Molecular Mechanisms and Regulation of Specific and Nonspecific Autophagy Pathways in Yeast

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The normal growth and development of eukaryotic cells requires a constant balance between biosynthetic and degradative processes. There are two highly conserved mechanisms for degradation in eukaryotes that are responsible for the majority of protein turnover: one is the ubiquitin-proteasome system and the other is autophagy. Under conditions of normal growth, the bulk of protein degradation occurs via the proteasomal machinery (reviewed in Ref. 1). Proteins that are to be degraded are post-translationally tagged with polyubiquitin and are subsequently broken down via the multisubunit 26 S proteosome present in the cytosol and nucleus. In contrast, autophagy is largely a nonspecific process that leads to the vacuolar/lysosomal degradation not just of proteins but also of other cytosolic macromolecular components. Importantly, autophagy is the only degradative pathway that has the capacity to degrade large aggregates and even entire organelles. Autophagy plays a role in normal development and differentiation; however, it is also involved in defense against pathogens, life span extension, and the prevention of certain types of cancer and neurodegeneration (reviewed in Ref. 2).

Autophagy Involves Dynamic Membrane Rearrangements

In macroautophagy (hereafter referred to as autophagy), double membrane vesicles called autophagosomes surround the cargo intended for degradation (Fig. 1). Once the autophagosomes are fully formed, the outer membrane fuses with the lysosomal/vacuolar membrane releasing the inner vesicle, which is subsequently broken down. The cargo is released into the lumen of these organelles for degradation, and the resulting macromolecules are then made available for reuse. Two other forms of autophagy, called chaperone-mediated autophagy and microautophagy, have been reviewed recently elsewhere (3, 4) and will not be covered here.

There are several comprehensive reviews on the molecular mechanism of autophagy (3, 5, 6). Nonetheless, the functions of most of the autophagy proteins are not known. For example, Atg1 was the first protein component of this pathway identified and one of the very few with an obvious functional motif; it appears to be a serine/threonine-protein kinase (7). Although Atg1 is thought to play a central role in autophagy, almost a decade after its discovery the protein appears to be a serine/threonine-protein kinase (7). Although Atg1 is thought to play a central role in autophagy, almost a decade after its discovery the protein appears to be a serine/threonine-protein kinase (7).

Although autophagy is a dynamic process, it can be conceptually divided into seven discrete steps: induction or a cue to start the formation of vesicles, selection and packaging of cargo, initiation of vesicle formation (nucleation), vesicle expansion and completion, retrieval of certain autophagy proteins during or after vesicle completion, fusion of the completed vesicle with the lysosome/vacuole, and the breakdown of the inner membrane of the vesicle and enclosed cargo within the lysosomal/vacuolar lumen (5). Mutants affecting different steps of this pathway have been isolated in yeast, and so far 27 different Atg (autophagy-related) genes specifically affecting various steps of this process have been identified. It is interesting to note that even though most of these genes were first identified in yeast, many of them are functionally conserved in higher eukaryotes (2).

Selective and Non-selective Autophagy

Contrary to its general mode of bulk cargo degradation, there are some instances in which autophagy plays a biosynthetic role by mediating the specific vesicular trafficking of some vacuolar enzymes (Fig. 1) or performs a homeostatic role (controlling biogenesis versus degradation) by selectively degrading unwanted organelles. A well studied example of the former is that of a selective, biosynthetic pathway called the cytoplasm to vacuole targeting (Cvt) pathway that operates constitutively under growing conditions in S. cerevisiae (8). The vacuolar enzymes in amniosensis l (Ape1) and e-mannosidase (Ams1) are synthesized in the cytoplasm as inactive precursors that assemble to form large homo-oligomeric complexes (9, 10). The oligomeric enzyme complexes are sequestered within double membrane Cvt vesicles and delivered into the vacuolar lumen where they function as resident hydrolases (11). Although Cvt vesicles are morphologically similar to autophagosomes, they are smaller and appear to exclude cytoplasmic contents other than their specific cargo.

