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Out with the Old, In with the New? Comparing Methods for Measuring Protein Degradation

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Abstract

Protein degradation is a key variable in controlling protein abundance in cells. Here, we compare classical methods to measure protein degradation rates with a novel GFP reporter library based method that characterizes degradation of thousands of individual proteins by flow cytometry. While no method is perfect, we conclude that chimeric gene reporter approaches should be applied cautiously due principally to GFP (or other tag) interference with organelle targeting or incorporation of chimeric proteins into macromolecular assemblies that results in spuriously high degradation rates.

Metabolic stability greatly influences the abundance of proteins in cells. Historically, protein degradation has been studied using methods that measure overall protein degradation or focus on a few individual proteins. With recent technical advances, it is possible to measure degradation rates of large numbers of defined proteins, with the ultimate goal of proteome-wide determination of protein stabilities under various conditions.

In reporter-dependent methods, open reading frames (ORFs) are expressed individually as fusion proteins with fluorescent protein or epitope tags, and their stabilities assayed based on tag detection. In reporter-independent methods, the fate of nascent proteins is followed by tagging them with isotopically labeled amino acids, chasing with unlabelled amino acids, and measuring the loss of the labeled cohort.

Yen *et al.* [1] describe a novel version of the reporter-dependent approach which they term global protein stability profiling (GPSP). After transducing cells with a biscistronic retroviral vector encoding red fluorescent protein (RFP) and a cDNA of the gene of interest fused to GFP, they determine the RFP/GFP ratio for each library member by flow cytometry, and convert values to a half-life using a panel of GFP variants with biochemically defined half-lives.

Doherty *et al.* [2] update the classical method of radioisotope amino acid pulse-cold amino acid chase studies by employing dynamic SILAC (stable isotope labeling with amino acids in cell culture), using [$^{13}C_6$] Arg labeling to measure the degradation of 576 proteins by quantitative mass spectrometry on proteins identified in 1D-SDS-PAGE gel slices..

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To evaluate the validity of these methods, we examined the extent to which their findings correlate. We identified the 339 gene products shared between SILAC and GPSP data sets. The output of the SILAC study $k_{deg,}$ fraction of degradation min^{-1}, whereas GPSP provides a protein stability index (PSI) from 1–7 (less stable to more stable). If both approaches accurately measure protein stability, there should be a clear negative correlation between k_{deg} and PSI.

Surprisingly, a scatter plot comparison of these two parameters demonstrates no significant correlation (Figure 1a, in Figure 1b convert the k_{deg} and PSI values to half-lives). What could account for such a dramatic difference?

One possibility is that the SILAC and GPSP studies were conducted using different human cell lines, A549 lung adenocarcinoma cells and 293T embryonic kidney cells, respectively. While some proteins, no doubt, are degraded in a cell type specific manner, this is unlikely to apply to most proteins, particularly, essential housekeeping proteins. Rechsteiner *et al.* [3] measured metabolic stabilities of six proteins injected into five different cell lines and found less than 4-fold difference in half-lives for each of the proteins. Qian *et al.* [4] observed similar overall protein degradation rates in HeLa, 293, and hamster E36 cells by standard [^{35}S]-Met pulse chase radiolabeling. Furthermore, cell type-specific differences would not be expected to yield a statistically *random* scatter of degradation profiles.

Notably, the SILAC data agree with the many reported pulse-chase studies performed in cultured cells with radiolabeled amino acids [5, 6]. By contrast, GPSP half-lives are typically ~10-fold shorter than determined by radiolabeled pulse chase studies. This suggests that one of the approaches exhibits intrinsic bias, and calls for consideration of the strengths and weaknesses associated with each method.

Amino acid isotope pulse chase experiments

The most direct approach to study protein degradation is to label nascent proteins and follow their fate using either amino acid analogs that can be identified by their chemical properties, or isotopically labeled forms of the natural amino acids that can be identified by their mass or radioactivity. Since the precise chemical properties of amino acid side chains tremendously influence protein folding, amino acid analogs will induce misfolding to some extent, limiting this approach to isotopic labeling.

Isotopic amino acid tagging makes a number of assumptions, however, that if unwarranted undermine its accuracy

The isotopic label is exclusively present in the intended amino acid and not chemical analogs that are incorporated into proteins and induce misfolding This is not a problem for SILAC, since the read out is limited to the predicted mass of the labeled amino acid. While this is a real concern for radiolabeled preparations, incorporation of a radiolabeled analog would diminish half-lives and not increase them, as would be required to explain the difference with GPSP.

