Out with the old, in with the new? Comparing methods for measuring protein degradation

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Abstract

Protein degradation is a critical factor in controlling cellular protein abundance. Here, we compare classical methods for determining protein degradation rates to a novel GFP fusion protein-based method that assesses the intrinsic stability of cloned cDNA library products by flow cytometry [Yen et al. (2008) Science 322, 918]. While no method is perfect, we conclude that chimeric gene reporter approaches, though powerful, should be applied cautiously, due principally to GFP (green fluorescent protein) (or other reporter tag) interference with protein organelle targeting or incorporation into macromolecular assemblies, both of which cause spuriously high degradation rates.

Keywords: GPSP; metabolic labelling; protein degradation; proteolysis; proteostasis; pulse chase; SILAC

1. Introduction

Metabolic stability is a major factor in regulating levels of a given protein in cells (Schimke and Doyle, 1970; Goldberg and Dice, 1974). Historically, protein degradation has been studied using methods that measure overall protein degradation or focus on a few individual proteins. With recent technical advances in two basic approaches (Yen et al., 2008; Doherty et al., 2009), it is now possible to measure degradation rates of large numbers of proteins, with the ultimate goal of proteome-wide determination of protein stabilities under various cellular conditions.

In reporter-dependent approaches (Yen et al., 2008), ORFs (open reading frames) are expressed individually as fusion proteins with fluorescent protein or epitope tags and their stabilities assayed based on tag detection. In reporter-independent approaches (Doherty et al., 2009), nascent proteins are tagged with isotopically labelled amino acids and loss of the labelled cohort is measured following the addition of excess unlabelled amino acid to prevent label reutilization.

Yen et al. (2008) describe a novel version of the reporter-dependent approach, which they term GPSP (global protein stability profiling). After transducing cells with a bicistronic retroviral vector encoding RFP (red fluorescent protein) immediately downstream of an internal ribosome entry site and an upstream cDNA of the gene of interest fused to GFP (green fluorescent protein), they determined the RFP/GFP ratio for each library member by flow cytometry. The RFP/GFP ratio is then converted to a protein half-life value algorithmically, using measured values from a panel of GFP variants with biochemically defined half-lives.

Doherty et al. (2009) update the classical method of radioisotope amino acid pulse-chase amino acid chase studies by employing dynamic SILAC (stable isotope labelling with amino acids in cell culture), using [15N]arginine labelling to measure the degradation of 576 proteins by quantitative mass spectrometry on proteins identified in 1D SDS/PAGE gel slices.

If each method accurately assesses protein metabolic stabilities, they should obtain similar values for the same gene product. Cross-correlating the SILAC and GPSP data sets, we scatter plotted 339 shared gene products against each other (Figure 1). The output of the SILAC study is represented by the \( k_{diss} \) variable, which represents the fractional degradation per minute, whereas GPSP utilizes a PSI (protein stability index) ranging from 1 to 7 (less stable to more stable). Surprisingly, the plot reveals no significant correlation between the common members of the two data sets. What could account for the poor correlation between the two methods?

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, which constitute a significant fraction of the common members plotted. In support of this contention, Rechsteiner et al. (1987) 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. (2006) observed similar overall protein degradation rates in HeLa, 293 and hamster E36 cells by standard \([35S]\)-methionine pulse chase radiolabelling. Perhaps more importantly, cell type-specific differences would not be expected to yield a statistically ‘random’ scatter of degradation profiles.

Notably, the SILAC data set in aggregate agrees with the many reported pulse-chase studies performed in cultured cells with radiolabelled amino acids that indicate that cellular proteins demonstrate an average half-life on the order of a day (Poole and Wibo, 1973; Rock et al., 1994). By contrast, GPSP half-lives are typically ~10-fold shorter than determined by radiolabelled

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Abbreviations: DRiPs, defective ribosomal products; eGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GPSP, global protein stability profiling; ORF, open reading frame; PSI, protein stability index; RFP, red fluorescent protein; SILAC, stable isotope labelling with amino acids in cell culture; TAP, tandem affinity purification.
2. 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 analogues that can be identified by their chemical properties or isotopically labelled 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 analogues will induce misfolding to some extent, precluding their use and limiting this approach to isotopic labelling.

