, it is not yet known if defective targeting of Notch by Itch is the cause of the phenotypes seen in the mice.

One of the well-studied Notch ligands is Delta (Dl). Recent studies have identified the RING finger-containing ubiquitin ligases Neuralized (Neur) and Mind Bomb (Mib) as regulators of Dl that act in a similar fashion to regulate Notch signaling (181, 228). Neuralized (Neur) belongs to a family of genes involved in neurogenesis (47, 477), and neur mutant in Drosophila cause embryonic hypertrophy of the nervous system, similar to notch mutants; accordingly, Neur was shown to promote Notch signals (477). Neur is localized to the plasma membrane, where Dl is located, binds DI, and promotes its ubiquitylation, internalization, and degradation (76, 228, 233, 318, 476).

A conundrum arising from these observations is how can Neur-dependent degradation of DI increase signaling via Notch? Although more than one model has been proposed (reviewed in Ref. 228), a recent model suggests that Neur accumulates (along with Numb) in signal sending (type IIB) cells. It upregulates endocytosis of DI in the type IIB cells and acts in the type IIB cells to promote Notch activation in the type IIA cells (236). This is similar to the function of Mib, which binds to the intracellular domain of DI, promoting its ubiquitylation and internalization. Mib function is essential in the signaling, sending cells to promote Notch activation in neighboring cells (181). Recently, work of Fischer and colleagues (312) has demonstrated that liquid facet (Lqf, or deEpstin) is also involved in endocytosis of DI. Lqf was previously shown by the same group to interact genetically and physically with Fat Facet (Faf), a deubiquitylation enzyme that deubiquitylates Lqf (52, 63). In cultured C2C12 muscle cells, Tyr-phosphorylated mammalian Notch1 is able to bind c-Cbl, which in turn ubiquitylates it and targets it to lysosomal degradation (185).

In addition to regulating Notch at or close to the plasma membrane, another ubiquitin ligase, Sel-10/Cdc-4, acts in the nucleus to regulate Notch ICD (intracellular domain). Sel-10, an F-box WD40-containing protein belonging to the SCF complex, binds the PEST sequence within the ICD of a phosphorylated form of Notch-ICD, ubiquitylates it, and targets it for proteasomal degradation (144, 304, 466). This negatively regulates Notch-mediated transcription. In support, knockout of Sel-10 in mice leads to embryonic lethality due to vascular defects, with marked elevation of Notch4 ICD expression (but no effect on Notch1, -2, -3) (425).

