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Control of autophagosome size and number by Atg7

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ABSTRACT

The induction of bulk autophagy by nitrogen starvation in baker's yeast (*S. cerevisiae*) involves the upregulation of many autophagy related proteins, including Atg7. One way to investigate the importance of this upregulation is to measure the size and number of autophagosomes formed when insufficient amounts of that protein are available. Atg8 is known to affect autophagosome size, consistent with its role in phagophore expansion. Atg7 is upstream of Atg8, and might therefore be expected to affect only autophagosome size. We used electron microscopy to measure the size and number of autophagosomes formed with limiting amounts of Atg7 and found them to be both smaller and fewer than normal. This suggests that Atg7 may have an Atg8-independent role in autophagosome initiation in addition to its Atg8-dependent role in autophagosome expansion. We also present an improved simulation for estimating original autophagic body number based on the number of cross-sections observed in ultrathin sections.

1. Introduction

Macroautophagy (hereafter simply "autophagy") is a conserved pathway for trafficking material from the cytoplasm to the vacuole/lysosome for degradation. This material is carried inside double-membrane vesicles termed autophagosomes, which form de novo in the cytoplasm, enveloping their cargo as they form [1]. The outer membrane of the autophagosome then fuses with the vacuole (in yeast and plants) or lysosomes (in animals), so the inner membrane and cargo can be degraded. When the degradative capacity of the yeast vacuole is blocked, the inner membrane and cargo are not broken down, instead accumulating inside the vacuole as autophagic bodies [2]. The size and number of these bodies can be used as a proxy for the size and number of the autophagosomes that were formed [3]. Starvation-induced non-selective autophagy transports bulk cytoplasmic components to the vacuole so that these can be broken down and the nutrients recycled to sustain cellular metabolism [4,5]. When baker's yeast (*S. cerevisiae*) are subjected to nitrogen starvation, they rapidly upregulate autophagy, and within an hour of nutrient withdrawal each cell is forming autophagosomes with an average radius of over 150 nm every 8–10 min [6–8].

Formation of these autophagosomes is accomplished by a suite of Autophagy Related (Atg) proteins, many of which are conserved across eukaryotes. In yeast, most *ATG* genes are upregulated upon nutrient starvation, with *ATG1*, *ATG7*, *ATG8*, *ATG14*, *ATG29* and *ATG41* being some of the most strongly upregulated [9]. In every case so far investigated, levels of these proteins impact overall autophagic flux, which means they must affect either the size or number of autophagosomes formed, or both [7,8,10–12]. Determining which proteins control autophagosome size and which control number not only helps clarify the importance of their expression levels, but also gives insight into their respective roles in the process of autophagosome formation.

The first protein to be studied in this way was Atg8, whose expression increases dramatically upon starvation [8,11]. Insufficient levels of Atg8 lead to a reduction in autophagosome size, but not to autophagosome number [11]. Mutations in Atg8 that lead to reduced autophagic activity or mutations in upstream proteins likewise decrease autophagosome size [13,14]. In contrast, insufficient levels of Atg9 lead to a decrease in autophagosome number, but not to autophagosome size [7]. Atg9 is a transmembrane protein that is trafficked through the secretory pathway to the site of autophagosome formation; mutations in genes necessary for the proper trafficking of Atg9 likewise lead to a reduction in autophagosome number, likely by reducing the amount of Atg9 available for autophagosome formation [12,15–17]. This distinct difference suggests that Atg9 plays an early role, in autophagosome initiation, while Atg8 plays a later role, in expansion of the autophagosome

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membrane. This is consistent with other data on the roles of these two proteins [13,18,19], and underlines the information that can be gained from this approach.

Atg8 is a ubiquitin-like protein that is conjugated to the headgroup of phosphatidylethanolamine (PE) in a similar manner to the conjugation of ubiquitin onto other proteins [20]. Like ubiquitin, the conjugation of Atg8 requires an E1 activating enzyme, (Atg7) an E2 conjugating enzyme (Atg3) and an E3 complex that stimulates this activity (Atg12–Atg5) [21]. Atg12 is also an ubiquitin-like protein, and is conjugated to Atg5 by a pathway requiring Atg7 and a different E2 enzyme, Atg10 [22]. Therefore, Atg7 is necessary for the conjugation of two different ubiquitin-like proteins, in both cases contributing to the final goal of attaching Atg8 to the autophagic membrane, where it participates in autophagosome expansion.

Atg7 protein levels increase two-fold upon starvation, and levels of Atg7 have been shown to correlate with overall autophagic flux [10]. Since Atg7 is necessary for Atg8 conjugation, we would hypothesize that Atg7 controls autophagosome size, but not number, just like Atg8. However, in Rph1-overexpressing cells, where induction of Atg7 by starvation is strongly repressed, autophagosomes were found to be the same size, but fewer in number [10]. Rph1 is a transcription factor that controls the expression levels of many genes, so this may be the combined effect of alterations in the levels of multiple Atg proteins. Therefore, it is critical to examine the effect of manipulating Atg7 levels alone, without the manipulation of any other Atg proteins.

