Protein Turnover Via Autophagy: Implications for Metabolism*

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Key Words

amino acids, neurodegeneration, protein degradation, proteolysis, starvation

Abstract

Autophagy is a process of cellular "self-eating" in which portions of cytoplasm are sequestered within double-membrane cytosolic vesicles termed autophagosomes. The autophagosome cargo is delivered to the lysosome, broken down, and the resulting amino acids recycled after release back into the cytosol. Autophagy occurs in all eukaryotes and can be up-regulated in response to various nutrient limitations. Under these conditions, autophagy may become essential for viability. In addition, autophagy plays a role in certain diseases, acting to prevent some types of neurodegeneration and cancer, and in the elimination of invading pathogens. We review the current information on the mechanism of autophagy, with a focus on its role in protein metabolism and intracellular homeostasis.

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INTRODUCTION

Autophagy/ macroautophagy: the sequestration of cytoplasm within double-membrane vesicles for delivery to, and degradation within, the lysosome followed by recycling

When most people consider the topic of nutrition, the first thoughts that come to mind concern the types of food we eat. Of course, we also know that food is consumed as a fuel source and as such it must be broken down to provide energy and building blocks for anabolism. When nutrients become limiting, the biosynthetic needs may alter, but they must still be met to maintain viability. The carbohydrate stores of most organisms can supply nutrients only for a few hours; during the initial phase of fasting, it is generally thought that proteins are broken down to provide substrates for gluconeogenesis (9, 125). Accordingly, once these stores are exhausted during starvation conditions, the cell employs autophagy as a means of reutilizing existing macromolecules to preserve essential functions (49). Autophagy involves the dynamic rearrangement of cellular membranes to allow portions of the cytoplasm to be delivered to the degradative compartment, broken down, and recycled. Protein degradation via autophagy therefore plays a major role in the body's response to nutritional stress. Autophagy, however, is more than a starvation response. For example, this process is involved in cellular remodeling, including modifications that adapt the cell to changes in the types of available nutrients. This role of autophagy is seen when particular organelles such as peroxisomes are rapidly and specifically degraded in response to shifting carbon sources (15, 30).

Autophagy also has important roles beyond nutrition, and it has been linked to various pathophysiological conditions. For example, autophagy is involved in tumor suppression, in eliminating invading viruses and bacteria from host cells, in antigen presentation, neurodegeneration, and some myopathies. In addition, autophagy can function as a type of programmed cell death, distinct from apoptosis. Many of these aspects of autophagy have recently been the subject of reviews (11, 12, 59, 68, 79, 98, 106). In this review, we focus our discussion on the connections between autophagy and nutrition. We elaborate on the recent advances in mammalian cells that reveal the importance of autophagy in cellular metabolism.

OVERVIEW OF AUTOPHAGY

Morphology and Different Types of Autophagy

In very general terms, autophagy means "self-eating" at the subcellular level. There

are different types of autophagy-related processes, but in regard to starvation two are most relevant: chaperone-mediated autophagy and macroautophagy. Chaperone-mediated autophagy involves the translocation of unfolded proteins directly across the lysosomal membrane (63). It is a secondary response to starvation in mammals, being induced after macroautophagy, and is not covered in this review. Macroautophagy (hereafter referred to as autophagy) is the primary response to nutrient limitation. During autophagy, cytoplasm is nonspecifically sequestered within a double-membrane cytosolic vesicle, an autophagosome, which fuses with the lysosome (the vacuole in yeast and plants) (**Figure 1**). The fusion event releases the inner vesicle, now termed an autophagic body, into the lysosome lumen where it, along with the cytoplasmic cargo, is broken down by hydrolases. The resulting macromolecules are released back into the cytosol via permeases in the lysosome membrane, where they can be reused for anabolic or catabolic reactions (123).

Autophagy is unique as a degradative mechanism in that it has the capacity for sequestration of entire organelles. That is, unlike other degradative pathways such as chaperone-mediated autophagy or proteasomal degradation, autophagic breakdown of substrates is essentially not limited by steric considerations. In addition, the process of autophagy involves a topological rearrangement of the cytoplasm; the mechanism of sequestration moves the cargo from the cytoplasm into the lysosome lumen, the topological equivalent of the extracellular space. It is the special mechanism of sequestration, enwrapment within a double-membrane vesicle, which is able to move cargo intact across a membrane (**Figure 1**).

Selective Types of Autophagy

Autophagy is generally considered a nonselective degradation system; however, there are many examples of selective autophagy in both yeasts and higher eukaryotes. In yeast, even bulk autophagy may have some selective capacity, as the Ald6 protein was shown to be rapidly degraded in an autophagy-dependent manner compared with other cytosolic proteins, although the precise mechanism remains unknown (87). One of the clearest examples of selective autophagy is seen with the degradation of superfluous peroxisomes via pexophagy (15). When yeast grows on carbon sources that require peroxisome function, such as oleic acid or methanol, these organelles proliferate. If the cells are subsequently fed a preferred carbon source such as glucose, the peroxisomes are rapidly and specifically degraded. The tag on the peroxisome membrane that appears to allow for specific recognition appears to be Pex14 (7). The autophagy-related (Atg) components that are needed for pexophagy are mostly common with those needed for another type of selective autophagy, the cytoplasm-to-vacuole targeting (Cvt) pathway (24, 40). In contrast to other autophagic processes, the Cvt pathway is biosynthetic and is used for delivery of at least two resident hydrolases to the vacuole (45).

In mammals, p62/SQSTM1 seems to be a selective substrate for autophagy that is mediated by microtubule-associated protein 1 light chain 3 (LC3) binding (discussed below) (8). Although information of selectivity for other endogenous cytosolic proteins is very limited, pathogenic bacteria invading host cytosol appear to be enclosed selectively in a process termed xenophagy (81, 84). In the case of autophagic degradation of intracellular *Shigella* species, VirG on the bacteria might be the target protein that allows specific sequestration (84). It was proposed that Atg5 is involved in the recognition process, but the unique localization of Atg5 on the outer side of the autophagosome implies the presence of a more complicated mechanism.