Other contaminants present in the isotope label do not alter metabolic stability

For example, commercially available radiolabeled amino acids prepared from bacteria contain bacterial components that trigger cellular innate immune receptors with unpredictable effects on protein stabilities [7].

Reutilization of labeled amino acids from protein turnover increases the apparent half-lives of proteins

This is rarely a concern using cultured cells where a saturating level of unlabelled amino acid is easy to achieve during the chase when rapidly incorporated labels, commonly [^{35}S]-Met, [^{35}S]-Cys, [^{3}H]-Leu, or [^{3}H]-Tyr are employed.

The method used to identify individual protein species (typically antibody based recovery from detergent lysates for radiolabeling, mass spectrometry for SILAC) accurately quantitates all forms of the labeled protein

This is a significant problem for radiolabeling, since misfolded proteins are typically poorly solubilized with the mild cell lysis conditions needed to maintain antigenicity. Further, misfolding may prevent antibody binding, resulting either in failure to detect nascent protein (and an overestimation of protein stability) or in the mistaken conclusion that the protein has been degraded (and an underestimation of stability). This is less of a problem for SILAC, but still an issue, since misfolded/aggregated proteins may not be solubilized or may migrate aberrantly in SDS-PAGE.

Given the uncertainties associated with isotope pulse-labeling (indeed with *any* single technique), it is essential to corroborate its findings with other techniques used to study protein degradation.

Post-synthetic Radiolabeling

Proteins can also be labeled for by post-translational modification of side chains with radioisotopes (*e.g.* Tyr/Lys radioiodination) or other tags (*e.g.* Lys-biotinylation). Modified amino acids are typically not recognized by tRNA aminoacyl synthetases and are therefore not reincorporated into proteins, eliminating the confounding effects of reutilization. An important limitation is that labeling can damage the protein, leading to underestimation of protein stability.

Plasma membrane proteins

Plasma membrane proteins offer unique post-synthetic labeling targets since they can be selectively labeled extracellularly. Chu and Doyle [8] radioiodinated rat hepatoma plasma membrane proteins and found that eleven prominently labeled proteins exhibited half-lives ranging from 16 to 100 h, highly similar to values obtained in the same study by traditional ^{35}S-Met pulse-chase labeling. Similarly, Hare and Taylor [9] used biotinylation to selectively label cell surface proteins of 3T3 fibroblasts and rat hepatoma cells, reporting that the vast majority of proteins exhibited half-lives greater than 75 hours.

Cytosolically delivered proteins

Rechsteiner and colleagues developed a method for cytosolically introducing radioiodinated proteins based on target cells fusion with protein-loaded erythrocyte ghosts [10–12] and found good agreement with pulse chase labeling methods for dozens of proteins measured [13, 14]. Although the injection approach provides useful information, it is best suited for abundant proteins with half lives of at least hours..

The reasonable agreement between metabolic stabilities measured by isotopic pulse labeling vs. post-synthetic labeling or microinjection cross validates these methods for measuring protein half-lives..

"Cycloheximide-Chase" Immunoblotting

Given an immunoblotting mAb or antiserum, it is relatively easy to accurately quantitate antigen decay following addition and continued incubation ("chase") with cycloheximide, a rapidly acting eukaryotic protein synthesis inhibitor (with the caveat that the data should be related to a standard curve to account for non-linearity in the immunoblot signal). A significant advantage of this method is its insensitivity to highly denaturing extraction buffers that allow for maximal recovery of proteins from cells, often including misfolded, and even aggregated forms.

The method is ill suited, however, for long-lived proteins due to the effects of prolonged protein synthesis inhibition on overall cell function. There are also possible rapid effects on degradation pathways themselves. For example, increased levels of free amino acids, aminoacyl-tRNAs or nucleotides could affect proteolytic systems or the conformations of specific substrates. If key components of the degradation pathway are themselves short-lived, their substrates may actually be stabilized by cycloheximide [14].

