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Minireview

Ubiquitin: Same Molecule, Different Degradation Pathways

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Ubiquitin is a common demoninator in the targeting of substrates to all three major protein degradation pathways in mammalian cells: the proteasome, the lysosome, and the autophagosome. The factors that direct a substrate toward a particular route of degradation likely include ubiquitin chain length and linkage type, which may favor interaction with particular receptors or confer differential susceptibility to deubiquitinase activities associated with each pathway.

The dynamic state of bodily proteins was established by analyzing the fate of stable isotope-labeled amino acids that had been fed to mice. These classic experiments, conducted by Rudolf Schoenheimer in the late 1930s, presage modern stable isotope labeling techniques (such as SILAC), which allow determination of the turnover rate of hundreds to thousands of individual proteins in a single mass spectrometry experiment (Kristensen et al., 2008). After its discovery, the lysosomal compartment was considered the principal site of degradation of cellular proteins, through the action of resident acid-dependent proteases. However, this view perished with the demonstration that the half-lives of most cellular proteins are insensitive to alkalinization of the lysosomes. The subsequent discovery of the ubiquitin-proteasome degradation system as the major route to protein degradation generated a new orthodoxy. Central to this model is the idea that covalent modification of substrate proteins with a polypeptide ubiquitin tag targets them to the large (26S) proteolytic complex known as the proteasome.

It came then as a surprise to discover that ubiquitin tagging also provides a signal to route endocytosed receptors to the lysosomal degradation pathway and more recently to mark organelles for disposal by the third major cellular degradative pathway of autophagocytosis. The role of ubiquitin in protein degradation is more ubiquitous than once thought (Figure 1). In this Minireview, we consider how a ubiquitin tag selects for specific degradation pathways and also highlight the interplay between these pathways that a shared dependence on ubiquitin engenders.

General Considerations

Substrate proteins are selected for modification of lysine residues by ubiquitin through interaction with an E3 ligase protein that recruits an E2-enzyme charged with ubiquitin. This can result in transfer of a single ubiquitin molecule (monoubiquitination) or coupling of further ubiquitin molecules, through integral lysine residues, to form a chain. The seven lysines of ubiquitin provide for the formation of different isopeptide chain linkages, which adopt different three-dimensional structures, and all of which are represented in eukaryotic cells (Xu et al., 2009). The specific combination of E2 and E3 enzymes recruited to a substrate dictates the chain linkage type. The human genome encodes more than 20 different types of ubiquitin-binding domains, and proof of principle for linkage specificity of binding has been established (see Essay by F. Ikeda, N. Crosetto, and I. Dikic on page 677 of this issue). One means to achieve this is through the spatial arrangement of tandem ubiquitin-binding domains (UBDs) either encoded in a single protein or by combining domains within a multimolecular complex, such that simultaneous occupancy of two binding sites is restricted to particular chain configurations.

Proteasomal Degradation

Early work suggested that proteasomal targeting requires a lysine 48 (K48)-linked ubiquitin chain consisting of at least four conjoined ubiquitin molecules. This was based first upon the biochemical analysis of chains formed on a model substrate, β-galactosidase, in a reticulocyte lysate system and second upon studies showing that unique among lysine mutant versions of ubiquitin, K48R cannot serve as the sole source of ubiquitin in yeast (Finley, 2009; Xu et al., 2009). The affinity of unanchored K48 polyubiquitin chains for the proteasome increases more than 100-fold from di- to tetraubiquitin (~170 nM) and less steeply thereafter (Thrower et al., 2000).

A body of work now suggests that in fact the proteasome happily accepts other ubiquitin chain types. Indirect evidence for this comes from the observation that acute proteasome inhibition does not lead to the selective accumulation of K48 chains. Rather, all chain types with the exception of K63 are increased (Jacobson et al., 2009; Xu et al., 2009). During cell division, the human anaphase-promoting complex (APC/C) recruits two E2 ligases (UbcH10 and Ube2S), which combine to exclusively generate K11-linked chains on substrates. Loss of this unit leads to strong defects in mitotic progression due to failure of the necessary degradation processes (Song and Rape, 2010). In vitro studies have even shown that K63-modified dihydrofolate reductase provides an efficient proteasome substrate (Hofmann and Pickart, 1999).

