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Measuring the overall rate of protein breakdown in cells and the contributions of the ubiquitin-proteasome and autophagy-lysosomal pathways

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Abstract

In certain physiological or pathological states (e.g. starvation, heat shock, or muscle atrophy) and upon drug treatments, the overall rate of protein degradation in cells may increase or decrease. These adaptations and pathological responses can occur through alterations in substrate flux through the ubiquitin-proteasome pathway (UPP), the autophagy-lysosomal system, or both. Therefore, it is important to precisely measure the activities of these degradation pathways in degrading cell proteins under different physiological states or upon treatment with drugs. In particular, proteasome inhibitors have become very important agents for treating multiple myeloma and very useful tools in basic research. To evaluate rigorously their efficacy and the cellular responses to other inhibitors, it is essential to know the degree of inhibition of protein breakdown. Unfortunately, commonly used assays of the activities of the UPP or autophagy rely on qualitative, indirect approaches that do not directly reflect the actual rates of protein degradation by these pathways. In this chapter, we describe isotopic pulse-chase methods to directly measure overall rates of protein degradation in cells by radiolabeling cell proteins and following their subsequent degradation to radioactive amino acids, which diffuse from cells into the medium and can be easily quantitated. While pulse-chase methods have often been used to follow degradation of specific proteins, the methods described here allow quantification of the total cellular activity in degrading either long-lived proteins (the great bulk of cell constituents) or the fraction with short half-lives. Moreover, by use of specific inhibitors of proteasomes or lysosomes, it is also possible to measure precisely the total contributions of the UPP or lysosomal proteases. These approaches have already been proven very useful in defining the effects of inhibitors, growth factors, nutrients, ubiquitination, and different proteasome activators on overall proteolysis and on substrate flux through the proteasomal and lysosomal pathways.

Keywords

Protein degradation; ubiquitin-proteasome pathway (UPP); autophagy-lysosomal system; radiolabeling; ³H-phenylalanine; pulse-chase; liquid scintillation counting; 26S proteasomes; ubiquitin

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1. Introduction

In mammalian cells under standard culture conditions, the ubiquitin-proteasome pathway (UPP) accounts for 70% or more of the protein degradation, while the autophagy-lysosomal system degrades the remainder [1,2]. It is now well established that the total amount of protein degradation by these two pathways is tightly regulated and can vary in different physiological and pathological conditions. For example, there is growing evidence that proteasome activity and protein degradation are diminished in several neurodegenerative diseases associated with the accumulation of misfolded aggregation-prone proteins, such as tau [3]. Conversely, a key event in muscle atrophy and cachexia is the increase in overall protein degradation through coordinate activation of both the UPP and autophagy [4,5]. Protein degradation can also be inhibited by many synthetic inhibitors of the proteasome, ubiquitin conjugation, or lysosomal function. The proteasome inhibitors, Bortezomib, Carfilzomib, and Ixazomib, are now the preferred treatments for multiple myeloma, and there are many ongoing clinical trials with these inhibitors for other diseases [6–8]. In addition, proteasome inhibitors (e.g. MG132) are very widely used as research tools in many areas of biology. In such studies, it can be very important to directly measure the actual rates of cellular protein breakdown and the specific contributions of the UPP and autophagy-lysosome pathways. The approaches described here provide the most physiologically relevant measurements of the cell's degradative activities and allow us to accurately evaluate how substrate flux through these pathways changes under different physiological conditions or with drug treatments.

Unfortunately, the methods commonly used to measure the activities of the proteasome or autophagy in cells or the extent of their inhibition do not directly measure the actual rates of proteolysis in cells. Proteasome activity is most commonly evaluated simply by assaying the peptidase activities of the 20S particles' catalytic β subunits using small fluorogenic peptides [9] or the recently-developed active-site probes that bind covalently to the β subunits' active sites [10–12]. However, in cells, proteasomes degrade ubiquitinated proteins, not short peptides, and peptidase assays often do not reflect the actual extent of changes in the cellular rate of protein breakdown by the UPP. Peptidase assays measure only the ability of peptides to enter the 20S's catalytic chamber and their subsequent hydrolysis [13], but there are many additional steps that are essential for the degradation of ubiquitinated proteins [14], and they can also change under different physiological conditions or upon drug treatment. These steps include the binding of ubiquitinated substrates to specific 26S receptor subunits, substrate deubiquitination, opening of the substrate entry channel into the 20S particle, and protein unfolding, translocation, and hydrolysis. Protocols to measure many of these steps are discussed in the Chapter 19 by Kim et al.; and Methods to purify 26S proteasomes for such studies are presented in the Chapter 18 by Kuo et al. Also, approaches to investigate the regulation of proteasome function by subunit phosphorylation are covered in the Chapter 20 by VerPlank and Goldberg.

