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Research Article

Autophagic bulk sequestration of cytosolic cargo is independent of LC3, but requires GABARAPs

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ABSTRACT

LC3, a mammalian homologue of yeast Atg8, is assumed to play an important part in bulk sequestration and degradation of cytoplasm (macroautophagy), and is widely used as an indicator of this process. To critically examine its role, we followed the autophagic flux of LC3 in rat hepatocytes during conditions of maximal macroautophagic activity (amino acid depletion), combined with analyses of macroautophagic cargo sequestration, measured as transfer of the cytosolic protein lactate dehydrogenase (LDH) to sedimentable organelles. To accurately determine LC3 turnover we developed a quantitative immunoblotting procedure that corrects for differential immunoreactivity of cytosolic and membrane-associated LC3 forms, and we included cycloheximide to block influx of newly synthesized LC3. As expected, LC3 was initially degraded by the autophagic-lysosomal pathway, but, surprisingly, autophagic LC3-flux ceased after ~2 h. In contrast, macroautophagic cargo flux was well maintained, and density gradient analysis showed that sequestered LDH partly accumulated in LC3-free autophagic vacuoles. Hepatocytic macroautophagy could thus proceed independently of LC3. Silencing of either of the two mammalian Atg8 subfamilies in LNCaP prostate cancer cells exposed to macroautophagy-inducing conditions (starvation or the mTOR-inhibitor Torin1) confirmed that macroautophagic sequestration did not require the LC3 subfamily, but, intriguingly, we found the GABARAP subfamily to be essential.

Abbreviations: 3MA, 3-methyladenine; Baf (or Baf A1), bafilomycin A1; BSA, bovine serum albumin; GABARAP, γ-aminobutyric acid receptor-associated protein; LC3, microtubule-associated protein 1 light chain 3; LDH, lactate dehydrogenase; MEFs, mouse embryonic fibroblasts; MMapp, apparent molecular mass; mTOR, mammalian target of rapamycin; NDK, nucleoside diphosphate kinase; TG, thapsigargin

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Introduction

Autophagic sequestration is performed by specialized organelles called *phagophores*, which are essentially compressed membrane cisterns capable of expanding to envelop pieces of whole cytoplasm (*macroautophagy*) or individual cytoplasmic elements (*selective/exclusive autophagy*) for shipment to lysosomes where the sequestered cargo is degraded [1]. The selective autophagies are usually named according to their cargo (*mitophagy*, *pexophagy*, *reticulophagy*, *xenophagy*, *aggrephagy*, *lipophagy* etc.) [2]. Macroautophagy is generally regarded as a nonspecific bulk process, although it has been suggested that it may encompass a selective sequestration of certain cytosolic proteins [3].

In yeast, phagophores are manufactured at distinct cytosolic locations (*pre-autophagosomal structures*, or *PAS*) [4], whereas mammalian phagophore assembly has been associated with various organelles such as mitochondria, endoplasmic reticulum or Golgi cisterns [5–7], which may be collectively referred to as *phagophore assembly sites* (*PAS*) to conform with the yeast terminology. At these sites, the products of various autophagy-related genes (*Atg*s) and other proteins (e.g., WIPIs) [8] cooperate to assemble membrane elements from various sources (endoplasmic reticulum, mitochondria, plasma membrane etc.) and proteins into functional phagophores, a process that culminates in the conjugation of Atg8 (or its mammalian orthologues, LC3s and GABARAPs) to phosphatidylethanolamine (PE) in the phagophore membrane [9].

LC3 was first described as an 18-kDa protein found in preparations of microtubule-associated proteins (MAPs) from bovine brain [10]. Following its recognition as a MAP-1 subunit [11], it was named MAP-1 light chain 3 (LC3). The protein was purified from bovine brain and partially sequenced, and eventually cloned from the rat with a deduced 142-amino acid sequence, a predicted size of 16.4 kDa and a predicted pI of 9.2 [12]. Five isoforms from four genes have been described in humans: LC3A (with the alternative splice variants LC3A1 and LC3A2), LC3B1 and LC3B2 (which are virtually identical in their gene coding region) and LC3C [13,14]. LC3A and LC3B are also found in the rat, both with wide tissue distributions, but only LC3B is significantly expressed in liver tissue [15]. Since LC3B is identical to the previously described LC3, the name "LC3B" is a junior synonym that should be generally discarded unless used to explicitly differentiate it from other LC3 isoforms.