The proteasome is composed of a core (20S) particle containing multiple proteolytic sites and a 19S regulatory particle that

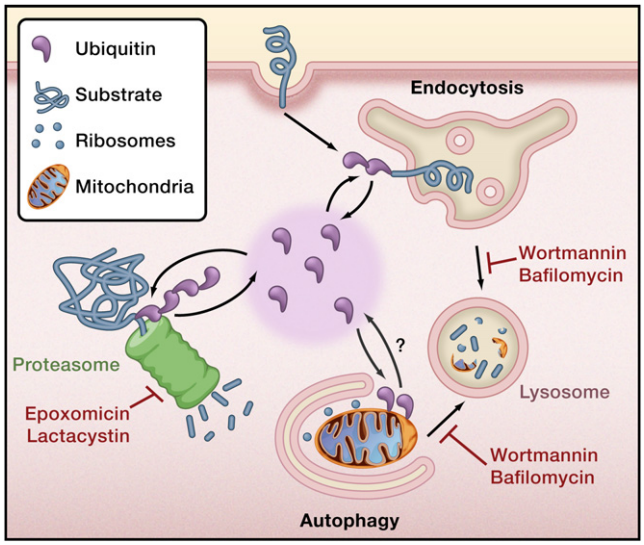

Figure 1. Ubiquitin Is a Common Denominator of Protein Degradation Pathways

Specific ubiquitin receptors are associated with each degradation pathway. Autophagosomal and multivesicular body (MVB) pathways merge at the lysosome and share a dependence on v-ATPase activity (inhibited by bafilomycin). Both pathways also share sensitivity to inhibitors of phosphoinositide 3-kinase activity, such as wortmannin or 3-methyladenine, as the family member hVPS34 is required both for recruitment of ESCRT (endosomal sorting complex required for transport) components to MVBs and for expansion of the double-membrane preautophagosomal structure. Proteasomal inhibitors include lactacystin and epoxomicin.