C) REGULATION OF COMMISSURELESS/ROUNDABOUT IN AXON GUIDANCE. Navigation of axons is a carefully controlled process, with intermediate targets serving as guidespots. The central nervous system (CNS) midline is one such guidespot, and CNS midline crossing by axons is tightly regulated by...
attractive and repulsive cues. In *Drosophila*, axons are initially attracted to the midline by Netrin, but once there, they respond to the repellent Slit, extending away from the midline and never recrossing it (150, 213, 282). Slit is the ligand for the receptor Roundabout (Robo), which is expressed at low levels in axons before crossing the midline and at high levels after crossing (213). Levels of Robo at the surface of axons are regulated by the fly transmembrane protein Commissureless (Comm) (419). In *comm* mutants, axons never cross the midline (419), suggesting Robo levels remain high. Conversely, overexpression of Comm exhibits a Robo phenotype, where Robo levels are low and too many axons cross and recross the midline (214). Recent work has demonstrated that Comm can regulate cell surface expression of Robo and that this regulation is mediated by recruitment of Drosophila Nedd4 (dNedd4) to the Comm:Robo complex (294). DNedd4 directly binds, via it WW domains, to the PY motifs present in the COOH terminus of Comm and ubiquitylates Comm. Comm ubiquitylation by dNedd4 is required for localization of the Comm:Robo complex in intracellular vesicles (endosomes) (294), thus removing Robo from the cell surface and permitting midline crossing. Indeed, overexpression of Comm and DNedd4 promotes excessive axon crossing, which is not seen upon overexpression of catalytically inactive dNedd4 or Comm lacking its PY motifs (294). Of the two PY motifs present in Comm, one (LPxY) binds Ned4-WW domains, especially the second WW domain, with relatively high affinity (~2 μM), and recent work provides a structural explanation for this higher affinity interaction, imparted by the presence of Ala-Pro-Asn sequence within the domain (199). This LPxY motif in Comm was proposed to be necessary for Comm-mediated sorting of Robo during midline crossing (212). In the latter study, it was suggested that Comm (but not Comm lacking the LPxY motif) diverts Robo from the biosynthetic to late endocytic pathway, thus preventing Robo from reaching the plasma membrane and allowing midline crossing. Although the mechanism involved was not demonstrated, it was initially proposed to be mediated by a Nedd4 family member, as shown in Reference 294. Regardless of whether the Comm:dNedd4 interaction prevents Robo from reaching the plasma membrane (212), or promotes its endocytosis from the plasma membrane (294), or both, the end result is reduced cell surface expression of Robo and stimulation of axon midline crossing. Interestingly, Comm is a *Drosophila* protein not found in other organisms, yet Slit and Robo regulate midline crossing in mammals as well. Recent reports suggest that this regulation is carried out by Robo3/Rig1, a divergent Robo family member, which functions in an analogous fashion to Comm in flies (186, 357). However, Robo3/Rig 1 is not similar to Comm in sequence, nor does it recruit Nedd4 to downregulate Robo1 expression as seen in flies. Rather, it appears to regulate axons’ sensitivity to the repellent Slit, and thus Robo3/Rig1 mutants are prematurely sensitive to Slit and unable to cross the midline (357). The importance of regulation of midline crossing is underscored by the recent identification of mutations in Robo3 that cause horizontal gaze palsy with progressive scoliosis (HGPPS) in humans, a disease in which hindbrain midline crossing is impaired (186).

**H. Viral Budding**

The process of viral budding appears to have many parallels with sorting of cellular proteins in the MVBs, with viruses utilizing numerous cellular components to promote their egress from infected cells to infect neighboring cells. Late domains in the Gag sequences of retroviruses (e.g., HIV-1, HTLV-1, RSV, MPMV, MMuLV), the VP40 domain of filoviruses (Ebola, Marburg), the M domain of rhabdoviruses (Rabies, VSV) or paramyxoviruses (SV5), and the Z region of arenaviruses (Lassa, LCMV), all contain one or more short recognition motifs for proteins involved in cargo sorting into MVBs (reviewed in Refs. 82, 288). These include the P(T/S)AP motif that binds the UEV domain of Tsg101, the PY motif (PPxY) that binds to WW domains of Nedd4 family members, and the YPx(n)L motif (or its variant ΦPxY) that binds AIP1/Alix (Table 1). MVB biogenesis and viral budding are similar processes, in that in both budding is directed away from the cytoplasm.

The realization that viral budding may utilize the MVB budding machinery came several years ago, when it was discovered that the PTAP motif within p6 (late domain) of HIV-1 binds to the UEV domain Tsg101 and that

**TABLE 1. Known motifs involved in viral budding present in the late domains of enveloped RNA viruses**

<table>
<thead>
<tr>
<th>Family</th>
<th>Virus</th>
<th>P(T/S)AP</th>
<th>PPxY</th>
<th>YPx(n)L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filoviruses</td>
<td>Ebola</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Marburg</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retroviruses</td>
<td>HIV-1</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HTLV-1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RSV</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPMV</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMuLV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>EIAV</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhabdoviruses</td>
<td>Rabies</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Arenaviruses</td>
<td>Lassa</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCMV</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tacaribe</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Paramyxoviruses</td>
<td>SV5</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