Autophagic Machinery

The formation of the autophagosome is a complex process and is said to be de novo,

Autophagosome: the double-membrane cytosolic vesicle that sequesters cytoplasm during macroautophagy

Autophagic body: the single-membrane vesicle that is generated by fusion of the autophagosome with the lysosome

Pexophagy: a specific type of macroautophagy involving the targeted sequestration and degradation of peroxisomes

Atg: autophagy related

Microtubule-associated protein 1 light chain 3 (LC3): the mammalian homologue of yeast ubiquitin-like Atg8, which is present as a proteolytically processed, LC3-I (cytosolic), or lipidated, LC3-II (autophagosome-associated) form

Phagophore: the initial membrane structure that initiates the sequestration process during macroautophagy, also called the isolation membrane

Atg proteins: the protein machinery of the process of macroautophagy

involving a range of unique machinery. Autophagosome formation is de novo in that a budding process does not directly generate the autophagosome as occurs with transient transport vesicles that operate in the secretory pathway. Rather, the autophagosome is formed by expansion of a membrane core of unknown origin, termed the phagophore or isolation membrane. The protein machinery of autophagy participates primarily at the step of autophagosome formation.

The Atg proteins were first identified in yeasts, and there are currently 29 proteins that are specific to autophagy (38, 46, 108). These proteins function at the various steps of the process including induction, vesicle formation, and breakdown of the autophagic body. Additional proteins play a role during targeting of the autophagosome to, and fusion with, the vacuole, the yeast analogue of the lysosome, but these proteins are common to all pathways that deliver material to the lysosome/vacuole and are not considered Atg proteins. Many of the Atg proteins have clear homologues in higher eukaryotes and, in some cases the proteins have been shown to function heterologously, indicating that they are true orthologues. The functions of these proteins have been the focus of several reviews (45, 120, 124).

Almost all of the Atg proteins associate at least transiently with the preautophagosomal structure (PAS), which is thought to be the site for assembly of the autophagosome in yeast (111). Thus, the PAS is essentially composed of the phagophore, or its precursor, and its associated Atg proteins. The current view is that the phagophore represents the nucleation membrane for autophagosome biogenesis. Additional membrane is delivered to the phagophore; although the origin of this membrane is not definitely known, it appears to include the early secretory pathway (14, 18, 28, 94, 95) and, in yeast, the mitochondria (94). The Atg proteins may function in part by directing membrane to the phagophore as well as in causing it to form into the three-dimensional double-membrane sphere that will become an autophagosome. Autophagosome biogenesis is a structurally complex process because it is a very large vesicle, 300–900 nm diameter in yeast and even larger in mammalian cells, and it is not clear how the curvature is enforced upon the expanding membrane.

For the purposes of this review, we briefly highlight a few of the Atg proteins that are relevant in sections below. One of the most striking features of the autophagic machinery is the involvement of more than one-quarter of the Atg proteins in two interconnected processes of protein conjugation (85, 86). Atg8 (LC3 in mammals) and Atg12 are ubiquitin-like proteins. Atg8/LC3 is processed by the proteolytic removal of a C-terminal residue(s) through the action of Atg4 (also referred to as autophagins in mammals), exposing a glycine as the ultimate amino acid, whereas Atg12 is synthesized with the glycine exposed (22, 23, 33, 34, 41, 42, 61, 71). Both proteins are activated by Atg7, which is homologous with the ubiquitin-activating (E1) enzyme (39, 115, 127). The activated intermediates are transferred to ubiquitin-conjugating (E2) analogues, Atg3 and Atg10 (27, 75, 82, 107), respectively. Atg8/LC3 is then covalently attached to phosphatidylethanolamine (in mammalian cells the precursor form is termed LC3-I, and the lipidated species is termed LC3-II), causing it to become membrane-associated, whereas Atg12 covalently modifies Atg5 via an isopeptide linkage to an internal lysine of the latter. These types of reactions are clearly reminiscent of ubiquitination; however, Atg8/LC3 and Atg12 are not ubiquitin homologues, although these proteins have some structural similarity (19, 109, 110, 112).

The functions of Atg8/LC3 and Atg12 are not known, although the proteins in both conjugation systems are normally needed for autophagosome formation. Atg8/LC3 is the only Atg protein that remains associated with the completed autophagosome in mammalian cells, and thus serves as one of the few markers for autophagy (33). LC3 can also be found

on the surface of autolysosomes, but at much lower levels than is seen with autophagosomes, because LC3-II on the outer surface of the autophagosome is deconjugated from phosphatidylethanolamine through a second cleavage event involving Atg4 (22, 34, 61, 114). Atg5 binds Atg16 noncovalently (69, 70), and the tetramerization of Atg16 results in the formation of a large complex (55, 69). These two protein conjugation systems are highly conserved from yeast to human (72).

Regulation

The in vivo regulation of autophagy is a very important topic; for example, to use autophagy therapeutically it will be critical to be able to finely regulate its induction because too high a level of autophagy can result in cell death. Autophagy occurs at a basal level in most or all cells, and it can be induced by a variety of conditions; however, many details of the regulatory process remain unclear. Regulation of autophagy has been extensively reviewed (1, 35, 36, 65, 76, 80), and we only briefly highlight this topic here. As a starvation response, autophagy is subject to control by a range of nutrients including nitrogen and carbon in yeast, and by amino acids and certain hormones such as insulin and glucagons in mammals. The autophagy-inhibitory effect of amino acids and insulin has been well established in cell culture and organ perfusion experiments (78). However, the blood amino acid concentration remains virtually unchanged or decreases only slightly during short-term starvation (10, 90, 105). On the other hand, insulin levels rise following a meal, causing the activation of plasma membrane insulin receptors. These receptors in turn activate downstream effectors such as the class I phosphatidylinositol 3-kinase, and Akt/protein kinase B, resulting in the eventual activation of mTor kinase, one of the primary negative regulators of autophagy; however, downstream effectors of mTor are largely unknown. In contrast, nutrient depletion results in mTor inhibition and activation of autophagy. The induction process also requires a class III phosphatidylinositol 3-kinase. There are many additional factors reported to affect autophagic activity (summarized in **Table 1**). Which of these are the critical factors under physiological conditions is not fully