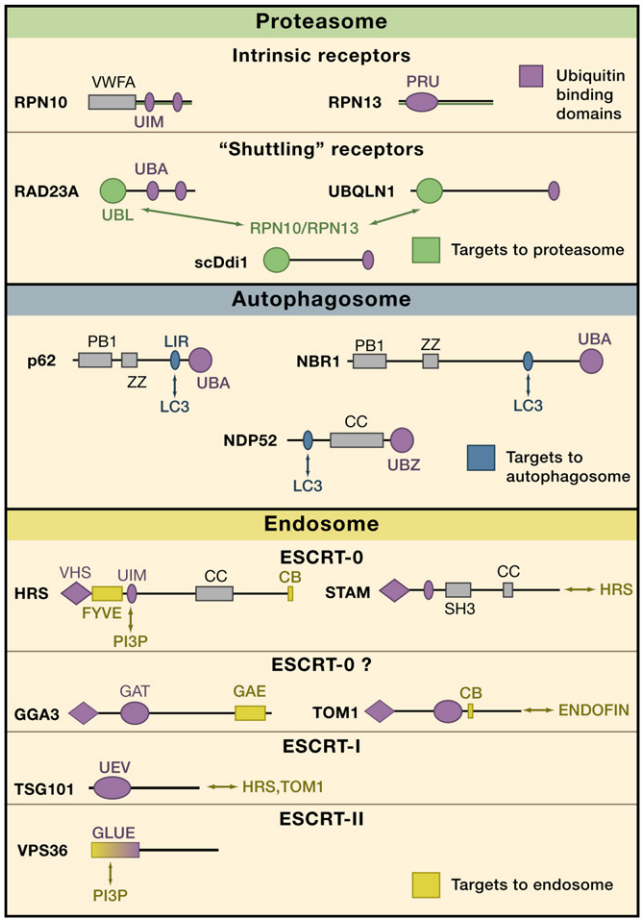

Figure 2. Ubiquitin Recognition by the Major Degradative Pathways

Depiction of the "ubiquitin receptors" associated with each degradative pathway. The domain structures shown are for the human representatives of each protein family, except for yeast Ddi1, the human ortholog of which does not contain a UBA domain. CB: clathrin-binding motif; CC: coiled coil; ESCRT: endosomal sorting complex required for transport; GGA: golgi-associated, gamma adaptin ear containing, ARF-binding protein; GAE: gamma adaptin ear; GAT: GGA and TOM1; GLUE: GRAM-like ubiquitin-binding in Eap45; HRS: HGF receptor tyrosine kinase substrate; LIR: LC3-interacting region; PB1: Phox and Bem1; PRU: Pleckstrin-like receptor for ubiquitin; SH3: Src homology domain 3; STAM: signal transducing adaptor molecule; TOM1: target of myb1; TSG101: tumor susceptibility gene 101; UBA: ubiquitin-associated domain; UBL: ubiquitin-like domain; UEV: ubiquitin E2 variant domain; UIM: ubiquitin-interacting motif; VHS: Vps27, HRS, and STAM; VPS36: vacuolar protein sorting 36; vWFA: von Willebrand Factor type A; ZZ: zinc finger. Note the following gene names and commonly used alternative names also apply: p62; SQSTM1 (sequestosome), NDP52; CALCOCO2, UBQLN1; PLIC1; DSK2. Domain annotation based on PFAM and UNIPROT.

governs access to the core. To enter the core, substrates must be amenable to unfolding by a hexamer of ATPases associated with the base of the regulatory particle. Other constituents of the regulatory particle are implicated in the recruitment of substrates (Finley, 2009). Rpn10 and Rpn13 interact with ubiquitinated substrates through UIM (ubiquitin-interacting motif) domains and a Pru (pleckstrin-like receptor for ubiquitin) domain, respectively. The UBL/UBA family of proteins are substoichiometric components of purified proteasomes that bind ubiquitin via their UBA (ubiquitin-associated) domain and the proteasome regulatory particle through its UBL (ubiquitin-like) domain. They are proposed to remotely scavenge ubiquitinated substrates and present them to the proteasome (Figure 2). Particular proteasome-associated ubiquitin receptors have been linked with the degradation of specific substrates (reviewed in Finley, 2009).

The mammalian regulatory particle has three associated deubiquitinating enzymes (DUBs), POH1/PSMD14, USP14, and UCH37 (Rpn11 and Ubp6 in budding yeast), which have distinct specificities for different chain linkages (Finley, 2009). What is the function of these DUB activities? One important function is to salvage ubiquitin in order to maintain the cellular ubiquitin pool. The JAMM/MPN$^+$ metalloprotease POH1 is thought to specifically disassemble K63-linked chains, as well as cleave the isopeptide bond that links the substrate and the proximal ubiquitin, allowing for en bloc removal of an ubiquitin chain. It also governs entry into the central proteolytic chamber, thereby coupling substrate degradation to recycling of ubiquitin (Yao and Cohen, 2002). Ubiquitin-aldehyde-sensitive cysteine protease activities (that is, USP14 and UCH37) account for all activity directed toward K48-linked chains and also contribute to K63-linked chain disassembly (Jacobson et al., 2009). One attractive notion is that the integration of these DUB activities may provide for a proof-reading mechanism, facilitating release from the proteasome if commitment to degradation is not accomplished within a given time window. For example, preferential proteasomal DUB activity against K63-linked chains has been proposed to select against these substrates for degradation (Jacobson et al., 2009). Also in line with this principal,

a specific chemical inhibitor of USP14 has recently been shown to enhance the rate of protein degradation (Lee et al., 2010). In yeast, a ubiquitin ligase, Hul5 (mammalian ortholog is KIAA10/E3a), that is associated with proteasomes can oppose Ubp6 activity through chain elongation (E4) (Crosas et al., 2006). Thus a balance between proteasome-associated ubiquitin ligase and DUB activity may determine receptor fate.

Endolysosomal Degradation

The lysosomal degradation pathway is the principle means by which a cell turns over plasma membrane proteins, such as receptors or channels. Its defining characteristic is a requirement for organelle acidification, mediated by the v-ATPase. Endocytosed proteins are either recycled to the plasma membrane or captured into lumenal vesicles of the multivesicular body (MVB) as it matures from the sorting endosome, before fusing directly with lysosomes. Some receptors use ubiquitin as an internalization signal, but for other ubiquitinated receptors, such as epidermal growth factor receptor, this is secondary to, or redundant with, other adaptor-binding motifs. Ubiquitination directs internalized proteins toward lysosomal degradation by engagement with endosomal sorting complexes required for transport (ESCRTs) (reviewed in Clague and Urbé, 2006). Monoubiquitination, in the form of an irreversible linear fusion appended to various receptors, is a sufficient signal for this sorting step. However, evidence suggests K63 as the primary ubiquitin chain type involved in endosomal sorting. Early studies in yeast cells, which suggested that appendage of K63-linked diubiquitin enhances vacuolar sorting, have been recently elaborated on with a detailed analysis of the downregulation of the Gap1 permease. These studies conclude that monoubiquitination is sufficient for initial internalization (at least so long as it is an irreversible linear fusion) but that efficient sorting at the endosome by the ESCRT machinery requires K63-linked polyubiquitin (Lauwers et al., 2009). Concordantly, studies of the mammalian TrkA and MHC class I proteins reveal their utilization of K63-linked polyubiquitination for routing to the lysosome (Duncan et al., 2006; Geetha and Wooten, 2008).