Nearly all of the myriad cycloheximide-chase studies focus on one or a few chosen proteins. Belle *et al.* [15], however, combined this approach with a reporter-dependent methodology to measure the half-lives of more than 3700 yeast gene ORFs encoding a tandem affinity purification (TAP) tag, a protein consisting of 184 amino acids. The mean and median half-lives of 3,751 yeast proteins were 43 min with a range from less than 4 min to greater than 400 min. Significantly, a comparison of the stabilities of tagged and untagged versions for 24 proteins revealed that two-thirds of the tagged proteins were degraded more rapidly than their wild-type forms, demonstrating that TAP-tagging, like GPSP, displays an intrinsic bias to decreasing protein stability.

GFP interferes with Protein Function

Based on their broad agreement with other approaches, it is clear that isotope based amino acid pulse chase studies, while imperfect, provide a reasonably accurate measure of protein turnover, and if anything, generally underestimate protein stability. How then, to explain the consistent overestimation of protein turnover that appears characteristic of reporter-dependent methods, such as GPSP?

Examining the most divergent proteins between SILAC and GPSP studies (Table 1) provides insight into this question. Ribosomal proteins, which represent 10% of the proteins shared between the studies, account for 35% of the top decile of most divergent proteins, with GPSP reporting much shorter half lives than dynamic SILAC and other methods [16, 17]., The stability of ribosomal subunits is dependent on assembly into functional ribosomes [16,18]. The likely explanation, then, is that GPSP underestimates protein stability by interfering with proteins assembly into macromolecular structures either structurally or stoichiometrically (*i.e.* by over expression, which is well known to result in the degradation of free subunits, whose stability depends on their integration). Consistent with this interpretation, many proteins in the subset of most divergent proteins between the data sets are components of larger macromolecular assemblies. Many of the other proteins in the top decile exhibit organelle-specific targeting for proper localization and function (into mitochondria, the endoplasmic reticulum, and the nucleus), consistent with mistargeting of the GFP-fusion proteins.

GFP-fusion, while a simple and powerful technique is fraught with artifacts as expounded by Snapp [19]. To wit:

GFP tagging impairs protein biogenesis

Table 1 gives reason to suspect that GFP reporter fusion sterically blocks incorporation of ribosomal, ATP synthase, and cytochrome oxidase subunits into their respective multimeric complexes. The eGFP tag could also prevent chaperones from binding and promoting proper folding the ORF-encoded protein, leading to artifactual destabilization. Conversely, high local concentrations of eGFP, such as in oligomers, can cause the eGFP tag to dimerize and potentially stabilize otherwise labile complexes [19].

GFP tagging blocks subcellular targeting signals

The discrepancies highlighted in Table 1 strongly suggest GFP-interference with intracellular trafficking. The N- and C-termini of proteins contain the majority of sub-cellular localization signals. Methods based on cassette-termini tagging will likely alter the localization and thereby stability of the chimeric product. Snapp [19] highlights the magnitude of this problem: 7500 of 30,000 annotated proteins are targeted to the ER or mitochondria, and would be expected to misfold if not properly exported from the cytosol. Yen *et al.* noted that membrane proteins were notably less stable in their GPSP data set. They also reported that C-terminally tagged versions of the proteins were equally unstable, and use randomly selected ORFs mAb epitope tagged at the C-terminus to validate their GPSP data. The concern remains that tagging either terminus similarly increases degradation rates.

GFP expression intrinsically interferes with polyubiquitylation and polyubiquitin-dependent signals

Baens *et al.* [20] reported that eGFP expressed either alone or as fusion proteins inhibits polyubiquitylation and modifies multiple cell signaling pathways. The authors cite other examples where eGFP disrupts cellular physiology by unknown mechanisms. Obviously, this will exert unpredictable effects on the stability of individual gene products.

A significant fraction of GFP is rapidly cleaved

In extensively using eGFP either alone or in multiple gene fusion contexts and expression scenarios, JWY's laboratory has found that that approximately 25% of GFP is rapidly cleaved (probably autocatalytically [21]) resulting in degradation of the fusion protein (*e.g.* Qian *et al.* [4]). If this process is affected by the nature of the fusion partner, it will variably affect the RFP/GFP ratio, and result in spurious degradation rates. Moreover, the cleaved fusion protein has a high chance of misfolding and acting in a dominant negative manner, interfering with cell function and accelerating degradation of folded versions of the fusion protein.

