A high-throughput FRET-based assay for determination of Atg4 activity

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; CBB, Coomassie Brilliant Blue; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; GABARAP, γ-aminobutyric acid receptor-associated protein; GATE-16, Golgi-associated ATPase enhancer-16; HTS, high throughput screening; LC3B, microtubule-associated protein 1 A/B light chain 3B; NEM, N-ethylmaleimide; RFU, relative fluorescence unit; TCEP, Tris-2-carboxyethyl-phosphine; YFP, yellow fluorescent protein

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Macropathology (hereafter referred to as autophagy) is a major intracellular degradation process conserved in all eukaryotic cells. Autophagy removes superfluous components, damaged organelles, misfolded proteins and certain intracellular pathogens via double-membraned autophagosomes, which degrade their content after fusing with lysosomes. Autophagy can also contribute to cellular dysfunction and cell death under specific conditions. The roles of autophagy in both cell survival and cell death indicate that the autophagy process can be an important therapeutic target for diseases such as neurodegeneration and cancer.

Autophagosome biogenesis requires two ubiquitin-like conjugation systems: the Atg12–Atg5 and the Atg8–phosphatidylethanolamine (PE) systems. The cysteine protease Atg4 cleaves newly synthesized Atg8 to reveal a glycine residue required for covalent attachment to PE (lipidation), and removes conjugated Atg8 from the autophagosome membranes (delipidation) to facilitate autophagosome biogenesis and Atg8 recycling.

While yeast express single genes encoding Atg4 and Atg8, there are six human Atg8 homologs. The LC3 subfamily of Atg8 includes MAP1A/B light chain 3 (LC3A, LC3B and LC3C), and the GABARAP subfamily consists of γ-aminobutyric acid receptor-associated proteins (GABARAP), Golgi-associated ATPase enhancer of 16 kDa (GATE-16/GABARAPL2) and Atg8L (GABARAPL3). It seems that molecules of the two subfamilies can differ in their functions in autophagosome biogenesis. In
mammals there are four Atg4 homologs. Atg4B is likely the principal human Atg4 homolog. It is able to cleave each of the human Atg8-related proteins tested so far in vitro and in living cells. Studier have also shown that Atg4A is a potent protease for the GABARAP subfamily, but not the LC3 subfamily, of Atg8 substrates, whereas Atg4C and Atg4D seem to possess marginal action on the Atg8 homology of both subfamilies. Blockage of Atg4-mediated Atg8 processing could be an effective way to interfere with autophagy. Toward that end it would be critical to develop an efficient, sensitive and specific method to measure Atg4 activity. Several methods have been used in previous studies. Assessment of Atg4 activity has been mainly based on an SDS-PAGE-based method. To determine Atg4 activity and kinetics, and for high throughput screening. We had previously determined the kinetic parameters of all four Atg4 homologs toward LC3B and GATE-16 using the SDS-PAGE-based method. To determine whether the FRET-based assay could also be used to measure the kinetics of cleavage, we incubated Atg4A or Atg4B with FRET-GATE-16 or FRET-LC3B, respectively, at different concentrations. The ratio of the fluorescence intensity (RFI) at 527 nm and 477 nm was in proportion to the substrate concentrations and the corresponding cleavage products, suggesting that the assay was suitable for kinetics analysis (Fig. 3A). The initial velocity of the cleavage was thus determined based on the change of the 527 nm/477 nm ratio and then plotted against the substrate concentrations. From the fitted curve, the key kinetic parameters, \( V_{\text{max}} \) and \( K_{\text{m}} \), were derived (Fig. 3B–D). The data indicated that Atg4B had a higher affinity and a higher catalytic efficiency than Atg4A toward the two substrates. GATE-16 was a better substrate than LC3B, particularly for Atg4A.
which could not cleave LC3B. These findings were consistent with previous determinations using LC3B-GST and GATE-16-GST as the substrates in an SDS-PAGE-based assay. The catalytic efficiency of Atg4B toward FRET-GATE-16 (221,000 mol \(^{-1}\) s\(^{-1}\), Fig. 3D) was in the same order of magnitude as that toward GATE-16-GST (107,000 mol \(^{-1}\) s\(^{-1}\)) despite the different assay formats used for the measurement. The catalytic efficiency of Atg4B toward LC3B-GST\(^{17}\) and FRET-LC3B (Fig. 3D) was also quite comparable (89,600 vs 120,000 mol \(^{-1}\) s\(^{-1}\)).