Isotopic amino acid tagging makes a number of assumptions, however, that should be considered in assessing its validity. The isotopic label is exclusively present in the intended amino acid and not chemical analogues that incorporate into proteins and induce misfolding. This is not a problem for SILAC, since the read out is limited to the predicted mass of the labelled amino acid. While this is a real concern for radiolabelled preparations, incorporation of a radiolabelled analogue would diminish half-lives and not increase them, as would be required to explain the differences apparent between the SILAC and GPSP data sets.

Other contaminants present in the isotope label do not alter metabolic stability. For example, commercially available radiolabelled amino acids prepared from bacteria contain bacterial components that trigger cellular innate immune receptors with unpredictable effects on protein stabilities (Lelouard et al., 2002).

Reincorporation of labelled 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 equilibrated labels, commonly [35S]-methionine, [35S]-cysteine, [3H]-leucine or [3H]-tyrosine are employed.

The method used to identify individual protein species (typically antibody-based recovery from detergent lysates for radiolabelling, mass spectrometry for SILAC) accurately quantitates all forms of the labelled protein. This is a significant problem for radiolabelling, 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 labelling (indeed with ‘any’ single technique), it is essential to corroborate its findings with other techniques used to study protein degradation.

3. Postsynthetic radiolabelling

Proteins can also be labelled by posttranslational modification of side chains with radioisotopes (e.g. tyrosine/lysine radioiodination) or other tags (e.g. lysine biotinylation). Such 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 labelling can damage the protein, leading to underestimation of protein stability.
3.1. Plasma membrane proteins

Plasma membrane proteins offer unique postsynthetic labelling targets, since they can be selectively labelled extracellularly. Chu and Doyle (1985) radioiodinated rat hepatoma plasma membrane proteins and found that 11 prominently labelled proteins exhibited half-lives ranging from 16 to 100 h, highly similar to values obtained in the same study by traditional $^{35}$S-methionine pulse-chase labelling. Similarly, Hare and Taylor (1991) 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 h.

3.2. Cytosolically delivered proteins

Rechsteiner and colleagues developed a method for cytosolically introducing radioiodinated proteins based on target cell fusion with protein-loaded erythrocyte ghosts (Schlegel and Rechsteiner, 1978; Neff et al., 1981; Rogers and Rechsteiner, 1988) and found good agreement with pulse-chase labelling methods for dozens of proteins measured (Goldberg and Dice, 1974; Goldberg and St John, 1976). 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 labelling compared with postsynthetic labelling or microinjection serves to cross-validate these methods for measuring protein half-lives.

3.3. ‘Cycloheximide-chase’ immunoblotting

Given an immunoblotting mAb (monoclonal antibody) or antisera, 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 nonlinearity 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, aminocarboxyl-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 (Goldberg and St John, 1976).

Nearly all of the myriad cycloheximide-chase studies focus on one or a few chosen proteins. Belle et al. (2006), however, combined this approach with a reporter-dependent methodology to measure the half-lives of more than 3700 yeast gene ORFs encoding a TAP (tandem affinity purification) tag, a protein consisting of 184 amino acids. The mean and median half-lives of 3751 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 displays an intrinsic bias to decreasing protein stability.

4. 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 (Lastick and McConkey, 1976; Warner, 1977). The stability of ribosomal subunits is dependent on assembly into functional ribosomes (Warner, 1977; Granneman and Tollervey, 2007). The likely explanation, then, is that GPSP underestimates protein stability by interfering with protein 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 for studying protein function in cells, is fraught with artifacts as expounded by Snapp (2009).

4.1. GFP tagging impairs protein biogenesis

A reason to suspect that eGFP (enhanced green fluorescent protein) fusion sterically blocks incorporation of ribosomal, ATP synthase and cytochrome oxidase subunits into their respective multimetric complexes is given (Table 1). The eGFP tag could also prevent chaperones from binding and promoting proper folding of 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 (Snapp, 2009).
4.2. GFP tagging blocks subcellular targeting signals

The discrepancies highlighted (Table 1) also strongly suggest GFP interference with intracellular trafficking. The N- and C-termini of proteins contain the majority of subcellular localization signals. Methods based on cassette-termini tagging can thus alter the localization and thereby stability of the chimeric product. Snapp (2009) highlights the magnitude of this problem: 7500 of 30000 annotated proteins are targeted to the ER (endoplasmic reticulum) 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.