Differential DRiP Fraction Lead to Spurious Values

Numerous studies point to the conclusion that a significant fraction of nascent proteins do not achieve their stable functional conformation, and are rapidly degraded [4, 22–26]. Such defective ribosomal products (DRiPs) provide the majority of antigenic peptides for the MHC class I immunosurveillance system [27, 28]. While little is known about differences in the DRiP fraction among gene products, this could vary significantly. GPSP substrates with a high intrinsic DRiP fraction, or a DRiP fraction increased by GFP fusion will score with aberrantly rapid degradation rates since the reference protein is only affected by its own intrinsic DRiP fraction, which should be constant. (It should be possible to study this effect by measuring the decay in the GFP signal via flow cytometry following addition of cycloheximide to cells. Gene products with a higher DRiP fraction will demonstrate less GFP decay than predicted by their steady state levels.)

Based on these considerations, we suggest that reporter-dependent methods for assessing intrinsic protein stability, such as GPSP, are ill suited for proteome-wide assessments of protein stability. At the same time, this approach is clearly applicable for exploring cell type differences in protein stability and for absolute measurements of proteins whose stabilities match those measured by alternative techniques. Further, Yen & Elledge [29] demonstrate the value of GPSP for high throughput screening to identify ubiquitin ligase substrates, pointing the way to applications in which relative stabilities yield important insights and information.

In comparison, SILAC is more laborious and requires expertise in a highly demanding technology based on expensive instrumentation. It does, however, provide an accurate measure of stability of hundreds to low thousands of proteins and is the method of choice for measuring absolute protein degradation rates in different cells and under different conditions.

Pressing Questions in Proteolysis

Renewed appreciation for the importance of proteolysis has accompanied the explosion in research on the ubiquitin-proteasome pathway in the past decade. In addition to gene product specific issues, a number of basic questions remain to be addressed.

What is the basis for the typical first order degradation kinetics of proteins?

Classic studies in the 60's correlated protein metabolic stability in cells with their *in vitro r*esistance to endoprotease digestion. This is consistent with the idea that stability is inversely proportional to protein dynamics. This correlation was observed using a limited set of proteins chosen for ease of purification, and it is important to update and extend these findings to more proteins and additional parameters of protein stability. Which of the myriad ubiquitin ligases are involved in normal protein turnover and how they select their substrates? The contribution of protein "aging", *i.e.* post-translational damage (*e.g.* oxidative damage) to stochastic recognition based on spontaneous unfolding remains a central question.

Why are 20% or more of translation products degraded so rapidly [4, 22–25]

Is this due to translation of many defective polypeptides?. What are the contributions of pioneer translation in nonsense mediated decay and translation of short mRNAs generated transcriptionally [30] or via microRNA mediated cleavage [31]? What exactly are cells translating (*i.e.* defining the translatome)? What are the inefficiencies in folding, assembling, or modifying otherwise normal proteins? Why are nascent proteins destined to become native proteins more sensitive to chemical or physical denaturation for the first hour post-synthesis [32]? Do proteins need to pass a final quality control step before being integrated into the cell, as originally suggested by Wheatley [33]?

To what extent is translation specialized based on the requirements of individual gene products?

Ribosomes are known to be highly heterogeneous [34]. Mauro and Edelman's *ribosome filter* hypothesis, posits that sequence specific targeting of mRNAs to ribosome subsets contributes to controlling gene expression [35]. This is supported by the report of Komili *et al.*[36], that duplicated ribosomal genes function to specialize ribosomes for translation of specific mRNAs. Could ribosome specialization also entail recruitment of chaperones and protein modification machinery tailored for classes of gene products? Does this contribute to the failure of genome wide gene fusion methods to recapitulate physiological biogenesis of the gene products of interest?

How do findings with cultured cells relate to cells in their natural state in organisms?

The original pulse chase radiolabeling studies of protein stability were largely performed in living animals [13, 14, 37]. Use of cultured cells offered obvious advantages in labor, expense, and experimental manipulation. Mammalian cells, however, did not evolve to grow in culture, and it is essential in future studies to develop methods for quantitatively studying protein degradation (and biogenesis*) in vivo*.