Other common approaches to evaluate the activities of the UPP and autophagy share the similar limitation of not being able to provide direct information on changes in the actual rates of protein degradation. For example, proteasome function and inhibition are also often monitored in cells and tissues by following the accumulation of ubiquitinated proteins.

However, the abundance of ubiquitin conjugates is affected not only by their rates of degradation by proteasomes, but also by the actions of cellular deubiquitinating enzymes (DUBs), and the rate of protein ubiquitination. Therefore, the accumulation of ubiquitinated proteins does not necessarily indicate proteasome inhibition. In fact, inhibition of mTOR or heat shock elevate Ub conjugate levels in cells by increasing protein ubiquitination, while also increasing overall degradation by the UPP [15]. Similarly, the activity of the autophagy-lysosome pathway is generally monitored by microscopic measurements of the abundance of autophagic vacuoles or by the western blot assays of marker proteins, such as lipidated LC3 [16]. However, neither approach measures the actual amount of cell proteins that are being degraded by lysosomes at any time.

This chapter describes our methods to overcome these limitations and to directly measure the actual rates of protein degradation in cells. For this purpose, we pre-label cell proteins with a radioactive amino acid and then determine the rate of the hydrolysis of these pre-labeled proteins to amino acids. In cells, proteins are hydrolyzed by the proteasome or lysosome to small peptides, which are further cleaved by cytosolic endo- and exopeptidases to amino acids that are transported across the plasma membrane into the extracellular medium. By using a large excess of nonradioactive amino acids in the medium as the chase, it is possible to prevent the rapid reincorporation of the labelled amino acid back into new proteins and also to promote the counter-transport of these labelled residues into the medium. The radioactive amino acids in the medium are then separated from any radiolabeled proteins by precipitating the proteins with trichloroacetic acid (TCA). Both the TCA-soluble radioactivity and the total radioactivity initially incorporated into cell proteins are measured by liquid scintillation counting. We can then calculate the mean rate of protein degradation in cells (% degradation / h) [4]. This pulse-chase approach not only measures the overall protein degradation rate, but it is also used to determine the degradation that is specifically catalyzed by the UPP or the autophagy-lysosome pathways by specifically inhibiting either proteasome or lysosomal function. Such information is very useful in evaluating mechanisms of cell growth and the effects of disease, hormones, or drugs. For example, these methods have recently enabled the discovery of the role of protein kinase A and Dyrk2 in enhancing proteasome activity and overall protein degradation ([17,18] See Chapter 20 by VerPlank and Goldberg). By contrast, the commonly used measures of proteasomal peptidases or LC3-II assays fail to provide such quantitative information, and such assays can be misleading. For example, the major proteasome inhibitors can block the chymotrypsin-like activity to near completion at nanomolar concentrations, but cause only about a 50% inhibition of the proteasomal degradation of cell proteins [19] due to the continued functioning of the other proteasome sites.

By varying the length of the cell's exposure to the radioactive precursors (the pulse) and introducing a chase period, it is possible to follow specifically the degradation of long-lived cell proteins, which are the overwhelming majority of cell constituents. Under normal growth conditions, these proteins are degraded mainly by the UPP [15] and to some small extent by autophagy. By using a short pulse of the precursor (without a chase), it is possible also to monitor the loss of the rapidly degraded (short-lived) proteins, which include misfolded proteins and regulatory proteins (e.g. transcription factors, rate-limiting enzymes, many oncogenes and tumor suppressors). Their degradation seems to be catalyzed solely by

UPP. For most studies, the pulse-chase method is most useful to follow the degradation of long-lived proteins since their mean rates are linear for hours. Therefore, the effects of inhibitors or physiological factors on their overall degradation can be very precisely measured. By contrast, short-lived proteins may disappear in minutes to 1–2 hours, and even a short-pulse also labels some long-lived stable proteins. Therefore, their mean degradation rates are continually changing and difficult to evaluate.