Table 1 Factors/reagents that can regulate autophagosome formation

Stimulators
Extracellular
Glucagon
TNFα
Intracellular
Rapamycin
G_{i3}, GAIP, Erk1/2
Ecdysone
Death-associated protein kinase (DAPK)
Death-associated related protein kinase-1 (DRP-1)
Bacterial/viral infection
Protein aggregates
C2-ceramide
BNIP3
Anticancer agents
TRAIL
FADD
Lithium
ER stress
$p19^{ARF}$
p53, DRAM
UVRAG
Inhibitors
Extracellular
Amino acids
Insulin
Intracellular
Amino acids
3-methyladenine
Cycloheximide
Insulin signaling pathway—mTOR
Phosphatidylinositol 3-kinase inhibitors
Bcl-2
myo-inositol-1,4,5-triphosphate (IP_3)

These factors are classified based on the localization of their targets. For example, ecdysone is a *Drosophila* steroid hormone and binds its receptor inside cells. This table does not cover factors functioning at the autophagosome maturation step and autophagosome-lysosome fusion step.

understood. For example, the involvement of one of the mTor substrates, S6 kinase, is very controversial (48).

Methods for Monitoring Autophagy

A wide range of methods exist for monitoring autophagy in yeasts, and several approaches can be used in higher eukaryotes; these have been covered in various reviews (43, 47, 67). Here we briefly mention the methods that are applicable to mammalian cells. To understand the physiology of autophagy, it is essential to use quantitative diagnostic/monitoring methods. Classic methods include electron microscopy and the measurement of long-lived protein degradation or lysosomotropic reagent-sensitive degradation. Electron microscopy has been useful for following the morphology of autophagy due to the relatively unique size and double-membrane nature of the autophagosome; however, immunoelectron microscopy using anti-LC3 is necessary for unequivocal identification. Various degradation and/or sequestration assays can be used that rely on the uptake of endogenous or exogenously introduced cytosolic markers. The simplest of these is to monitor the degradation of long-lived radioactively labeled cytosolic proteins in the absence and presence of an inhibitor of lysosomal degradation to differentiate autophagy from cytosolic (e.g., proteasomal) degradation events. Alternatively, the measurement of autophagic lactolysis (i.e., the degradation of lactose) is a specific method for monitoring autophagic degradation because there is no degradative enzyme in the cytosol (47). Sequestration assays include the lysosomal uptake of raffinose or long-lived enzymes such as lactate dehydrogenase (measured in the presence of a lysosomal protease inhibitor) (47). Sequestration assays typically provide a more specific means of measuring autophagy; however, these require subcellular fractionation to purify the lysosome.

Additional methods for monitoring autophagosome formation, which rely on fluorescent microscopy or monitoring protein modifications, have been developed since the identification of the Atg proteins (47, 67). One such approach is to monitor the change in localization of LC3 that has been tagged with the green fluorescent protein (GFP) or a similar fluorophore. During autophagosome formation, LC3 is recruited from the cytosol to autophagosomes (33). Thus, the number of autophagosomes is easily estimated by counting the GFP-LC3 dots or ring-shaped structures, if they are large enough. Alternatively, the total dot area can be measured using computer software (e.g., Meta Morph Series, Molecular Device). This method has been applied to whole animals by generating GFP-LC3 transgenic mice (74). Possible pitfalls and limitations of this method are listed in **Table 2**. It should be noted that the (GFP-)LC3 protein itself is nonspecifically incorporated into protein aggregates independent of autophagy (54a). This is a serious problem if one would like to analyze whether protein aggregates can be engulfed by autophagosomes. Accordingly, other methods should be used at the same time for this purpose.

The other method of monitoring autophagy with LC3 is through immunoblotting of the endogenous protein. As indicated above, nascent LC3 is modified posttranslationally similar to yeast Atg8 (27, 42, 85). The two forms of LC3, LC3-I and LC3-II, are easily separated by SDS-PAGE; LC3-II migrates faster than LC3-I because of its extreme hydrophobicity (33). Consequently, immunoblotting of LC3 usually gives two bands: LC3-I (apparent mobility, 16 kD) and LC3-II (apparent mobility, 14 kD). The amount of LC3-II correlates well with the number of autophagosomes. It was recently found that the immunoreactivity of LC3-II is higher than that of LC3-I due to the conformational change produced by the conjugation to phosphatidylethanolamine (26, 34). Therefore, the amount of LC3-II on immunoblots is overestimated. Accordingly, the LC3-II/LC3-I ratio is not a good indicator of

autophagy (**Table 2**). On the other hand, it is reasonable to compare the amounts of LC3-II between samples. One important caveat is that an increase in LC3-II levels can result from either the induction of or a block in autophagy; LC3-II that is localized on the autophagosome inner membrane is normally degraded by lysosomal hydrolases. Thus, to monitor the autophagic flux, it is necessary to measure the amount of LC3-II delivered to lysosomes by comparing LC3-II levels in the presence and absence of lysosomal protease inhibitors (114).

Table 2 Pitfalls in autophagy monitoring using LC3

Pitfalls	Solution
(GFP-)LC3 localization	
(GFP-)LC3 protein can be incorporated into protein aggregates or inclusion bodies in an autophagy-independent manner.	1. Conjugation-defective LC3 (the C-terminal glycine mutant, $LC3^{G120A}$) can be used as a negative control. 2. Immunoelectron microscopy if necessary.
Some structures with autofluorescent signal such as lipofuscin might be misrecognized.	1. True GFP-LC3 signals should not be detected with other fluorescence filter sets such as rhodamine, Cy5, and UV. 2. Nontransgenic control should be prepared.
Autophagy flux cannot be monitored because most (GFP-)LC3 is not present in autolysosomes.	Inhibitors of lysosomal enzymes may be used to inhibit (GFP-)LC3 degradation in lysosomes (50, 114).
LC3 immunoblotting	
LC3-II is more reactive to anti-LC3 antibody than LC3-I.	The amount of LC3-II among samples should be compared. The LC3-II/LC3-I ratio is not very meaningful.
Autophagy flux cannot be monitored because LC3-II is degraded in autolysosomes.	Inhibitors of lysosomal enzymes can be used to inhibit LC3-II degradation in lysosomes (50, 114).
The amount of LC3-II fluctuates even using the same cell line.	Strict controls should be prepared in each experiment. It is difficult to compare independently cultured cell lines.