2. Methods

2.1. General methods

pRS406 vectors containing protein A-tagged Atg7 (*ATG7-PA*) under the control of distinct promoters were made from their pRS416 counterparts by deletion of the *CEN6* sequence by PCR using phosphorylated primers followed by ligation. Additional details of plasmid and strain construction, growth and western blotting conditions are available in the supplemental methods.

The Pho8Δ60 assay was performed essentially as previously described [23], with the exception that PMSF was omitted in order to avoid interference with the protein quantification, which was performed using a BCA assay (ThermoFisher) in a 96-well plate format with absorbances measured on a BioTek Synergy 2 plate reader.

2.2. TEM analysis

Transmission Electron Microscopy (TEM) and estimation of autophagic body size and number distribution was performed as previously described [24]. In brief, log phase *pep4Δ vps4Δ atg7Δ* cells expressing Atg7-PA under various promoters (or containing just an empty vector with no Atg7-PA) were starved in parallel for 3 h in SD-N, fixed with $KMnO_4$, dehydrated in acetone, embedded in Spurr's resin, sectioned, and systematically imaged at 30,000x for measuring autophagic bodies and 6,000x for measuring vacuoles. Body cross-sections were circled manually and the distribution of the size of these cross-sections was used to estimate the size distribution of the original bodies by numeric methods as described. Similar methods were used to estimate the size of the vacuoles, and additional numeric methods were used to estimate the average number of original bodies based on body size, vacuole size, and the observed number of cross-sections per vacuole.

Additional details of the TEM and image analysis are available in the supplemental methods.

2.3. "R" simulations

Simulation of the sectioning of vacuoles with autophagic bodies was performed in R v 3.4.3 [25] with packages glpkAPI [26] and MultiRNG [27] using successive modifications of the previously published "old (2014)" simulation [24]. The most updated simulation, "Autophagic Body Sectioning Simulation v3.36", referred to here as the "new (2018) simulation", is included in the Supplementary Material as "Simulation Code". This simulation begins by generating a set of spherical vacuoles with lognormal distributed radii based on mu and sigma estimated by numerical methods from measurements of actual vacuoles in that strain. These vacuoles are arranged randomly along the Z axis, and then a 70 nm virtual slice is taken, representing the ultrathin section. Vacuoles not included by the slice are discarded, as are vacuoles where the slice results in a section below the vacuole recognition limit. In vacuoles cut by the slice, autophagic bodies are generated, positioned randomly, and clustered. The radii of the bodies follow a lognormal distribution based on mu and sigma estimated by numerical methods from measurements of actual body sections, and the number of bodies used follows a normal distribution with a given mean and standard deviation. The simulation uses the position of the slice and the clustered bodies to calculate the observed cross-section for each body, discarding any cross-sections smaller than the body recognition limit, and records both the radii of the observed cross-sections and the number of observed cross-sections per vacuole.

The resulting simulated distributions of the number of observed body cross sections per cell were compared to the experimental data, with the goodness of fit measured by the difference between the means and standard deviations of the two data sets and the calculation of the Kolmogorov Smirnov (KS) "D" statistic. The KS test was performed in R v. 3.4.3 b y creating an empirical cumulative density function from the simulated data and comparing the TEM data to it using the command ks. test from the dgof package, which allows use of the one-sample KS test with discrete distributions [28]. Multiple simulations were run, each generating ~4000 measurements, with mean and standard deviation of the number of original bodies systematically varied, starting with a mean equal to the to the value estimated from the numerical methods and a range of standard deviations. We looked for values that minimized both measures of goodness of fit; this typically occurred over a small range of means (within one or two) and a slightly larger range of standard deviations (within three to five).

3. Results

3.1. Atg7 controls both autophagosome size and number

To examine the effect of altering the levels of Atg7, we created yeast strains expressing C-terminally protein A (PA)-tagged Atg7 under the control of various promoters in a *Pho8Δ60* background. The promoters chosen were the same ones used previously [10], however, we chose to integrate our constructs into the *URA3* locus to give stable transformants instead of expressing them on centromeric plasmid [10]. We felt this would give the most consistent results when using TEM to measure the size and number of autophagosomes formed in each cell, as centromeric plasmids can vary in copy number from cell to cell [29].

We integrated *ATG7-PA* under the control of ~800bp of the native *FLO5*, *GAL3* and *SEF1* promoters into a pho8Δ60, *atg7Δ* strain to measure autophagic activity. Western blotting verified that these strains gave a wide range of Atg7 expression levels, with the *SEF1* promoter yielding mild overexpression, *GAL3p* a 10-fold underexpression, and *FLO5p* a 50-fold underexpression (Fig. 1A).

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