This method is actually an adaptation for cultured mammalian cells of approaches introduced almost 50 years ago for studying proteins degradation in *E. coli* [20,21] and the acceleration of degradation of long-lived proteins upon starvation for amino acids, carbon source, or charged tRNAs [22]. Also, similar-pulse chase studies with a brief pulse made possible discovery of the rapid selective degradation of misfolded abnormal proteins containing amino acid analogs or puromycin in *E. coli* [20,21], mitochondria [20], and reticulocytes [23,24]. In fact, the existence of the non-lysosomal proteolytic degradative system, which we now call the UPP, was first demonstrated through measurement of the degradation of radiolabeled proteins to TCA-soluble peptides in cell free systems [23].

Analogous non-radioactive methods has also often been used to assay hydrolysis of cell proteins to specific amino acids, which are assayed by specific fluorometric assays or HPLC [21]. These non-radioactive methods have been widely used in physiological studies to evaluate overall protein degradation in isolated rodent tissues, as well as the actual contributions of lysosomes or the UPP [25]. For example, such approaches first indicated that protein degradation by the UPP is activated in skeletal muscle upon fasting, glucocorticoid treatment, cancer cachexia, or denervation [26]. A recently-developed non-radioactive pulse-chase method to follow protein degradation uses azidohomoalanine (AHA) [27,28], which is incorporated into proteins in place of methionine. This method allows quantification by mass spectrometry of labelled proteins after binding to avidin beads with a “click” reaction between the alkyne-bearing biotin and the azide moiety of AHA. However, this method is less accurate for determining the rates of protein degradation, because it measures the net decline in the cellular content of AHA-containing proteins (which is very slow for long-lived proteins). Although incorporation of AHA does not seem to cause large changes in protein stability [29], cautions in using such methods seem necessary because incorporation of amino acid analogs can markedly perturb protein conformations and promote rapid degradation [21,23].

2. Materials

1. The appropriate complete cell growth media for your cells (*see* Note 1).
2. [³H] Phenylalanine (Phe L-[3,4,5 ³H]; American Radiolabeled Chemicals, ART0614), stock 1 mCi/ml in 0.01M HCl.
3. Phenylalanine.
4. Scintillation Vials (RPI, 125514-SP).

¹.The methods described in this protocol can be used to measure degradation rate in any cell in culture and related methods have been used in isolated tissues.

5. Bio-Safe II™ Complete Counting Cocktail (scintillation fluid) (RPI, 111195-CS).
6. Trichloroacetic acid.
7. PerkinElmer Tri-Carb 2910TR Liquid Scintillation Analyzer controlled with QuantaSmart™ software.
8. 4 M NaOH.
9. Bortezomib (LC Laboratories, B-1408).
10. TAK-243 (a.k.a. MLN-7243, Active Biochem, A-1384).
11. Concanamycin A..
12. Cycloheximide.

3. Methods

The duration of the exposure to radioactive precursors and of the chase period determines which types of cell proteins are preferentially labeled. Protocol **3.1** describes a method to label selectively long-lived cellular proteins by first exposing cells to the radioactive amino acid for 20 hours and then chasing with an excess of non-radioactive amino acid for 2 hours to allow breakdown of the short-lived proteins. Although the long lived proteins are heterogeneous mixtures, their mean half-lives are similar and therefore this approach gives a highly reproducible degradation rate that appears linear for up to ten hours. The slope of this line represents the percent of cell proteins hydrolyzed per hour (i.e. degradation rate) (Fig 1A). On the other hand, a brief pulse-labeling (for 5–20 minutes), followed immediately by resuspension in the chase media for measurement, allows measurement of the degradation of newly synthesized proteins, which also includes the short-lived cell proteins. Protocol **3.2** describes our standard method to label proteins using a 20-minute pulse to follow the degradation of this highly heterogeneous group of proteins (Fig 1C). Consequently, their degradation is rapid, non-linear and harder to follow. Initially these curves reflect the loss of most short-lived proteins and then the more stable and finally the long-lived components. Finally, Protocol **3.3** describes a method to measure the amount of proteins that is degraded: 1) via the proteasome; 2) that is ubiquitin-dependent; and 3) that is by the autophagy-lysosome pathway by using highly specific inhibitors to completely block each of these pathways. Subtracting the degradation rate in the inhibitor-treated cells from that in the control (untreated) cells yields the amount of degradation catalyzed by the inhibited pathway (Fig 1E). In our extensive experience, these rates remain constant for at least six hours even in the presence of inhibitors of the proteasome or lysosome, which implies that blocking one pathway does not cause rapid compensatory changes in the other pathways. Figure 1 also provides examples where these protocols have been used to answer key research questions (Fig 1B, D, F).