CONTRIBUTION OF AUTOPHAGY TO INTRACELLULAR PROTEIN DEGRADATION

General Concepts of Protein Degradation

Intracellular proteins can be classified into two groups: short-lived proteins (half-life, 10–20 min) and long-lived proteins (25, 37). In hepatocytes, although less than 1% of the total proteins are short-lived, they contribute to as much as one-third of the total protein degradation because of their rapid turnover (37, 78). It is generally considered that most short-lived proteins are degraded by the ubiquitin-proteasome system, whereas most long-lived proteins are degraded in lysosomes via the autophagic pathway; however, this statement sometimes leads to a misunderstanding that autophagy selectively degrades long-lived proteins. Because more than 99% of intracellular proteins are long-lived (37), random sequestration by autophagosomes could provide a simple explanation for the above observation. Furthermore, short-lived proteins can also be degraded in lysosomes (4).

The diameter of mammalian autophagosomes is usually 0.5–1.5 micrometers. Therefore, the volume of each autophagosome represents less than 0.1% of the total cellular volume; however, as the half-life of autophagosomes is considered to be very short (approximately 10 min) (91, 102), the total degradative capacity of autophagy could be large. In perfused rat liver, the rate of protein degradation varies from 1.5%/h (basal) to 4.5%/h (accelerated by starvation) of total cellular protein (102). Similar rates were reported for isolated hepatocytes; 4%–5% of total protein was degraded under amino acid–free conditions (104). In fibroblasts, 3-methyladenine-sensitive degradation, which would account for autophagy, is estimated to be 0.5%–1%/h (16).

Although these degradation rates likely represent autophagy, the contribution of

autophagy was demonstrated in a more specific manner using autophagy-deficient cells. The rate of autophagy in isolated hepatocytes could be estimated as about 2%/h by comparison between wild-type and Atg7-deficient hepatocytes (53). In Atg7-deficient hepatocytes, accelerated proteolysis under starvation conditions is abolished almost completely. Similarly, the contribution of autophagy in embryonic stem cells is estimated to be about 2%/h (73). These values might be underestimated, however, because some other degradation systems may be up-regulated when macroautophagy is defective. This type of cross-talk is clearly seen, for example, with the up-regulation of macroautophagy to partially compensate for defects in chaperone-mediated autophagy (62). In addition, the relative contribution of autophagy depends on the cell type. Under starvation conditions, autophagic degradation represents less than 1%/h in transformed fibroblasts (N. Mizushima, unpublished data) and about 1%/h in yeast (117).

In the following sections, we discuss the role of autophagy focusing on protein metabolism. Because of space limitations, the roles of autophagy in cell death, cancer, antigen presentation, killing of intracellular bacteria, support of viral replication, and pathogenesis of human diseases are not covered.

ROLE OF INDUCED AUTOPHAGY AS A STARVATION RESPONSE

Induction of Autophagy Following Starvation

Much attention has been paid to the role of autophagy during starvation because autophagy is drastically induced by nutrient limitation. The most striking demonstration of starvation-dependent induction was provided by yeast studies. Autophagy is induced in yeast cells most efficiently by nitrogen starvation and to a lesser extent by starvation of carbon sources, auxotrophic amino acids, or sulfate (113). When haploid yeast cells defective in autophagy are subjected to nitrogen starvation, most of them die within five days, which is much faster than occurs with wild-type cells (117). These observations clearly indicate that autophagic degradation of cytoplasm (self-eating) is critically important to maintain cell viability during nitrogen starvation.

Autophagy is also induced in mammalian cells cultured in various starvation media such as amino acid–free and serum-free media. Glucose starvation also can induce autophagy, but the effect is milder. Unlike yeast cells, it is very difficult to see phenotypic differences between wild-type and autophagy-defective cells cultured in vitro. As far as we have tested, there is no significant difference in sensitivity to stresses such as various nutrient starvation and endoplasmic reticulum (ER) stress (N. Mizushima, unpublished data); however, as discussed below, the role of induced autophagy was clearly demonstrated in whole animals.

Starvation Response in Whole Animals

Differential levels of autophagy in tissues. Although much attention has been paid to autophagy in liver, where the protein turnover rate is very high, autophagy occurs in almost all tissues. Autophagic activity is enhanced following starvation. This phenomenon was confirmed by studies with GFP-LC3 transgenic mice and by monitoring endogenous LC3. Active autophagosome formation was observed in skeletal muscle, liver, heart, exocrine glands such as pancreatic acinar cells and seminal gland cells, and podocytes in kidney after 24-h food withdrawal (74). Interestingly, autophagy is differentially regulated among organs. In most tissues, the autophagic activity reaches maximal levels within 24 h and then gradually decreases, whereas it is further accelerated even after 24 h in some tissues, such as the heart, and in slow-twitching

muscles, such as the soleus. On the other hand, autophagy induction in liver seems to be quicker than in other tissues (A. Kuma & N. Mizushima, unpublished observation). This observation is consistent with earlier findings on total proteolysis (78). Some tissues show constitutively active autophagy. Thymic epithelial cells are the best example: autophagy actively occurs under nutrient-rich conditions (74) and even during embryogenesis (N. Mizushima, unpublished observation). In this case, autophagy might be involved in presentation of cytosolic antigens onto major histocompatibility complex (MHC) class II proteins for lymphocyte selection (79).

In contrast, autophagy is not observed in the brain even after 48-h food withdrawal. This might be because the brain is nutritionally protected under physiological conditions. For example, the brain can utilize nutrients such as glucose and ketone bodies supplied by the liver and other tissues. Nonetheless, neural cells retain autophagic ability, because high levels of autophagy (or accumulation of autophagosomes) are observed in other adverse conditions, such as cerebral ischemia (2) and neurodegeneration (51, 122, 126).

Autophagy in the neonate. Autophagy is also up-regulated during the early neonatal period in response to the nutrient limitations imposed by the sudden termination of the transplacental supply of nutrients (54). Although autophagy seems to be suppressed throughout the embryonic period, except in some tissues such as thymic epithelial cells (discussed above), autophagy is rapidly and extensively induced in various tissues soon after birth. In particular, the heart muscle, diaphragm, alveolar cells, and skin, but not the brain, display massive autophagy. The autophagic activity reaches the maximal level 3–6 h after birth, although the neonatal mice begin suckling before that time. The number of autophagic vacuoles gradually decreases to basal levels by day 1 or 2.