The molecular components involved in the selection of the Cvt cargo and the temporal sequence of their action have recently been clarified (Fig. 2). A peripheral protein Atg19 serves as a receptor for the Cvt cargo and directly binds to Ape1 or Ams1 oligomers (12, 13). Another protein, Atg11, then binds to the C terminus of Atg19 and tethers the Cvt complex to a peri-vacuolar site called the pre-autophagosomal structure (PAS) (13). The PAS is thought to be the site from which both Cvt vesicles and autophagosomes originate (14, 15), and the expansion of the intermediate structures is thought to occur by subsequent membrane addition. The source of membrane for the formation and expansion of these vesicles is still unknown, although recent data suggest a role for the early secretory pathway (16–18) and possibly the mitochondria (19). Three proteins, prApe1, Atg19, and Atg11, are required for formation of the PAS in nutrient-rich conditions (20, 21). Once the cargo complex arrives at the PAS, Atg19 interacts with a phosphatidylinositolanolaminol (PE)-conjugated protein, Atg8–PE, which is initially present on both the inner and outer membrane of the growing vesicle (13). The interaction between Atg19 and Atg8–PE may mediate the completion of the Cvt vesicle. When the membrane sequestration event is completed, the Cvt vesicle fuses with the vacuole releasing its inner membrane and cargo into the vacuolar lumen. Atg19 and the luminal oriented Atg8–PE remain inside the vesicle and are degraded, whereas Atg11 is retrieved at some stage prior to vesicle completion and recycled (22). The Atg8–PE on the outer vesicle surface is released from the vesicle by an Atg4-dependent cleavage event prior to fusion.

In addition to binding Atg19, Atg11 appears to be a part of the putative Atg1 regulatory complex (Fig. 3). We refer to this complex as putative because a holocomplex containing all of these proteins has not been demonstrated to exist. In addition to Atg1 and Atg11, the proteins that interact as part of this complex include Atg13 (23), which appears to modulate Atg1 kinase activity, Atg17 (24, 25), a protein that is specific for autophagy, and three proteins of unknown function that are relatively specific for the Cvt pathway, Atg20, Atg24, and Vac8 (26). Deletion of the ATG1 gene results in a block in both the Cvt pathway and autophagy (7, 8, 27). Indeed, Atg1 may play a role in more than one step of both transport routes, as discussed later in this review. The function of the interaction between Atg11 and Atg1 is currently unknown, although it may serve to coordinate delivery of the cargo with the arrival of the vesicle-forming machinery. For example, the Atg1–Atg13 complex is needed for the localization or assembly/disassembly of Atg11 homo-oligomers at the PAS (Fig. 2) (21).

The best example of specific organelle degradation is seen with peroxisomes. Peroxisomes accumulate in the methyloptropic yeasts Hansenula polymorpha and Pichia pastoris when these species are grown in media containing methanol as the sole carbon source. When cells are shifted to media containing preferred carbon sources, peroxisomes become superfluous and are selectively degraded via pexophagy (reviewed in Ref. 3). Homologs of S. cerevisiae Atg1 and Atg11 are present in both species, and both gene products are required for pexophagy (28, 29).

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In yeast, autophagy appears to operate at a basal level in vegetative conditions; it is a biosynthetic pathway used to deliver the inactive form of the resident hydrolase aminopeptidase 1 (Ape1) to the vacuole. The Cvt vesicle ranges from 140 to 160 nm in diameter and, in contrast to the autophagosome, appears to exclude bulk cytoplasm. Following completion, the outer membrane of the sequestering vesicle fuses with the vacuolar membrane. The inner vesicle along with its contents is released into the vacuolar lumen where the vesicle membrane is degraded, allowing processing and activation of prApe1. Under starvation conditions, prApe1 is packaged into autophagosomes and is transported to the vacuole along with other cargo.