EIAV, equine infectious anemia virus; HIV, human immunodeficiency virus; HTLV, human T cell leukemia virus; LCMV, lymphocytic choriomeningitis virus; MMuLV, Moloney murine leukemia virus; MPMV, Mason-Pfizer monkey virus; RSV, Rous sarcoma virus; SV5, parainfluenza virus 5; VSV, vesicular somatitis virus; Φ, hydrophobic residue.
this interaction is required for viral budding (83, 128, 268, 333, 442). Tsg101 UEV domain binds the PTAP motif and ubiquitin simultaneously (333). Binding of HIV-1 PTAP motif to Tsg101 mimics the normal binding of Hrs to Tsg101, allowing recruitment of the virus to the ESCRT I complex (334) (Fig. 4B). As indicated above, ESCRT I complex can bind ubiquitin and acts as a receptor-sorting machine to select ubiquitylated proteins for incorporation into MVBs (38, 128, 206). ESCRT I also promotes the recruitment of the ESCRT II and III complexes. In addition, bridging between the ESCRT complexes is enhanced by AIP1/Alix, which can bind Tsg101 and CHMP4 of the ESCRT III complex (204, 306, 406). AIP1 recognizes the YP(x)nL motif found in EIAV, HIV-1, and MMuLV and, accordingly, is required for budding of these viruses (370, 406, 445). Both AIP1 and the ESCRT III-associated AAA ATPase Vps4 induce membrane invagination, and indeed, Vps4 mutants cause the accumulation of large vesicles and inhibit cargo and virus sorting into MVBs (128, 245, 269, 445). Thus the association of the virus with the ESCRT complexes allows it to gain entry into the MVB, from where it can bud out of the cell via a “Trojan exosome” (e.g., in macrophages) or by assembly of the ESCRT complexes at the plasma membrane and subsequent viral budding from there (136, 300, 309, reviewed in Refs. 82, 288).

Exactly how ubiquitin is involved in viral budding is not entirely clear, but its involvement is underscored by the finding that proteasome inhibitors (which decrease cellular pool of free ubiquitin) lead to inhibition of viral release (151, 365), and a substantial fraction of viral Gag proteins are monoubiquitylated (311, 337). As indicated above, ubiquitin may enhance the interaction between the viral late domains and Tsg101/ESCRT proteins. Moreover, the late domains of several enveloped viruses (e.g., Ebola, Marburg, HTLV-1, RSV, MPMV; see Table 1) contain PY motifs that directly bind the WW domains of the ubiquitin ligase Nedd4 or its family members.

Human Nedd4–2 (LDI-1) has been demonstrated to bind the PY motif of RSV, and this binding is required for viral budding (215, 431). Moreover, intact HECT domain of LDI-1/Nedd4–2 is required for Gag ubiquitylation, as do specific Lys residues that serve as ubiquitin acceptor sites (431), suggesting that viral Gag ubiquitylation by Nedd4 family members is needed for its budding. A similar scenario exists for HTLV-1, which contains both a PTAP and PY motifs. The latter appears to play a critical role in binding to Nedd4 family members (Nedd4–1, WWP1), allowing ubiquitylation of Gag by these ubiquitin ligases, a necessary step before Tsg101 recruitment, sorting to MVBs, and viral budding (41, 431). Like HTLV-1, budding of MPMV relies on both Nedd4-like and Tsg101 (135, 474). The Ebola virus contains overlapping PTAP and PY motifs (PTAPPEY) in its VP40 protein (late domain). Both motifs, as well as Vps4, are necessary for viral budding (245). Nedd4 (or its relatives WWP1, WWP2, or Itch) binding to the PY motif of Ebola, as well as its catalytic activity, are needed for viral egress, as does the interaction with Tsg101 (152, 267, 424, 475). Interestingly, recent work suggests that Ebola and Marburg viruses can utilize lipid rafts for entry, assembly, and exit from cells (29, 315). Although it is not known whether ubiquitylation plays a role in this process, it is curious that Nedd4 has been found to localize to lipid rafts via its C2 domain (326), suggesting it may have a role in regulating viral entry or egress in that compartment as well.