These observations are consistent with the notion that Atg4B is less selective toward the substrates and their configurations. On the other hand, it is noted that Atg4A is more selective than Atg4B in substrate preference, only being able to hydrolyze the GABARAP subfamily substrates, and is therefore likely more sensitive to the configuration of the substrate. Consistently, the catalytic efficiency of Atg4A toward FRET-GATE-16 (1,310 mol \(^{-1}\) s\(^{-1}\), Fig. 3D) seemed to be particularly lower than that toward GATE-16-GST (12,800 mol \(^{-1}\) s\(^{-1}\)), showing a preference toward GATE-16-GST over FRET-GATE-16.

Measurement of Atg4 activities in cell lysates using the FRET-based assay. While the above studies used purified recombinant Atg4 proteins, we sought to determine whether the FRET assay would be also effective for the measurement of Atg4 activity in biological samples. We investigated this issue by examining the activity of Atg4B in the following studies. Lysates prepared from HEK-293A cells with or without various overexpressed Atg4 constructs were incubated with the FRET-LC3B substrate. While the SDS-PAGE-based assay clearly revealed the difference in the extent of FRET-LC3B cleavage with the overexpressed Atg4B (Fig. 4A), the FRET-assay provided a more quantitative measurement (Fig. 4B). Thus, the FRET assay could specifically and quantitatively measure the Atg4B activity in a biological sample.

To determine whether the assay could discriminate Atg4B activity in cells constitutively expressing different levels of Atg4B, we analyzed a panel of 4 breast cancer lines and found that the endogenous Atg4B in 20 μg of lysates in 5 min, nearly 100% of the same amount of FRET-LC3B could be cleaved by 20 μg of lysates from Atg4B-overexpressing cells, and nearly 90% of cleavage was reached with 5 μg of each lysate. The overexpression of neither Atg4BC74S nor Atg4A (Fig. 4A) caused an elevation of the cleavage above the level reached by the endogenous Atg4B. Conversely, knockdown of Atg4B by about 50% led to the reduction of the cleavage by 60% in a 30 min reaction (Fig. 4B). Thus, the FRET assay could specifically and quantitatively measure the Atg4B activity in a biological sample.
cleavage activity was fully suppressed by N-ethylmaleimide (NEM), a general cysteine protease inhibitor. Considering these data, we next sought to determine whether the Atg4B expression and activity could change during autophagy. Incubation of cells in EBSS, a starvation medium, did not alter the expression of Atg4B in the breast cancer cell lines (Fig. 4C, a) and in HEK-293A cells (Fig. 4D, a). Consistently, application of other chemicals that affect autophagy, such as rapamycin, N-acetylcysteine, or bafilomycin A1, did not change the Atg4B expression level (Fig. 4D, a), or