Environmental Sensing and Induction of Autophagy

It is now becoming clear that the specific targeting of cargo is not an isolated phenomenon restricted to unicellular eukaryotes. For example, the degradation of abnormal, proliferated peroxisomes in rat liver cells treated with di(2-ethylhexyl)phthalate is suggestive of selective autophagy (30). Certain bacterial pathogens including Group A Streptococcus, Shigella flexneri, and Mycobacterium tuberculosis are exclusively targeted for degradation via the autophagic machinery reviewed in Ref. 31. Similarly, some viruses are cleared from the host cell through the action of Atg4 (not shown) and released from the outer membrane.

FIGURE 1. Schematic representation of macroautophagy (autophagy) and the Cvt pathway in yeast. In both autophagy and the Cvt pathway, cargoes are engulfed by double membrane vesicles. During autophagy, various cytoplasmic components such as organelles and cytoplasm are sequestered into a large, double membrane autophagosome that is 300–900 nm in diameter. The Cvt pathway occurs during vegetative conditions; it is a biosynthetic pathway used to deliver the inactive form of the resident hydrolase aminopeptidase 1 (Ape1) to the vacuole. The Cvt vesicle ranges from 140 to 160 nm in diameter and, in contrast to the autophagosome, appears to exclude bulk cytoplasm. Following completion, the outer membrane of the sequestering vesicle fuses with the vacuolar membrane. The inner vesicle along with its contents is released into the vacuolar lumen where the vesicle membrane is degraded, allowing processing and activation of prApe1. Under starvation conditions, prApe1 is packaged into autophagosomes and is transported to the vacuole along with other cargo.

FIGURE 2. Temporal order for packing cargo components in the Cvt pathway. Precursor Ape1 monomers assemble into dodecamers and then into a large Ape1 complex. The receptor protein Atg19 binds to the propeptide of prApe1 to generate the Cvt complex. The binding of Atg11 is necessary for this complex to be targeted to the PAS where the disassembly of Atg11 oligomers occurs. Consequently, Atg8 conjugated to phosphatidylethanolamine binds to Atg19 and also to the growing membrane. In the final step of vesicle formation, Atg8–PE that is not enclosed within the vesicle is cleaved by the action of Atg4 (not shown) and released from the outer membrane.

FIGURE 3. Schematic representation of the putative Atg1 regulatory complex. Components required primarily for autophagy are shown in blue, those required mainly for the Cvt pathway are shown in green, and those needed for both are in pink. Tor kinase is a major regulatory protein controlling induction of autophagy. When nutrients are not limiting, Tor kinase is active. Under this condition, Atg13 and Atg1 are more highly phosphorylated resulting in their reduced affinity for each other and an inhibition of autophagy. In the absence of nutrients or when challenged with rapamycin, Tor is inactivated resulting in the partial dephosphorylation of Atg13 and Atg1, increasing their affinity for each other and inducing autophagy. All the interactions of proteins in the Atg1 regulatory complex have been detected by yeast two-hybrid analysis, communoprecipitation experiments, or affinity isolation, which have indicated pairs of interacting proteins; there is no evidence for a single holocomplex.

phagic capacity is clearly important for overcoming starvation because mutants that are defective in autophagy lose viability during stationary phase. Autophagosomes are not simply large Cvt vesicles; not only do they differ from each other in size and the nature of their cargoes but also in the components needed for vesicle formation; as mentioned above, several components of the Atg1 complex that seem to be required for vesicle formation are specific for either the Cvt pathway or autophagy. A major unresolved question is how the cell induces the formation of autophagosomes.

One of the major signal transduction pathways for the induction of autophagy results in the inactivation of two highly homologous serine/threonine-protein kinases, Tor1 and Tor2 (reviewed in Ref. 36). Under growing conditions, these kinases redundantly inhibit autophagy while regulating several aspects of cell growth. The inactivation of these enzymes, either because of nutrient deprivation or treatment with the specific inhibitor rapamycin even in rich media, leads to a cell cycle arrest in the G1 phase and the induction of autophagy. Currently our knowledge of the mechanism that leads to autophagosome formation is very limited. In growing conditions, the Tor kinases either directly or indirectly hyperphosphorylate Atg13, a component of the proposed Atg1 complex (3). Hyperphosphorylated Atg13 has reduced affinity for Atg1, and this reduced interaction is thought to prevent the induction of autophagy (37). Under conditions of nitrogen deprivation, Atg13 is largely dephosphorylated by an unknown phosphatase(s), thereby increasing its affinity for

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Atg1 (37, 38). The interaction between these two proteins might act as a molecular trigger that initiates a switch from the Cvt pathway to autophagy.