3.1. Measurement of the degradation of long-lived proteins.

Day 1 Radiolabeling of long-lived proteins with ³H-phenylalanine (*see* Note 2).

1. Cells are grown to 60–80% confluence in six-well plates. To obtain sufficient replicates, 4 wells of cells for each condition are seeded.
2. Prepare complete media supplemented with ^3H -Phenylalanine at a final concentration of 5 $\mu\text{Ci/ml}$. Add 1 ml to each well (*see Note 3*).
3. Incubate cells for 20 hours to allow incorporation of ^3H -phenylalanine into cellular proteins.

Day 2 Sample collection for the measurement of degradation of long-lived proteins.

4. Prepare complete media containing 2 mM non-radioactive phenylalanine (chase medium). This chase medium will be used in all subsequent steps (*see Note 4*).
5. Remove radioactive media and thoroughly wash cells twice with 2 ml chase medium. It is essential to thoroughly wash the cells in order to remove as completely as possible the ^3H -phenylalanine remaining in the cells and media. The amount of un-incorporated ^3H -phenylalanine is much larger than the amount incorporated into cell proteins. Therefore, if not removed thoroughly, it can cause a high background radioactivity that will prevent accurate assay of proteolysis.
6. Add 2 ml of the chase medium to each well and incubate for 2 hours. This step allows short-lived radiolabeled proteins to be degraded and makes possible the measurement specifically of the degradation of long-lived proteins.
7. Remove media from cells completely after step 6. Wash once more with 2 ml the chase medium and remove the wash media completely.
8. Add 2 ml chase medium to each well, and begin sample collection for the measurements of degradation. The volumes of the samples taken must be very precise and consistent for accurate determination of degradation rates.
9. At 0, 1–4 hours (or longer), collect precisely 200 μl aliquots of the media from each well, and transfer to a clean microcentrifuge tube. Briefly centrifuge the media samples (if necessary) to ensure that they are free of floating cells. These samples can be kept at 4 $^{\circ}\text{C}$ for up to 3 days before subjecting to trichloroacetic acid (TCA) precipitation (*see Note 5*).

²Several other radioactive amino acids, especially ^3H -leucine, ^{14}C -leucine, and ^{35}S -methionine, have long been used to follow turnover of cell proteins. In the past, we and others have also used ^3H -tyrosine especially for measuring the degradation of long-lived cell proteins in muscle [4]. However, since treatment of cells with high concentrations of leucine (as in the chase media) can activate mTORC1 and suppress protein degradation by both autophagy and the UPP [15,30], it is inappropriate to use this amino acid for measurements of protein turnover. Our extensive experience comparing different amino acids in pulse-chase protocols ([15] and Garcia, Zhao, et al., unpublished) further demonstrated that after pulse, labelling the amount of intracellular radioactive free methionine and tyrosine declines over time much more slowly than phenylalanine. Since these intracellular radioactive amino acids can continue to be incorporated into newly-synthesized proteins, the higher amounts of intracellular labeled methionine and tyrosine are more prone than phenylalanine to an erroneously low degradation rate, particularly after a short pulse. Therefore, phenylalanine is a much better choice than leucine, methionine, or tyrosine as the radioactive precursor for such experiments.

³Cell proteins can be efficiently labelled with ^3H -phenylalanine despite the presence of cold phenylalanine in the standard tissue culture media and FBS. Removal of this nonradioactive phenylalanine to enhance specific activity of the ^3H -precursor during radiolabeling and the cpm incorporated is not recommended in order to avoid the potential cell stress caused by depletion of phenylalanine and reduction of protein synthesis, especially in the 20-hour labelling protocols.