The first point of engagement of ubiquitinated cargo with the MVB sorting machinery is proposed to be the ESCRT-0 complex, comprising HRS and STAM, both of which possess UIM and VHS (Vps27, HRS, and STAM) domains, which can bind ubiquitin (Figure 2). Intact ESCRT-0 binds 50 times more tightly to K63-linked tetraubiquitin than to monoubiquitin, but only 2-fold more tightly than to K48-tetraubiquitin (Ren and Hurley, 2010). ESCRT-0 is recruited to endosomes through binding to phosphatidylinositol 3-phosphate but also binds to clathrin and the downstream ESCRT-I complex. An alternative ESCRT-0 complex comprising TOM1, Tollip, and Endofin possesses all these salient features of the HRS-STAM complex. It is currently unclear whether these two complexes are redundant or used to receive different cargoes. In a further striking parallel to the proteasomal system, the ESCRT machinery has known associations with at least two DUB activities, AMSH and USP8 (UBPY), drawn from the JAMM/MPN$^+$ and USP families, respectively. In yeast, the dominant endocytic E3 ligase activity Rsp5 can also associate with the STAM ortholog Hse1, providing a counterbalance to Ubp2 and Ubp7 (Ren et al., 2007), while a third ESCRT-associated DUB Doa4 is required for ubiquitin recycling of receptors that are committed to degradation. Although deubiquitination is not an obligate step for MVB sorting, proof-reading and ubiquitin recycling roles akin to those suggested for proteasomal DUBs are consistent with available data (Clague and Urbé, 2006).

Autophagy

The signature of autophagy is the capture of cytosol and organelles through envelopment within a double-membrane compartment derived from the preautophagosomal structure. In common with the MVB, the autophagosome can then directly fuse with late endosomes or lysosomes to form the autolysosome, wherein the double-membrane structure is digested. It is well suited for the digestion of cytosolic entities, which are incompatible with unfolding by the proteasome, such as organelles or protein aggregates.

Identification of autophagy (Atg) genes and subsequent biochemical characterization revealed two essential posttranslational modification pathways, which resemble ubiquitination. In one case, Atg12 is stably conjugated to Atg5 in a constitutive fashion. In the second case, Atg8 is conjugated to the lipid phosphatidylethanolamine by transfer from an E2 enzyme following the onset of autophagy (for example, as induced by amino acid depravation). This is a prerequisite for the expansion of the preautophagosomal structure, perhaps by facilitating fusion between membranes. In mammalian cells, Atg8 is known as LC3 and its lipidated form as LC3-II. In fact, there are six Atg8 homologs in the human genome collectively known as the LC3/GABARAP family.

Whereas autophagy is generally thought of as a nonselective degradation process, certain structures and organelles are selectively removed by this pathway. For example, mitochondria are lost during reticulocyte maturation and as a consequence of uncoupling (disconnecting the electron transport chain from ATP production) in cultured cells. Ribosomes, peroxisomes, and pathophysiological protein aggregates can also be degraded by autophagy. Recent studies have led to the proposal of a common principle involved in "selective autophagies" and once again ubiquitin plays a critical role (Kirkin et al., 2009). In general if the body to be cleared is ubiquitinated, then an adaptor molecule is required to couple this to the preautophagosomal membrane rich in Atg8/LC3. The prototypical adaptor of this class is p62/sequestosome 1, which contains both a ubiquitin-interacting domain (UBA) and a LIR motif (LC3-interacting region), a domain structure shared with Neighbor of BRCA1 gene 1 (NBR1) (Figure 2) (Pankiv et al., 2007). p62 has been previously implicated in the clearing of protein aggregates, which are known to be concentrated in ubiquitin. Recent data have indicated an essential role for ubiquitin (K63 and K27 polyubiquitin chain linkages have been implicated) in the selective autophagy of depolarized mitochondria, which become ubiquitinated following recruitment of the E3 ubiquitin ligase Parkin (Geisler et al., 2010). Using a lysine-less mutant of ubiquitin fused with red fluorescent protein, Kim et al. established that irreversible monoubiquitination is sufficient to concentrate a soluble protein within autophagosomal structures in a p62-dependent manner (Kim et al., 2008).