![Figure 2](image-url). Verification of the cleavage of FRET substrate by Atg4 using the FRET-based assay. (A) Schematic representation of the assay principle. The FRET signal (Em = 527 nm) is reduced as the result of cleavage, which separates the CFP (donor) moiety from the YFP (acceptor) moiety. (B) The fluorescence emission spectra of FRET-GATE-16 or FRET-LC3B before and after cleavage. Substrate (500 µg/ml) were mixed with buffer, Atg4A (100 µg/ml) or Atg4B (2 µg/ml) in a cuvette in the volume of 0.5 ml for 30 min. Data from representative experiments were collected on a Cary Eclipse spectrophotometer. The excitation wavelength was 484 nm. Emission peaked at 477 nm with Atg4 present, but at 527 nm with no Atg4 present in the reactions. In the presence of the corresponding Atg4 enzyme, the ratio of 527 nm/477 nm for FRET-GATE-16 decreased from 1.8 to 0.6 and that for FRET-LC3B was reduced from 1.65 to 0.69. (C) FRET-LC3B, FRET-GATE-16 and FRET-LC3BG120A (100 µg/ml) were incubated with Atg4A, Atg4B, or Atg4BC74S (2 µg/ml) in a volume of 200 µl for 10 min. The fluorescence ratios of 527 nm/477 nm at the beginning and at the 10 min point were determined. Data represent the mean ± SD from three independent experiments. ***p < 0.001 (paired t-test, panel C).
the activity (Fig. 4D, b). These data indicate that the cleavage potency of Atg4B was more dependent on the expression level than on the autophagy status. This notion, however, does not rule out the possibility that Atg4 activity could vary inside the cells following proper autophagy stimulation, but failed to be detected in the in vitro condition.

Configuration of the FRET-based assay for high-throughput screening (HTS). One major advantage of the FRET assay over the SDS-PAGE-based assay is that it can be easily configured to a high-throughput format for analysis or for screening. To ascertain HTS compatibility and robustness, we examined the performance of the FRET assay in multiple replicates in 384-well plates.

An important parameter for evaluating the reliability and robustness of the high-throughput assay is the Z’ factor. This factor evaluates the size of the errors in the data relative to the difference in the maximum and minimum values in the assay, which gives rise to an estimate of the assay window. The Z’ factor can assume any value $\leq 1$. A Z’ factor of 1 denotes an ideal assay with complete separation of positive and negative controls. Z’ factor $> 0.5$ denotes separation between the means ± 3 SD of the positive and negative control. In HTS, Z’ factors of $> 0.5$ are considered excellent. We determined that the Z’ factors for the Atg4A FRET-GATE-16 assay and the Atg4B FRET-LC3B assay were 0.78 and 0.81, respectively (Fig. 5), which indicated a robust performance of these assays. Thus, the FRET-based Atg4 assay has the quality to be successfully employed in HTS.

Analysis of chemical interference of Atg4 activity using the FRET-based assay. The FRET assay would allow for a better quantitative determination and comparison of various enhancing or inhibiting modulators of Atg4 enzymes. Using this assay, we first examined the effect of six representative protease inhibitors on Atg4A and Atg4B activities. These chemicals include a disulfide bond reducer, tris-2-carboxyethyl-phosphine (TCEP), a sulfhydryl group blocker, N-ethylmaleimide (NEM), a serine protease inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), a general cysteine protease inhibitor, E64-D, a general...
Figure 4. For figure legend, see page 407.
Figure 4 (See opposite page). Measurements of Atg4B activity in cell lysates using the FRET-based assay. (A) HEK-293A cells were transfected with Flag-Atg4A, Flag-Atg4B or vector for 24 h. Cells were harvested and cell lysates (25 μg) were mixed with FRET-LC3B (5 μg) for 20 min before the reaction was stopped by the addition of sample loading buffer, and separated by SDS-PAGE. The expression level of Flag-Atg4A and Flag-Atg4B was detected by immunoblot assay using an anti-Flag antibody. The cleavage was assessed after CBB staining. (B) HEK-293A cells were transfected with Flag-Atg4B, Flag-Atg4B<sup>74S</sup> or the control vectors. Alternatively, they were transfected with a control siRNA or a siRNA against Atg4B. Cell lysates (20 μg) were prepared and subjected to immunoblot analysis (a), or incubated with FRET-LC3B (3 μg) for the cleavage assay (b). Five micrograms (indicated) or 20 μg (all others) of lysates were used. WT, control; KD, control or Atg4B siRNA-treated. The percentage of substrate cleavage (c) was calculated. (C) Four breast cancer cell lines were cultured in complete medium (CM) or EBSS for 4 h. Cell lysates (20 μg) were prepared and subjected to immunoblot analysis (a), or incubated with FRET-LC3B for the cleavage assay (b). A positive control group with purified recombinant Atg4B (2 μg) was included. A generic cysteine protease inhibitor, NEM (100 μM), was included in some reactions as indicated. The percentage of substrate cleavage (c) was calculated. (D) HEK-293A cells were incubated in EBSS, or in complete medium alone (CM) or with rapamycin (Rap, 2 μM), N-acetylcysteine (NAC, 20 mM) or bafilomycin A₁ (Baf, 1 μM) for 4 h. Cell lysates were prepared and subjected to immunoblot analysis (a), or incubated with FRET-LC3B for the cleavage assay (b). A positive control group with purified recombinant Atg4B (2 μg) was included (indicated as Atg4B). Data represent the mean ± SD from three independent experiments. ***p < 0.001 (one way ANOVA, B, c; and C, c).

