Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Macroautophagic cargo sequestration assays

Per O. Seglen [a], Morten Luhr [a], Ian G. Mills [a,b], Frank Sætre [a], Paula Szalai [a], Nikolai Engedal [a,*]

[a] Centre for Molecular Medicine Norway, Nordic EMBL Partnership, University of Oslo and Oslo University Hospital, N-0318 Oslo, Norway
[b] Department of Cancer Prevention and the Department of Urology, Oslo University Hospital, N-0424 Oslo, Norway

ARTICLE INFO

Article history:
Received 31 October 2014
Received in revised form 17 December 2014
Accepted 31 December 2014
Available online xxxx

Keywords:
Autophagy
Macroautophagy
LC3
Cargo sequestration
Lactate dehydrogenase
LDH

ABSTRACT

Macroautophagy, the process responsible for bulk sequestration and lysosomal degradation of cytoplasm, is often monitored by means of the autophagy-related marker protein LC3. This protein is linked to the phagophoric membrane by lipidation during the final steps of phagophore assembly, and it remains associated with autophagic organelles until it is degraded in the lysosomes. The transfer of LC3 from cytosol to membranes and organelles can be measured by immunoblotting or immunofluorescence microscopy, but these assays provide no information about functional macroautophagic activity, i.e., whether the phagophores are actually engaged in the sequestration of cytoplasmic cargo and enclosing this cargo into sealed autophagosomes. Moreover, accumulating evidence suggest that macroautophagy can proceed independently of LC3. There is therefore a need for alternative methods, preferably effective cargo sequestration assays, which can monitor actual macroautophagic activity. Here, we provide an overview of various approaches that have been used over the last four decades to measure macroautophagic sequestration activity in mammalian cells. Particular emphasis is given to the so-called "LDH sequestration assay", which measures the transfer of the autophagic cargo marker enzyme LDH (lactate dehydrogenase) from the cytosol to autophagic vacuoles. The LDH sequestration assay was originally developed to measure macroautophagic activity in primary rat hepatocytes. Subsequently, it has found use in several other cell types, and in this article we demonstrate a further validation and simplification of the method, and show that it is applicable to several cell lines that are commonly used to study autophagy.

1. Introduction

Autophagy was originally defined by de Duve as the process by which portions of the cell are sequestered and transferred to lysosomes for degradation, and he suggested the term *autophagosome* for the prelysosomal vacuole that contained the sequestered cytoplasm [1,2]. Eventually the "isolation membrane" [3] that performs the actual sequestration, folding and enclosure of the cytoplasm was recognized as a distinct organelle, called a *phagophore* [4].

For the following twenty years, autophagy was unambiguously understood as the nonselective bulk sequestration and degradation of cytoplasm. However, in 1983 Mortimore [5] suggested that the term *macroautophagy* might be useful to distinguish the autophagosome-mediated bulk sequestration from the direct lysosomal uptake of cytoplasm by membrane invagination, previously called *microautophagy* by de Duve [2]. Subsequent uncovering of numerous mechanisms for the selective sequestration of organelles, protein aggregates and microorganisms [6] have made the term macroautophagy increasingly useful for distinguishing bulk autophagy of cytoplasm from these "selective autophagies", and the term macroautophagy will be used as such here.

Autophagy was first observed and investigated using electron microscopy, and ultrastructural methods have played an indispensable role throughout the history of autophagy research [7]. However, although ultrastructural information can be quantified by morphometric methods, it is a very time-consuming and not very precise method for studying the dynamics of the autophagic-lysosomal pathway in a quantitative manner [8–10]. Accurate identification of the various autophagic organelles can be very difficult, and it is also very hard to determine whether observed autophagosome-like structures represent sealed entities or not. The latter is an important point, since a crucial step in macroautophagy is the ability of the sequestering phagophore to close and thereby envelop cytoplasmic material within the double-membrane

Abbreviations: Atg, autophagy-related gene; Baf A1, bafilomycin A1; BHMT, betaine homocysteine methyltransferase; ^{14}C, radiocarbon; Con A, concanamycin A; F, farad; GFP, green fluorescent protein; GST, gluthathione S-transferase; ^{3}H, tritium; KO, knockout; LDH, lactate dehydrogenase; 3MA, 3-methyladenine; MEFs, mouse embryonic fibroblasts; mTOR, mammalian target of rapamycin; RFP, red fluorescent protein; RNAi, RNA interference.

* Corresponding author. Fax: +47 22840598.
E-mail address: k.n.engedal@ncmm.uio.no (N. Engedal).

http://dx.doi.org/10.1016/j.ymeth.2014.12.021
1046-2023/

autophagosomal structure. Assays that specifically monitor macroautophagic cargo sequestration activity are therefore needed.

By far the most widely used approach to monitor the autophagic process in mammalian cells involves the autophagy-related marker LC3 (microtubule-associated protein1 light chain 3). LC3 is a ubiquitin-like protein that attaches to the phagophore membrane upon covalent conjugation to phosphatidylethanolamine. This conjugation has been termed "LC3 lipidation". The cytosolic form of LC3 is called LC3-I, and the membrane-bound, lipidated form is called LC3-II. LC3-II stays attached to the membrane even after the autophagosome has been completed, and whereas the LC3-II that faces the cytosol is de-lipidated before or upon fusion with the lysosome, LC3-II on the inner membrane is degraded in the lysosome. Alterations in cellular LC3-II levels can be assessed by Western blotting, and the concentration of LC3 on membranes can be detected as bright LC3-positive "puncta" by fluorescence imaging of GFP-LC3-expressing cells or by indirect immunofluorescent staining of endogenous LC3. The so called "autophagic flux" or "LC3 turnover" assay measures the accumulation of LC3-II or the accumulation of LC3- or GFP-LC3-positive puncta in response to a particular treatment condition combined with an inhibitor of lysosomal degradation of LC3-II, such as the proton pump inhibitor Bafilomycin A1 (Baf A1). This assay does not actually measure autophagic flux, but rather indicates whether a particular treatment condition increases the amount of lipidated LC3 or LC3-positive puncta by means other than inhibition of lysosomal LC3-II degradation. The assay is often interpreted as measuring *de novo* formation of autophagosomes, LC3-II levels and LC3-positive puncta being used as surrogate markers for completed autophagosomes. However, since both phagophores and unclosed autophagosomes can be decorated with LC3-II and will appear as LC3-positive puncta, this interpretation is not valid.