When Atg8/Aut7 was identified as a gene required for yeast autophagy and subsequently cloned, it was pointed out that its protein sequence was 28% identical to that of rat LC3 [16]. Soon after, LC3 was shown to be present in animal cells both as a soluble, ~18-kDa (MMapp) form (LC3-I), generated by proteolytic cleavage from a slightly larger precursor, and as a structure-associated, ~16-kDa (MMapp) form (LC3-II) [17], the latter being derived from LC3-I by lipidation (conjugation to PE) [18,19]. Upon amino acid starvation, the amount of LC3-II associated with autophagic organelles was found to increase rapidly, subject to strong antagonism by autophagic sequestration inhibitors such as 3-methyladenine and wortmannin [17]. LC3-II would thus seem to be suitable as an autophagic organelle marker, and the conversion of LC3-I to LC3-II, detectable by immunoblotting, might conceivably serve as an autophagy assay. However, it should be emphasized that LC3 lipidation is a measure of phagophore maturation rather than of autophagic sequestration activity (autophagosome formation); the latter process can only be measured by an appropriate cargo assay [1,20]. Furthermore, since LC3-I displays much weaker immunoreactivity than LC3-II [18], it is not only difficult to detect and quantify, but uncorrected LC3-II/LC3-I ratio values hold no quantitative meaning. It has been suggested that LC3-II values should instead be related to some invariant household protein such as tubulin [20], but the distinction between altered lipidation and changes in total LC3 levels would then be lost.

Besides the LC3s, mammalian cells harbor another subfamily of orthologues to yeast Atg8, i.e., the GABARAP (γ-aminobutyric acid receptor-associated protein) family. Its family members GABARAP, GABARAPL1 and GABARAPL2 are, like LC3, subject to constitutive proteolytic processing as well as to enhanced lipidation under autophagy-inducing conditions [18,19,21,22]. The GABARAPs have received much less attention than the LC3s, but both families have been reported to be involved in cargo recognition during selective autophagy [23] and in autophagosome formation [24,25]. In the latter process, LC3 has been associated with phagophore expansion, whereas the GABARAPs have been associated with phagophore closure [25]. However, in contrast to LC3, the GABARAPs have not found general use as autophagy markers.

An increase in the steady-state level of LC3-II can result from enhanced phagophore/autophagosome biogenesis, or from an inhibition of autophagosome turnover. Therefore, measurements of LC3 turnover have been proposed to provide a more dynamic picture of "autophagic flux" [26,27], but since LC3 is part of the autophagic "cart" rather than of its cargo, its turnover tells little about actual autophagic activity. The use of fluorescence microscopy to monitor the transfer of LC3 from a diffuse, cytosolic to a dot-like distribution (assumed to represent organelle-associated LC3-II) [20] also has its limitations: only the dots can be quantified, the lipidation step thus being indistinguishable from overall changes in the quantities of autophagic organelles.

Clearly, quantitative measurements of both LC3-I and LC3-II, and a correction for their different immunoreactivities [18] would be required for a meaningful analysis of autophagic-lysosomal LC3 flux and its relation to macroautophagic cargo flux. In the present study, we have, therefore, developed an immunoblotting strategy that allows a satisfactory and comparable quantification of both LC3 forms. To simplify the analysis of LC3 dynamics, we have used the protein synthesis inhibitor, cycloheximide, to block de novo influx of LC3, allowing us to measure the turnover of this short-lived protein in isolated rat hepatocytes. By comparing the autophagic-lysosomal flux of LC3 with actual macroautophagic sequestration activity, using the enzyme LDH as a cytosolic cargo marker [28], we have been able to demonstrate that macroautophagic-lysosomal cargo flux can take place in the absence of an autophagic-lysosomal LC3 flux. Furthermore, the accumulation of sequestered LDH in LC3-free autophagic vacuoles suggested that macroautophagy may not require LC3 at all. This conclusion was confirmed by an RNAi-mediated approach in LNCaP prostate cancer cells: Knock-down of the LC3s did not reduce macroautophagic cargo sequestration induced by either starvation for serum and amino acids or by the mTOR-inhibitor Torin1. In contrast, autophagic bulk sequestration of cytosolic cargo was strongly suppressed by selective knock-down of the GABARAPs, indicating that this subfamily of human Atg8 orthologues, but not the LC3 subfamily, serves an essential function in macroautophagy.

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