Figure 5. Determination of the performance of the FRET-based Atg4 assay in a high-throughput format. Atg4A (10 μg/ml, A) or Atg4B (2 μg/ml, B) were mixed with 60 μg/ml of FRET-GATE-16 (A) or FRET-LC3B (B) in a total volume of 20 μl in 384-well plates. After 60 min incubation, the RFU ratio of 527 nm/477 nm was determined for each of the reactions in 192 wells (solid circle). Control reactions with no Atg4 enzymes were set up in the same way (open circle). The ranges of the maximal and minimal values ± three standard deviations were indicated and the Z’ factors for both Atg4A and Atg4B assays were calculated.
aspartic peptidase inhibitor, pepstatin A, and an aminopeptidase inhibitor, bestatin.

We found that only NEM, but not other chemicals at the tested concentrations, was able to inhibit the activity of Atg4A and Atg4B (Fig. 6A and 6B), consistent with previous findings.\(^{15,18}\) This indicates that while the cysteine residue of Atg4A and Atg4B is important for enzymatic activity, generic cysteine protease inhibitors, such as E64-D, are not effective in suppressing this activity. Consistently,
TCEP showed an enhancing effect on the reaction, especially for Atg4A, suggesting the importance of maintaining cysteine residues in reduced status.

To determine the potency of NEM, different concentrations of NEM were applied and the RFU ratio of 527 nm/477 nm of the cleavage reaction was reversed in a concentration-dependent manner (Fig. 6E). The IC_{50} was determined to be 10 μM for Atg4A and 17.7 μM for Atg4B (Fig. 6E and F). Thus, the FRET-based Atg4 assay can quantitatively determine the effects of small molecule modulators of Atg4A and Atg4B.

Discussion

The mammalian Atg8 homologs are a group of proteins with diverse sequences, belonging to two subfamilies, the LC3 subfamily and the GABARAP subfamily. Diversity in their function has been reported in which LC3B seems to be mostly involved in the elongation of the phagophore membrane, whereas GATE-16 is more important for autophagosomal maturation at a later stage. The initial processing of Atg8 molecules requires the cysteine protease Atg4, which has multiple processing sites, suggesting that its function could be exerted at this level of autophagy and be complemented by other Atg4 homologs. The latter is able to process the GABARAP subfamily of Atg8 proteins, but not the LC3 subfamily of Atg8 proteins. Thus the functional complementarity of Atg8 homologs could be based on the functional complementarity of the two Atg8 subfamilies. It also seems important that both Atg4A and Atg4B are prone to changes not related to the Atg4 activity. In addition, the introduction of the luciferase fusion construct to the cell can be labor intensive. Recently, a simplified one-reaction design has been proposed. This system uses FITC-labeled Atg8 substrate peptides conjugated to polymeric nanoparticles, which are highly permeable to cells. Once inside the cells, the peptides are cleaved by Atg4, releasing a carboxy-terminal FITC-labeled subunit, which can be detected using a CCD camera. The method has been used widely with no complications methods needed to introduce the substrate into cells. A different concern is the use of a 4-amino acid peptide based on the consensus sequence of several Atg8 homologs, instead of the full length proteins, as the substrate of Atg4 will likely reduce the efficiency of the cleavage reaction because of the requirement for conformational change of the enzyme triggered only by the full-length substrates.