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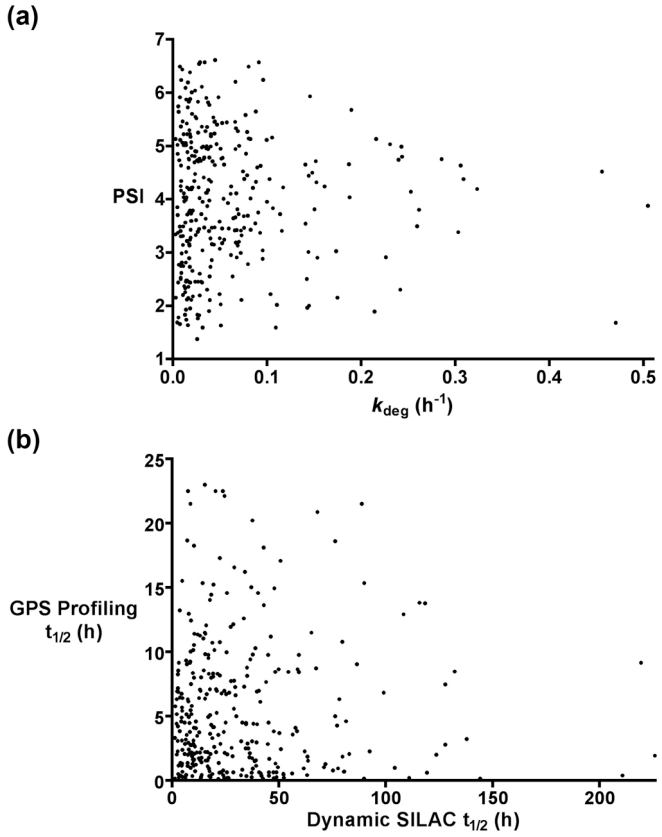

Figure 1.

Comparisons of protein stability constants determined by dynamic SILAC versus global protein stability (GPSP) profiling. Correlation analysis of protein stability values determined by SILAC and global protein stability (GPSP) profiling **(a)** Proteins common to the dynamic SILAC [2] and GPSP [1] data sets were identified (339 in total) along with their associated degradation rate (*k*deg) and protein stability index (PSI) value, respectively. The *k*deg and corresponding PSI value for each protein is shown on a scatter plot. In this plot, data set correlation would be indicated by a scatter plot with negative slope. As is apparent, no significant correlation is seen (Spearman's rank correlation coefficient, $\rho = 0.028$, $p = 0.61$). To clarify the graphical presentation, 14 outlier values (*k*deg < 0.00307 and *k*deg > 0.5042) that were included in the statistical analysis are not shown on the scatter plot. **(b)** Comparison of the half-life values calculated from *k*deg based on [2] and from PSI using a modification of the regression analysis in [1]. While one would predict a positive correlation between the two half-life measurements, again, no significant correlation is seen (Spearman's rank correlation coefficient, $\rho = -0.28$, $p = 0.61$). The same outlier values from (a) are not shown in the scatter plot, but were included in the statistical analysis. All statistical analyses were performed with GraphPad Prism 4. It should be noted that this analysis required recalculation of the protein half-lives initially reported by Yen *et al.* Following their method yields a linear regression where PSI values of less than 2.96 yield negative half-life values, a biological impossibility. We instead fit the published PSI data to the half-life values of the various eGFP-ornithine decarboxylase degron fusions used as standards by these authors. The best fit is described by the equation $t1/2 = 0.0243 \times (PSI)^{3.6281}$, which gives an R^2 of 0.9974 for the provided data.

Table 1

Examples from the top 10% of "worst offenders" showing divergent stability between the dynamic SILAC and GPS profiling data sets.

Proteins	SILAC stability percentile[a]	GPS stability percentile[a]	Notes	Entrez Gene IDs
L14, L15, L23, L23A, L27, L28, L35, S10, S13, S14, S15A, S16	88th (75th-99th)	11th (2nd-21st)	Ribosomal proteins	9045, 6138, 9349, 6147, 6155, 6158, 11224, 6204, 6207, 6208, 6210, 6217
F0 complex subunit G and F1 complex subunit O	98th and 99th	7th and 18th	Components of ATP synthase	10632, 539
Cytochrome c oxidase subunit Va	88th	17th	Mitochondrial inner membrane protein	9377
GRP94	97th	9th	ER chaperone	7184
RRC1	7th	91st	Ran Guanine exchange factor	1104

[a]Stability is expressed as a percentile of the rank ordered stability of the 339 proteins shared between the SILAC and GPS data sets. High percentiles correspond to stable proteins, while low percentiles correspond to unstable proteins. For the ribosomal proteins, the mean percentile of the group is shown with the percentile range in parentheses.