⁴Excessive non-radioactive phenylalanine in the media is essential to competitively prevent the re-incorporation of ^3H -phenylalanine back into cellular proteins. The excess amount of non-radioactive phenylalanine in the media also stimulates exchange with ^3H -phenylalanine that is generated by cells due to degradation.

10. After the last time point (4 hour), completely remove the media from each well.
11. Prepare 0.2M NaOH solution, and add 2 ml to each well to dissolve the cells. Make sure that the cell lysate is homogeneous by pipetting up and down 10–20 times until the lysate is no longer viscous.

Day 3 Preparation of samples of degradation products by precipitation with trichloroacetic acid (TCA).

12. Prepare scintillation vials containing 3 ml scintillation fluid before TCA precipitation.
13. To each microcentrifuge tube containing the 200 μ l media samples (from step 9), add 20 μ l TCA (100 % W/V, 6.12 M), which precipitates proteins, but not amino acids. Therefore, the radioactivity that is TCA-soluble reflects ^3H -phenylalanine generated by protein degradation.
14. Mix well and incubate on ice for 30 minutes.
15. Centrifuge at 20,000 g for 15 min at 4°C in a table-top centrifuge.
16. Take the supernatants being careful not to disturb the pellets. Add supernatants to a vial containing 3 ml scintillation fluid for measurement of radiolabeled amino acids released from proteins.
17. Take 100 μ l dissolved cell lysate (from step 11) and add to a scintillation vial containing 3 ml scintillation fluid. Amounts of total radiolabeled cell proteins are measured with these samples.
18. Prepare four blank control vials that contain only scintillation fluid, but no samples.

Measurement of protein degradation rates by liquid scintillation counting.

19. Place scintillation vials on an 18-vial cassette and place the cassette in the chamber of a Liquid Scintillation Analyzer.
20. With the software of the Liquid Scintillation Analyzer (we typically use the QuantaSmart™ software), set up a protocol that specifies the optimal counting time for ^3H (e.g. 2 min) and output measurement (Counts per minute, Cpm).
21. Place the correct protocol flag (with protocol number) on the first cassette, and start running the program from the software.

Calculation of degradation rates.

To calculate the total degradation rate (percent per hour), we need to plot the percentage of cell proteins hydrolyzed at each time point against the time. The slope of this plot, which should be linear, gives the rate. To calculate the percentage of degradation at each time point, we need to divide the ^3H -phenylalanine cpm released into the medium by the cpm

⁵.TCA precipitation can be performed immediately after media collection, or up to three days after collection if the media is stored at 4°C. However, before storing the media, remove any floating cells by centrifugation. These floating cells may lyse during storage, thus raising the radioactivity in the media.

initially incorporated into protein (which is the sum of the cpm in the cell sample plus the total in the medium at the final time point). Figure 2 demonstrates how to calculate the rates of degradation from raw cpm values (Fig 2A).

22. Follow equations in Fig 2B and Fig 2C to calculate the cumulative release of ^3H -phenylalanine Cpm at 1–4 hours, which reflects the amount of degraded radiolabeled proteins at each time (D0, D1, D2, D3, D4 values in Fig 2C) (see Note 6).
23. Follow equations in Fig 2D to calculate the remaining ^3H -Phenylalanine cpm (R) in cells, which reflects the amount of remaining radiolabeled proteins. Then sum up R and D4 to obtain the total radioactivity (Cpm) initially in cell proteins.
24. The percentage of protein degradation at each hour can now be calculated by dividing D0, D1, D2, D3, and D4 values by the total cpm as in Fig 2E. These percentages are plotted over time to get a linear degradation curve (characteristic for long-lived proteins), whose slope is the degradation rate in these specific cell lines under the tested incubation conditions.
25. Calculate the degradation rate for the other 3 wells of cells, in order to obtain the average and standard deviation of degradation rate for each conditions. With this approach, degradation rates can now be compared following different physiological conditions or drug-treatments.

3.2. Measurement of the degradation of mostly short-lived proteins labelled in a brief pulse

The protocol for measuring the degradation of short-lived proteins is almost the same as that for long-lived proteins with the exception for the length of cell exposure to the radioactive precursor, the lack of a chase period, and other precautions, as stated below.