Physiological Significance of Starvation-Induced Autophagy

Autophagy in the liver. The studies described above suggest that induced autophagy is a fundamental response to adapt to starvation. However, in contrast to the well-established roles of carbohydrate and lipid catabolism, the contribution of proteolysis to nutrient and energy homeostasis is less clear. Analyses of *ATG* gene–deficient mice have provided valuable information along these lines (53, 54). Komatsu et al. (53) generated inducible liver-specific Atg7 knockout mice. As discussed, Atg7 is an ubiquitin-activating enzyme (E1)-like protein that catalyzes the first step of both Atg12—Atg5 conjugation and LC3—PE conjugation (85, 116). Mice homozygous for the *Atg7* flox allele ($Atg7^{flox/flox}$) were crossed with Mx1-Cre transgenic mice. In the resulting mice, exon 14 of the *Atg7* gene is excised by intraperitoneal injection of interferon γ or poly-inosinic acid-polycytidylic acid (pIpC). In wild-type mice, liver proteins in the cytosol and organelles such as mitochondria decrease to about 70% after one-day starvation, whereas the decrease is not significant in $Atg7^{flox/flox}$;Mx1 mice, suggesting that autophagy accounts for the majority of starvation-induced protein degradation in the liver (53).

Flox: a DNA segment utilizing repetitive sequences to facilitate directed gene removal by recombination mediated via Cre recombinase

Maintenance of amino acid pools in yeast. The contribution of autophagy in the maintenance of amino acid pools has been directly shown in yeast. When yeast cells are cultured in nitrogen-deficient media, autophagy is induced within one hour and reaches a maximal level by three hours (113). During the first two hours of starvation, the intracellular total amino acid level rapidly decreases (88). The amino acid pool is partially restored thereafter. Such restoration is not observed in *atg* mutants, clearly indicating that autophagy is critical for the maintenance of the cytosolic amino acid pool during starvation. Onodera & Ohsumi (88) further demonstrated that synthesis of total proteins as well

as certain specific proteins whose expression is up-regulated in response to starvation was markedly inhibited in *atg* mutants under starvation conditions. Similarly, release of vacuolar amino acids derived from autophagic proteolysis via membrane permeases is the critical final step of autophagy (123). Thus, yeast cells utilize amino acids produced by enhanced autophagy for new protein synthesis, which would account, at least in part, for the loss of viability phenotype of starved *atg* mutants. The contribution of amino acids to energy production in yeast is considered less important than in mammals.

The role of autophagy in maintaining plasma amino acids in the newborn. Likewise, the role of autophagy has been examined in neonatal mice using *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice. As noted above, Atg5 is an acceptor molecule for the ubiquitin-like modifier, Atg12 (71, 72, 85). It has been already found that Atg5 and its proper modification with Atg12 are required for the elongation of the phagophore/isolation membrane (73). *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice are born at the expected Mendelian frequency, and they appear almost normal at birth (53, 54). These findings suggest that autophagy could be dispensable for mammalian embryogenesis, although many studies have suggested possible roles for autophagy in development and cell death in other species (59).

Despite the minimal abnormalities present at birth, most *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ neonates die within one day of delivery (53, 54; **Figure 2**). The cause of death, however, is not straightforward. *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ neonates have a suckling defect of unknown etiology (probably due to neurological defects, as discussed below); however, the early death of the homozygous mice was not simply due to the suckling failure because the survival time of these knockout mice was still much shorter than that of wild-type mice when compared under nonsuckling conditions after cesarean delivery. The survival time of the knockout neonates could be delayed by forced milk feeding, suggesting that they suffer from a nutritional problem (54). Indeed, plasma amino acid concentrations of *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ neonates rapidly decrease after birth (53, 54). In particular, the plasma concentrations of essential amino acids and branched-chain amino acids (BCAAs) show large differences. A similar pattern is also observed for amino acid concentrations in various tissues such as liver, heart, and brain. Therefore, autophagy-defective neonates suffer from systemic amino acid insufficiency. Taken together, these studies emphasize the point that increased intracellular generation of amino acids by autophagy is a physiologically important starvation response.

Autophagic versus proteasomal contributions. Recently, the critical contribution of the ubiquitin-proteasome system to the maintenance of the intracellular amino acid pool also has been shown (119). Upon acute amino acid restriction up to 3 h, the amino acid supply mostly relies on proteasome function rather than autophagy. After prolonged starvation, however, amino acids are primarily generated by autophagy. Subsequently, chaperone-mediated autophagy may be induced as a secondary response (63). Therefore, the ubiquitin-proteasome system and autophagy differentially contribute to maintenance of the amino acid pool, dependent on nutrient conditions.

Use of autophagy-derived amino acids. How amino acids produced by autophagy during starvation are utilized in mammals remains to be determined. Although amino acids are not generally considered a good fuel source, such amino acids, particularly BCAA, can be used to generate energy. The activity of the branched-chain α-ketoacid dehydrogenase complex, which is the most important regulatory enzyme for BCAA catabolism, is up-regulated in starvation (21). In addition, in disease conditions such as liver cirrhosis, BCAA is reduced, probably due to

enhanced consumption as an energy source (77). The preferential reduction of BCAA in *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ neonates may indicate an increase in BCAA consumption during this period. In addition, tissue energy levels estimated by the activity of AMP-activated protein kinase seem to be low in 10-h-fasting *Atg5*$^{-/-}$ mice, suggesting that neonates could use the amino acids produced by autophagy for energy homeostasis (54). The significance of amino acid production by autophagy in energy metabolism was also shown in an in vitro study. In an IL-3-dependent hematopoietic cell line established from *Bax*$^{-/-}$*Bak*$^{-/-}$ mice, amino acid availability from the media relies on IL-3 stimulation (60); however, these cells can maintain viability even after IL-3 withdrawal by up-regulating autophagy. RNAi-mediated inhibition of autophagy is lethal to these cells; however, the effect of autophagy inhibition can be overcome by the addition of methylpyruvate, a cell-permeable tricarboxylic acid cycle substrate. These results suggest that cultured cells use amino acids as an energy source. In addition to direct energy production, amino acids produced by autophagy can be used for gluconeogenesis and ketogenesis in the liver or for new protein synthesis required for the proper starvation response, as discussed above in yeast cells (88, 123).