The role of Atg1 (and Atg13) in initiating the formation of autophagosomes is speculative based on the fact that they are the only proteins in the putative Atg1 complex that are essential for both the Cvt pathway and autophagy. However, the role of Atg1 kinase activity is currently under debate: two different studies have come to opposite conclusions about its role in autophagosome versus Cvt vesicle formation. Because the physiological target of Atg1 is currently unknown, an artificial substrate was used to measure its kinase activity in vitro. Under starvation conditions, the ability of Atg1 to phosphorylate an artificial substrate (myelin basic protein) increases (37), whereas its autophosphorylation activity decreases (7). This activity is dependent on Tor and on the Atg1 interacting partners, Atg13 and Atg17, and to a lesser extent on Atg1 (37).

A separate chemical genetics approach was utilized to examine the requirement for Atg1 kinase activity in vivo. The Atg1M102A allele is sensitive to inhibition only in the presence of 1-NA-PP1 (25, 38). In this case, inhibition of kinase activity shows a greater block in the Cvt pathway than in autophagy. Similarly, an Atg2M102A allele containing a mutation in the kinase domain (Atg2K54A) is not defective for the vacuolar import of precursor Ape1 through autophagy (38). The interpretation of these studies has likely been complicated by the fact that both Atg1M102A and Atg2K54A appear to retain a low level of activity. Considering all of the data together, we conclude that kinase activity is needed for both the Cvt pathway and autophagy, although a higher level of activity appears to be more important for the former. In addition, Atg1 may play a structural role in autophagy (38). Furthermore, these results cast doubts on the role of Atg1 kinase activity in the initiation of autophagosome formation per se and instead suggest that it may have a role in regulating vesicle expansion, similar to Atg17 (24, 25). Additionally, because Atg1 is not required for the formation of the PAS (at least the localization of some Atg proteins to this site), it is unlikely that it is involved in a signal transduction event to initiate autophagy (14).

Interestingly, in higher eukaryotes, unlike in S. cerevisiae, the size of autophagosomes does not appear to increase during starvation conditions (39). Thus, the regulation of the size of the degrading vesicle appears to be a property unique to S. cerevisiae and possibly other fungi. It has been shown that in S. cerevisiae, the expansion, but not the biogenesis, of autophagosomes requires de novo protein synthesis during starvation (40). Further experiments will be required to identify the starvation-specific factor(s) that regulate autophagosome size and to determine whether they modulate Atg1 kinase activity or interact with other components of the Atg1 complex.

**Regulation of the Retrieval Transport of Atg9 and Atg23 before Vesicle Completion**

Among the proteins that are directly involved in the Cvt pathway or autophagy, only two, Atg8 and Atg19, remain associated with the completed vesicles and are degraded in the vacuole. The other components, including an integral membrane protein, Atg9, are retrieved for later reuse at a step prior to the completion of vesicle formation or fusion of the completed vesicle with the vacuole. Because most of the Atg proteins are soluble, it is easy to imagine that they can simply dissociate from the completed vesicle and remain in the cytosol until they are required; however, the retrieval of membrane proteins like Atg9 presumably include a vesicular mechanism. Atg9 is essential for both Cvt and autophagy and functions at an early stage of both pathways (41). Although most other Atg proteins localize to the PAS in a single dot, Atg9 and a peripheral membrane protein Atg23 (42) localize to several cytoplasmic sites as punctate dots in addition to the PAS, in both growing and starvation conditions (43). Recently for Atg9, this non-PAS location was shown to be in part the mitochondria (44), but the organelle to which Atg23 localizes is not known. These results suggest that the cycling of Atg9 between the PAS and the mitochondrial may have a role in membrane flow from that organelle to the growing vesicles. Indeed, biochemical fractionation experiments show that both the mitochondrial and the cytosolic/PAS populations of Atg9 are associated with membrane vesicles (44).