Collectively, these findings demonstrate a role for ubiquitin-protein ligases of the Nedd4 family and their ubiquitylation of viral Gag proteins, as well as interactions with Tsg101 and the ESCRT complexes, the ESCRT associated Vps4 and possibly AIP1, as critical steps in MVB sorting and viral budding. It is worth noting that in addition to regulating viral budding, Nedd4 family members and ubiquitylation play an important role in regulating signal transduction in B cells infected with Epstein-Barr virus (EBV). The EBV latent membrane protein LMP2A contains two PY motifs that serve as recruitment sites for Nedd4 family members, as well as binding sites for the tyrosine kinases Lyn and Syk, which are involved in B-cell signaling. The recruitment of Nedd4 family members leads to ubiquitylation and degradation of these kinases, thus inhibiting B-cell signaling in the host and promoting viral survival (176, 177, 462).

VI. PERSPECTIVES

The regulation of transmembrane proteins by the ubiquitin system has received much attention in recent years. Consequently, great strides have been made towards the understanding of the role of ubiquitin in trafficking and sorting of transmembrane proteins, in its recognition by ubiquitin binding domains/motifs present in endocytic and sorting proteins, and in the discovery of numerous human diseases associated with defects in ubiquitylation of such transmembrane proteins or with infectious agents (viruses) that usurp the ubiquitin-vesicular transport system to propagate themselves. In this review, we provided a series of examples of plasma membrane proteins whose internalization, sorting, and recycling are regulated by the ubiquitin system. These examples represent different families of proteins (e.g., receptors, transporters, ion channels) often with important functions in specific tissues (e.g., the kidney, nervous system, the immune system) and span a very large evolutionary spectrum, from yeast to humans. Although all these plasma membrane proteins have unique characteristics and mode of regulation, common themes in their regulation by the ubiquitin system can be observed. 1) Ubiquitylation of the transmembrane protein itself, or of
an associated protein, promotes either the initial step of internalization, subsequent steps of sorting, or both. The sorting events may include diverting from the recycling pathway into late endosomes, entry from the limiting membrane of the MVB into the MVB itself for delivery to the lysosomes, or sorting from the Golgi to the late endosomes. 2) In many cases (although not always), the plasma membrane protein is mono- or multimonoubiquitylated, resulting in its targeting to the endosomal/lysosomal pathway and not to the proteasome. 3) The endocytic/sorting steps of ubiquitylated transmembrane proteins are facilitated by a series of proteins that contain ubiquitin binding motifs/domains. 4) Much like the proteasomal-mediated degradation of polyubiquitylated proteins, the final fate of mono- or multimonoubiquitylated proteins is also destruction, which, however, takes place in the lysosome.

As is evident from the examples provided in this review, there are also many differences in the mode of regulation of the various transmembrane proteins by the ubiquitin system. For example, different ubiquitin ligases (e.g., RING-containing or Hect-containing E3s), or deubiquitylation enzymes, are involved in regulating the different transmembrane proteins. The intracellular site of regulation may differ between these proteins as well, as is the involvement of the proteasome, which is seen in some cases (e.g., the GHR, β2-AR, GlnR), although not most.

Despite the immense progress in our understanding of the regulation of plasma membrane proteins by the ubiquitin system in recent years, many questions remain unanswered. For example, how is the catalytic activity of E3 ligases, such as Cbl or Nedd4, regulated? What is their temporal and spatial regulation of and specificity towards substrates? How exactly does monoubiquitylation of transmembrane proteins protect them from polyubiquitylation? Does binding to ubiquitin binding domains/motifs protect ubiquitylated proteins from deubiquitylation? How is substrate specificity determined for deubiquitylation enzymes? What is the precise role of ubiquitin in the trafficking machinery and in viral budding, and is it playing a role also in viral entry into cells? How many more domains and proteins are there in cells that recognize ubiquitin, and to what pathways do they belong? Does modification by ubiquitin-like molecules (e.g., SUMO, Nedd8) also serve a role in endocytosis and sorting of transmembrane proteins? These and many other questions await future studies.

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