Developmental steps related to nutrient limitation. Autophagy-defective mutants have been generated in various species. Thus far, a number of developmental defects have been reported in the mutants (59). In *Saccharomyces cerevisiae*, autophagy mutants are unable to sporulate (117). Autophagy mutants of *Dictyostelium discoideum* are defective in normal multicellular developmental processes such as aggregate formation and fruiting body formation (89). In *Drosophila melanogaster*, lethality from the third larval to pupal stages was reported in autophagy mutants (32, 103). Finally, dauer formation is abnormal in *Caenorhabditis elegans* autophagy mutants (66). As described above, *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice die soon after birth (53, 54). In contrast, mice deficient for Beclin 1, a mammalian homolog of Atg6/Vps30, die at about embryonic day 7.5 (93, 128). Beclin 1 is a subunit of the class III phosphatidylinositol 3-kinase and probably has multiple functions. Thus, the very early embryonic lethality could be explained by some additional roles beyond its function in autophagy. These phenotypes of loss of autophagy seem to be very divergent, but all of them are highly related to nutrient starvation. Therefore, these studies likely indicate the stages at which endogenous amino acid production by autophagy is critically important, probably for both energy metabolism and remodeling.

ROLE OF BASAL AUTOPHAGY IN INTRACELLULAR QUALITY CONTROL

Autophagy and Protein Quality Control

Basal autophagy as a homeostatic mechanism. Although autophagy is characteristically induced upon starvation, it occurs constitutively at low levels even under nutrient-rich conditions. Because yeast cells defective in autophagy do not show obvious abnormalities unless they are cultured under various starvation conditions (117), the role of basal autophagy has not been clarified in this system. However, recent mouse genetic studies have provided evidence that basal autophagy functions as an important quality-control system, particularly in hepatocytes and neural cells.

Liver-specific Atg7 knockout mice. The role of basal autophagy was first demonstrated in liver-specific knockout mice. As discussed above, liver-specific conditional Atg7 knockout (*Atg7*$^{flox/flox}$;Mx1) mice are defective in starvation-induced proteolysis. However, even if maintained in nutrient-rich conditions, *Atg7*$^{flox/flox}$;Mx1 mice develop various abnormalities in the liver (53). For example, these mice show hepatomegaly

20 days after gene targeting by pIpC injection. Some abnormal organelles such as deformed mitochondria and endoplasmic reticulum also accumulate in hepatocytes. Finally, *Atg7*$^{flox/flox}$;Mx1 mice at 90 days after pIpC injection show severe hepatomegaly with disorganized hepatic lobules, cell swelling, and cell death. Serum alanine aminotransferase and aspartate aminotransferase are significantly elevated at this stage. The most unexpected finding with these mice, however, is that many ubiquitin-positive aggregates are generated in hepatocytes (53).

Knockout embryos. Although phenotypic abnormality of *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice was minimal at birth, they showed some significant defects (53, 54). The body size of *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ neonates was slightly reduced and they displayed a suckling defect. Because embryos are not starved and autophagy seems to be maintained at low levels during embryogenesis, autophagy presumably plays a role other than starvation adaptation. Indeed, accumulation of protein aggregates is already detected in the liver of *Atg5*$^{-/-}$ neonates (20). However, systematic analysis of *Atg5*$^{-/-}$ neonates revealed that the importance of basal autophagy differs among cell and tissue types. Ubiquitin-positive aggregates accumulate vigorously only in hepatocytes, a subset of neurons, the anterior lobe of the pituitary gland, and the adrenal gland (20). As for the nervous system, aggregates are observed in large neurons of dorsal root ganglion (DRG), pons, spinal cord (ventral horn cells), hypothalamus, midbrain, and trigeminal ganglia. The neurons in DRG showed the most extensive accumulation of the inclusions; more than 10 aggregates were observed in a single cell slice. In contrast, very few aggregates are seen in skeletal muscle, heart, and kidney.

It is unclear why autophagy is so important in certain cell types. There was no apparent correlation between the level of basal autophagic activity and the extent of aggregate accumulation (A. Kuma & N. Mizushima, unpublished observation), and autophagic activity in the brain is very low in both embryos and adult mice (74). One possible explanation for cell-specific differences is that intracellular quality control is more important in nondividing, postmitotic cells than in rapidly dividing cells, in which abnormal constituents can be quickly diluted even if they are not degraded. In addition, endogenous aggregation-prone proteins may be more highly expressed in certain affected cell types. Another factor may be cell volume. Larger neurons, such as DRG neurons, tend to accumulate inclusion bodies more readily. These cells may contain greater quantities of proteins per cell that must be turned over through autophagy. Finally, autophagy may occur at higher rates than were previously determined in some cells due to an acceleration of autophagosome turnover (3).

Neural cell–specific Atg5 and Atg7 knockouts. The role of basal autophagy in the nervous system has been further analyzed in neural cell–specific Atg5 and Atg7 knockout mice (*Atg5*$^{flox/flox}$;*Nestin-Cre* mice and *Atg7*$^{flox/flox}$;*Nestin-Cre* mice). These mice are born normally but exhibit growth retardation. After three weeks, they develop progressive motor and behavioral deficits, including ataxic gait, impaired motor coordination, abnormal limb clasping reflexes, and systemic tremor (20, 52). Histological examination revealed the presence of degenerative changes such as partial loss of cerebellar Purkinje cells and cerebral pyramidal cells, and axonal swelling in various brain regions. There is a difference in survival rate between *Atg5*$^{flox/flox}$;*Nestin-Cre* mice and *Atg7*$^{flox/flox}$;*Nestin-Cre* mice. Most *Atg7*$^{flox/flox}$;*Nestin-Cre* mice die within three weeks, whereas only sporadic death is observed for *Atg5*$^{flox/flox}$;*Nestin-Cre* mice. This might be due to the difference in the number of backcrossings; the phenotype seems to be severe in the C57BL/6 background (T. Hara, M. Komatsu, & N. Mizushima, unpublished observation). Alternatively, Atg7 may have some functions other than autophagy because it is a

common activating enzyme for mammalian Atg8 homologs such as γ-aminobutyric acid $(GABA)_A$-receptor-associated protein (121) and GATE-16 (99). Ubiquitin-positive protein aggregates extensively accumulated in the cytoplasm of neurons in many regions. Accumulation of aggregates is time-dependent because the distribution of aggregate-positive cells is more limited in autophagy-deficient neonates. Finally, aggregates do not accumulate in glial cells. The studies of Atg5 and Atg7 knockout mice indicate that autophagy is required to prevent neurodegeneration, even in the absence of disease-associated mutant proteins.