1. Cells are grown to 60–80% confluence in six-well plates. To obtain sufficient replicates, 4 wells of cells were seeded.
2. Prepare complete media supplemented with ^3H -phenylalanine at a final concentration of 5 $\mu\text{Ci/ml}$. Add 1 ml to each well.
3. Incubate cells for 5–20 minutes to allow cellular proteins to be radiolabeled (Shorter pulses cause fewer proteins to be labelled, but increase the fraction that is rapidly degraded).
4. Prepare nonradioactive chase media as in the long-lived protein degradation in 3.1, except that 100 $\mu\text{g/ml}$ cycloheximide should be added (see Note 7) to prevent reincorporation of released amino acids into proteins. Cycloheximide

⁶The TCA-soluble cpm in the media at 0-time (T0, Fig 2A) is caused by ^3H -phenylalanine that failed to be washed out, and thus should be subtracted so that D0 is set to 0 (Fig 2C). When calculating the cumulative release of ^3H -phenylalanine cpm, take into account the reduction of total media volume over time due to the removal of 200 μl samples at each hour (Fig 2B).

⁷Inhibitors of protein synthesis, such as cycloheximide, should be added during the chase to eliminate the incorporation of radioactive amino acid into cell proteins during the chase after short pulse labeling. Cycloheximide should not be used in measuring the degradation of long-lived proteins, because with time it reduces the overall degradation of these proteins.

does not significantly reduce degradation rates for 1–2 h, but with longer incubations, cycloheximide reduces the degradation of long-lived proteins.

5. Remove radioactive media and thoroughly wash cells twice with 2 ml chase medium.
6. Add 2 ml chase medium to each well, and immediately begin sample collection for the measurements of degradation. The volumes of media taken must be very precise.
7. At 0, 15, 30, 45, and 60 minutes, collect 200 μ l aliquots of media from each well and transfer to a clean microcentrifuge tube. Briefly centrifuge the media, if necessary, to make sure it is free of floating cells. The media can be kept at 4 °C for up to 3 days before subjecting to TCA precipitation.
8. After the last time point (60 minutes), completely remove media from each well, and dissolve cells with 0.2M NaOH solution as in step 11 in **3.1**.
9. TCA precipitation and liquid scintillation counting are performed as in protocol **3.1** for measurements of the degradation of long-lived protein (*see* Note 8).

3.3. Measuring the degradation of long-lived proteins by the UPP and the autophagy-lysosomal system (*see* Note 9).

To quantitate the amount of protein degradation by a particular pathway under various conditions, we use small molecule inhibitors to block selectively and completely proteasome function (with 1 μ M Bortezomib to block its multiple peptidase activities), ubiquitin conjugation (with 10 μ M TAK243, which inactivates the ubiquitin activating enzyme, E1), or lysosomal degradation (with 200 nM Concanamycin A to block the lysosomal V-ATPase and prevent lysosomal acidification) (*see* Note 10). The net decrease in protein degradation that is blocked by these inhibitors indicates the rate of degradation through the corresponding pathway (*see* Note 11). Prior studies [15] have indicated that these

⁸Because the plot of degradation of long-lived proteins remains linear for many hours, precise degradation rates can be measured. However, with a short pulse, the proteins labelled at initial time points are primarily short-lived. Because they are rapidly degraded, the later time points will be increasingly dominated by proteins with longer half-lives. Consequently, the degradation rate drops significantly during the 1 hour measurement period, and mean half-lives of the short-lived fraction cannot be precisely calculated. In addition, although 5–20 minute pulses label differentially short-lived proteins, still a considerable amount of long-lived proteins are radiolabeled. Therefore, the degradation of pulse-labelled proteins is a combination of the rapid hydrolysis of short-lived components and the slower degradation of long-lived proteins, which predominate with time (after a 2–3 h chase). Therefore, rigorous conclusions about the changes in degradation rates of short-lived protein can only be made under conditions, where rates of degradation of long-lived proteins are unchanged (as measured in **3.1**).

⁹One difficulty in measuring the role of proteasomes in the degradation of short-lived proteins is that the proteasome inhibitors take time to enter the cells and to achieve maximal inhibition (especially at low inhibitor concentrations). Also, the degradation assay for short-lived proteins is generally complete within 1 hour after radiolabeling, which is too short for maximal inhibition. Therefore, it is necessary to pretreat the cells with the inhibitor for at least 30 minutes before exposure to the ³H-phenylalanine.