Subsequent assay development has been focused on the aspects of being more quantitative and sensitive, while adopting the principle of using fusion substrates. A successful example is the use of phospho-lipase A2 (PLA2) as the fusion partner of LC3B. After cleavage by Atg4B, PLA2 is separated from LC3B and regains its enzymatic activity, which is detected with a secondary fluorogenic assay. Thus the activity of PLA2 is in proportion to the level of LC3B-PLA2 cleavage, or Atg4B activity. This method seems to be very sensitive and specific, and is suitable for high throughput screening. However, one possible concern for the two-reaction design is that interference with the PLA2 reaction may be mistakenly attributed to the change in the Atg4 activities.

To detect Atg4 activity in situ, two other methods have been developed. One uses a LC3B fusion construct that contains Gaussia luciferase and β-actin, which would allow the hybrid molecule to be sequestered inside the cells in the actin network. The cleavage by Atg4B results in the release of Gaussia luciferase, which is exported to the extracellular space where it can be measured by luminometry. This assay is also a coupled assay in which the luciferase reaction may render the assay prone to changes not related to the Atg4 activity.
With these properties, this assay has been successfully used to determine the kinetic properties of the recombinant Atg4A and Atg4B enzymes, to determine Atg4 activity in cell lysates, and to assess the potency of chemical inhibitors. The robust performance of the assay in high throughput format suggests that it is suitable for screening chemical modulators. For example, this study, the FRET-substrate construct can be transfected into cells to analyze Atg4 activity in situ. It is thus believed that this FRET-based assay has overcome some of the key limitations of current methodology and offers an efficient way to determine Atg4 activity under a variety of conditions.

Materials and Methods

Chemicals, antibodies, bacterial stains and plasmids. All chemical reagents were obtained from Sigma-Aldrich unless otherwise stated. Anti-Flag was obtained from Sigma-Aldrich (F3165), anti-Atg4B was from Novus (NB800-221) and secondary antibodies conjugated with HRP was from Novus (NB300-221) and sec-ondary antibodies conjugated with HRP was from Abgent (Ap1809c), anti-GAPDH was from Sigma-Aldrich (F3165), anti-Atg4B was obtained from Sigma-Aldrich unless otherwise stated. Anti-Flag was obtained from Sigma-Aldrich unless otherwise stated. 17 To express HEK-293A cells were grown in DMEM containing 10% fetal bovine serum and 1% antibiotics. To express the cDNA fragments encoding Atg4A, Atg4B and Atg4BC74S or 25 g/ml. Atg4A or Atg4B were incubated in FRET-LC3B or FRET-GATE-16 and YFP by the proper Atg4. The percentage of cleavage was calculated as previously described.17 Briefly, the optical density (OD) of the product protein bands is divided by the OD of all the protein bands percentage of cleavage (%) = (ODYFP + ODCEFPLC3B-YFP-180b)/ ODCEFPLC3B-YFP-180b*100%, in which the ODs are the intensity of the total RFU change, which is calculated as following: percentage of cleavage (% at a given condition (x) = (RFUmin - RFUs)/(RFUmax - RFUmin) *100%, in which the RFUs are the RFU ratio of 527 nm/477 nm of reactions under no cleavage (RFUmin), under highest cleavage (RFUmax), and under a given condition (RFUs). Measurement of Atg4 activity using the FRET-based assay. Purified Atg4A and Atg4B proteins or cell lysate were mixed with FRET-LC3B or FRET-GATE-16 in Buffer B. The assay volumes were 400 μl (cuvette format), 200 μl (96-well format) or 20–50 μl (384-well format, respectively). After incubation at 37°C for a given time, the cleavage of the substrates was measured using a fluorescence spectrometer (Cary Eclips, Agilent, or M5, Molecular Device). The excitation wavelength was 434 nm. Emission was measured at 477 nm (CFP) and 527 nm (YFP or FRET). The RFU ratio of 527 nm/477 nm was calculated and used to determine the degree of cleavage since it decreased proportionally to the extent of substrate cleavage. The percentage of cleavage of the substrates was thus determined based on the proportion of the total RFU change, which is calculated as following: the RFU min), under highest cleavage (RFUmax), and under a given condition (RFU).