A further limitation with using LC3 lipidation or puncta formation as universal surrogate markers for phagophores and autophagosomes is the accumulating evidence that LC3-II can interact with a number of non-autophagy-related proteins and is involved in a number of non-autophagic processes (for reviews, see [11,12]), and importantly, that macroautophagy can proceed in the absence of LC3 lipidation. In mouse embryonic fibroblasts (MEFs) devoid of Atg5, LC3 was not lipidated, yet similar-sized autophagosome-like structures were efficiently formed, etoposide-induced degradation of long-lived proteins (which occurs in the course of macroautophagy) was not altered, and a substantial proportion of the protein degradation induced by amino acid starvation was still maintained [13]. Similarly, macroautophagic flux, as measured using the LC3-independent probe Keima (see also Section 4.2.3) was found to proceed well in Atg5$^{-/-}$ MEFs under both nutrient-rich and amino acid free conditions [14]. Moreover, RNAi-mediated silencing of LC3 showed incomplete inhibition of starvation-induced degradation of long-lived proteins in HeLa cells [15] and had no effect on basal autophagic degradation of long-lived proteins in HEK293T cells [16]. In LNCaP prostate cancer cells we found that LC3 silencing did not alter basal degradation of long-lived protein, nor the degradation induced by amino acid starvation or by the mTOR inhibitor Torin1 (our unpublished results). Finally, we have recently observed that even under conditions where substantial levels of LC3 and lipidated LC3 are present, but when the autophagic-lysosomal flux of LC3-II is halted, macroautophagic-lysosomal cargo sequestration and degradation can still proceed efficiently (submitted manuscript). Thus, tandem fluorescent LC3 constructs, such as mRFP-GFP-LC3, which can be used to trace the autophagic flux of LC3, may not reliably reflect macroautophagic cargo flux. In summary, LC3 has limited value as a probe to study macroautophagy, and alternative methods are needed.

2. Strategies to analyze macroautophagic cargo sequestration

Strategies to analyze macroautophagic sequestration are generally based on the use of soluble cytosolic probes. Although macroautophagy may include many selective elements [17,18] it still shows a large degree of non-selectivity [19], and many cytosolic proteins with different half-lives have been shown to be autophagically sequestered at conspicuously similar rates [20]. Thus, during bulk sequestration of cytoplasm, cytosolic and membrane-impermeant probes will be non-selectively sequestered along with other cargo, and can be used as indicators of overall macroautophagic sequestration activity. The various probes that have been used for this purpose, and which will be described in the following sections, range from radioactively labelled sugars to fluorescently-conjugated or fluorescent proteins, GST-tagged proteins, and endogenous proteins.

3. Analyzing macroautophagic sequestration using exogenously administered probes

3.1. Introduction of fluorescently conjugated proteins as macroautophagic sequestration probes

In an early study, various purified rhodamine-conjugated exogenous cytoplasmic proteins were introduced into living HeLa cells by microinjection, and the fluorescence followed by microscopy [21]. For most proteins a gradual segregation of fluorescence into distinct granular structures was observed with time. Cells injected with rhodamine-conjugated BSA were found to form fluorescent granular structures, which at 3 h after injection were spread throughout the cytoplasm and were devoid of acid phosphatase (a lysosomally located enzyme) activity, whereas at later time-points the fluorescent granular structures co-localized with acid phosphatase activity in juxtanuclear areas, where the fluorescence gradually disappeared [21]. This indicated that the injected cytoplasmic proteins were sequestered and degraded by autophagy. However, direct evidence for this was not presented. For example, it was not determined whether the fluorescent granular structures were associated with membranes or whether the fluorescence was sequestered from the cytosol. One method that could be used to assess the latter was introduced in 2006 as the fluorescence protease protection (FPP) assay [22], where the sequestration of fluorescent proteins was assessed by whether the fluorescence was protected from tryptic proteolysis after selective permeabilization of the plasma membrane with digitonin. This assay has been used to assess autophagic sequestration of fluorescence in COS-7 cells expressing GFP-LC3 and Ubiquitin-RFP proteins [23], see Section 4.1.

3.2. Introduction of fluorescently conjugated or radioactively labelled dextran as macroautophagic sequestration probes

Dextrans are highly water soluble, membrane-impermeable and biologically inert polysaccharides that are widely used as biological tracers. Since microinjection is a rather tedious process, other techniques have been developed to load cells with exogenous probes. A simple method called "scrape loading" exploits the transient permeability of adherent cells upon scraping off the culture plate [24]. Cells are chilled on ice, scrape-loaded with the exogenous probe and washed under cold conditions in order to avoid fluid-phase endocytosis of dextran. Scrape-loading was used to introduce FITC-conjugated dextran into the cytoplasm of MCF-7 breast cancer cells [25]. Under control conditions, bright punctuate fluorescent structures appeared, whereas such structures were