¹⁰Other inhibitors of these pathways may also be used, but pilot experiments with different inhibitor concentrations or durations should be done to ensure that the degradation pathway of interest is completely inhibited.

¹¹When choosing the type of inhibitor, the concentration and duration to be used, there are several important considerations. A) Cell death must be avoided. Dying cells frequently lose membrane integrity and spill radiolabeled small peptides or proteins out into the media, or allow Ca⁺⁺ into enter the cells triggering calpain activation and breakdown of cell proteins. Dead cells also elevate protein degradation by caspases. These artefacts erroneously increase the apparent rates of degradation and make data uninterpretable. Therefore, pilot experiments with LDH release assay must be performed to ensure that there is negligible cell death during inhibitor treatment. B) It is important to ensure that efficient inhibition of the degradation pathway is achieved before collecting media samples for TCA precipitation, because a certain duration of inhibitor treatment is required to achieve maximal inhibition of the cellular process. For example, concanamycin A inhibits lysosomal degradation by raising the pH of lysosomes, and at least 90 minutes of treatment is required for lysosomal proteases to be efficiently inhibited by this agent.

concentrations cause maximal selective inhibition of these pathways. The bortezomib-sensitive (proteasomal) and concanamycin A-sensitive (lysosomal) degradation rates should add up to account for nearly all protein degradation occurring in cells.

1. Cells are grown to 60–80% confluent in six-well plates. 4 wells of cells should be seeded for each inhibitor-treated condition as well as the untreated cells.
2. Radiolabel cellular proteins with ^3H -phenylalanine for 20 hours, as described above.
3. Remove radioactive media, thoroughly wash cells twice with 2 ml chase medium, and chase away short-lived proteins by incubating cells for 2h with 2 ml chase medium as in **3.1**.
4. Remove chase medium, thoroughly wash cells again, and add 2ml chase medium with or without Bortezomib (1 μM), TAK243 (10 μM), or Concanamycin A (200 nM).
5. Allow cells to be pre-treated for 2 hours to allow efficient inhibition.
6. After the pre-treatment, begin media collection (0h). At 0, 1 – 4 hours, collect 200 μl (must be very precise) aliquots of media from each well, and transfer to a clean microcentrifuge tube. Briefly centrifuge the media if necessary to make sure it is free of floating cells. The media can be kept at 4 $^{\circ}\text{C}$ for up to 3 days before subjecting to TCA precipitation.
7. After the last time point (4 hour), completely remove the media from each well and dissolve cells with 0.2M NaOH solution.
8. TCA precipitation and liquid scintillation analysis are performed, as in **3.1** for the measurement of the degradation of long-lived proteins.
9. Calculate the degradation rate in cells treated with Bortezomib as in Figure 2. Subtract this rate from the degradation rate in untreated cells to obtain the rate of proteasome-dependent degradation. In the same way, calculate the rates of ubiquitin-dependent degradation and degradation by the autophagy-lysosome system.

4. Conclusions

The widely used methods to evaluate these degradative processes (i.e. measurement of a proteasomal peptidase activity, the cellular level of ubiquitinated proteins, the amount of autophagosomes visualized, or the levels of lipidated LC3), although often useful for estimating the activity of these pathways, are not direct measurements of the amount of proteins being degraded by these systems and under many conditions can yield misleading impressions. This chapter describes methods to directly measure cellular protein degradation rates by radiolabeling long-lived or short-lived proteins with ^3H -phenylalanine. By further selective inhibition of ubiquitination, proteasome activity, or lysosomal function, it is possible to precisely calculate the fraction degraded by each system under different experimental conditions. Therefore, this powerful and versatile approach makes it possible

to accurately test whether protein degradation by the UPP or autophagy-lysosomes is altered under specific conditions or efficiently inhibited upon treatment with different drugs.

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Figure 1. Application of protein degradation assay protocols described in sections 3.1–3.3.

A. Protocol 3.1 measures the degradation of long-lived proteins (radiolabeled for 20 hours then chased for 2 hours to deplete short-lived radiolabeled proteins), whose degradation rate is constant. Therefore, precise degradation rate can be measured from the slope of the linear degradation curve.

B. Application of Protocol 3.1: By measuring long-lived protein degradation in HEK293A cells treated with two mTOR inhibitors (Rapamycin or Torin1, which are anticipated to activate autophagy), we validated that the ability of cells to degrade protein was indeed elevated by these inhibitors. Figure adapted from [31].