A retrieval mechanism involving Atg1, Atg13, Atg2, and the phosphatidylinositol (PtdIns)-3-P-binding protein Atg18 has been demonstrated recently for Atg9 (43) (Fig. 4). The data suggest that membrane vesicles containing Atg9 and derived from the mitochondria arrive at the PAS that is already decorated with PtdIns-3-P, Atg5, and Atg16 (14). The Atg1-Atg13 complex arrives at the PAS at a later step, in a mechanism independent of the PtdIns 3-kinase complex (14, 43). The conjugation of the ubiquitin-like Atg12 with Atg5 appears to be the signal that triggers the enlargement of the growing vesicle. Atg12–Atg5 and Atg16 form large oligomeric complexes that are recruited to the growing autophagosome along with Atg8–PE and membrane-bound Atg9. Atg1 and Atg13 mediate the retrieval of Atg9 from the PAS, upon its interaction with Atg2 and Atg18, in the absence of Atg1. Atg2 is not recruited to the PAS (45). When the autophagosome is completed, the Atg12–Atg5 conjugate as well as Atg16 dissociate from the vesicle, and Atg8–PE on the outer membrane of the autophagosome is cleaved by Atg4 and released into the cytosol. In contrast, Atg8–PE, which decorates the inner lipid bilayer of the double membrane Cvt vesicle or autophagosome is not released.

In the case of Atg23, this protein interacts with Atg9 and localizes to the PAS in an Atg9-dependent manner (42). It is possible that similar to Atg9, Atg23 also supplies membrane to the growing vesicle. Under growing conditions, the retrieval of Atg23 is dependent only on the kinase activity of Atg1; by contrast, the kinase activity of Atg1 is not needed for the retrograde transport of Atg9 (43). This result suggests that the kinase activity of Atg1 is more important in the Cvt pathway because, first, Atg23 is essential only for the Cvt pathway, and second, Atg1 kinase activity is required for the retrieval of Atg23 only in growing conditions. Under starvation conditions, Atg23 is localized within the cell in multiple punctate dots, bypassing the requirement for Atg1 kinase activity for its retrieval (43), although the mechanism involved is not known. Finally, because Atg1 acts at a late stage of vesicle formation and has a role in retrieving proteins such as Atg11, Atg9, and Atg23, it is possible that it acts as a sensor of vesicle completion.

**Atg1 in Higher Eukaryotes**

Genes encoding serine/threonine-protein kinases similar to S. cerevisiae ATG1 have recently been found in other model organisms such as Caenorhabditis elegans (unc-51), Dictyostelium discoideum (DdAtg1), and Drosophila melanogaster (CG10967) and have been experimentally shown to function in autophagy (46–48). There is a high percentage of sequence similarity between the N-terminal kinase domains of these proteins and that of S. cerevisiae Atg1; however, their C-terminal sequences are poorly conserved. The atg1 mutants in these other organisms not only have defects in autophagy but also in development: for example, defective dauer development in C. elegans, defective fruiting body formation in D. discoideum, and larval and pupal lethality in D. melanogaster. The mechanism of action of Atg1 in these organisms is likely to differ from their yeast ortholog because components of the putative Atg1 complex are absent in these organisms. For example, Atg11 and Atg17 are not conserved outside of fungi, and Atg13 is only found in plants. It will be interesting to find out whether an Atg1 holocomplex exists in S. cerevisiae or whether Atg1 functions by forming smaller subcomplexes with its interacting partners in a nutrient-dependent manner. Future experiments in the near term...
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will likely reveal the precise role of *S. cerevisiae* Atg1 in autophagy, as well as the identity and function of its physiological substrate(s).

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