4.3. GFP expression intrinsically interferes with polyubiquitylation and polyubiquitin-dependent signals

Baens et al. (2006) reported that eGFP expressed either alone or as fusion proteins inhibits polyubiquitylation and modifies multiple cell signalling 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.

4.4. 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 (Barondeau et al., 2006)] resulting in degradation of the fusion protein (e.g. Qian et al., 2006). If this process is modulated 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 potentially acting in a dominant negative manner, interfering with cell function and accelerating degradation of folded versions of the fusion protein.

4.5. Differential DRIP fraction leads to spurious values

Numerous studies point to the conclusion that a significant fraction of nascent proteins (upwards of 15%) do not achieve their stable functional conformation and are rapidly degraded (Wheatley et al., 1980; Schubert et al., 2000; Turner and Varshavsky, 2000; Fuertes et al., 2003; Vabulas and Hartl, 2005; Qian et al., 2006) (though ref. 24 questions the magnitude of this effect, Figure 4D in this study clearly demonstrates the phenomenon). Such DRIPs (defective ribosomal products) provide the majority of antigenic peptides for the MHC class I immunosurveillance system (Yewdell et al., 2003; Yewdell and Nicchitta, 2006). While little is known about differences between the DRIP fractions 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.)

5. Old compared with new

Based on these considerations, we believe 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 and Elledge (2008) demonstrated 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 provide, however, an accurate measure of
stability of hundreds to low thousands of proteins and is the method of choice for the time being for measuring absolute protein degradation rates in different cells and under different conditions.

6. 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 conditions, 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 1960s correlated protein metabolic stability in cells with their in vitro resistance to endoprotease digestion. This is consistent with the idea that metabolic stability is inversely proportional to protein structural flexibility (i.e. their propensity to spontaneously unfold locally or globally). 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 flexibility. Which of the myriad ubiquitin ligases are involved in normal protein turnover, and how do they select their substrates? The contribution of protein ‘aging’, i.e. posttranslational damage (e.g. oxidative damage) to stochastic recognition based on spontaneous unfolding remains a central question.

Why are so many translation products degraded so rapidly (Wheatley et al., 1980; Schubert et al., 2000; Turner and Varshavsky, 2000; Vabulas and Hartl, 2005; Qian et al., 2006)? What are the contributions of truly defective polypeptides, intrinsically disordered proteins that fail to find suitable ligands for stability (Tompa, 2002; Vladimir, 2002; Uversky et al., 2008) and excess subunits of proteins destined for immediate integration into multimeric assemblies? What are the contributions of pioneer rounds of translation in nonsense-mediated decay (Maquat, 2004) and translation of short mRNAs generated transcriptionally (Xu and Ganem) or via microRNA-mediated cleavage (Gu et al., 2009)? Indeed, what exactly are cells translating? (i.e. defining the translatome).

What are the inefficiencies in folding, assembling or modifying otherwise normal, stable proteins? Why are nascent proteins destined to become native proteins more sensitive to chemical or physical denaturation for the first hour postsynthesis (Medicherla and Goldberg, 2008)? Do proteins need to pass a final quality control step before being fully ‘integrated’ into the cell, as first suggested by Wheatley et al. (1980, 1982)? Is there some general mechanism for sensing the appropriate copy number of proteins that acts as a gatekeeper for nascent protein integration (Wheatley, 1984)?

To what extent is translation specialized based on the requirements of individual gene products? Ribosomes are known to be highly heterogeneous (Dimman, 2009). Mauro and Edelman’s ‘ribosome filter’ hypothesis, posits that sequence-specific targeting of mRNAs to ribosome subsets contributes to controlling gene expression (Mauro and Edelman, 2002). This concept is supported by the report of Komili et al. (2007) 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 radiolabelling studies of protein stability in mammalian cells were largely performed in living animals (Schimke and Doyle, 1970; Goldberg and Dice, 1974; Goldberg and St John, 1976). Use of cultured cells offered obvious advantages in labour, 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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