Measurement of Atg4 activity in situ. It is thus believed that this FRET-based assay has overcome some of the key limitations of current methodology and offers an efficient way to determine Atg4 activity under a variety of conditions. For immunoblot assay, lysates were separated by SDS-PAGE and transferred to PVDF membranes. After the application of the appropriate antibodies, signals were developed using Immobilon Western detection kit (Millipore, WBKLS0500) according to the manufacturer’s instructions.

Analysis of the Atg4-mediated cleavage of FRET-substrates by SDS-PAGE. Purified FRET-LC3B, FRET-LC3B(G120A) and FRET-GATE-16 (5 μg) were mixed with 0.25 μg of purified Atg4A, Atg4B or Atg4BC74S or 25 μg of HEK293A cell lysate in Buffer B (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM glycerophosphate, 1 mM Na3VO4, 1% Triton X-100, 50 mM TRIS-HCL, pH 7.5). To suppress the endogenous expression level of Atg4B, siRNA against Atg4B (SantaCruz Biotech, sc-72584) was transfected into HEK293A cells using Oligofectamine (Invitrogen).

For immunoblot assay, lysates were separated by SDS-PAGE and transferred to PVDF membranes. After the application of the appropriate antibodies, signals were developed using Immobilon Western detection kit (Millipore, WBKLS0500) according to the manufacturer’s instructions.
substances at 527 nm ([S]t-527nm) as well as on the fluorescence change of the substrate at 477 nm ([S]t-477nm), whereas [S]t-477nm was calculated as (RFUmax-477nm – RFUmin-477nm)/2. [S]t-527nm was calculated as ([RFUmin-527nm]/RFUmax-527nm) - 1. Fluorescence intensity at a given time (t).

The percentage of cleavage (see above): which was calculated using the formula of inhibition of Atg4 cleavage activity, which was calculated using the formula modified from the one used for calculating the percentage of cleavage (see above):

High-throughput screening format of the Atg4 assay. Atg4A (10 µg/ml) or Atg4B (2 µg/ml) were first incubated with chemical compounds (10 µM) for 30 min at 57°C in 384-well plates. FRET-GATE-16 or FRET-LC3B (60–100 µM) was then added to a total volume of 20–50 µl. After 30 min (for the Atg4B/FRET-LC3B reaction) or 60 min (for the Atg4A/FRET-GATE-16 reaction), the fluorescence intensity was recorded. The relative cleavage activity of Atg4A or Atg4B was calculated based on the change of 527 nm/477 nm ratio as described above. The positive control for the cleavage was without any chemicals, whereas the negative control was without the Atg4 enzyme. The performance of the screening was measured by the Z′ factor: Z′ = (σ – μ)/σp, where σ is the standard deviation and μ is the mean for positive (p) and negative (n) controls. The potency of inhibition by a given chemical was measured by the percentage of the inhibition of Atg4 cleavage activity, which was calculated using the formula modified from the one used for calculating the percentage of cleavage (see above):

percentage of inhibition (%) = (RFUx – RFUmin/RFUx)*100%, in which RFUx is the RFU ratio of 527 nm/477 nm in the presence of a given concentration of the chemical, RFUmin is the ratio in the absence of chemical and RFUx is the ratio in the presence of a maximal inhibitory concentration of the chemical. IC50 was determined by plotting the percentage of inhibition against the concentration of the chemical, which was fitted with a nonlinear 4-parameter fitting method using SigmaPlot 10.0.

Statistical analysis. All experiments have been performed at least three times. Data shown are the mean ± SD from three experiments and were subjected to t-test or one-way ANOVA followed by Holm-Sidak’s post-hoc analysis as indicated in the figure legend. A p-value of < 0.001 was considered significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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