The mechanism underlying the accumulation of ubiquitin-positive aggregates is unknown. Loss of autophagy first leads to accumulation of diffuse ubiquitinated proteins in the cytoplasm and is followed by the generation of protein aggregates (20). Therefore, aggregate formation is likely a secondary result of a general protein turnover defect. This idea is in contrast with the hypothesis that autophagosomes specifically degrade inclusion bodies that might be continuously formed under normal conditions. It has been suggested that large aggregates themselves may not be pathogenic, whereas mutant proteins diffusely present in the cytosol could be the primary source of toxicity (5). Thus, continuous clearance of diffuse cytosolic proteins, not protein aggregates themselves, by basal autophagy is important for preventing the accumulation of abnormal proteins that can disrupt neural function.

In this scenario, accumulation of soluble ubiquitinated proteins could be interpreted as a secondary result of impaired protein turnover in the absence of autophagy. Turnover of most cytosolic proteins would be impaired, which provides more opportunities to be damaged or misfolded. In that situation, they have a greater chance to be both ubiquitinated and aggregated. This is consistent with the general agreement that autophagic sequestration is random and nonselective. However, there is yet another possibility that ubiquitinated proteins are recognized via the inner membrane of autophagosomes and thus are preferentially degraded. A number of studies suggest that ubiquitinated proteins are delivered to lysosomes (13, 56, 58, 101, 118). Recently, it was proposed that p62/SQSTM1 mediates the specific recognition of ubiquitinated aggregates by autophagosomes (8). p62/SQSTM1 has been reported to function in various processes such as in the IL-1/TNF signaling pathways toward NF-κB and MAPK9 that is involved in the RNAK-signaling pathway (100), protein kinase C-ζ signaling (92), oxidative stress response (29), and p56lck signaling (31). Because p62/SQSTM1 can bind both ubiquitin and LC3, it could function as an adaptor protein linking ubiquitinated proteins and autophagosomes (8). The p62/SQSTM1 protein is selectively degraded by the autophagy pathway: p62/SQSTM1 is extensively enriched in various autophagy-deficient cells and organs (122) (T. Hara, A. Kuma & N. Mizushima, unpublished observation; M. Komatsu, personal communication). However, it has not been determined how much this pathway contributes to the total degradation of ubiquitinated proteins under normal conditions. The p62/SQSTM1 mutation is associated with Paget disease of the bone in human (57), and p62 knockout mice develop mature-onset obesity (96). Because no apparent neurological abnormality is reported in these human and mouse models, abnormal protein metabolism in autophagy-defective neural cells would not be explained solely by a defect in the p62/SQSTM1-mediated ubiquitinated protein turnover. As almost all proteins in autophagosomes do not directly associate with the autophagosome membrane, selective substrate recognition would account, at most, for only a small fraction of total autophagic degradation.

Clearance of Intracellular Organelles

In contrast to the ubiquitin-proteasome system, autophagy (including microautophagy)

can degrade not only proteins but also intracellular organelles such as mitochondria (44, 97, 113), peroxisomes (15), and endoplasmic reticulum (17). Some of these organelles seem to be selectively recognized by autophagy. More recently, the physiological importance of peroxisome degradation by autophagy was directly shown using *Atg7*$^{flox/flox}$;Mx1 mice (30). That study estimates that 70%–80% of peroxisomes induced by phthalate ester are degraded via autophagy during one week after secession of phthalate ester. Finally, even under steady state conditions, abnormal organelles were observed in autophagy-deficient hepatocytes (53). Taken together, these observations suggest that both basal autophagy and induced autophagy are important for control of number and quality of organelles.

Drastic degradation of organelles is also observed in the processes of lens and erythroid development. The lens fiber cells originate from lens epithelial cells. During this process, membrane-bound organelles within the epithelial cells are rapidly lost, which allows fiber cells to be transparent (6). Similarly, intracellular organelles are eliminated during erythroid cell maturation, and the possible involvement of autophagy has been suggested in these processes. More recently, it was reported that the degradation of nuclei of lens cells depends on DNase II-like acid DNase/DNase IIb (83), suggesting that chromatin degradation occurs in an acidic organelle, possibly in the lysosome. However, organelle degradation in lens and erythroid cells occurs normally in autophagy-deficient *Atg5*$^{-/-}$ mice (64). Therefore, a degradation system(s) other than autophagy appears to play a major role in organelle degradation during these processes.

CONCLUDING REMARKS

In this review, we have discussed the molecular mechanism and physiological role of autophagy in protein metabolism. Recent studies have demonstrated that autophagy has many physiological roles and that this process is more pleiotropic than ever expected. Because autophagy is one of the major degradation systems, all of the proposed roles of autophagy are presumably linked to the degradation of intracellular components. Three modes of autophagy, specific, induced nonspecific, and basal, are all critical for nutrient regulation and intracellular quality control/homeostasis. Further investigations into the mechanism of autophagy and the effects of autophagic dysfunction will likely continue to provide important insights into this complex but ubiquitous process.

SUMMARY POINTS

1. Autophagy occurs in all eukaryotes and is induced to higher levels in response to certain types of stress, particularly nutrient starvation.
2. The process of autophagy is typically nonspecific, but can involve specific targeting of cargo. The basic process involves the sequestration of cytoplasm within a double-membrane cytosolic vesicle, followed by delivery to, and degradation within, the lysosome.
3. The autophagosome has an essentially unlimited capacity for sequestering cargo, allowing for the degradation of large protein complexes and entire organelles.
4. Autophagy is involved in tumor suppression, preventing some types of neurodegeneration, and in removing pathogenic bacteria and viruses from host cells.
5. Autophagy occurs constitutively at basal levels, which is important for homeostatic purposes.