A selective pathway requiring the Ubp3:Bre5 DUB complex in *Saccharomyces cerevisiae* operates in the removal of mature ribosomes (Kraft and Peter, 2008). In cells deficient in Ubp3, ribosomal fractions are enriched with ubiquitin. Although an intimate

connection has been established, the exact role of ubiquitin in ribophagy is unclear. One model posits that ubiquitin may be protecting ribosomes from autophagy, which is then promoted by Ubp3 activity. Alternatively, a dynamic modification with ubiquitin may be required, perhaps as an engulfment signal similar to that of mitochondria. In support of this notion, a temperature-sensitive defect in the E3 ligase Rsp5 shows a synthetic ribophagy defect with loss of Ubp3 as compared with cells lacking Ubp3 alone (Kraft and Peter, 2008). If correct, then the principle of ensuring ubiquitin homeostasis through deubiquitination may be conserved by each of the selective degradation pathways we have discussed.

The Interdependence of Degradation Pathways

The relative contribution of degradation pathways may vary greatly between cell types. In most cases of cells cultured under stress-free conditions, proteasomal degradation predominates, but in muscle cells, lysosomal pathways (principally autophagy) can account for 40% of degradation of long-lived proteins. In atrophying muscle cells, both pathways are proposed to be co-ordinately upregulated under the transcriptional control of FOXO3 (Zhao et al., 2007). However, the proteasome is itself degraded by starvation-induced bulk autophagy (Kristensen et al., 2008).

The reliance of three major cellular degradation pathways upon ubiquitination suggests that specific inhibition of any one pathway may perturb the ubiquitin economy of the cell and hence indirectly affect other degradation events (Figure 1). A clear example of this is the activated Met receptor, for which its lysosomal degradation is exquisitely sensitive to the depletion in free ubiquitin caused by proteasomal inhibition (Carter et al., 2004). Proteasome inhibition may also induce autophagy as a compensatory response. The autophagy adaptor protein p62 has also been implicated in proteasomal degradation, whereas the E3 ligase Parkin generates an autophagy tag on mitochondria but elsewhere can target proteins to the proteasome. VCP/p97 co-ordinates a number of ubiquitin-dependent processes that include the proteasome-dependent ERAD (endoplasmic reticulum-associated degradation) pathway but interestingly has recently been identified as a necessary factor for autophagosome maturation under basal conditions and following proteasome inhibition (Tresse et al., 2010).

The MVB and autophagy pathways merge at the late endosome/lysosome and are both sensitive to proton pump and phosphoinositide 3-kinase inhibitors. Autophagosome formation is inherently sensitive to perturbations earlier in the endocytic pathway, which change the character of later endosomal compartments (such as the composition of SNARE proteins). Occasionally, teleological distinctions between these systems blur, such that some ubiquitinated cytosolic proteins may be degraded in the lysosome and cytoplasm-exposed domains of receptors may be nibbled by the proteasome. Mounting evidence suggests that there is a proteasome pool associated with endosomes that influences receptor sorting (Geetha and Wooten, 2008; Gorbea et al., 2010).

Concluding Remarks

Ubiquitin tagging is common to the three major cellular pathways for protein degradation. Herein lies a conundrum: how is a given substrate targeted to a particular pathway? Variable parameters include location, chain length, and linkage type. A clear bias of the endosomal pathway toward K63-linked chains has emerged. This may simply reflect the subcellular localization of specific E3 ligases in combination with a high local concentration of ubiquitin-binding proteins, which couple to the ESCRT-machinery rather than the proteasome. New techniques allow for the determination of individual protein turnover on a global scale (Kristensen et al., 2008). This will enable the generation of a comprehensive annotation of turnover rates as a function of experimental perturbations or disease states, opening the door to a systems-level understanding of protein degradation.

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