C. Protocol 3.2 measures the degradation of mostly short-lived proteins labeled by a short pulse (5–20 min). Their mean degradation rate decreases rapidly over time due to the depletion of short-lived proteins, so that the degradation curve is non-linear, and it is not possible to calculate a constant degradation rate.

D. Application of Protocol 3.2: The degradation of proteins pulse-labelled for 10 minutes was measured. Treatment with a Protein Kinase A (PKA) activator Forskolin greatly increased the amount of pulse-labelled proteins that were degraded over 1 hour. Note that the degradation curve is non-linear and no degradation rate could be calculated. The percentage of degraded proteins should be compared at each time points to draw conclusions. Figure adapted from [18].

E. Protocol 3.3 measures the degradation of long-lived proteins by a specific degradation pathway, by using a specific inhibitor to completely block this pathway. Subtracting these two degradation rates allow us to calculate the rate of inhibitor-sensitive degradation, which represents the ability of cells to degrade long-lived proteins through this particular pathway.

F. Application of Protocol 3.3: To determine whether the increased degradation in cells treated with mTOR inhibitors (as in B) is caused by the lysosome pathway, the long-lived protein degradation rate was measured in cells whose lysosomes are completely inhibited by concanamycin A. This allow us to compare concanamycin A-sensitive (or lysosomal)

degradation rates between control cells or cells treated mTOR inhibitor. We found using this approach, that lysosome contributes to the increased degradation in wild-type HEK293A or MEF cells, but not in cells with deficient autophagy (Atg5^{-/-}). Figure adapted from [31].

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A

Raw Cpm (Count per minute)						
Blank (average of four)	TCA supernatant from 200 µl media sample					100 µl cell lysate in 0.2 N NaOH
	0h	1h	2h	3h	4h	
	T0	T1	T2	T3	T4	
B						L

B

Calculate how many Cpm is released in each hour due to protein degradation

	0-1h	1-2h	2-3h	3-4h
Cpm increase	T1-T0	T2-T1	T3-T2	T4-T3
Sample volume (ml)	0.2	0.2	0.2	0.2
Media volume (ml)	1.8	1.6	1.4	1.2
Total Cpm released in this hour	$9 \times (T1 - T0)$	$8 \times (T2 - T1)$	$7 \times (T3 - T2)$	$6 \times (T4 - T3)$

C

Calculate the cumulative Cpm release after 1, 2, 3, 4 hours

	0h	1h	2h	3h	4h
Cumulative Cpm released (degraded)	D0 = 0	D1 = $9 \times T1 - 9 \times T0$	D2 = $8 \times T2 + 7 \times T3 + 6 \times T4 + 9 \times T0$	D3 = $7 \times T3 + 6 \times T4 + 5 \times T0$	D4 = $6 \times T4 + 5 \times T3 + 4 \times T2 + 3 \times T1 + 2 \times T0$

D

Cpm in remaining
protein (R)

=

$$\frac{(L - B) \times 2 \text{ ml}}{100 \mu\text{l}}$$

= 20 × (L-B)

Total Cpm (Total) = R + D4

E

Calculate percent degradation at 0, 1, 2, 3, 4 hours

	0h	1h	2h	3h	4h
Percent Degradation	0	$100 \times D1$	$100 \times D2$	$100 \times D3$	$100 \times D4$
		Total	Total	Total	Total

Figure 2. Calculation of the degradation rate of long-lived proteins

- A. Raw Cpm counts for blank, TCA-soluble media samples, and cell lysate samples.
- B. To calculate how much ^3H -Phenylalanine is released by protein degradation in each hour, multiply cpm increments by the volume of media during that hour.
- C. Combine the values from Fig 2B to calculate cumulative ^3H -Phenylalanine released in 1–4 hours, which reflects the amount of degraded proteins (D0, D1, D2, D3, and D4).
- D. Calculate the ^3H -Phenylalanine cpm remaining in proteins (R) and in total proteins (Total).
- E. Divide D0-D4 values by Total Cpm to calculate the percent degradation over 0–4 hours. Plotting these values (% cell protein degraded) will generate a linear curve, whose slope is the rate of protein degradation (% cell protein degraded / h).