6. Induced autophagy is important for maintaining the appropriate amino acid levels for both protein synthesis and supplying energy during starvation.
7. Intracellular quality control is one function of autophagy, which can remove misfolded and aggregated proteins as well as damaged organelles before they become toxic to the cell.

FUTURE ISSUES

1. The regulation of autophagy needs to be better understood to allow its modulation for potential therapeutic purposes.
2. The interactions among, and functions of, the Atg proteins along with structural data from crystallographic studies will provide important information about mechanism and about ways to stimulate and/or inhibit autophagy.
3. Continued analyses with tissue-specific and temporally controlled autophagy genes will allow an assessment of the different roles of autophagy in the whole organism.

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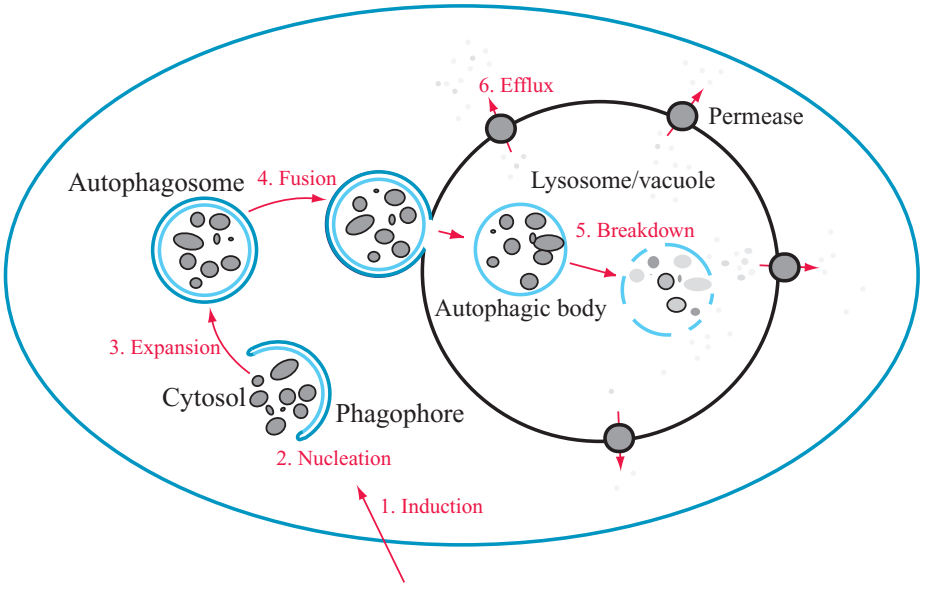

Figure 1

Schematic model of macroautophagy. Autophagy occurs at basal levels and can be induced (*1*) by certain environmental or intracellular cues. The process begins with the nucleation step (*2*) in which a membrane core of unknown origin, termed the phagophore or isolation membrane, sequesters a portion of cytoplasm. The phagophore expands (*3*), probably through the vesicle-mediated addition of membrane (not shown) to generate the double-membrane autophagosome. Upon completion, the autophagosome outer membrane fuses (*4*) with the lysosome, releasing the inner single-membrane vesicle. The autophagic body is broken down (*5*) by lysosomal hydrolases and the resulting macromolecules are released back into the cytosol via membrane permeases (*6*) for reuse in the cytosol in catabolic or anabolic reactions. The steps of cargo recognition and packaging needed for specific types of autophagy are not depicted. The numbers correspond to the individual steps in the figure.

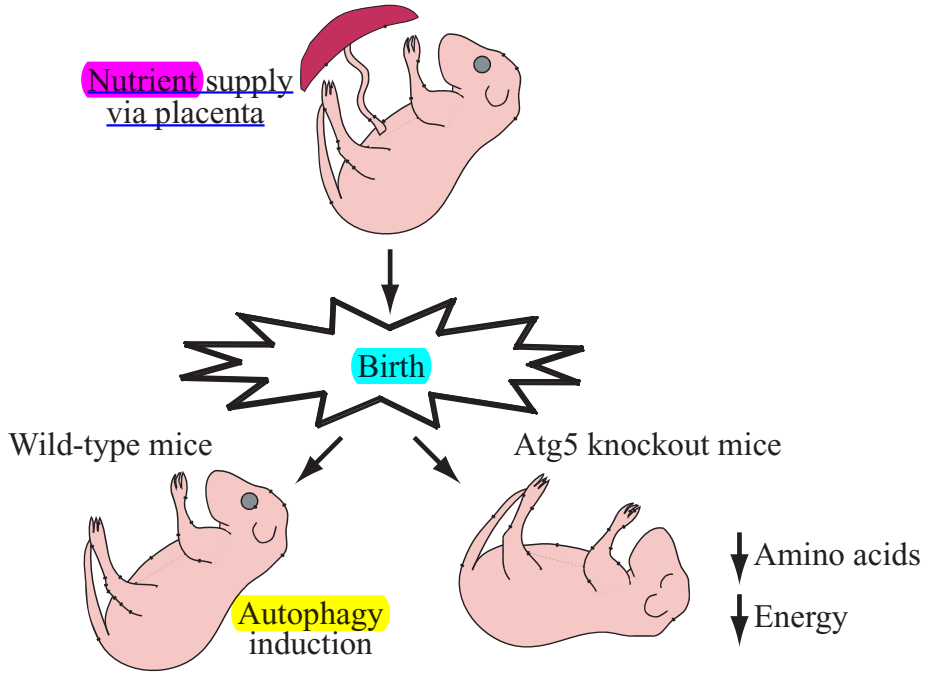

Figure 2

The role of autophagy during the early neonatal starvation period. Embryos receive a transplacental nutrient supply, but the supply becomes suddenly interrupted upon birth, leaving neonates to face severe starvation until the supply is restored through milk nutrients. Atg5 knockout neonates appear grossly normal at birth, but plasma and tissue amino acid levels decrease immediately and the newborns rapidly lose viability. In contrast, wild-type animals survive this unique kind of starvation by inducing autophagy, in addition to the use of carbohydrates and fat stocks.

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Errata

An online log of corrections to *Annual Review of Nutrition* chapters (if any, 1997 to the present) may be found at http://nutr.annualreviews.org/errata.shtml